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Formation of Heteromeric γ-Aminobutyric Acid Type A Receptors Containing Two Different α Subunits

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SUMMARY

The functional properties of recombinant γ-aminobutyric acid (GABA) receptors expressed transiently in human embryonic kidney 293 cells were examined. Combinations of α1β2γ2, α3β2γ2, and α1α3β2γ2 subunits were transiently expressed and the properties of the resulting receptors were studied with patch-clamp electrophysiology. Each subunit combination produced receptors having a unique set of functional properties. Concentration-response experiments showed that receptors composed of α1β2γ2 subunit combinations were more sensitive to GABA (EC_{50} = 17.4 μM) than were either α3β2γ2 (EC_{50} = 103 μM) or α1α3β2γ2 (EC_{50} = 55.8 μM) receptors. Consistent with its action at native GABA-A receptors, diazepam (1 μM) potentiated the effect of GABA by shifting the GABA concentration-response curve to the left. The magnitude of the diazepam shift also differed between subunit combinations. The apparent potency of GABA was increased 2-fold by diazepam with α1β2γ2 receptors, 3-fold with α3β2γ2 receptors, and 5-fold with α1α3β2γ2 receptors. Brief applications (6–25 msec) of 3 mM GABA to outside-out patches revealed that currents decayed predominantly with double-exponential time courses. The decay time courses of currents mediated by α1β2γ2 and α1α3β2γ2 receptors were similar, whereas the α3β2γ2 receptor response decayed more slowly. The distinct properties observed in cells expressing each of these subunit combinations suggest that the subunits form unique receptors. The possibility that some neuronal GABA receptors contain two different α subunits is discussed.

The known subunits of the neuronal GABA-A/benzodiazipine receptor complex represent a relatively large family of homologous polypeptides. These subunits have been divided into five structural classes, i.e., α, β, γ, δ, and ρ. Based on the presumed structure of the neuromuscular junction nicotinic acetylcholine receptor, it is thought that GABA receptors are heteroligomers made up of five subunits. Unlike the nicotinic acetylcholine receptor, the exact subunit structure and stoichiometry of native GABA receptors have not been elucidated. However, heterologous expression of GABA receptor subunits has defined which subunits are needed to reproduce some aspects of native function and has determined some of the "rules" that govern the assembly of different subunit combinations. It is known that ternary combinations of α, β, and γ subunits are required to confer high affinity benzodiazipine binding to recombinant receptors (1), and different α subunits produce receptors having different benzodiazipine pharmacology (2). Measurements of functional GABA-activated ion channels have shown that active receptors are efficiently formed from αβ, αγ, and αβγ combinations, whereas homomeric α, β, or γ subunits and βγ combinations are expressed poorly. Moreover, both the sensitivity to GABA (3) and the amount of potentiation induced by benzodiazipine agonists (4, 5) have been shown to depend upon subunit combination.

The subunit structure of native GABA receptors also has been examined biochemically in rat brain, using antibodies directed against particular subunits. Immunoprecipitation, immunoaffinity purification, and Western blot analysis using these antibodies have shown that at least two different α subunit variants may assemble into a single receptor (6–8). The α1 and α3 subunits were among the α subunit pairs postulated to coassemble in some native receptor configurations. RNAs encoding the α1 and α3 subunits also have been co-localized by *in situ* hybridization in a number of brain regions in adult rats (9, 10). Therefore, GABA receptors containing both α subunit variants may constitute an important GABA receptor subtype in certain brain regions.

Xenopus oocyte expression of recombinant subunits revealed that when α1 and α3 subunits were coexpressed with β and γ subunits unique receptor properties were observed (11). However, it was not clear from that study whether a single receptor entity was expressed or whether multiple structures were formed. In the present study, the properties of GABA receptors

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ABBREVIATIONS: GABA, γ-aminobutyric acid; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

constructed from the $\alpha1\alpha3\beta2\gamma2$ subunit combination were examined and compared with the properties of receptors made of either $\alpha1\beta2\gamma2$ or $\alpha3\beta2\gamma2$ combinations. The results suggest that a homogeneous population of receptors having unique functional features is formed after expression of all four subunits. The properties of this particular subunit configuration are consistent with those of native GABA receptors.

Materials and Methods

Cell culture and transfection. HEK cells were cultured in minimum essential medium, supplemented with 10% fetal bovine serum, until ready for transfections. Transient expression of GABA receptors was accomplished by transfection of plasmids carrying full-length cDNAs encoding the appropriate subunits downstream from a human cytomegalovirus promoter. Transfections were done by $CaPO_4$ precipitation (12). Forty-eight hours after transfection, the cells were assayed electrophysiologically.

Electrophysiology. Functional channels were measured at room temperature by patch-clamp electrophysiology in the whole-cell and outside-out configurations. The growth medium was replaced by Ringer solution consisting of (in mM) 140 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, and 5 HEPES, pH 7.2. Patch electrodes were filled with a solution consisting of (in mM) 140 CsCl, 10 CsEGTA, and 10 HEPES, pH 7.3, for both whole-cell and outside-out configurations. Agonists were rapidly applied to cells or outside-out patches using a piezo-driven θ-tubing pipet (13). After the cell or patch was lifted clear of the bottom of the culture dish, control solution continuously flowed (driven by gravity) over it through one side of a glass θ-tubing pipet while solution containing agonist flowed through the other side. The solution bathing the cell was exchanged by stepping the application pipet with a piezo electric manipulator so that the agonist-containing side faced the cell. The time required for complete exchange of solutions was usually between 20 and 50 msec for whole cells and <1 msec for outside-out patches. Solutions flowing through either the control or agonist side of the θ-pipet were changed upstream with an eight-way Hamilton valve. GABA was dissolved directly in the extracellular solution. A 10 mM stock solution of diazepam was made in dimethylsulfoxide and diluted to 1 μM in the extracellular solution. Currents were measured using an Axopatch-200 amplifier (Axon Instruments). For whole-cell recording the currents were filtered at 750 Hz (−3 dB) using an eight-pole Bessel filter (Frequency Devices) and were digitized on-line at 1500 Hz with an ITC-16 analog/digital interface and a Macintosh-IIfx computer. The series resistance in whole-cell recordings ranged from 4 to 10 MΩ and was compensated using the prediction and correction circuits of the amplifier (setting, 65–75%). Outside-out patch currents were filtered at 2000 Hz before being digitized at 5000 Hz.

Data analysis. Concentration-response curves were constructed by applying different concentrations of GABA in random order. The maximum current evoked by each concentration was used for analysis. Data from cells in which the current amplitudes changed by >5% over the course of the experiment were discarded. The parameters I_{max} (maximum response amplitude), EC_{50} (concentration of GABA producing half of the maximum response), and n (Hill coefficient) were estimated by fitting data from individual cells to the logistic equation (eq. 1):

$$I = \frac{I_{max}}{1 + \left(\frac{[GABA]}{EC_{50}}\right)^{n}} \quad (1)$$

The best fit was obtained by minimizing the sum of squared differences using a computer algorithm (IGOR; WaveMetrics Inc.). The means and confidence intervals of the EC_{50} values were calculated after the logarithms of the individual EC_{50} values were taken. For the GABA pulse experiments, three to five raw current traces were usually averaged before least squares fitting of the current traces to exponential functions to estimate the kinetic parameters. The current records shown in the figures were not averaged.

Results

Expression of each of the three subunit combinations ($\alpha1\beta2\gamma2$, $\alpha3\beta2\gamma2$, and $\alpha1\alpha3\beta2\gamma2$) resulted in efficient assembly of functional GABA-activated channels on the plasma membrane of the HEK cells. The current density (current evoked by a saturating concentration of GABA) was somewhat higher in cells expressing the $\alpha1\beta2\gamma2$ subunit combination than in cells expressing the other structural isoforms (mean amplitudes were as follows: $\alpha1\beta2\gamma2$, 4870 ± 1237 pA, range = 888–13,250 pA, n = 12; $\alpha3\beta2\gamma2$, 2229 ± 487 pA, range = 473–6311 pA, n = 12; $\alpha1\alpha3\beta2\gamma2$, 3062 ± 1101 pA, range = 316–8815 pA, n = 9). Differences in the current density could arise from differences in the number of functional cell surface receptors or the overall probability of channel opening at high GABA concentrations. Although each receptor type exhibited similar conductance states of 30 and 16 pS (data not shown, but see Ref. 14), the amount of time spent in each of those states was not measured and could underlie the variations in current density.

GABA has been shown to be more potent in activating $\alpha1\beta1$ receptors than $\alpha3\beta1$ receptors (3). To test whether this difference was observed with ternary complexes containing the $\beta2$ and $\gamma2$ subunits, complete GABA concentration-response curves (Fig. 1) were constructed. The $\alpha1\beta2\gamma2$ subunit combination was found to be the most sensitive to GABA. $\alpha3\beta2\gamma2$ GABA receptors were markedly less sensitive and $\alpha1\alpha3\beta2\gamma2$ showed an EC_{50} between those two extremes (Table 1). The Hill coefficients were greater than unity in each case, suggesting that more than one GABA molecule must bind before the channels open. Notably, the Hill coefficient for the $\alpha1\alpha3\beta2\gamma2$ combination was not different from that for $\alpha3\beta2\gamma2$ receptors, suggesting that the currents did not arise from some combination of independent populations of $\alpha1\beta2\gamma2$ and $\alpha3\beta2\gamma2$.

A number of tests were performed to confirm that the GABA concentration-response curve for $\alpha1\alpha3\beta2\gamma2$ receptors did not arise from two independent populations of $\alpha1\beta2\gamma2$ and $\alpha3\beta2\gamma2$ receptors. Data from individual $\alpha1\alpha3\beta2\gamma2$-expressing cells were fitted to two-component logistic equations in which the average EC_{50} values and Hill coefficients for $\alpha1\beta2\gamma2$ and $\alpha3\beta2\gamma2$ were fixed and the relative contribution of each receptor was allowed to float. The resulting fits were always poorer than single-site fits. Similar results were obtained with curves generated from mean EC_{50} and Hill coefficients (Fig. 2). When individual $\alpha1\alpha3\beta2\gamma2$ concentration-response curves were fitted with two-component logistic equations in which all parameters were allowed to float, the parameters of the two components differed by <20%, suggesting that two-component fits do not describe the data better than single-component fits (data not shown).

The α subunit appears to determine the pharmacology of the benzodiazepine binding site (2) and also affects the overall efficacy of benzodiazepine agonists (4, 5). The effect of a benzodiazepine agonist, diazepam, was quantified by constructing GABA concentration-response curves in the presence of 1 μM diazepam. Diazepam caused a leftward shift in the GABA concentration-response curve for each receptor type, but the magnitude of the shift differed between them (Fig. 3; Table 1). In the presence of 1 μM diazepam the average GABA EC_{50} for $\alpha1\beta2\gamma2$ was reduced <2-fold, from 17.4 to 9.62 μM. The shift was more dramatic for $\alpha3\beta2\gamma2$ (107 to 18.8 μM), but $\alpha1\alpha3\beta2\gamma2$

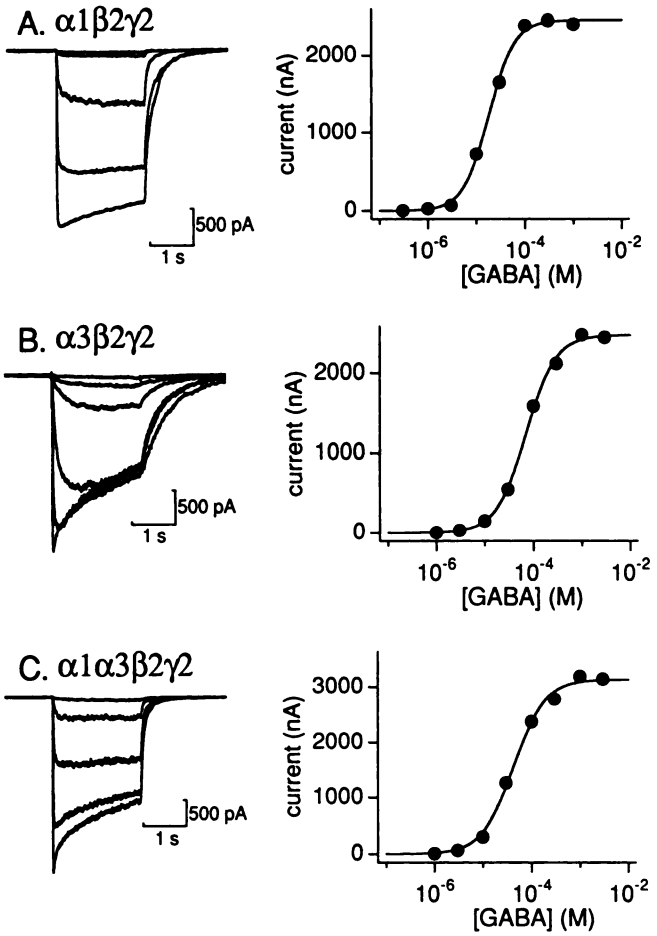

Fig. 1. GABA receptors made from different subunit combinations have different sensitivities to GABA. A, *Left*, superimposed currents evoked by 1, 3, 10, 30, and 300 μM GABA, recorded with whole-cell patch-clamp techniques at −60 mV, in a HEK cell expressing $\alpha1\beta2\gamma2$ GABA receptors. *Right*, GABA concentration-response relationship derived from that cell. *Points*, amplitudes of the peak currents evoked by different concentrations of GABA; *line*, results of fitting the data to eq. 1. B and C, Results from identical experiments done with cells expressing $\alpha3\beta2\gamma2$ (B) and $\alpha1\alpha3\beta2\gamma2$ (C). The GABA EC_{50} values differ between the receptor types. Note the markedly slower return to base-line values seen with the $\alpha3\beta2\gamma2$-expressing cell.

receptors were potentiated to the greatest extent, showing a shift from 55.8 to 10.5 μM. The maximum currents in the presence of 1 μM diazepam, derived from fitting the concentration-response data to eq. 1 (I_{max}), were slightly smaller than the current evoked by 3 mM GABA under control conditions. The fitted maximum was 84 ± 6% ($n = 4$) of control for $\alpha1\beta2\gamma2$ receptors and 83 ± 8% for $\alpha3\beta2\gamma2$ receptors ($n = 4$). The reduction was not significant for $\alpha1\alpha3\beta2\gamma2$ receptors (91 ± 10%, $n = 4$). Hill coefficients were not altered by diazepam (Table 1).

While the concentration-response curves were being measured, it became apparent that the currents mediated by each subunit combination decayed with different rates after the removal of GABA (see current traces in Fig. 1). Diffusion slows the time it takes to exchange solutions around a whole cell, so rapid application experiments using outside-out membrane patches were performed. A saturating concentration of GABA (3 mM) was pulsed onto the patches, simultaneously activating most of the receptors in the patch. Patches contained between

TABLE 1

GABA concentration-response parameters in the absence and presence of 1 μM diazepam

The values represent the means from fits of individual concentration-response experiments (see Materials and Methods).

	Control	+1 μM Diazepam
$\alpha1\beta2\gamma2$		
EC_{50} (μM)[a]	17.4 (12.9–19.8)	9.62 (5.13–18.0)[b]
Hill coefficient[c]	1.91 ± 0.10	1.78 ± 0.17
Number of cells	8	4
$\alpha3\beta2\gamma2$		
EC_{50} (μM)[a]	103 (82.8–130)	28.8 (11.6–71.4)[b]
Hill coefficient[c]	1.33 ± 0.06	1.42 ± 0.12
Number of cells	8	4
$\alpha1\alpha3\beta2\gamma2$		
EC_{50} (μM)[a]	55.8 (42.6–73.2)	10.5 (5.32–20.6)[b]
Hill coefficient[c]	1.28 ± 0.06	1.54 ± 0.12
Number of cells	5	4

[a] The statistical analyses were performed on the logarithm of the estimated EC_{50} values, and the numbers in parentheses represent the 95% confidence intervals.
[b] Significantly different from control ($p < 0.05$, Student's t test).
[c] Values are mean ± standard error.

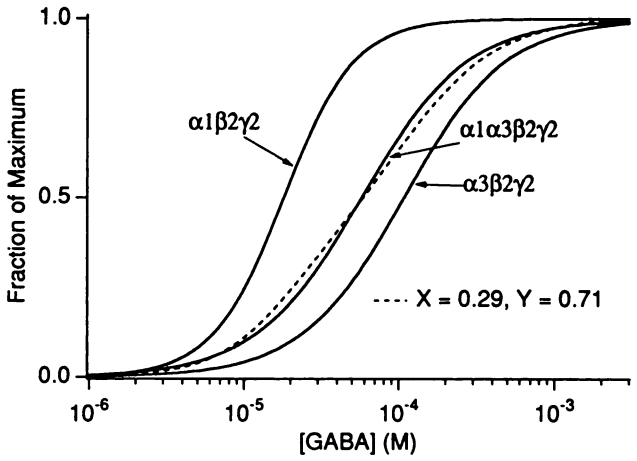

Fig. 2. Data derived from cells expressing $\alpha1\alpha3\beta2\gamma2$ do not arise from independent populations of $\alpha1\beta2\gamma2$ and $\alpha3\beta2\gamma2$ receptors. The *solid lines* were generated from eq. 1 using the mean Hill coefficients and EC_{50} values observed in the present experiments for each subunit combination (Table 1). The *dashed line* was generated by fitting the $\alpha1\alpha3\beta2\gamma2$ curve using a two-component variant of the logistic equation and mean GABA EC_{50} values and Hill coefficients for $\alpha1\beta2\gamma2$ and $\alpha3\beta2\gamma2$ receptors, as follows:

$$\text{Fraction of maximum} = \frac{X}{1 + \left(\frac{[\text{GABA}]}{17.4}\right)^{1.91}} + \frac{Y}{1 + \left(\frac{[\text{GABA}]}{103}\right)^{1.33}}$$

in which X and Y are various fractional values representing different proportional contributions of each receptor type, such that $X + Y = 1$. The fitting procedure estimated the best values for X and Y and kept the other parameters constant. Note the poor fit and the shallower slope of the dual-component curve.

30 and 300 active channels, and thus relatively large macroscopic currents were usually evoked by GABA pulses (Fig. 4). After the rapid removal of GABA the currents decayed back to base-line values with exponential kinetics. The time course of the solution change was determined at the end of each experiment by disrupting the patch and measuring the current evoked by pulsing Ringer solution diluted to half-strength with water. The rise and decay times of these test currents were always <1 msec (average 10–90% rise time = 0.79 ± 0.07 msec, $n = 16$) and the length of the pulse varied between 5 and 25 msec (mean

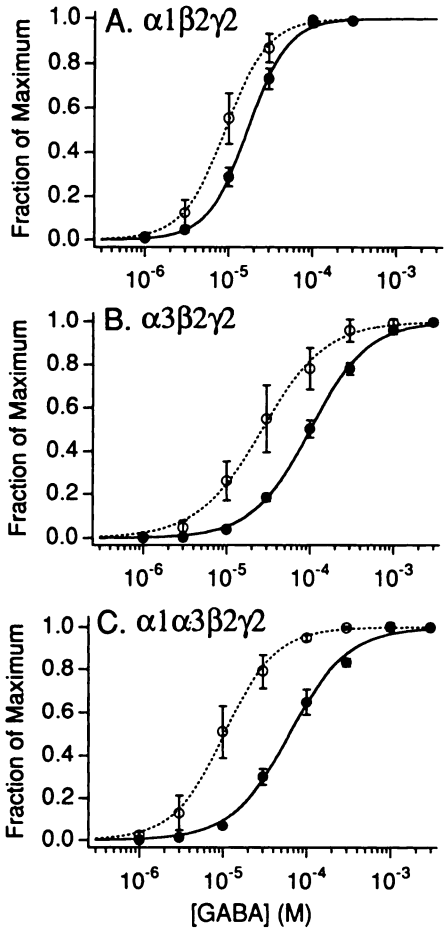

Fig. 3. The effect of 1 μM diazepam differs between subunit combinations. Shown are averaged concentration-response relationships for $\alpha1\beta2\gamma2$ (A), $\alpha3\beta2\gamma2$ (B), and $\alpha1\alpha3\beta2\gamma2$ (C) under control conditions (●) and in the presence of 1 μM diazepam (○). *Lines*, results of fitting these data to the following variant of the logistic equation:

$$\text{Fraction of maximum} = \frac{1}{1 + \left(\frac{[\text{GABA}]}{EC_{50}}\right)^{n}}$$

Diazepam shifted the GABA concentration-response relationship to the left in each case, but the magnitude of that shift was largest in cells expressing $\alpha1\alpha3\beta2\gamma2$ receptors and smallest in cells expressing $\alpha1\beta2\gamma2$ receptors. The diazepam curves are plotted relative to the maximum current measured in the presence of diazepam and do not reflect the slight decrease in the maximum current amplitude caused by diazepam (see Results).

length = 12.4 ± 1.6 msec, n = 16). There was no correlation between the decay rate of the GABA-activated current and the length of the pulse, either over the entire data set or within currents measured from a single receptor type.

In the majority of patches, the time course of the current decay showed double-exponential kinetics after the GABA pulse (Figs. 4 and 5). In patches containing $\alpha1\beta2\gamma2$ receptors, an average of 62 ± 4% of the current decayed with a time constant of 22.6 ± 4.6 msec (n = 4). The slow component had a mean time constant of 132 ± 30.5 msec. Currents mediated by $\alpha3\beta2\gamma2$ receptors decayed more slowly, with time constants of 40.4 ± 8.5 msec (54 ± 6% of the current) and 546 ± 55.7 msec (n = 6). In one $\alpha3\beta2\gamma2$ patch a single-exponential decay with a time constant of 572 msec was observed. Receptors consisting of $\alpha1\alpha3\beta2\gamma2$ showed decay kinetics similar to those

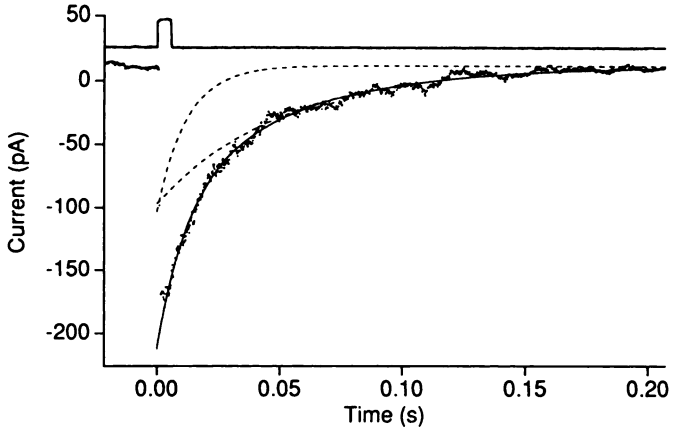

Fig. 4. Currents decay with a double-exponential time course after a brief pulse of 3 mM GABA. *Dots*, digitized data points recorded from an outside-out patch at −50 mV, taken from a cell expressing $\alpha1\beta2\gamma2$ receptors. *Solid line*, result of fitting the data points to a double-exponential equation; *dashed lines*, individual exponential components. The time constants estimated for this current were 12.0 and 50.5 msec. After the patch was ruptured, half-concentrated Ringer solution was pulsed onto the open pipet using the same protocol as used for the GABA pulses. The resulting current closely approximates the time course of the solution change around the intact patch and is shown above the GABA-evoked current.

of $\alpha1\beta2\gamma2$ receptors. The fast component comprised 71 ± 4% of the current and had an average time constant of 29.8 ± 3.7 msec (n = 6), whereas the slow component had a time constant of 182 ± 43 msec. GABA-activated currents had rise times that were slightly slower (between 2.5 and 5 msec for 10–90% rise times) than the average exchange time of the GABA application system. However, because these values were not markedly slower than the time required for the solution to change, they probably do not accurately reflect receptor activation kinetics.

Discussion

The three subunit combinations examined here show different sensitivities to GABA, potentiation by diazepam, and decay kinetics. The unique functional properties of $\alpha1\beta2\gamma2$, $\alpha3\beta2\gamma2$, and $\alpha1\alpha3\beta2\gamma2$ subunit combinations indicate that the subunits assemble into three different GABA receptor subtypes. Receptors composed of $\alpha1\beta2\gamma2$ and $\alpha3\beta2\gamma2$ subunit combinations have been shown previously to assemble in host cells (4, 5, 11, 14–16). The properties of these two GABA receptor subtypes clearly distinguish them from each other and from receptors consisting of subsets of subunits (e.g., homomeric receptors and $\alpha\beta$, $\alpha\gamma$, or $\beta\gamma$ combinations). The studies described here were designed to determine whether cells expressing multiple α subunit variants along with β and γ subunits expressed a single unique receptor type or multiple receptors made of different subunit complexes. The results indicate that currents measured in cells transfected with $\alpha1$, $\alpha3$, $\beta2$, and $\gamma2$ probably were mediated by a single structural entity. Moreover, this receptor showed a novel set of functional properties, suggesting that it contained both α subunits.

As expected from previous studies (3), $\alpha1\beta2\gamma2$ and $\alpha3\beta2\gamma2$ receptors had markedly different affinities for GABA. The apparent Hill coefficients were greater than unity in each case, but the value was significantly lower for $\alpha3\beta2\gamma2$ than for $\alpha1\beta2\gamma2$ receptors, suggesting that the degree of cooperativity differs for these receptor subtypes. The GABA EC_{50} for

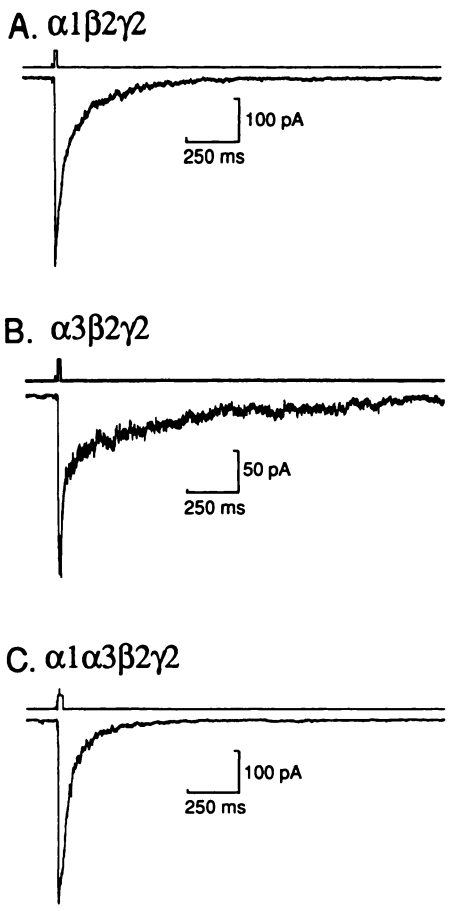

Fig. 5. GABA-activated currents decay with different rates in outside-out patches containing different GABA receptor subtypes. A, Inward current evoked by a brief application of 3 mM GABA to an outside-out patch at −60 mV, taken from a cell expressing $\alpha1\beta2\gamma2$ GABA receptors. The *square pulse* above the GABA-activated current illustrates the time course of the solution change in this patch. B and C, Results of identical experiments from patches containing $\alpha3\beta2\gamma2$ (B) and $\alpha1\alpha3\beta2\gamma2$ (C). Note the markedly slower decay kinetics exhibited by $\alpha3\beta2\gamma2$ receptors.

$\alpha1\alpha3\beta2\gamma2$ receptors was intermediate between those of the ternary complexes, and the Hill coefficient was identical to that shown by $\alpha3\beta2\gamma2$ receptors. Markedly lower Hill coefficients would be expected if the currents arose from independent populations of $\alpha1\beta2\gamma2$ and $\alpha3\beta2\gamma2$ receptors, because the EC_{50} values for these receptors are >5-fold different from each other. Thus, $\alpha1\alpha3\beta2\gamma2$ currents are probably mediated by a single population of receptors.

The effect of the benzodiazipine diazepam was examined by measuring the magnitude of the leftward shift of the GABA concentration-response curve induced by diazepam. Currents mediated by $\alpha1\alpha3\beta2\gamma2$ receptors were potentiated by diazepam to a greater degree than were currents mediated by either of the ternary complexes. The present results are similar to previous work in which benzodiazipine agonists had greater potentiating effects on $\alpha3\beta2\gamma2$ receptors than on $\alpha1\beta2\gamma2$ receptors (4, 5). This probably is the result of differences in the maximal effect of diazepam and is not due to differences in the potency of diazepam. Binding studies with $\alpha1\beta2\gamma2$ and $\alpha3\beta2\gamma2$ receptors indicated that diazepam bound to the two receptors with nearly identical K_i values of about 16.5 nM (2). That report concluded that $\alpha1\beta2\gamma2$ and $\alpha3\beta2\gamma2$ represented type I and type II benzodiazipine receptors, respectively, based on differences in the affinities of CL 218872 and zolpidem. It would be of interest to determine whether receptors containing both $\alpha1$ and $\alpha3$ subunits had type I, type II, or hybrid pharmacological properties.

The brief application of saturating concentrations of GABA to outside-out patches was used to measure the time course of current decay after the removal of GABA. Each receptor mediated currents that decayed with double-exponential kinetics. The decay kinetics were generally slower for $\alpha3\beta2\gamma2$ receptors than for the other two subunit combinations. The rise times of the GABA-activated current could not be accurately measured because the solution exchange time was not quite fast enough. The length of the GABA pulse varied from 6 to 25 msec between patches, and in this range the decay kinetics did not seem to depend on the length of time the patches were exposed to GABA. The slower decay kinetics of $\alpha3\beta2\gamma2$ GABA receptors suggest that inhibitory synaptic currents mediated by such receptors may show slower kinetics than currents mediated by $\alpha1\beta2\gamma2$ or $\alpha1\alpha3\beta2\gamma2$ receptors. However, the much shorter duration (1 msec or less) of the GABA pulse thought to underlie inhibitory synaptic transmission could result in very different decay kinetics than those observed here.

The unique mixture of functional features exhibited by cells expressing $\alpha1\alpha3\beta2\gamma2$ subunits supports the assertion that these four subunits assemble into a separate GABA receptor subtype. Independent populations of $\alpha1\beta2\gamma2$ and $\alpha3\beta2\gamma2$ GABA receptors do not contribute to the currents measured in cells transfected with all four subunits. However, the data cannot rule out the possibility that the preferred assembly contains a smaller subset of the four subunits that were transfected. Both $\alpha\beta$ and $\alpha\gamma$ combinations readily form functional channels when expressed in HEK cells (14), and thus $\alpha1\alpha3\beta2$ or $\alpha1\alpha3\gamma2$ combinations are the most likely candidates. However, combinations lacking a γ subunit are probably not strongly represented because of the benzodiazipine sensitivity exhibited by each subunit combination, and assembly of $\alpha1\beta2\gamma2$ combinations seems to be favored over assembly of either $\alpha1\beta2$ or $\alpha1\gamma2$ receptors. Homomeric receptors and the $\beta2\gamma2$ combination are expressed quite poorly in HEK cells. Thus, if these rules for assembly apply to $\alpha3$-containing receptors, then one would expect that ternary or quaternary complexes predominate in HEK cells transfected with mixtures of α, β, and γ subunits.

Interestingly, recent studies using mutant GABA-A receptor subunits (including $\alpha3$, $\beta2$, and $\gamma2$) suggest that in host cells the functional receptor is a pentamer containing two α, one β, and two γ subunits (17). The results presented here indicate that the receptor may prefer to assemble with two different α subunits in host cells. If assembly of GABA receptors in neurons is determined by the structural features of the subunits themselves, then the rules for assembly in transfected HEK cells may shed light on the subunit structure of native receptors. For example, from our results one might conclude that GABA receptors in a neuron that expresses $\alpha1$, $\alpha3$, $\beta2$, and $\gamma2$ subunits would probably consist of a single population of receptors containing all four subunits in some as yet unknown configuration. Alternatively, neurons themselves may possess some enzymatic machinery that actively assembles particular subunit combinations. However, the present and previous (14, 18) results suggest that assembly of GABA receptors in transfected mammalian cells is not random. Random assembly would produce multiple receptors, the functional properties of which

should have been detected in these experiments. It is unlikely (although formally possible) that HEK cells possess the same subunit assembly machinery as do neurons. Thus, the nonrandom assembly of GABA receptor subunits suggests that some information about how to assemble them can be found on the subunits themselves. Supporting this postulate is the finding that information controlling the assembly of heteromeric acetylcholine receptors (19) and potassium channels (20, 21) has been localized to certain regions of the subunit polypeptides.

The assembly of GABA receptors containing multiple different α subunits also seems to occur in brain. Using antibodies specific for particular α subunit variants and either immunoprecipitation (7) or immunoaffinity purification (6, 8), GABA receptor subpopulations that contain two different α subunits have been detected in the rat brain. In each case combinations of α1 and α3 subunits were clearly identified. *In situ* hybridization studies (9, 10) have shown that a number of rat brain structures and neuronal cell populations express mRNAs encoding both α1 and α3 subunits. These include most layers of the neocortex, the pyriform cortex, dentate granule cells, the mitral and tufted cells of the olfactory bulb, and certain thalamic and basal nuclei. Thus, the potential exists in these areas for the expression of GABA receptors that contain both α1 and α3 subunits.

The formation of GABA receptors with multiple α subunit variants represents another mechanism for generating GABA receptor diversity. The receptor heterogeneity that could potentially arise simply from the basic template of α, β, and γ subunits is enormous. Moreover, neuronal GABA receptors composed of $\alpha\beta$ or $\alpha\gamma$ combinations and those containing two different α subunits add significantly to the possible number of different GABA receptors. Such extreme receptor heterogeneity has not been observed in neuronal systems to date, and the difficult task of making sense of the potential diversity still lies before us. Perhaps the functional properties of different structural isoforms are similar to each other or neurons express relatively few different structural motifs. However, it is also possible that functional heterogeneity has not been detected because functional properties that differ with subunit configuration were not measured. Each of the three subunit combinations examined gave functional properties that were quite similar to those of neuronal GABA receptors, but subtle differences between them were apparent. Similar experiments carried out on neuronal preparations will likely reveal analogous functional heterogeneity. Thus, detailed functional comparisons, together with biochemical and molecular biological analyses, will eventually shed light on native subunit configurations and help make sense of GABA receptor diversity.

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