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# Endogenous Subunits Can Cause Ambiguities in the Pharmacology of Exogenous γ-Aminobutyric Acid$_A$ Receptors Expressed in Human Embryonic Kidney 293 Cells

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## SUMMARY

The physiological and pharmacological properties of GABA$_A$ receptors have been studied extensively after the expression of subunits in non-neural cells. Many of these studies have used the human embryonic kidney cell line HEK 293. We examined the properties of subunits that result in the expression of low levels of functional receptors and found that the properties of the γ-aminobutyric acid (GABA)-elicited responses in transfected HEK 293 cells differ from expectations based on previous work and are consistent with the idea that the expressed receptors do not necessarily contain the expected subunits. In particular, expression of a mutated β2 subunit [β2(Y205S)] in combination with α1 and γ2L results in cells that have large responses to pentobarbital (as expected) but also show appreciable responses to GABA (contrary to expectation). Furthermore, transfection of HEK 293 cells with α1 plus γ2L subunits results in responses to GABA that are potentiated by the drug loreclezole, suggesting that a subunit resembling the β2 or β3 subunit had assembled with the α1γ2L subunits. In addition, some nontransfected HEK 293 cells respond to applications of GABA, and transfection of cells with α1, β1, or γ2L subunits alone can result in the expression of GABA-elicited currents. In comparison, when QT6 quail fibroblasts are used as the expression system, no responses were seen in untransfected cells or in cells transfected with α1, β1, or γ2L subunits alone or α1γ2L subunits. Furthermore, no response to GABA was seen in QT6 cells transfected with α1β2(Y205S)γ2L subunits, although cells gave strong responses to pentobarbital. These observations indicate that caution must be taken in interpreting the results of studies of the properties of GABA$_A$ receptors expressed in HEK 293 cells if the exogenous subunits result in the expression of low levels of functional GABA$_A$ receptors.

The action of the neurotransmitter GABA underlies most rapid inhibitory synaptic transmission in the brain as a result of activation of the A-type receptor-gated chloride channel (the GABA$_A$ receptor). The GABA$_A$ receptor is a multimeric protein, and molecular cloning studies have isolated and characterized a number of subunits (for a review, see Ref. 1). Studies of receptor subunits expressed in non-neural cells have shown that the physiological and pharmacological properties of the GABA$_A$ receptor are determined by the subunit composition (2, 3). One expression system that has been used extensively is the human embryonic kidney cell line HEK 293 (4). This line can express exogenous receptor subunits efficiently and is very good for electrophysiological studies.

We have been examining the properties of receptors formed from several types of GABA$_A$ receptor subunits, including the α1, β1, β2, γ2L, and mutated β2 subunits. To our surprise, the data indicated that the properties of the GABA-elicited responses differed in some cases from the properties predicted from previous results. In light of these observations, we tested a second somatic cell expression system, quail QT6 fibroblasts, and found that in this system, there is no indication of these discrepancies. The observations indicate that HEK 293 cells may express functional GABA$_A$ receptors that do not include all of the transfected subunits.

## Materials and Methods

All chemicals were obtained from Sigma Chemical (St. Louis, MO) unless otherwise specified. Loreclezole was provided by Janssen Pharmaceuticals (Antwerp, Belgium).

cDNA constructs for GABA receptor subunits were provided by A. Tobin, University of California Los Angeles (rat α1 and rat α6), D. Weiss, University of Alabama at Birmingham [human β1, rat β2, mutated rat β2(Y205S) and rat β2(Y157S), rat γ2L, and human ρ1], and G. White, Neurogen, Branford, CT (human β3). GABA receptor subunit cDNAs were transferred to the eukaryotic expression vector

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**ABBREVIATION:** GABA, γ-aminobutyric acid; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

pcDNA3 (InVitrogen, San Diego, CA) for expression in HEK 293 and QT6 cells. A cDNA construct for CD8 was provided by Dr. B. Seed (Massachusetts General Hospital, Boston, MA) in the eukaryotic expression vector piH3. Direct sequencing of the mutated β2 subunits confirmed that the constructs contained the appropriate base changes (Sequenase kit version 2, Amersham, Arlington Heights, IL).

cDNA constructs were introduced into HEK 293 cells (American Type Culture Collection, Rockville, MD) by electroporation (Gene Pulser, BioRad, Hercules, CA). HEK 293 cells were maintained in a mixture of Dulbecco's modified Eagle's medium (plus 5 mM HEPES) and Ham's F12 (1:1) with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% $CO_2$. HEK 293 cells were passaged twice a week and were used for 8–16 passages. Care was taken to passage cells before they reached confluence. For electroporation, cells were plated at ~$10^6$ cells/10-cm plate, the day before electroporation. They were dissociated and resuspended at $10^6$ cells/ml in a calcium/magnesium-free saline (139 mM NaCl, 2.7 mM KCl 2.7, 8.1 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$, pH 7.4). Cell suspension (0.7 ml) was mixed with cDNA (20 μg of each subunit plus, if necessary, 1 μg of CD8 cDNA), placed into a 0.4-cm gap electroporation cuvette, and electroporated at settings of 290 V and 250 μF (time constant, ~3.5 msec). The cell suspension was removed, added to 8 ml of growth medium, and plated onto 35-mm dishes for electrophysiology. In a few experiments, HEK 293 cells were transfected according to the calcium phosphate method of Chen and Okayama (5).

Quail fibroblasts (QT6 cells; initially provided by Dr. J. Merlie, Washington University, St. Louis, MO) were maintained in Medium 199 (Earle's salts) containing 5% fetal bovine serum (Hyclone), 10% tryptose phosphate broth (GIBCO, Grand Island, NY), 1% DMSO, and 100 units/ml penicillin plus 100 μg/ml streptomycin in a humidified atmosphere containing 5% $CO_2$. QT6 cells were passaged twice a week and were used for 8–16 passages. Care was taken to passage cells before they reached confluence. Calcium phosphate precipitation was used to transfect QT6 cells according to the method of Chen and Okayama (5), with the additional step of an initial wash to remove tryptose phosphate broth (6).

In some cases (see Results), cells were plated in medium with 5 mM sodium butyrate added in an effort to enhance expression of transfected cDNAs (7, 8).

Recordings were made using standard whole-cell methods (9) at 24–72 hr after transfection. All experiments were performed at room temperature (21–23°), and drugs were dissolved in external solution. In all cases, data were obtained from isolated single cells. Experiments were performed in two laboratories with identical results. In the Steinbach laboratory (10), drugs were applied with a polyethylene Y-tube. The bath was continuously perfused with normal external solution from a separate perfusion line, and solution was removed from the bath with a Leiden aspirator (Medical Systems, Greenvale, NY). In the Zorumski laboratory (11), drugs were applied by pressure ejection from "puffer" pipettes positioned within 5 μm of the clamped cell, using a 500-msec pulse of air pressure (10–20 p.s.i.) to the back of the pipette.

To allow comparison of the GABA-elicited responses (see Results), currents were converted to conductances assuming a linear current-voltage relationship. Conductances were then normalized by the cell input capacitance measured from the settings on the amplifier compensation circuitry. The maximal capacitance setting was 100 pF, and some of the largest cells could not be fully compensated. In this case, the input capacitance was arbitrarily set at 100 pF. The cell size can be estimated from the capacitance, assuming a specific membrane capacitance of 1 μF/cm² (12). A typical HEK 293 cell had an input capacitance of ~40 pF, corresponding to a surface area of ~4000 μm² or a hemisphere of radius of 21 μm. The largest HEK 293 cells (used to maximize the surface area and increase the size of recorded currents) have a calculated radius of ~33 μm as a hemisphere and correspond to the occasional large cells seen in cultures (perhaps 1% of the total cells).

The fraction of responsive cells was estimated based on the observed fraction of responding cells, and 95% confidence intervals on the fraction were taken from Beyer (13, Table 3.5). Comparisons of the fractions of responsive cells were made with the $\chi^2$ test. Comparisons of the responsiveness of cells in various groups were made with the Wilcoxon rank sum test.

Cells that expressed a high level of protein from exogenous cDNA were identified using the bead-labeling technique described by Jurman *et al.* (14). In some cases, cDNA coding for CD8 was coexpressed with GABA subunits, and cells expressing high levels of CD8 were identified with beads that had covalently coupled antibody to CD8 (Dynabeads, Dynal, Lake Success, NY). An alternative approach was also used to identify cells by engineering an epitope tag into the amino-terminal region of the α1 subunit. An oligonucleotide coding for the FLAG epitope (15) was inserted into an *Afl*II restriction enzyme site in codon 34 of the translated sequence (the ninth amino acid residue in the mature protein). The sequence of the insert used was TT AAG GAT TAC AAG GAC GAC GAT GAC AAG CTT AA. The nucleotide sequence of the modified sequence was confirmed by sequencing (Sequenase kit version 2), and the new construct will be referred to as α1F. The predicted sequence of the resulting peptide is, starting with the amino terminus of the predicted mature peptide, YGQPSQDELKDYKDDDDKLKDNTT, in which the introduced residues are underlined. The particular site that was used resulted in a duplication of the LK residues at the end of the FLAG insert. This construct could be identified on the surface of intact cells using a mouse monoclonal antibody to the FLAG epitope (M2, Eastman Kodak Scientific Imaging Systems, New Haven, CT), which had been adsorbed to beads with covalently attached goat anti-mouse IgG antibody (Dynal).

## Results

**Expression of subunits and selection of cells by bead labeling.** To identify cells for physiological experiments, we used a surface marker for cells that expressed membrane proteins coded by transfected nucleic acid sequences. Marker expression on the surface was detected by the binding of small beads coated with monoclonal antibody to the marker (14). One marker was a surface protein, CD8. We also used an α1 subunit of the $GABA_A$ receptor with a FLAG epitope engineered into the amino-terminal region (termed α1F; see Materials and Methods). Control experiments indicated that similar amplitude responses were seen after transfection with α1β2γ2L subunits (cells selected by beads coated with antibody to CD8) or α1Fβ2γ2L subunits (cells selected by beads coated with antibody to FLAG) (Table 1). There also was no difference in the GABA concentration-response curves; when fit with the Hill equation, the $EC_{50}$ values were 13 ± 12 μM for α1β2γ2L subunits and 16 ± 9 μM for α1Fβ2γ2L subunits, with Hill coefficients of 1.3 ± 0.2 in both cases (mean ± standard deviation for data from five cells with each subunit combination). These values were obtained using Y-tube applications and agree well with the data shown in Fig. 2 obtained with puffer applications and the α1Fβ2γ2L subunit combination.

Bead binding was used only as a qualitative assay because the number of bound beads may depend on many factors, such as the number and distribution of binding sites on the surface. Cells were chosen that had three or more bound beads, and no correlation between responsiveness and number of beads was seen over this range. With the control trimeric subunit combinations (e.g., α1Fβ2γ2L), ~5-15% of

TABLE 1

**Normalized responses to 100 μM GABA of HEK 293 cells**

Results were obtained from single, isolated cells. GABA (100 μM) was used as a test dose because it gave maximal or close-to-maximal responses for many of the subunit combinations tested (see Results). The subunit combinations tested are listed in the first column; the row headed "Pooled" gives overall values if no significant difference was found for cells in different conditions that had been transfected with that subunit combination. The responses are given in units of conductance divided by membrane capacitance (pS/pF) to account for effects of holding potential and cell size (see Methods). Cells were identified using the cotransfected marker CD8 or epitope-tagged α1F subunit (column headed "tag"), except for "untransfected" cells. In this case, randomly chosen large cells were studied. "Mock-transfected" cells were electroporated in the presence of the expression construct for CD8 but no $GABA_A$ receptor subunits, and cells were selected that bound beads coated with antibody to CD8. In some cases, cultures had been treated with 5 mM sodium butyrate to enhance expression (column headed "butyrate"; see Methods). The median and range of the normalized response are given for the number of cells tested. The fraction of responsive cells is also presented, with the 95% confidence limits for the fraction. The limit of detection for responses was ~10 pA (see Fig. 1); the corresponding normalized conductance depends on both holding potential and cell size.

| HEK 293 cells | Butyrate | Tag | Response median | Response range | Responding/tested | Fraction responding | 95% Limits on fraction |
|---|---|---|---|---|---|---|---|
| | | | | *pS/pF* | | | |
| α1β2γ2L | No | CD8 | 1549 | 55–2728 | 5/5 | 1.00 | 0.48, 1.00 |
| α1Fβ2γ2L | No | Flag | 1267 | 229–6842 | 10/10 | 1.00 | 0.69, 1.00 |
| Pooled | No | | 1299 | 55–6842 | 15/15 | 1.00 | 0.78, 1.00 |
| α1Fβ2(Y157S)γ2L | No | Flag | 60 | 57–312 | 3/3 | 1.00 | 0.29, 1.00 |
| α1Fβ2(Y205S)γ2L | No | Flag | 7 | 3–344 | 3/3 | 1.00 | 0.29, 1.00 |
| α1Fγ2L | No | Flag | 382 | 35–1273 | 3/3 | 1.00 | 0.29, 1.00 |
| α1F | Yes | Flag | 257 | 21–527 | 4/4 | 1.00 | 0.40, 1.00 |
| γ2L | Yes | CD8 | 152 | 14–183 | 5/5 | 1.00 | 0.48, 1.00 |
| β2 | No | CD8 | 0 | 0–7 | 1/6 | 0.17 | 0.00, 0.64 |
| β2 | Yes | CD8 | 46 | 11–81 | 5/5 | 1.00 | 0.48, 1.00 |
| β1 | No | CD8 | 0 | 0–19 | 2/9 | 0.22 | 0.03, 0.60 |
| β1 | Yes | CD8 | 0 | 0–91 | 9/22 | 0.41 | 0.21, 0.64 |
| Pooled | | CD8 | 0 | 0–91 | 11/31 | 0.35 | 0.19, 0.55 |
| Untransfected | No | None | 0 | 0–21 | 6/18 | 0.33 | 0.14, 0.59 |
| Mock transfected | No | CD8 | 0 | 0–10 | 2/7 | 0.29 | 0.06, 0.71 |
| Pooled | No | | 0 | 0–21 | 8/25 | 0.32 | 0.14, 0.54 |
| Mock transfected | Yes | CD8 | 8 | 0–59 | 9/12 | 0.75 | 0.50, 0.95 |

cells in the culture had beads bound, and labeled cells gave robust responses. However, it was qualitatively apparent that some combinations of subunits [e.g., α1Fγ2L or α1Fβ2(Y205S)γ2L] resulted in fewer cells with beads bound (<1%) as well as lower levels of responses on cells with beads bound (see below). Bead-labeled cells were normal in appearance aside from the presence of beads.

The overall efficacy of bead selection was estimated from a comparison of the frequencies of responses in cells transfected with subunit combinations that gave reliable responses (ρ1, α1β2γ2L, and α6β2γ2L). Several GABA concentrations were used in the tests, so the sizes of the responses are not comparable. However, the selection method could be scored based simply on the presence or absence of a response. For randomly selected cells, 22 of 50 cells responded, giving a fraction responsive of 0.44 (95% confidence limits, 0.30, 0.59). This fraction is larger than the fraction of cells labeled with beads coated with antibody to the FLAG epitope, suggesting that bead binding selects for cells with a higher-than-average level of expression. When CD8 was used as a cotransfected expression marker, 74 of 83 cells responded, giving a fraction responsive of 0.89 (0.82, 0.95). The fraction of responsive cells differed from randomly selected cells at a level of $p < 0.005$ ($\chi^2$ test). When α1F subunit was used as a marker, 63 of 65 cells responded, giving a fraction responsive of 0.97 (0.89, 1.00). The fraction responsive differed from results with the CD8 marker at a level of $p < 0.05$ and from randomly selected cells at a level of $p < 0.005$.

**An example of unexpected properties of transfected $GABA_A$ receptor subunits in HEK 293 cells.** We were interested in defining ligand-binding sites on the $GABA_A$ receptor. In initial experiments, we transfected HEK 293 cells with subunits for three $GABA_A$ receptor subunits: α1F (the epitope-tagged α1 subunit described in Materials and Methods), γ2L, and one of two β2 subunits. We used wild-type β2 and the point mutant β2(Y205S) (16). The mutated subunit has been shown to produce $GABA_A$ receptors that show no response to GABA at concentrations of ≤20 mM but essentially normal gating by pentobarbital after expression in *Xenopus laevis* oocytes (16).

In each case, cells expressing a high level of transfected α1F subunit were selected by the binding of beads that had been coated with antibody to the FLAG epitope. Expression of wild-type subunits resulted in receptors that were strongly activated by GABA (Table 1 and Fig. 1A) or pentobarbital (not shown). However, expression of the α1Fβ2(Y205S)γ2L subunits also resulted in currents that could be activated by GABA (Table 1 and Fig. 1B) as well as pentobarbital (Fig. 1B). The concentration-response relationship for GABA activation was determined for responses from cells transfected with α1Fβ2γ2L or α1Fβ2(Y205S)γ2L subunits (Fig. 2) and showed that the $EC_{50}$ was shifted by only ~10-fold in the mutant rather than the expected factor of >1000-fold (16). The median response elicited from cells transfected with α1Fβ2(Y205S)γ2L subunits was significantly less than that for cells transfected with α1Fβ2γ2L subunits ($p < 0.05$, Wilcoxon rank sum test). However, responses were significantly larger than those for untransfected or mock-transfected HEK 293 cells (see below and Table 1; $p < 0.05$ compared with pooled cells not treated with butyrate).

Transfection of HEK 293 cells with α1Fβ2(Y157S)γ2L subunits also resulted in greater responsiveness to GABA than

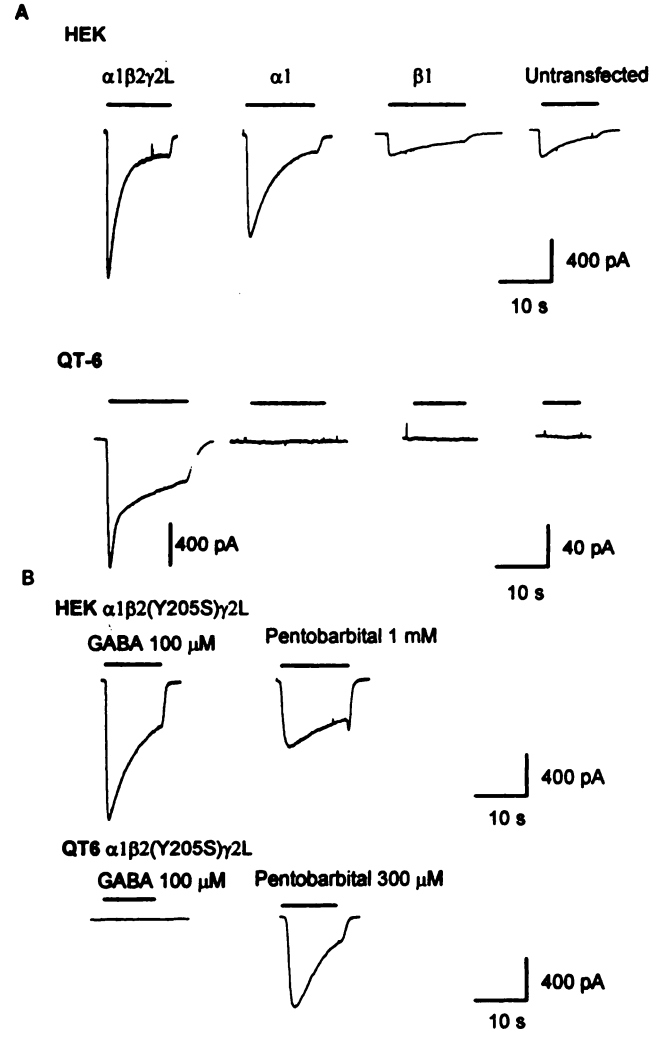


**Fig. 1.** Responses of cells to 100 μM GABA. A, Representative current traces for HEK 293 (*top*) and QT6 (*bottom*) cells transfected with the subunit combinations indicated. *Scale bars on right of top row* (HEK 293 cells) apply to all responses in the *top row*. Note that the current scale differs for the first response in the second row (QT6); *vertical scale on right of second row* applies to the other responses from QT6 cells. B, Responses of a HEK 293 cell (*top*) and a QT6 cell (*bottom*) transfected with the α1Fβ2(Y205S)γ2L subunit combination. The same cell was tested first with an application of 100 μM GABA and then with 1000 or 300 μM pentobarbital. Cells were voltage-clamped to −70 mV, and agonists were applied by a Y-tube (*bars above each record*). Data were obtained from HEK 293 cells transfected by electroporation.

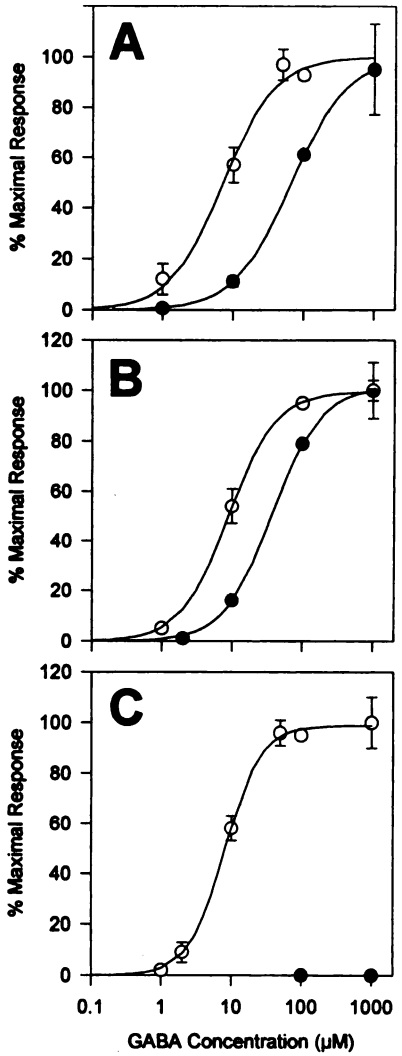


**Fig. 2.** GABA concentration-response curves for cells transfected with wild-type or mutated β2 subunits. HEK 293 cells (A and B) or QT6 cells (C) were transfected with various subunits for $GABA_A$ receptors, and the concentration-response curves for GABA-elicited currents were determined. A, Results obtained from HEK 293 cells transfected with α1Fβ2γ2L subunits (○; mean ± standard error for data from four to six cells at various concentrations) or α1Fβ2(Y205S)γ2L subunits (●; data from four to nine cells). When the Hill equation was fit to the data shown, the $EC_{50}$ values were 7 μM for α1Fβ2γ2L and 67 μM for α1Fβ2(Y205S)γ2L, and Hill coefficients were 1.2 and 1.1, respectively (predictions shown by *lines*). B, Data obtained from HEK 293 cells transfected with α1Fγ2L subunits (●; data from three or four cells), and α1Fβ3γ2L (○; data from three or four cells) subunits. The concentration-response curve is shifted to higher GABA concentrations for cells transfected with only α1Fγ2L subunits compared with either α1Fβ2γ2L (A) or α1Fβ3γ2L subunits, although to a lesser extent than that for α1Fβ2(Y205S)γ2L subunits (A). When the Hill equation was fit to the data shown, the $EC_{50}$ values were 37 μM for α1Fγ2L and 9 μM for α1Fβ3γ2L, and Hill coefficients were 1.3 in each case. C, Data from QT6 cells transfected with α1Fβ2γ2L (○; data from three to five cells). When fit with the Hill equation, the GABA concentration-response curve had a similar $EC_{50}$ to data obtained from HEK 293 cells (8 μM), but the Hill coefficient was somewhat larger (1.7). No responses were obtained from QT6 cells transfected with α1Fβ2(Y205S)γ2L to either 100 μM or 1000 μM GABA (●). Data were acquired by applying GABA from matched "puffer" pipettes to a single cell. The response to a given concentration of GABA was then normalized to the response of the same cell to 100 μM GABA, and the mean normalized response was calculated for each concentration applied. Data were obtained from HEK 293 cells transfected by electroporation. Data for responsiveness are presented in Table 4.

expected (Table 1; compare with Ref. 16). Again, the median response was less than that for cells transfected with α1Fβ2γ2L subunits ($p < 0.05$) and larger than that for untransfected or mock-transfected HEK 293 cells ($p < 0.01$).

These observations suggested that $GABA_A$ receptors expressed by HEK 293 might have properties that differed significantly from those expected based on previous studies of the transfected subunits.

**Responses of untransfected or mock-transfected HEK 293 cells.** It has previously been reported that untransfected HEK 293 cells did not respond to applied GABA (4). However, we found that some untransfected cells did respond to applied GABA (Table 1 and Fig. 1A). The responses were small and were detected in fewer than half of cells tested (Table 1). We obtained responses from cells of

normal size and shape, but to increase the size of the evoked currents, we often selected large, flat cells for study. These large cells make up <1% of the cells in our low density cultures.

Responses did not depend on transfection because cells that were not electroporated ("untransfected") or that were transfected with CD8 (and selected by bead binding) ("mock-transfected") could give responses (Table 1). The median responses did not differ between these two groups ($p > 0.9$, Wilcoxon test). Therefore, results obtained from untransfected and mock-transfected cells in the absence of butyrate were pooled for further comparisons. Treatment with sodium butyrate increased the size of responses from mock-transfected cells ($p < 0.01$, compared with cells not treated with butyrate). However, the frequencies of responsive cells did not differ significantly for butyrate-treated cells ($p > 0.1$, $\chi^2$ test).

Because the responses were so small, we did not undertake a study of the pharmacology of the responses. However, pentobarbital (1 mM) could also elicit a conductance increase in untransfected cells that responded to GABA.

**Responses of HEK 293 cells after transfection of single $GABA_A$ receptor subunits.** We found small responses to GABA after transfection of HEK 293 cells with human β1 (Fig. 1A and Table 1). To enhance the probability of obtaining responses, we recorded from cells that had expressed transfected cDNAs by using a cotransfected marker, CD8 (see Materials and Methods), and by recording from large cells. In some cases, we treated cells with 5 mM sodium butyrate after transfection to enhance expression (see Materials and Methods and Table 1). When we examined the pharmacology of the responses recorded from cells transfected with β1 alone, we determined an $EC_{50}$ value for GABA activation of ~10 μM (10 ± 4 μM; mean ± standard deviation for eight cells). Furthermore, we found that 10 μM bicuculline reduced the responses to 10 μM GABA to 10% of control (10 ± 10%, five cells). Picrotoxin (10 μM) effectively blocked the responses elicited by GABA but had no detectable effect on the holding current, suggesting that activity of $GABA_A$ receptors was low in the absence of applied GABA. These results differed from previous observations on homomeric β1 receptors expressed in *X. laevis* oocytes (17–19). Expression of β1 subunits from rat (17) or mouse (19) brain results in expression of a membrane conductance that is not activated by GABA but seems to result from the presence of a constantly open $GABA_A$ receptor channel because the conductance can be blocked by picrotoxin. On the other hand, expression of β1 subunits from human brain (18) results in a GABA-activated conductance with an $EC_{50}$ value for activation by GABA of ~120 μM. However, bicuculline was ineffective at blocking GABA-elicited currents, and picrotoxin blocked part of the holding current (suggesting the existence of channels open in the absence of GABA) (18). Our observations differed from either of these reports. The differences might arise from the expression system or the exact subunit sequence we used, but the discrepancies suggested to us that the responses we saw might not arise from homomeric human β1 receptors. The median responses and frequencies of responses did not differ between cells transfected with β1 subunits and the appropriate mock-transfected control cell group ($p > 0.1$). Transfections with rat β2 subunit (plus CD8) also gave small responses to GABA (Table 1).

We then transfected cells with the α1 subunit or the γ2L subunit with the expectation that transfected cells would show no response to GABA. Cells expressing a high level of transfected gene products were selected with an antibody against CD8 (see Materials and Methods). In both cases, the cultures were treated with sodium butyrate, and all of the cells tested gave responses to applied GABA (Table 1 and see Fig. 1A), although the responses were only 10–20% of the level seen when α1β2γ2L subunits were transfected (Table 1). However, the median responses were significantly larger than those for mock-transfected cells or cells transfected with β1 subunit alone (treated with butyrate; $p < 0.05$), although they did not differ significantly from responses of cells transfected with β2 subunit alone (treated with butyrate; $p > 0.1$).

**Potentiation by loreclezole of responses of HEK 293 cells transfected with α1 plus γ2L subunits.** Taken together, the observations obtained with α1β2(Y205S)γ2L subunits and with α1 or γ2L subunits alone suggested that HEK 293 cells might express an endogenous β subunit that could assemble with α or γ subunits and potentially replace a β subunit. The drug loreclezole provides a possible pharmacological marker in that the action of loreclezole has been associated specifically with a residue in the β2 and β3 subunits (20, 21). To examine this possibility, we transfected cells with α1F plus γ2L subunits and determined whether loreclezole potentiated the GABA-elicited currents. We found that HEK 293 cells transfected with α1Fγ2L subunits responded to applied GABA (Table 1; cultures were not treated with sodium butyrate). The median response was not significantly different from that for cells transfected with α1Fβ2γ2L subunits ($p > 0.1$), but it was significantly larger than that after transfections with β1 or β2 subunits alone ($p < 0.05$ for each comparison). Furthermore, the GABA responses were strongly potentiated by loreclezole (Table 2). This suggests that a β2- or β3-like subunit had assembled with the transfected subunits. However, we found that the GABA concentration-response curve for cells transfected with α1Fγ2L subunits differed from that for cells transfected with the other three subunit combinations we tested (Fig. 2). The $EC_{50}$ values were 7 μM for cells transfected with α1Fβ2γ2L, 9 μM for cells transfected with α1Fβ3γ2L, and 37 μM for α1Fγ2L with no transfected β subunit.

**Comparison with quail QT6 fibroblasts.** We performed a number of transfections of QT6 cells, a second somatic cell

TABLE 2

**Loreclezole potentiates responses to GABA**

HEK 293 cells were transfected with α1Fβ2γ2L subunits or α1Fγ2L subunits, and the responses to GABA and GABA plus loreclezole were measured. The first column lists the subunit combinations transfected and the GABA and loreclezole concentrations that were applied. The relative response gives the ratio of the response to GABA plus loreclezole to the response of that cell to GABA alone (mean ± standard deviation for *n* cells tested). In all cases, cells were identified by the binding of beads coated with antibody to the FLAG epitope, and cultures were not treated with sodium butyrate. Experiments with α1Fγ2L subunits using 3 μM GABA were performed with the Y-tube; all other experiments were performed using matched "puffer" pipettes.

| Subunit transfected | Loreclezole | Relative response | *n* |
|---|---|---|---|
| | μM | | |
| α1Fβ2γ2L (2 μM GABA) | 1 | 1.80 ± 0.27 | 5 |
| | 10 | 3.64 ± 0.77 | 5 |
| α1Fγ2L (10 μM GABA) | 1 | 1.60 ± 0.34 | 8 |
| | 10 | 3.76 ± 1.12 | 8 |
| α1Fγ2L (3 μM GABA) | 1 | 3.00 ± 0.44 | 4 |

TABLE 3

**Normalized responses to 100 μM GABA of QT6 cells**

Data are presented as described in the legend to Table 1.

| QT6 CELLS | Butyrate | Tag | Response median | Response range | Responding/ tested | Fraction responding | 95% Limits on fraction |
|---|---|---|---|---|---|---|---|
| | | | | *pS/pF* | | | |
| α1Fβ2γ2L | No | Flag | 1787 | 1034–2408 | 7/7 | 1.00 | 0.59, 1.00 |
| α1Fβ2γ2L | Yes | Flag | 1169 | 810–1528 | 2/2 | 1.00 | 0.16, 1.00 |
| Pooled | | Flag | 1536 | 810–2408 | 9/9 | 1.00 | 0.64, 1.00 |
| α1Fβ2(Y157S)γ2L | No | Flag | 3 | 0–15 | 4/6 | 0.67 | 0.22, 0.96 |
| α1Fβ2(Y157S)γ2L | Yes | Flag | 0 | 0–0 | 0/2 | 0.00 | 0.00, 0.84 |
| Pooled | | Flag | 1 | 0–15 | 4/8 | 0.50 | 0.16, 0.84 |
| α1Fβ2(Y205S)γ2L | No | Flag | 0 | 0–0 | 0/2 | 0.00 | 0.00, 0.84 |
| α1Fβ2(Y205S)γ2L | Yes | Flag | 0 | 0–0 | 0/6 | 0.00 | 0.00, 0.46 |
| Pooled | | Flag | 0 | 0–0 | 0/8 | 0.00 | 0.00, 0.37 |
| α1Fγ2L | No | CD8 | 0 | 0–0 | 0/3 | 0.00 | 0.00, 0.71 |
| α1Fγ2L | Yes | CD8 | 0 | 0–0 | 0/2 | 0.00 | 0.00, 0.84 |
| Pooled | | CD8 | 0 | 0–0 | 0/5 | 0.00 | 0.00, 0.52 |
| β1 | No | CD8 | 0 | 0–0 | 0/6 | 0.00 | 0.00, 0.46 |
| β1 | Yes | CD8 | 0 | 0–0 | 0/8 | 0.00 | 0.00, 0.37 |
| Pooled | | CD8 | 0 | 0–0 | 0/14 | 0.00 | 0.00, 0.22 |
| Untransfected | No | None | 0 | 0–0 | 0/4 | 0.00 | 0.00, 0.60 |
| Untransfected | Yes | None | 0 | 0–0 | 0/3 | 0.00 | 0.00, 0.71 |
| Pooled | | None | 0 | 0–0 | 0/7 | 0.00 | 0.00, 0.41 |

expression system in use in our laboratory. The goal was to determine whether the unexpected observations we had made with HEK 293 cells were more likely to result from the cDNAs we had used or the particular expression system we were using. Transfection with α1Fβ2γ2L subunits resulted in robust responses to applied GABA (Table 3 and Fig. 1A). Untransfected QT6 cells showed no response to GABA (Table 3 and Fig. 1A). When only α1F subunit was transfected, there was no binding of beads coated with antibodies to FLAG, and two randomly selected cells showed no response to GABA (Fig. 1A). QT6 cells were then transfected with α1F, γ2L, and CD8. There was no binding of beads coated with antibodies to FLAG, so cells were selected that bound beads coated with antibody to CD8; again, there was no response to GABA (Table 3). When β1 subunit and CD8 were cotransfected, cells that bound beads coated with antibody to CD8 showed no responses to GABA (Table 3). Finally, QT6 cells transfected with α1Fβ2(Y205S)γ2L showed large responses to pentobarbital (Fig. 1B) but no responses to 100 μM GABA (Table 3 and Fig. 1B). Treatment with butyrate had no effect of responses from QT6 cells (Table 3).

To determine whether the precise transfection method had any effect, we also transfected HEK 293 cells with α1Fβ2γ2L, α1Fβ2(Y205S)γ2L, and α1Fγ2L subunit combinations using the calcium phosphate method. No differences in the pattern of responses were seen for HEK 293 cells transfected using electroporation or calcium phosphate methods (Table 4).

## Discussion

Our observations indicate that some untransfected HEK 293 cells respond to GABA, although at a very low level. This suggests that there is endogenous expression of some $GABA_A$ receptor subunit or subunits in HEK 293 cells, which might be available to assemble with the products of exogenous cDNAs. The observation that HEK 293 cells transfected with α1β2(Y205S)γ2L subunits respond well to GABA suggests that in some cells the majority of the functional $GABA_A$ receptors do not include the transfected β2(Y205S) subunit. The possible assembly of an endogenous β subunit is suggested by the observation that transfection with the α1Fγ2L subunit combination results in expression on the surface of the epitope-tagged α1 subunit and responses to GABA.

The possibility that an endogenous β subunit might assemble with transfected subunits is supported by the results obtained with loreclezole. It has previously been reported that the ability of loreclezole to potentiate GABA-elicited currents is is large part due to the particular β subunit present (21). In particular, the β2 and β3 subunits have an asparagine at residue 289 or 290, respectively, and confer a high sensitivity to potentiation by loreclezole, whereas the β1 subunit has a serine at the homologous position and results in low sensitivity (20). Point mutation of the β1 subunit to convert this serine residue to asparagine produces a shift in the sensitivity to loreclezole to a level close to that conferred by the β2 subunit (20). Therefore, the observed potentiation by loreclezole when HEK 293 cells are transfected with α1Fγ2L subunits suggests that HEK 293 cells express a subunit similar to a β2 or β3 subunit. However, we found that GABA concentration-response curves differed when cells were transfected with α1Fβ2γ2L or α1Fβ3γ2L subunits compared with α1Fγ2L subunits alone. We do not have an explanation for this observation; two possible alternatives are that HEK 293 cells express a β subunit that differs from the human β3 or rat β2 subunit clones we used or that the GABA concentration-response curve might depend on the subunit stoichiometry in the assembled $GABA_A$ receptor.

We conclude that the results obtained with HEK 293 cells are a result of the expression system because we have used the same constructs for transfections of the quail fibroblast line QT6. In this case, untransfected cells or cells transfected with α1, γ2L, or α1γ2L subunits do not respond to GABA. Transfection of QT6 cells with α1β2(Y205S)γ2L subunits produces receptors that respond well to pentobarbital but show no detectable response to GABA. The method of transfection

TABLE 4

**Comparisons of HEK 293 cells transfected by electroporation or calcium phosphate precipitation**

Data are presented as described in the legend to Table 1. All of these data were obtained using 100 μM GABA applied by a "puffer" pipette. There are no significant differences in the median responses for a given subunit combination to the data obtained using Y-tube applications of GABA to cells expressing that combination, for cells transfected by electroporation, or calcium phosphate precipitation (see Table 1; $p > 0.05$ for all comparisons). However, in a comparison of data obtained with puffer pipettes, cells transfected using the calcium phosphate precipitation method gave significantly larger responses for the $\alpha1F\beta2\gamma2L$ subunits ($p < 0.01$) and the $\alpha1F\gamma2L$ subunits ($p < 0.05$). No significant differences were seen in the frequencies of responsive cells. All cells were identified with beads coated with antibody to the FLAG epitope. No cultures were treated with butyrate.

| HEK 293 CELLS | Response median | Response range | Responding/ tested | Fraction responding | 95% Limits on fraction |
|---|---|---|---|---|---|
| | | *pS/pF* | | | |
| Electroporation | | | | | |
| $\alpha1F\beta2\gamma2L$ | 550 | 19–1353 | 6/6 | 1.00 | 0.54, 1.00 |
| $\alpha1F\beta2(Y205S)\gamma2L$ | 96 | 11–476 | 9/9 | 1.00 | 0.64, 1.00 |
| $\alpha1F\gamma2L$ | 97 | 62–128 | 3/3 | 1.00 | 0.29, 1.00 |
| Calcium phosphate | | | | | |
| $\alpha1F\beta2\gamma2L$ | 3353 | 1965–3992 | 4/4 | 1.00 | 0.40, 1.00 |
| $\alpha1F\beta2(Y205S)\gamma2L$ | 145 | 0–1388 | 5/6 | 0.83 | 0.36, 1.00 |
| $\alpha1F\gamma2L$ | 478 | 105–1330 | 8/8 | 1.00 | 0.63, 1.00 |

apparently has no qualitative effect on the difference between HEK 293 and QT6 cells because the calcium phosphate method also resulted in GABA responses from HEK 293 cells transfected with $\alpha1\beta2(Y205S)\gamma2L$ or $\alpha1\gamma2L$ subunits.

Assembly of functional $GABA_A$ receptors has been reported to occur in a number of expression systems, but the minimal subunit requirements are not clear. In *X. laevis* oocytes, there is general agreement that after injection of cDNAs for α subunits alone, γ subunits alone, or α plus γ subunits, there is poor expression of functional surface receptors, if any (2, 17, 18, 22, 23, but see 24). Similarly, injection of most β subunits also results in low expression, with the exception of the β1 subunit, which apparently forms a homomeric receptor with unusual properties (2, 17–19). Mouse L cells have also been studied, and transfection with $\alpha1\gamma2$, $\beta1\gamma2$, or δ subunits does not result in detectable currents (25, 26), suggesting that robust expression of functional receptors needs transfection with both an α and a β subunit.

HEK 293 cells have been used most extensively in studies of $GABA_A$ subunit expression and have provided rather contradictory results. In records from small groups of electrically coupled cells, transfections of cells with single cDNAs for α1, β1, β2, β3, γ2, or δ subunits have been reported to generate weak responses to GABA (3, 4, 27–29). These observations agree with our present findings, although other studies have reported no response after transfection with single subunits (30) or with the $\beta1\gamma2$ subunit combination (31).

Previous studies of untransfected HEK 293 cells have reported that the cells produced no responses to applications of 10 μM GABA (4). The current data indicate that ~30% of untransfected HEK 293 cells will generate a small response to 100 μM GABA, although the response was <1% of the response after transfection with the $\alpha1\beta2\gamma2L$ subunit combination. One difference between the observations is that we used a higher concentration of GABA to test responsiveness. We also often selected large cells for testing, to increase the available surface area of the cells and so increase the size of the response. However, responses to 100 μM GABA were seen in cells of normal appearance, and we have no evidence that cells of a particular appearance are more likely to express an endogenous response. It did appear, however, that treatment of cultures with 5 mM sodium butyrate enhanced the size of endogenous responses to GABA, perhaps by increasing expression or assembly of endogenous subunits. Electroporation alone (mock transfection with CD8) did not affect the frequency or median amplitude of responses.

There have been conflicting reports on the existence, in untransfected HEK 293 cells, of mRNA that hybridizes to probes for $GABA_A$ receptor subunit sequences. Early studies found no evidence for sequences hybridizing to probes for α1, β1, or γ2 subunits (4, 32, 33), but two recent studies have reported low levels of mRNA for α1, β3, and γ2 subunits (34, 35). However, in one study, no evidence was found to indicate that endogenous subunits were actually expressed by untransfected HEK 293 cells (29). In a recent elegant study, the expression of epitope-tagged mouse brain $GABA_A$ receptor subunits was examined in HEK 293 cells (36). Using immunofluorescence of cells 18 hr after transfection, epitopes were confined to the endoplasmic reticulum when cells were transfected with the α1, β2, or γ2L subunits alone (36). These observations clearly support the idea that surface expression is low after transfection with these single subunits, as is also indicated by the small responses observed in the current study. Fluorescence was also not observed on intact (nonpermeabilized) cells after transfection with the $\alpha1\gamma2L$ subunit combination, suggesting that surface expression was low for this pair as well (36). However, the responses from bead-selected cells in the current study were relatively strong after transfection of these subunits into HEK 293 cells. We do not have an explanation for these differences. It does not result from differences in transfection protocol because we obtained similar results with electroporation or calcium phosphate methods, and HEK 293 cells were obtained from the American Type Culture Collection for both studies. There are three possibly significant differences in procedures. One is that Connolly *et al.* (36) used subunits from mouse brain. We also usually waited for 48 to 72 hr after transfection before recording, perhaps allowing for increased transport from internal compartments to the surface. Finally, the two methods of assay are both qualitatively and quantitatively different. The level of surface fluorescence is not given quantitatively by Connolly *et al.* (36), so it is not clear what level would be defined as null expression. Probably of more importance, however, is that our use of bead binding selected for cells that were expressing surface receptors. Therefore, a reduced level of responses, even in selected cells, might result in an apparent absence of expression for the entire population.

We used markers to choose cells for physiological studies to increase the proportion of cells that express proteins coded for by transfected sequences. Thus, our results may not be representative of the entire population of cells (which might be studied, for example, in biochemical studies of drug binding). However, the use of markers should result in studies of cells that have a higher-than-average expression of the transfected genes. Therefore, the observation of responses to GABA after transfection with $\alpha1\beta2(Y205S)\gamma2L$ subunits suggests that an endogenous subunit may make a significant contribution, even in the presence of a competing exogenous subunit.

The major point of this work is the observation that the use of HEK 293 cells can significantly affect the properties of the $GABA_A$ receptors expressed after transfection of subunit cDNAs. The effect is clear in the case of subunits that result in either low levels of expression of assembled receptors (e.g., single subunits) or low levels of functional receptors (e.g., $\alpha1\beta2(Y205S)\gamma2L$, when activated by GABA). We are concerned that endogenous subunits might also contribute to variability in the pharmacological properties of any combination of $GABA_A$ receptor subunits when expressed in HEK 293 cells, but the extent of the contribution would have to be assessed for each subunit combination.

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