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Mutating the Highly Conserved Second Membrane-Spanning Region 9′ Leucine Residue in the α_1 or β_1 Subunit Produces Subunit-Specific Changes in the Function of Human $\alpha_1\beta_1$ γ-Aminobutyric Acid$_A$ Receptors

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ABSTRACT

The properties of the human $\alpha_1\beta_1$ γ-aminobutyric acid (GABA)$_A$ receptors were investigated after mutation of a highly conserved leucine residue at the 9′ position in the second membrane-spanning region (TM2). The role of this residue in α_1 and β_1 subunits was examined by mutating the 9′ leucine to phenylalanine, tyrosine, or alanine. The mutations were in either the α_1 subunit ($\alpha*\beta$), the β_1 subunit ($\alpha\beta*$), or in both subunits ($\alpha*\beta*$), and the receptors were expressed in *Sf*9 cells. Our results show that the rate of desensitization is increased as the size and hydrophobicity of the 9′ residue in the α_1 subunit is increased: Y, F > L > A, T. Mutation of L9′ in only the β_1 subunit ($\alpha\beta*$) to either phenylalanine or tyrosine increased the EC_{50} value for GABA at least 100 times, but the EC_{50} was unchanged in $\alpha\beta*$ alanine mutants. In the 9′ α_1 mutants ($\alpha*\beta$, $\alpha*\beta*$) the GABA EC_{50} was minimally affected. In $\alpha*\beta$ and $\alpha*\beta*$, but not $\alpha\beta*$, the peak currents evoked by millimolar concentrations of GABA were greatly reduced. The reduction in currents could only be partially accounted for by decreased expression of the receptors These findings suggest different roles for the two types of subunits in GABA activation and later desensitization of $\alpha_1\beta_1$ receptors. In addition, an increase in the resting membrane conductance was recorded in alanine but not in phenylalanine and tyrosine mutants, indicating that the side chain size at the 9′ position is a major determinant of current flow in the closed conformation.

γ-Aminobutyric acid (GABA) binds at an extracellular site on the GABA$_A$ receptor and activates an integral chloride ion channel. How GABA binding is coupled to channel opening is not well understood. The receptors are thought to be heterooligomeric pentamers with each subunit contributing a transmembrane segment to line the pore. Residues in the second membrane-spanning region (TM2) contribute to the ion permeation pathway. A leucine residue at the 9′ position (L9′) in the middle of the TM2 region is highly conserved in GABA$_A$ receptor subunits and across other members of the C-C loop receptor family. Mutation of L9′ in rat $\alpha_1\beta_2\gamma_2$ GABA$_A$ (Chang et al., 1996), 5-hydroxytryptamine type 3 (Yakel et al., 1993), and nicotinic acetylcholine (nACh) (Revah et al., 1991, 1995; Filatov and White, 1995; Labarca et al., 1995) receptors can alter the agonist EC_{50} value and the rate of desensitization, suggesting functional roles for L9′ in desensitization and gating. Mutation of L9′ to threonine (L9′T) in the α_1 subunit of the human $\alpha_1\beta_1$ GABA$_A$ receptors slows receptor activation and desensitization (Tierney et al., 1996). When the L9′T mutation is in either the β_1 subunit or both α_1 and β_1 subunits together, the response to GABA is abolished and the channel becomes constitutively open. The differential effects of mutating the 9′ residue on the response to GABA suggest that there are functional differences between subunits in the response of the receptor to GABA, despite the high conservation of L9′ across subunits and the high sequence homology of the TM2 region. Mutation of L9′ to serine in subunits of the nACh receptor resulted in a reduction in the EC_{50} that was approximately proportional to the number of mutated subunits in the receptor complex (Filatov and White, 1995; Labarca et al., 1995). In contrast, mutation of L9′ to serine in rat $\alpha_1\beta_2\gamma_2$ GABA$_A$ subunits showed differences in the degree of shift in EC_{50} depending on which subunit was mutated (Chang et al., 1996). It is possible that unlike in nACh receptors, GABA$_A$ subunits do not contribute in an equivalent manner to the mechanisms

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ABBREVIATIONS: GABA, γ-aminobutyric acid; TM2, second membrane-spanning region; nACh, nicotinic acetylcholine; MES, 2-(*N*-morpholino)ethanesulfonic acid; *Sf*9, *Spodoptera frugiperda*; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

involved in receptor activation. An additional effect of mutating L9′ to smaller or more hydrophilic residues in $GABA_A$ and nACh receptors is current flow in the absence of agonist (Labarca et al., 1995; Tierney et al., 1996; Bertrand et al., 1997; Mihic et al., 1997; Pan et al., 1997; Chang and Weiss, 1998, 1999). Given the apparent importance of TM2 9′ position in $GABA_A$ receptor function, we investigated what effects different amino acids at this location had on the properties of the receptors and whether any subunit specificity could be detected.

Materials and Methods

Construction and Expression of Mutated Receptors. Double-stranded mutagenesis (Pharmacia Biotech, Piscataway, NJ) was used to introduce site-directed mutations to either α_1 (L9′: amino acid 264) or β_1 (L9′: amino acid 259) human $GABA_A$ cDNA in the dual promoter baculovirus transfer vector pAcUW31 (ClONTECH, Palo Alto, CA). Plasmids with mutations in both α_1 and β_1 subunits were produced by restriction enzyme digestion with *Bgl*2 and *Nhe*I, gel purification, and ligation of mutated fragments. Alternatively, a plasmid previously mutated in the α_1 subunit cDNA was used as a template in a subsequent mutagenesis reaction to mutate the homologous residue in the β_1 subunit .

Recombinant $\alpha\beta$(L9′A) and α(L9′A)β(L9′A) baculoviruses for L9′A mutated sequences were generated using the Bac-to-Bac expression system (Life Technologies, Grand Island, NY). The presence of mutations was confirmed by DNA sequencing across the mutated regions. Although an α(L9′A)β mutant plasmid was generated, we could not isolate a recombinant α(L9′A)β baculovirus.

Techniques for general handling of *Sf*9 (*Spodoptera frugiperda*) cells, production of high titer viral stock, and infection procedures have been described previously (Birnir et al., 1995).

Muscimol Binding. The method used to measure high-affinity muscimol binding in cells infected with recombinant baculovirus was as described previously (Tierney et al., 1996). The concentrations of muscimol used consisted of 10% radioactively labeled [^{3}H]muscimol and 90% cold muscimol (Sigma Chemical Co., St. Louis, MO) measured over a concentration range of 1 to 512 nM. Scintillation count values were multiplied by 10 to account for the 1:10 dilution of [^{3}H]muscimol.

Flow Cytometry. Antibody labeling methods to detect α_1 subunit expression were similar to that described previously (Tierney et al., 1996). To determine the level of α_1 subunit present in the plasma membrane, *Sf*9 cells were infected with recombinant baculovirus 40 to 48 h before use in experiments. Nonpermeabilized cells were labeled with primary monoclonal antibody bd24 (1:50 dilution), fixed in Zamboni's solution (2% formaldehyde, 15% picric acid, 0.1 M phosphate buffer, pH 7.4) for 90 min, and then labeled with secondary antibody using a fluorescein isothiocyanate-conjugated sheep anti-mouse Ig antibody (1:40 dilution; Silenus Laboratories, Hawthorn, Australia). To detect the total level of α_1 subunit present in the plasma membrane and within the cell, cells were permeabilized in PBS buffer containing 0.1% SDS plus 1% BSA (Boehringer-Mannheim Biochemica, Mannheim, Germany) for 10 min before labeling with the primary antibody. The level of fluorescence was detected using a FACStar Plus flow cytometer (Becton Dickinson, Mountain View, CA), and results were analyzed with the WinMDI 2.7 computer program (courtesy of Joseph Trotter, Scripps Cancer Research Institute, La Jolla, CA). The level of background fluorescence was determined from cells infected with the wild-type parent baculovirus (AcNPV) and subtracted from all other values. These were then calculated as a percent of wild-type fluorescence.

Electrophysiology. Cells were infected with virus when growing at a density of 1 to 3 $\times$ 10^6 cells/ml and incubated at 25 $\pm$ 1°C for 33 to 45 h before use in electrophysiological experiments. Whole-cell currents were recorded from voltage-clamped cells with a pipette potential of −40 mV. At this potential background, chloride current was minimized (Birnir et al., 1995). Cells were perfused with bath solution (14 ml/min) containing 180 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM MES adjusted to pH 6.2 with NaOH (330 mOsmol/liter). pH 6.2 is the normal pH for growth and maintenance of *Sf*9 cells. Pipettes were made from borosilicate glass with resistances of 3 to 10 MΩ and filled with a solution containing 178 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM EGTA, 4 mM ATP, and 10 mM TES, adjusted to pH 7.2 with NaOH. GABA (Sigma Chemical Co.) was dissolved in bath solution, serially diluted, and rapidly applied to cells by gravity feed through tubes aimed at cells. The rate of solution exchange across the cell surface was examined by monitoring the whole-cell current when the Cl^- concentration was changed around the cell. When the bath solution contained 184 mM Cl^- and a jet of solution containing 34 mM Cl^- was switched through the tubes, an inward current that reached a plateau in less than 1 ms was evoked (Birnir et al., 1995). The current increased from 10 to 90% of the final steady-state current in approximately 0.5 ms. Currents were monitored with a current-to-voltage converter (Axopatch-1D; Axon Instruments, Foster City, CA) using series resistance compensation. Data were digitized using an analog-to-digital converter (TL-1, DMA interface; Axon Instruments) and the Capture data acquisition program and then analyzed using the Channel 2 data analysis program (M. Smith, John Curtin School of Medical Research). To account for rundown of whole-cell currents over successive agonist applications, a control concentration of GABA was applied before and after a test concentration of GABA. Results were only used from cells in which the two control concentrations gave currents that differed by less than 20% in amplitude. Responses were calculated as a fraction of the averaged control currents.

Equations. Concentration-response data were averaged for each concentration and fitted using a Hill-type equation (nonlinear least-squares):

$$I = I_{max} \cdot [GABA]^h / [(EC_{50})^h + [GABA]^h] \quad (1)$$

where I is the peak current (pA) produced after the application of GABA, I_{max} is the value of the estimated maximal or "saturating" peak current response, [GABA] is the concentration of GABA, and h is the Hill coefficient. EC_{50} is the GABA concentration that gave half-maximal current response. Data from some concentration-response experiments were best fitted by the sum of two Hill equations.

Ligand-binding data were fitted by the Michaelis-Menten equation (nonlinear least-squares):

$$B = B_{max} \cdot [muscimol] / (K_d + [muscimol]) \quad (2)$$

where B is the amount of [^{3}H]muscimol bound (pmol/10^6 cells), B_{max} is the maximum bound concentration (pmol/10^6 cells), [muscimol] is the concentration of muscimol, and K_d is the concentration that yields half-maximal binding, the dissociation constant.

Statistics. The various equations were fitted to the data using SlideWrite software (version 4.0). Results are presented as mean $\pm$ 1 S.E. A two-tailed Student's t test was used to determine whether values were significantly different ($P \leq .05$).

Results

Possible subunit-specific effects on receptor function were assessed using *Sf9* cells infected with recombinant baculovirus containing α_1 and β_1 subunit cDNAs that had been mutated in either the α_1 subunit or in the β_1 subunit or in both α_1 and β_1 subunits (referred to as α and β, respectively). We mutated L9′ to phenylalanine (L9′F), tyrosine (L9′Y), or alanine (L9′A). Receptors mutated to phenylalanine in the α_1 subunit were designated α(L9′F)β, those mutated in the β_1 subunit were designated $\alpha\beta$(L9′F), and those carrying the mutation in both subunits were designated α(L9′F)β(L9′F). This format was also used for the L9′Y and L9′A mutations.

α-Subunit-Mutated Receptors Have Reduced Peak Current Amplitude and Altered Desensitization. Whole-cell current responses to high concentrations of GABA in mutated and wild-type receptors are shown in Fig. 1 and summarized in Table 1. The average maximum peak current produced in response to 10 mM GABA in wild-type receptors was 3370 pA and was similar in amplitude to currents obtained in cells expressing αβ(L9′F) receptors, where it was 3958 pA. When the receptors were mutated in the α_1 subunit either alone or in both subunits, the peak currents were greatly reduced in the L9′F mutants (Fig. 1A). The average maximum peak currents in cells expressing α(L9′F)β or α(L9′F)β(L9′F) receptors were 765 and 365 pA, respectively. These values are only about 23 and 11% of the maximum peak current amplitude recorded in cells expressing wild-type receptors. The results were similar when L9′ was mutated to tyrosine (Y, Fig. 1B). The largest peak currents were again obtained when the mutation was present only in the β_1 subunit (αβ(L9′Y), 1428 pA) and the average response was about 40% of the value for wild-type receptors. In α(L9′Y)β and α(L9′Y)β(L9′Y) receptors, the average peak currents were 672 and 172 pA, respectively. These values are only 20 and 5% of the maximum current obtained in cells expressing wild-type receptors. When L9′ was mutated to alanine (Fig. 1C), the α_1 subunit mutation had a greater effect on the current amplitude than when the mutation was only in the β_1 subunit. The average maximum currents were 1017 and 107 pA in the αβ(L9′A) and α(L9′A)β (L9′A) receptors, respectively. Hence, when L9′ is mutated to phenylalanine, tyrosine, or alanine in the α_1 subunit in $\alpha_1\beta_1$ receptors, the maximal peak current amplitude is reduced.

Current activation times and decay times in saturating GABA concentrations are summarized in Table 1. The rise time of the currents is the time it takes for the whole-cell current to increase from 10 to 90% of the peak current value ($T_{10-90\%}$). Millimolar GABA concentrations were used because it has been shown (Akaike et al., 1986) that rise times decrease rapidly and level off as the GABA concentration is raised. The 50% decay time is the time taken for the peak current to decay by half (T_{50}). The current rise times were similar for wild-type, L9′F, and L9′Y receptors, ranging from 7 to 16 ms, but the rate of current decay varied. The current rise time in αβ(L9′A) receptors was similar to that for wild type (7 ms), but for the α(L9′A)β (L9′A) receptors, it was much slower and more similar to that observed for the α(L9′T)β mutants (116 ms, Tierney et al., 1996). It took on average about 0.5 s to rise from 10 to 90% of the peak current value. Normalized whole-cell currents are shown in Fig. 2 and allow a comparison of the time course of the current decay between the different mutants. The average T_{50} after the application of 10 mM GABA was 223 ms in wild-type receptors. When L9′ was mutated to F in the α_1 subunit [α(L9′F)β and α(L9′F)β(L9′F)], the T_{50} after the application of 10 mM GABA was 55 and 27 ms, or about 25 and 12% of that in wild-type receptors (Fig. 2A). Similarly, when L9′ was mutated to Y in the α_1 subunit [α(L9′Y)β and α(L9′Y)β(L9′Y)], the average T_{50} was 78 and 12 ms, respectively, or about 35 and 5% of that in wild-type receptors (Fig. 2B). In contrast, when the mutation was in the β_1 subunit only, the average T_{50} values were 290 ms for αβ(L9′F) and 337 ms for αβ(L9′Y) receptors, similar to the T_{50} values in wild-type receptors (Fig. 2, A and B). The rate of current decay was much slower when L9′ was mutated to alanine than was observed when the 9′ leucine was mutated to an aromatic residue (Fig. 2C). For the double mutant, α(L9′A)β(L9′A), the current did not decay (Fig. 1C), and the average T_{50} was 1.4 s for the αβ(L9′A) mutant receptors. This is similar to the T_{50} value for the α(L9′T)β mutant receptors, where it was about 2.1 s. The results show that in the $\alpha_1\beta_1$ $GABA_A$ receptor, an aromatic residue at the 9′ TM2 location in the α_1 subunit increases the rate of current decay, whereas the smaller alanine or threonine (Tierney et al., 1996) residues decrease it.

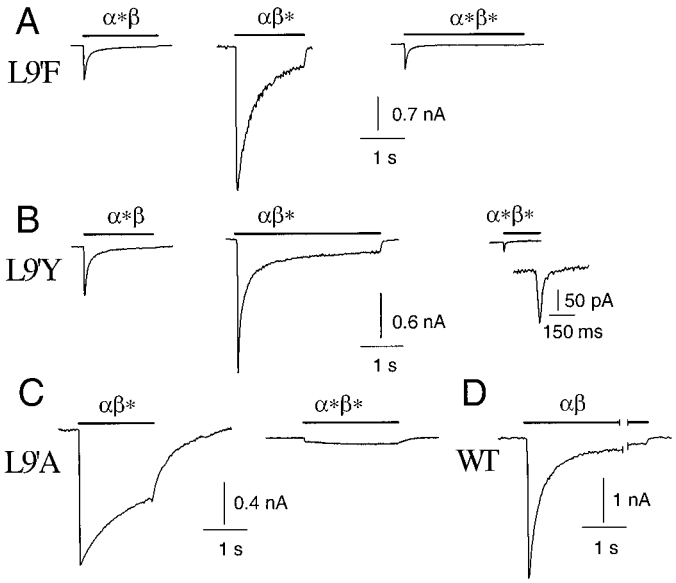

Fig. 1. Whole-cell currents activated by GABA in L9′ mutated receptors. GABA (10 mM) was applied to cells expressing mutants (A–D). The bar represents the period in which GABA was applied. D, GABA was applied for 4.5 s but only the first 2.2 s and the last 0.5 s are shown. A, α(L9′F)β, αβ(L9′F), and α(L9′F)β(L9′F). B, α(L9′Y)β, αβ(L9′Y), and α(L9′Y)β(L9′Y). C, αβ(L9′A) and α(L9′A)β(L9′A). D, αβ. The pipette potential was −40 mV. Calibrations for A, B, and C are shown beside the αβ* current traces in each case. In B, the α*β* current trace is shown at a larger scale next to the 50-pA, 150-ms calibrations bars. For the wild type (WT) (αβ; D), calibrations are next to the current trace.

EC_{50} for GABA Is Increased in αβ(L9′F) and αβ(L9′Y) Receptors. The reduced current amplitudes observed in the α_1 mutants could be due to a shift in the GABA concentration-response relationship to higher concentrations for these mu-

TABLE 1

Whole-cell currents activated by GABA

Characteristics of whole-cell currents activated by 10 mM or [a]30 mM

GABA concentrations are summarized for cells expressing either mutated or wild-type (WT) receptors. Values are expressed as mean ± 1 S.E. The number of cells used is shown in parentheses. I_p is the value of the peak current; T_{10-90} is the time taken for the peak current to increase from 10 to 90% of the maximum current; T_{50} is the time taken for the peak current to decay by 50%.

Receptor	I_p	T_{10-90}	T_{50}
	pA	*ms*	
αβ (WT)	3370 ± 612	16 ± 2 (6)	223 ± 43 (6)
α(L9′F)β	765 ± 90 (15)	7 ± 1 (15)	55 ± 5 (15)
αβ(L9′F)[a]	3958 ± 687 (6)	12 ± 1 (6)	290 ± 27 (6)
α(L9′F)β(L9′F)	365 ± 68 (8)	9 ± 1 (8)	27 ± 3 (8)
α(L9′Y)β	672 ± 148 (12)	10 ± 1 (12)	78 ± 10 (12)
αβ(L9′Y)[a]	1428 ± 204 (5)	11 ± 1 (5)	337 ± 29 (5)
α(L9′Y)β(L9′Y)	172 ± 26 (7)	9 ± 1 (7)	12 ± 1 (7)
αβ(L9′A)	1017 ± 223 (8)	7 ± 1 (3)	1400 ± 200 (3)
α(L9′A)β(L9′A)	107 ± 15 (6)	559 ± 220 (5)	No desensitization
α(L9′T)β[b]	407 ± 53 (5)	116 ± 15 (5)	2140 ± 200 (5)

[a] Activated by 30 mM.
[b] From Tierney et al., 1996. GABA concentration was 100 μM.

tants. This was examined by applying GABA concentrations ranging from 1 μM to 30 mM to cells expressing the mutant receptors. The results are shown in Fig. 3. Data were fitted using a Hill-type equation (eq. 1). GABA EC_{50} values and Hill coefficients are listed in Table 2. When the aromatic residues replaced the 9′ leucine in the α_1 subunit only, the GABA EC_{50} values were somewhat increased relative to wild-type receptors (Fig. 3, A and B): 57 μM for α(L9′F)β and 25 μM for α(L9′Y)β receptors compared with 11 μM for wild-type receptors. When the L9′F mutation was present in both subunits, α(L9′F)β(L9′F), the half-maximal concentration was 12 μM (Fig. 3A), similar to wild-type receptors. In cells expressing receptors with aromatic residues at 9′ in the α_1 subunit , the peak current amplitude was significantly less than that in wild-type receptors (Fig. 1 and Table 2). The small current amplitude in α(L9′Y)β(L9′Y) receptors in particular made it difficult to construct a concentration-response curve. Therefore, to examine whether there were changes in the GABA sensitivity of the α(L9′Y)β(L9′Y) receptors relative to wild type, we measured the relative current amplitudes produced in response to GABA concentrations that produce maximal (10 mM) and half-maximal (10 μM) currents in wild-type receptors. The current evoked in response to 10 μM GABA as a ratio of the response to 10 mM GABA in cells expressing α(L9′Y)β(L9′Y) receptors was 0.36 ± 0.01 (n = 3). This is similar to the ratio of 0.31 ± 0.03 (n = 6) for α(L9′Y)β receptors for the same GABA concentrations. When the aromatic mutations were present in the β_1 subunit only, the data could not be fitted by a single sigmoidal function: two sigmoidal functions were required to fit the data (Fig. 3, A and B, diamonds). The GABA EC_{50} values for the higher-affinity component of the concentration-response curve were 11 for $\alpha\beta$(L9′F) and 17 μM for $\alpha\beta$(L9′Y) receptors. The maximum current corresponding to the higher affinity component was about 20% of the maximal peak current value. The GABA EC_{50} value for the lower-affinity component of the concentration-response curve was in the millimolar range for both mutants [$\alpha\beta$(L9′F), 1.131 mM; $\alpha\beta$(L9′Y), 2.453 mM]. The double-component nature of the EC_{50} curves observed for $\alpha\beta$(L9′Y) and $\alpha\beta$(L9′F) receptors was surprising because the receptors are generally assumed to form a population of functional channels with a common stoichiometry of three α-subunits and two β-subunits (Im et al., 1995). We therefore examined whether the effect was related to the nature of the amino acid present at the 9′ position in the β_1 subunit. When alanine was present at this position [$\alpha\beta$(L9′A), Fig. 3C], the

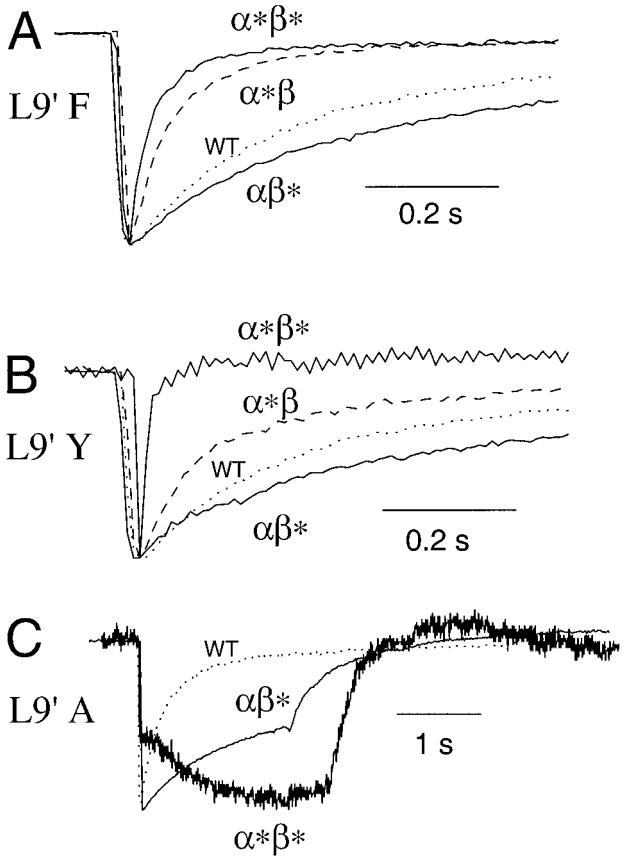

Fig. 2. Comparison of current decay in L9′ mutants. Whole-cell currents activated by millimolar concentrations of GABA (10 or 30 mM) at −40 mV are shown. The currents have been scaled to the same amplitude as in wild-type receptors (dotted line) to illustrate the differences in time courses. A, α(L9′F)β, $\alpha\beta$(L9′F), and α(L9′F)β(L9′F). B, α(L9′Y)β, $\alpha\beta$(L9′Y), and α(L9′Y)β(L9′Y). C, $\alpha\beta$(L9′A) and α(L9′A)β(L9′A).

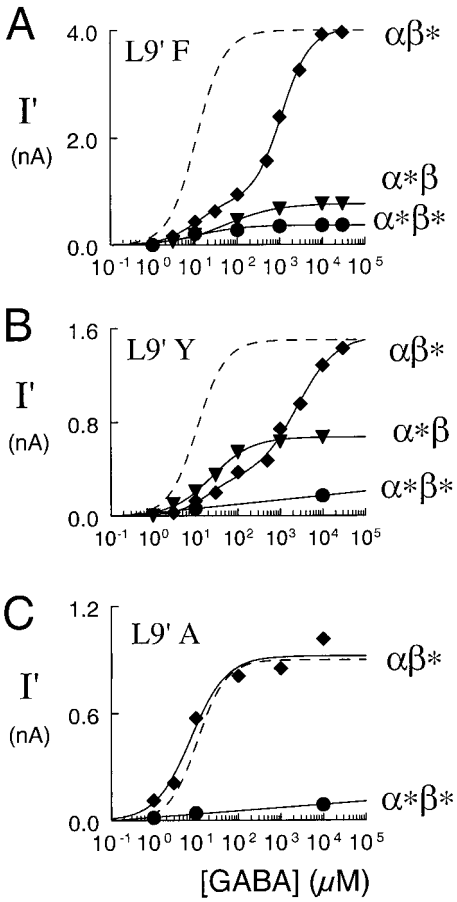

Fig. 3. Dose-response curve of whole-cell currents generated by GABA. The value of the peak current normalized to 10 mM GABA in the same cell and then multiplied by the average 10 mM GABA peak current value from all cells used to generate the curve (I′) is plotted against GABA concentration. The pipette potential was −40 mV. The data points are the mean ± 1 S.E. in three or more cells. The vertical bars are shown if larger than the symbol. The curves are a fit of a Hill-type equation (see *Materials and Methods*) to the data. The broken line represents the dose-response curve for wild-type receptors (Birnir et al., 1995). A, α(L9′F)β (▼), $\alpha\beta$(L9′F) (♦), and α(L9′F)β(L9′F) (●). B, α(L9′Y)β (▼), $\alpha\beta$(L9′Y) (♦), and α(L9′Y)β(L9′Y) (●). C, $\alpha\beta$(L9′A) (♦) and α(L9′A)β(L9′A) (●). EC_{50} values and Hill coefficients for the different mutants are given in Table 2.

concentration-response curve was fitted by a Hill-type equation with an EC_{50} value of 6 μM, similar to wild-type receptors. To examine whether there were changes in the GABA sensitivity of the α(L9′A)β(L9′A) receptors relative to wild type, we determined the relative current amplitudes produced in response to GABA concentrations that produce maximal and half-maximal currents in wild-type receptors. The current produced in response to 10 μM GABA as a ratio of the response to 10 mM GABA in cells expressing α(L9′A)β(L9′A) receptors was 0.45. Because α_1 mutants and wild-type receptors appear to have similar GABA EC_{50} values, the smaller peak current responses to millimolar concentrations of GABA in α_1 mutants cannot be explained by an increase in the EC_{50} values that give submaximum responses to 10 mM GABA.

Muscimol Binding and α_1 Subunit Expression in Plasma Membrane. It was possible that the smaller whole-cell peak current amplitudes in the α_1 mutants were caused by decreased expression of the receptors. Furthermore, the heterogeneity observed in the GABA EC_{50} values of the aromatic double mutants could indicate that the GABA binding affinity of the mutated receptors is altered. Muscimol is a high-affinity $GABA_A$ agonist that binds at the GABA binding site. Both α_1 and β_1 subunits must be present for specific muscimol binding to be detected (Pritchett et al., 1988; Pregenzer et al., 1993). Using radiolabeled muscimol, receptor expression can be quantified and the muscimol dissociation constant can be determined. We examined whether the binding of muscimol was decreased or impaired in the mutated receptors; the results are shown in Fig. 4. The data were fitted using the Michaelis-Menten equation (eq. 2). The muscimol dissociation constant (K_d) and the maximum binding values for the mutants are shown in Table 3. In cells expressing wild-type receptors (Fig. 4D), the K_d value was 39 nM, whereas in the mutant receptors, the values for the K_d ranged from 18 to 92 nM, and all could be fitted with a single hyperbolic curve (Fig. 4, A–C). The small alterations in the binding constants for the mutants did not appear to depend on whether the mutation was in the α_1 or the β_1 subunit . The maximum binding values ranged from 2.7 to 6.9 pmol/10^6 cells in the mutants with wild-type values of 5.8 pmol/10^6 cells. The lowest level of binding was observed in the double aromatic mutants: 2.7 pmol/10^6 cells for α(L9′F)β(L9′F) receptors (Fig. 4A) and 3.4 pmol/10^6 cells for α(L9′Y)β(L9′Y) receptors (Fig. 4B), suggesting a lower level of functional expression of these mutant receptors.

To further examine the level of mutated receptors in the plasma membrane, flow cytometry experiments were carried out. The level of the α_1 subunit in the plasma membrane and the total expression in the cell were determined in nonpermeabilized and permeabilized cells, respectively. Fluorescence was measured as a percent of the level for wild type in each experiment; the results are shown in Table 3 for the 9′ aromatic mutants. For receptors mutated in either the α_1 or the β_1 subunit, the level of the α_1 subunit in the plasma membrane and the total cell expression were similar to those measured in wild-type receptors. The total α_1 in cells expressing either α(L9′F)β(L9′F) or α(L9′Y)β(L9′Y) receptors was again similar or somewhat reduced from wild-type levels, whereas the plasma membrane expression was significantly decreased and was only 51 and 60% of the wild-type level, respectively (see Table 3). The decrease in the α_1 expression was specific for the plasma membrane as a similar decrease was not measured for the total cell α_1 expression. The change in expression level therefore cannot be explained by the mutations somehow altering the ability of the antibody to recognize the receptors. These results are consistent with a significant reduction in the maximum muscimol binding that was observed only for the double phenylalanine and tyrosine mutants. It therefore appears that in the double aromatic mutants, the reduction in peak current amplitudes can be in part accounted for by reduction in the surface expression of the receptors.

L9′A Mutated Receptors Have a High Resting Conductance. We reported previously that the L9′T mutation in either or both subunits produced constitutively active receptors resulting in a high resting cell conductance (Tierney et al., 1996). We therefore examined whether the resting conductance was affected when either the aromatic residues or alanine replaced leucine at the 9′ position. The data are shown in Fig. 5. In wild-type receptors, the resting conductance was about 4 nS. In cells expressing receptors containing either the L9′F or L9′Y mutation in the α_1 subunit , the β_1 subunit, or both subunits, the average resting membrane conductance ranged from 4 to 7 nS and was not significantly different from that in cells expressing wild-type receptors. In cells expressing L9′A receptors mutated either in the β_1 subunit or both subunits, however, the resting membrane conductance was greatly increased [due to difficulties in isolating recombinant α(L9′A)β virus, no data were obtained on the α-only mutant]. In cells expressing the $\alpha\beta$(L9′A) receptors, it was about 26 ± 4 nS (n = 10), and in cells expressing α(L9′A)β(L9′A) receptors, it was about 38 ± 3 nS (n = 8). This is similar to the resting conductance induced by the L9′T mutation when expressed in the *Sf*9 cells (Tierney et al., 1996).

TABLE 2

GABA dose-response relationship

Dose-response data were fitted using eq. 1. EC_{50} is the concentration of GABA that gave a half-maximal current response, and h is the Hill coefficient. r^2 is the coefficient of determination for the curve fits to each set of data, and n is the number of cells used to construct each curve.

Receptor	EC_{50}	h	r^2	n
	μM			
WT[a]	11 ± 2	1.3		12
α(L9′F)β	57 ± 5	0.7 ± 0.1	0.99	17
$\alpha\beta$(L9′F)[b]	11 ± 9	1.1 ± 0.7	0.99	25
	1131 ±	1.3 ± 0.2		
α(L9′F)β(L9′F)	12 ± 4	0.8 ± 0.4	0.95	6
α(L9′Y)β	25 ± 2	0.9 ± 0.1	0.99	13
$\alpha\beta$(L9′Y)[b]	17 ± 26	1.2 ± 1.1	0.99	24
	2453 ± 10	0.9 ± 0.5		
α(L9′Y)β(L9′Y)	N.D.			
$\alpha\beta$(L9′A)	8 ± 2	1.0 ± 0.3	0.97	6
α(L9′A)β(L9′A)	N.D.			
α(L9′T)β[c]	5.0	1.2		

N.D., not determined due to the small amplitude of current responses.
[a] From Birnir et al., 1995.
[b] Dose-response data for some receptors were best fitted using a sum of two Hill equations.
[c] From Tierney et al., 1996.

Discussion

Although the leucine at the 9′ TM2 location has received considerable attention in studies of the nACh and $GABA_A$ receptors, results are conflicting regarding subunit-specific

effects when the L9′ is replaced (Revah et al., 1991; Yakel et al., 1993; Filatov and White, 1995; Labarca et al., 1995; Chang et al., 1996; Tierney et al., 1996; Xu and Akabas, 1996; Mihic et al., 1997; Pan et al., 1997; Chang and Weiss, 1998). Furthermore, it often is not known whether the effects observed are associated with the TM2 9′ location itself or whether they are specifically related to the properties of the replacement amino acid. The second transmembrane region has been shown to be important in ion permeation, but its role in activation and desensitization of the receptors is still unclear. We have compared the effects of leucine, phenylalanine, tyrosine, threonine, and alanine at the 9′ TM2 location on the functional properties of the $\alpha_1\beta_1$ receptor and examined whether consistent subunit-specific effects were observed. The α- and β-subunits are the basic building blocks of all heteromeric $GABA_A$ receptors, whereas other types of subunits in the receptors vary (i.e., γ, δ, ϵ, χ, and ρ). Homomeric α_1 or β_1 receptors are not formed in the *Sf*9/baculovirus system and therefore do not contribute to the results (Birnir et al., 1992). The amino acids we used vary in size, hydrophobicity, and side chain properties. The aromatic residues phenylalanine (F) and tyrosine (Y) are larger in size than leucine, whereas alanine (A) and threonine (T) are smaller. Because of the aromatic ring, both phenylalanine and tyrosine have polar characteristics, although they are considered to be hydrophobic (Dougherty, 1996). Tyrosine and threonine both have a hydroxyl group on their side chain.

Effects of Mutations in α_1 Subunit

Current Decay. The size and hydrophobicity of the amino acid at the 9′ α_1 TM2 position in $\alpha_1\beta_1$ $GABA_A$ receptors determine the rate of desensitization of the receptors whether in the α_1 subunit only ($\alpha*\beta$) or together with the β_1 mutation ($\alpha*\beta*$, see Table 4). The following sequence de-

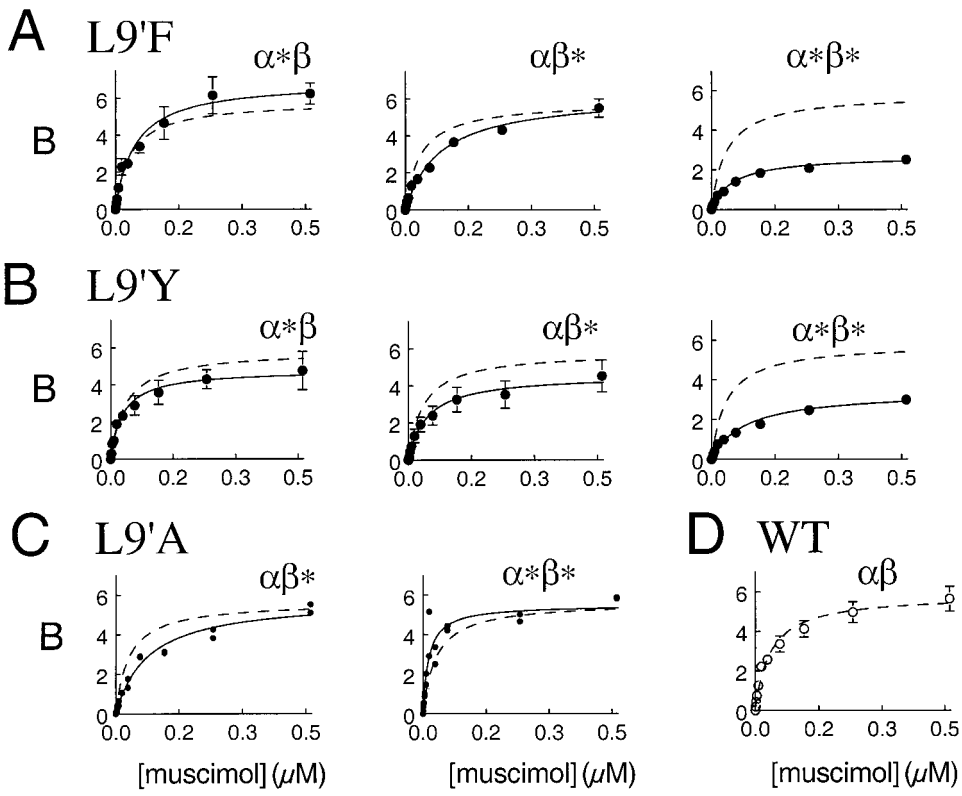

Fig. 4. [^{3}H]Muscimol binding to L9′ mutants. Cells were assayed 42 to 48 h after infection for binding of [^{3}H]muscimol. Cells were incubated in a range of muscimol concentrations (1–512 nM) containing 10% [^{3}H]muscimol and 90% nonlabeled muscimol for 30 min at 4°C. Data were fitted by the Michaelis-Menten equation for a single class of binding site. The broken line represents the binding curve for wild-type (WT) receptors obtained from the data shown in D. A, α(L9′F)β, $\alpha\beta$(L9′F), and α(L9′F)β(L9′F). B, α(L9′Y)β, $\alpha\beta$(L9′Y), and α(L9′Y)β(L9′Y). C, $\alpha\beta$(L9′A) and α(L9′A)β(L9′A). The data points are the averages of 6 to 16 measurements except in C, where the individual data points from two experiments for both mutants are shown. The vertical bars are shown if larger than the symbol and represent ± 1 S.E. for six or more measurements. B is the amount of [^{3}H]muscimol-bound (pmol/10^6 cells). The maximum binding values (B_{max}) and the dissociation constants (K_d) are given in Table 3.

TABLE 3

Muscimol binding and receptor expression at the cell surface

Muscimol binding parameters were calculated using eq. 2 for a single class of binding sites. K_d is the muscimol dissociation constant, and B_{max} is maximal binding (pmol/10^6 cells). Values represent the mean ± S.E. for $n \geq 3$. For detection of α_1 in the plasma membrane and within the cell, *Sf*9 cells were labeled with the α_1-specific monoclonal antibody, bd24, and the fluorescence of a FITC-conjugated secondary antibody was detected by flow cytometry. The level of plasma membrane (E_{PM}) and the total cell (E_{TC}) immunofluorescence is expressed as a percent of wild-type ($\alpha\beta$) immunofluorescence. The number of experiments carried out is shown in parentheses.

Receptor	K_d	B_{max}	E_{PM}	E_{TC}
	nM		%	
$\alpha\beta$ (WT)	39 ± 5 (16)	5.8 ± 0.2 (16)	100	100
α(L9′F)β	52 ± 9 (6)	6.9 ± 0.3 (6)[a]	109 ± 9 (5)	98 ± 6 (5)
$\alpha\beta$(L9′F)	92 ± 13 (8)[a]	6.2 ± 0.3 (8)	84 ± 5 (5)	101 ± 15 (5)
α(L9′F)β(L9′F)	55 ± 6 (8)[a]	2.7 ± 0.3 (8)[a]	51 ± 5 (10)	75 ± 6 (10)
α(L9′Y)β	32 ± 5 (6)	4.8 ± 0.2 (6)[a]	106 ± 17 (5)	110 ± 8 (5)
$\alpha\beta$(L9′Y)	49 ± 7 (6)	4.6 ± 0.2 (6)[a]	83 ± 8 (5)	105 ± 12 (5)
α(L9′Y)β(L9′Y)	88 ± 14 (6)[a]	3.4 ± 0.2 (6)[a]	60 ± 2 (5)	82 ± 7 (5)
$\alpha\beta$(L9′A)	77, 96	5.6, 6.0		
α(L9′A)β(L9′A)	13, 23	6.0, 6.4		

[a] Values that are significantly different from wild type (WT), $P \leq .05$.

scribes the effect on the rate of current decay of the 9′ α_1 residue: F, Y > L > T, A. Although similar trends have been described for the homomeric α_7 nACh, 5-hydroxytryptamine type 3, and ρ_1 $GABA_A$ receptors (Revah et al., 1991; Yakel et al., 1993; Chang and Weiss, 1998), subunit-dominant effects on current decay in heteromeric $GABA_A$ receptors have not been reported previously.

Current Amplitude. The α-dominant effect on the peak current amplitude was strong and has not been demonstrated previously. In $\alpha*\beta$ and $\alpha*\beta*$ receptors, the peak current amplitude was much smaller than in wild-type receptors (see Table 4). The reason for the reduction in current amplitude is not clear. In some mutants, it can be in part accounted for by a reduction in the expression of the receptors (e.g., L9′Y and L9′T). In others, the increased rate of desensitization (e.g., L9′F and L9′Y) possibly makes detection of the true current amplitude difficult to detect. However, the L9′A double mutant did not desensitize and was expressed similar to wild type. These results suggest that some other property of the ion channel has been affected by mutating the L9′ in the α-subunit. In the homomeric α_7 (L9′F) nACh receptors, there also was a reduction in the peak current amplitude (Revah et al., 1991), but it was not reported whether expression of the mutant receptors had decreased relative to wild-type receptors.

Effects of Mutations in β_1 Subunit

That the side chain properties of the amino acid at the 9′ location affect the apparent affinity for GABA was clear only in the $\alpha\beta*$ mutants (see Table 4). The apparent affinity for GABA followed the sequence L, A > F, Y ≫ T and had decreased about 100-fold in the aromatic mutants compared with wild-type receptors. In rat L9′S $\alpha1\beta2\gamma2$ $GABA_A$ receptors, the largest shift in the GABA EC_{50} value was also observed when the β_2 subunit was mutated (Chang et al., 1996). The change was an increase in the apparent affinity compared with the decrease recorded in $\alpha\beta*$ receptors. Unlike the $\alpha\beta$ receptors, the shift in the EC_{50} depended on the number of mutated subunits and saturated when three or more subunits carried the L9′S mutation (Chang and Weiss, 1999). In homomeric ρ_1 $GABA_A$ receptors, no shift in the EC_{50} value was reported when the L9′ was mutated (Chang and Weiss, 1998) similar to $\alpha_1\beta_1$(L9′A) and the $\alpha*\beta*$ receptors. It is surprising that the degree and direction of change in the apparent affinity of the receptors depend not only on the amino acid that replaces the leucine but also on the subunit that carries the mutation and the $GABA_A$ receptor subtype. It is not known whether ligand binding is affected in the mutant $\alpha_1\beta_2\gamma_2$ and ρ_1 $GABA_A$ receptors (Chang et al., 1996; Chang and Weiss, 1998), but the $\alpha\beta*$ $GABA_A$ receptors still bind muscimol similar to wild-type receptors. This may suggest that the conformational changes leading to gating of the receptors have been affected rather than GABA binding itself.

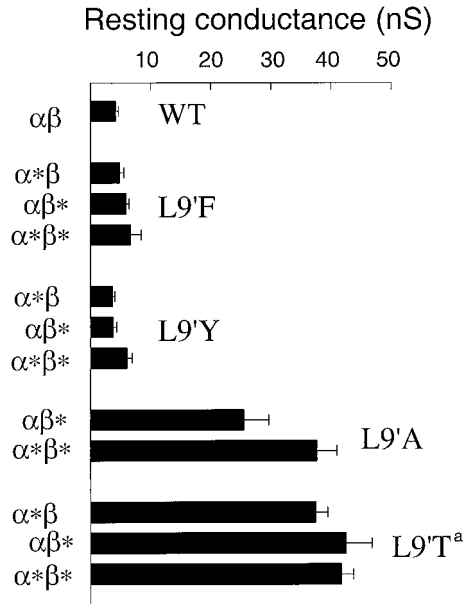

Fig. 5. Resting membrane conductance in the L9′ mutants. Leak currents were measured in the absence of GABA. Each column represents the average current ± 1 S.E. from 8 to 66 measurements. a, current values for L9′T mutants are from Tierney et al., 1996. WT, wild type.

The high-affinity component of the $\alpha\beta*$ aromatic mutants' concentration-response curves was associated with about 20% of the saturating peak current amplitude. Homomeric receptors are not functional in the *Sf*9/baculovirus expression system (Birnir et al., 1992) and therefore cannot be used to explain the presence of either the low- or high-affinity component. If the L9′ $\alpha\beta*$ aromatic mutations have caused a change in the subunit organization of the receptors, it may have resulted in the appearance of either the high- or low-affinity receptors. Perhaps it is more likely simply that in 20% of the receptors, the effects of the mutation were compensated for by some structural changes during folding or assembly and resulted in an apparent wild-type EC_{50} value. The large shift in the EC_{50} values associated with 80% of the saturating peak current in the aromatic $\alpha\beta*$ mutants was compensated for when the mutation was also present in the α-subunit ($\alpha*\beta*$), supporting the idea of a specific, coordinated interaction between the α- and β-subunits during channel activation.

Changing residues at other TM2 locations has generally not changed the EC_{50} value of the $\alpha_1\beta_1$ mutated receptors. Mutations at TM2 locations 5′, 6′, 10′, 12′, and 13′ did not change the GABA EC_{50} value of functional receptors (Birnir et al., 1997a,b; Cromer, 1998; Tierney et al., 1998; Dalziel et al., 1999). However, in a β-dominating manner, T13′A abolished and T12′Q modified GABA activation of the receptors. Although agonist binding is thought to involve both the α- and β-subunits (Sigel et al., 1992; Amin and Weiss, 1993), our results may suggest that the β_1 subunit of $\alpha_1\beta_1$ $GABA_A$ receptors transmits conformational changes to the membrane-spanning regions that result in gating of the receptor, a role analogous to that proposed by Unwin (1995) for the α-subunit of the muscle nACh receptor.

Subunit-Independent Effects: Constitutive Activity

Effects on channel opening in the absence of GABA were independent of the subunit in which the L9′ mutation was located (see Table 4). The increase in resting membrane conductance in cells expressing L9′A or L9′T receptors but lack of change in those expressing L9′Y or L9′F receptors indicate that the size of the 9′ residue is important for constitutive activity. The results also show that the presence of an hydroxyl group (L9′Y) is insufficient to increase the resting conductance. These effects of the L9′ mutants are somewhat similar to those reported in a study by Chang and Weiss

TABLE 4

Summary of effects of L9′ mutations on properties of $\alpha_1\beta_1$ receptors

T_{10-90}, time it takes for the whole-cell current to increase from 10 to 90% of the peak current amplitude; T_{50}, time it takes for the whole-cell current to decay 50%; P_I, peak current response to millimolar concentrations of GABA; EC_{50}, GABA concentration that gave half-maximal activation; K_d, muscimol dissociation constant; B_{max}, maximum muscimol binding; constutive I, leak current in the absence of GABA; −, a decreased value or faster current decay than wild-type receptors; +, an increased value or slower current decay than wild-type receptors; N.R., no current response; N.C., similar to wild-type receptors. Symbols qualitatively represent the values shown in Tables 1, 2, and 3.

	α-Only Mutation			β-Only Mutation				α + β Mutations			
	F $\alpha*\beta$	Y $\alpha*\beta$	T[a] $\alpha*\beta$	F $\alpha\beta*$	Y $\alpha\beta*$	A $\alpha\beta*$	T[a] $\alpha\beta*$	F $\alpha*\beta*$	Y $\alpha*\beta*$	A $\alpha*\beta*$	T[a] $\alpha*\beta*$
T_{10-90}	N.C.	N.C.	++++	N.C.	N.C.	N.C.	N.R.	N.C.	N.C.	++++	N.R.
T_{50}	−−−	−−−	+++	N.C.	N.C.	+++	N.R.	−−−	−−−	++++	N.R.
P_I	−−−	−−−	−−−	N.C.	−	−−	N.R.	−−−	−−−−	−−−−	N.R.
EC_{50}	+	+	N.C.	++++	++++	N.C.	N.R.	N.C.	N.C.	N.C.	N.R.
K_d	N.C.	N.C.	−	+	N.C.	+	−	N.C.	+	−	−
B_{max}	N.C.	−	−−	N.C.	−	N.C.	−−	−−	−−	N.C.	−−
Constitutive I	N.C.	N.C.	++++	N.C.	N.C.	++++	++++	N.C.	N.C.	++++	++++

[a] From Tierney et al., 1996.

(1998) in human ρ_1 $GABA_A$ receptors in which ρ_1(L9′T), ρ_1(L9′Y), and ρ_1(L9′A) receptors had increased resting current. The reason for the differences in constitutive activity between α_1(L9′Y)β_1(L9′Y) and ρ_1 (L9′Y) receptors is not clear but suggests structural differences between homomeric and heteromeric receptors. Residues mutated at other locations in the membrane-spanning regions of $GABA_A$ receptors have also been reported to increase the resting membrane conductance (Mihic et al., 1997; Pan et al., 1997) demonstrating that L9′ is not a unique site for this effect. Whether L9′ prevents ion flow across the membrane in the absence of GABA by physically occluding the ion channel or whether it contributes to stabilization of a closed conformation (Chang and Weiss, 1999) remains to be determined. Experiments by Xu and Akabas (1996) on α_1L9′C mutated $\alpha_1\beta_1\gamma_2$ $GABA_A$ receptors indicated that at least the $\alpha_1$9′C was exposed in the channel in both the closed and open conformations. Our results do not exclude the existence of a gating structure at a more intracellular location than 9′.

Conclusions

The different nature of the L9′ replacement amino acids helped determine the specific roles of the α_1 and β_1 $GABA_A$ subunits in receptor function and demonstrates the importance of restrained interpretation of results when a residue is replaced with only one type of amino acid.

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