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Effects of Mutating Leucine to Threonine in the M2 Segment of α_1 and β_1 Subunits of $GABA_A$ $\alpha_1\beta_1$ Receptors

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Abstract. The conserved leucine residues at the 9′ positions in the M2 segments of α_1 (L264) and β_1 (L259) subunits of the human $GABA_A$ receptor were replaced with threonine. Normal or mutant α_1 subunits were coexpressed with normal or mutant β_1 subunits in Sf9 cells using the baculovirus/Sf9 expression system. Cells in which one or both subunits were mutated had a higher ''resting'' chloride conductance than cells expressing wild-type $\alpha_1\beta_1$ receptors. This chloride conductance was blocked by 10 mM penicillin, a recognized blocker of $GABA_A$ channels, but not by bicuculline (100 μM) or picrotoxin (100 μM) which normally inhibit the chloride current activated by GABA: nor was it potentiated by pentobarbitone (100 μM). In cells expressing wild-type β_1 with mutated α_1 subunits, an additional chloride current could be elicited by GABA but the rise time and decay were slower than for wild-type $\alpha_1\beta_1$ receptors. In cells expressing mutated β_1 subunits with wild-type or mutated α_1 subunits ($\alpha\beta$(L9′T) and α(L9′T)β(L9′T)), no response to GABA could be elicited: this was not due to an absence of $GABA_A$ receptors in the plasmalemma because the cells bound [^{3}H]-muscimol. It was concluded that in $GABA_A$ channels containing the L9′T mutation in the β_1 subunit, GABA-binding does not cause opening of channels, and that the L9′T mutation in either or both subunits gives an open-channel state of the $GABA_A$ receptor in the absence of ligand.

Key words: $GABA_A$ receptors — Subunits — Mutations — Baculovirus — Sf9 cells — Whole-cell current

Introduction

Rapid signaling in the mammalian central nervous system is mediated by a family of neurotransmitter receptors that includes the inhibitory γ-aminobutyric acid type A ($GABA_A$) and glycine receptors, and the excitatory nicotinic acetylcholine (nACh) and 5-hydroxytryptamine$_3$ (5-HT_3) receptors (Schofield et al., 1987; Barnard, Darlison & Seeburg, 1987; Maricq et al., 1991). Homology at the primary sequence level and conserved structural features have led to the proposal that members of this family of receptors form similar topological arrangements in the membrane and that the structures of their ion channels are very similar (Unwin, 1989). Site-directed mutagenesis studies (Imoto et al., 1986; Imoto et al., 1991; Leonard et al., 1988; Charnet et al., 1990; Revah et al., 1991; Villarroel et al., 1991; Villarroel et al., 1992; Cohen et al., 1992; Akabas et al., 1992; Akabas et al., 1994) and photoaffinity labeling (Giraudat et al., 1986; Giraudat et al., 1987; Hucho, Oberthur & Lottspeich, 1986; Revah et al., 1990; White & Cohen, 1992) of the nicotine acetylcholine receptor have implicated the second hydrophobic region, M2, in forming the ion channel. Electron diffraction images of the *Torpedo* acetylcholine receptor at 9Å resolution depict a single kinked transmembrane helix contributed by each of the five subunits (Unwin, 1993). It has been suggested that the helix is M2 and that the side chain of the conserved leucine residues, located at the point where the helix bends, forms the channel gate (Unwin, 1993; Unwin, 1995).

Most subunits of the receptors in this family contain a leucine residue in M2 at the 9′ position about halfway across M2 (Miller, 1989). Mutation of this 9′ leucine residue to one of several other residues has been shown to have striking effects on desensitization and on the sensitivity of the receptor to agonists and antagonists (Revah et al., 1991; Yakel et al., 1993; Labarca et al., 1995; Filatov & White, 1995). The first report of effects of mutating this 9′ leucine residue described experiments on homomeric α7 neuronal nACh receptors (Revah et al., 1991). Replacing the 9′ leucine residue with polar amino acids, serine or threonine, slowed the rate of decay

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of the whole-cell current activated by acetylcholine and increased the apparent agonist affinity (EC_{50}) approximately 160-fold. Similarly, the homomeric 5-HT_3 receptor containing a mutation at the 9′ position in M2 also displayed a change in the rate of desensitization which was slowed by the introduction of polar amino acids (Yakel et al., 1993). The apparent ligand affinity of the mutant 5-HT_3 receptors was also increased, but by less than one order of magnitude, and the change was sensitive to the nature of the replacement amino acid. In the heteromeric nAChR, the introduction of a serine residue at the 9′ position in M2 increased the EC_{50} approx imately 10-fold for each additional mutant subunit in the pentameric nACh receptor (Labarca et al., 1995). Whole-cell currents displayed a slower rate of desensitization when receptors contained two or more mutated subunits per receptor complex. Single channels activated by acetylcholine in oocytes expressing the homomeric α7 L9′T mutant ACh receptor displayed a higher conductance at low agonist concentrations (<3 μM) (Revah et al., 1991): in contrast, no alteration in the single-channel conductance of heteromeric nAChR L9′S mutant receptors was detected (Labarca et al., 1995). There have been no reports of the effects of mutating the 9′ leucine in the M2 segment of subunits of the $GABA_A$ receptor.

Using the baculovirus-insect cell expression system, we have examined the effects of replacing the M2 9′ leucine residue with threonine in receptors formed by a combination of mutated and nonmutated α_1 and β_1 subunits of the human $GABA_A$ receptor. Threonine was substituted for leucine because it has been shown that a polar residue at this position produces significant changes in the function of cation-selective ligand-gated ion channels in this family of receptors (Revah et al., 1991; Yakel et al., 1993; Labarca et al., 1995; Filatov & White, 1995). We have found that the mutation has subunit-specific effects on the response to GABA and produces receptors that form open chloride channels in cells not exposed to GABA.

Materials and Methods

Construction of Plasmids and Isolation of Recombinant Baculoviruses

The human $GABA_A$ α_1 and β_1 cDNA sequences (Schofield et al., 1989) were subcloned into the dual promoter baculovirus transfer vector pAcUW31 (Clontech), as described previously (Birnir et al., 1995). The two mutations previously present in the α_1 subunit (I121V and I290F) were corrected and the α_1 protein could now be detected in the plasma membrane of Sf9 cells without expression of the β_1 subunit (*see* Table 1) whereas previously it was detected in the cytoplasm but not in the plasma membrane (Birnir et al., 1992). This could have been due to the change in promotor (P10 instead of polyhedrin), vector (pAcUW31 instead of the pBlueBacII) or the presence of the mutations. The α_1 cDNA was inserted under the control of the P10 promoter and the β_1 cDNA under the polyhedrin promoter in all pAcUW31 plasmid constructs described. These promoters are of similar strength and both are active in the very late phase of the viral infection cycle. Site-specific mutations were introduced into single-stranded DNA, prepared from M13 phage constructs, using the Amersham Sulptor™ *in vitro* mutagenesis system. Mutations were confirmed by single-stranded DNA sequencing. Mutant α_1 fragments were subcloned as 1.3 kb *Bgl* II/*Eco* R1 replicative form (RF) fragments into the compatible cloning site in the plasmid β_1pAcUW31 while mutant β_1 RF fragments were subcloned as 0.9 kb *Nco* 1/*Nhe* 1 fragments into *Nco* 1/*Nhe* 1 digested $\alpha_1\beta_1$pAcUW31 plasmid DNA. A plasmid carrying the mutated α_1 subunit alone was also constructed. The authenticity of the final plasmid constructs was confirmed by double stranded sequencing across the mutated region of the insert DNA and by restriction enzyme digestion of the plasmid. The final constructs which contained threonine instead of leucine at position 264 of the mature α_1 protein and/or at the equivalent position in the β_1 protein (position 259) were designated α(L9′T)β, αβ(L9′T) and α(L9′T)β(L9′T). Cells infected with viruses bearing these constructs are called α(L9′T)β, αβ(L9′T) and α(L9′T)β(L9′T) cells, respectively.

Sf9 (*Spodoptera frugiperda*) cells were cultured in spinner bottles in complete TNM-FH insect media (Sigma) at 25°C, as described previously (Birnir et al., 1995). The production, isolation and propagation of recombinant viruses was as described previously (Birnir et al., 1995) after Summers and Smith (1988) and King and Possee (1992). Recombinant viruses were produced using the lipofection-based method (Felgner et al., 1987) to cotransfect insect cells with transfer plasmid DNA and a linearized deleted form of the wild-type parent *Autographa californica* nuclear polyhedrosis virus (AcNPV) (BakPak 6, Clontech). Recombinant viral isolates were screened for the expression of the $GABA_A$ α_1 protein by Western blot analysis using the α_1-specific monoclonal antibody bd24 (Haring et al., 1985; Ewert et al., 1990). If Sf9 cells infected with a recombinant virus displayed [^{3}H]-muscimol binding, the presence of a β_1 subunit was assumed as singly expressed subunits do not bind muscimol with high affinity (Pritchett et al., 1988; Pregenzer et al., 1993; Tierney, 1995). In general, two recombinant isolates, derived from each cotransfection assay, were analyzed to determine the level of plasma membrane expression relative to the level for wild type ($\alpha_1\beta_1$) receptors. One isolate was further tested for [^{3}H]-muscimol binding and electrophysiological properties.

Flow Cytometry

Sf9 cells infected with the appropriate recombinant virus at a multiplicity of infection (MOI) > 10 plaque-forming units (pfu)/cell were harvested at about 28 to 30 hr post infection (hpi) by centrifugation at 1,000 rpm for 10 min. Cells were washed twice in 10 mM phosphate buffer pH 6.2 containing 0.9% NaCl (PBS) with intervening slow speed (1,000 rpm) centrifugation steps. The samples were fixed in Zamboni's solution (2% formaldehyde, 15% picric acid in 0.1 M phosphate buffer, pH 7.4) for 90 min and then washed as above. For cytoplasmic protein detection, cells were permeabilized in PBS buffer containing 0.1% SDS plus 1.0% BSA for 90 min at room temperature. SDS and non-SDS treated samples were then incubated overnight at room temperature in the primary monoclonal antibody bd24. The bd24 monoclonal antibody epitope has been partially mapped to the first four amino-terminal residues of the mature protein (Ewert et al., 1990); in the membrane assembled receptor these residues are located extracellularly. Secondary antibody labeling was subsequently performed for 60 min in the dark using a fluoroscein isothiocyanate (FITC) conjugated sheep anti-mouse Ig antibody (Selinus) diluted fortyfold in PBS.

The cell pellet following washout of the secondary antibody, as described above, was resuspended in 0.4 ml PBS. Samples were analyzed on a FACStar Plus flow cytometer (Becton Dickinson). Results were analyzed with the WinMDI computer program (courtesy of Joseph Trotter, Scripps Cancer Research Institute) run on an IBM-compatible computer. Data were displayed as histograms of fluorescence intensity (*x*-axis) *vs.* relative cell number (*y*-axis).

[^{3}H]-muscimol Binding to $GABA_A$ Receptors on the Cell Surface

Sf9 cells (1.4×10^7 cells) were seeded into 75-cm^2 tissue culture flasks (Corning) from a spinner culture growing at $0.6–1.0 \times 10^6$ cells/ml. The monolayer of cells was infected at an MOI > 5 pfu/cell. The experimental protocol for the binding of [^{3}H]-muscimol to cell surface receptors was based on the procedure described elsewhere (Kawamoto et al., 1991). At 42–50 hpi, the infected cells were harvested by centrifugation at 1,000 rpm for 10 min and washed in 30 mM Tris-HCl (pH 7.2), 2.5 mM $CaCl_2$ and 0.04% Triton X-100 at room temperature for 20 min. Following a second centrifugation step (1,000 rpm, 10 min) cells were resuspended in the binding buffer (containing in mM) 180 NaCl, 1 $MgCl_2$, 1 $CaCl_2$ and 10 MES, pH 6.2 at a final density of 1.5×10^6 cells/ml. [^{3}H]-muscimol binding was measured in duplicate at ten different concentrations over a radioligand range from 5 to 200 nM. Aliquots of 0.6×10^6 cells were dispensed into sterile eppendorfs and diluted radioligand added to a final volume of 0.5 ml. After incubation at 4°C for 30 min, bound radioactivity from a 0.2-ml sample aliquot was collected on 5-μm filters (Millipore) using a fast filtration apparatus. The filters were washed with 2 ml of ice-cold binding buffer and the radioactivity counted in a Packard scintillation counter. Data were presented as fmol of [^{3}H]-muscimol bound per 10^6 cells or normalized to the B_{max} value obtained by fitting the equation for a single class of binding site to the data. Nonspecific binding was estimated from the amount of [^{3}H]-muscimol bound to Sf9 cells infected with the wild-type parent baculovirus (AcNPV) assayed under identical conditions. This level of background radioactivity was subtracted from the total counts to give specific counts. Specific binding data were plotted using the software Prism™ (version 1.03, San Diego, CA) and the binding parameters calculated from the hyperbolic curves fitted for a single class of binding sites according to the equation: Bound = $B_{max} \times [A]/(K_d + [A])$ where B_{max} is the maximum level of [^{3}H]-muscimol bound (fmol bound per 10^6 cells), [A] the concentration of radioligand added and K_d the dissociation constant.

Electrophysiology

Currents were recorded from Sf9 cells using standard whole-cell, tight-seal recording techniques (Hamill et al., 1981). Patch electrodes were made from borosilicate glass (GC150F-15, Clark Electromedical Instruments, Reading, UK) and had a resistance of 5 to 10 MΩ after fire-polishing and filling with pipette solution. The ground electrode was a 3 M KCl agar bridge connected to a chlorided silver wire. Current was monitored with a current-to-voltage converter (Axopatch 200, Axon Instruments, Foster City, CA) using series resistance compensation. Signals were filtered at 1 kHz, digitized at 44 kHz using a Sony PCM and stored on videotape for later analysis. Pipettes were filled with a solution containing (mM): 180 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 EGTA, 10 TES (N-tris[Hydroxy]methyl-2-aminoethanesulfonic acid), pH 7.2 (335 m. osmoles/l). In some experiments, 4 mM ATP was added to the pipette solution. Cells were continuously perfused with bath solution (2–4 ml/min) containing (mM): 180 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 MES (2-[N-morpholino]ethanesulfonic acid) pH 6.2 (330 m. osmoles/l). In some experiments (*see* Results and figure legends) 150 mM of the NaCl in the extracellular solution was replaced by 150 mM Na gluconate or 160 mM choline chloride to give solutions with a $[Cl^-]_o$ of 34 mM or $[Na^+]_o$ of 30 mM. GABA and other drugs were dissolved in the bath solution and rapidly applied to cells by gravity feed through a stainless steel delivery tube as described previously (Birnir et al., 1995). Experiments were carried out at room temperature (19 to 22°C).

Data are given as the mean ± 1 SEM. Numbers in brackets indicate the number of experimental determinations, *n*.. The Student's *t*-test was used to determine the significance of differences between means.

Results

The aim of this study was to determine the effects of the mutation of leucine to threonine at the 9′ position in the M2 segment of α_1 and β_1 subunits of the $GABA_A$ receptor. As both α_1 and β_1 subunits are required to form functional receptors activated by GABA in Sf9 cells (Birnir et al., 1995), the effects of the mutation in combinations of α_1 and β_1 subunits of the $GABA_A$ receptor were tested. Three different recombinant viruses were prepared to give the three possible combinations of mutant and wild-type α_1 and β_1 subunits so that any subunit-specific effects could be evaluated. Hence, the 9′ leucine residue in M2 was replaced by threonine in the α_1 subunit and expressed with the wild-type β_1 subunit, the mutated β_1 subunit was expressed with the wild-type α_1 subunit, and both subunits carrying the L9′T mutation were co-expressed. Sf9 cells infected with these recombinant viruses are called α(L9′T)β, αβ(L9′T) and α(L9′T)β(L9′T) cells, respectively. In addition to the double subunit constructs, a recombinant virus containing the cDNA for only the α_1 subunit mutant (α(L9′T)) was prepared to test for the possible formation of α(L9′T) homomeric receptors and channels.

Only α(L9′T)β Cells Respond to GABA

In cells in which one or both of the subunits contained the L9′T mutation, a current could be generated by GABA only in α(L9′T)β cells. No response to GABA was obtained in 49 cells expressing αβ(L9′T) receptors or in 66 cells expressing α(L9′T)β(L9′T) receptors. A typical current elicited by GABA in an α(L9′T)β cell is shown in Fig. 1*A*. In this experiment, the membrane potential was stepped to −40 mV several seconds before applying the GABA.

The response in the α(L9′T)β cell is shown together with a typical current generated by GABA in a cell expressing wild-type (WT, $\alpha_1\beta_1$) receptors to illustrate the much slower time course of the current in an α(L9′T)β cell. The difference in early time course can be seen more easily in Fig. 1*B*. In 9 α(L9′T)β cells, the average rise time of the current (10 to 90% of the peak) generated by 100 μM GABA was 116 ± 15 msec, much slower than

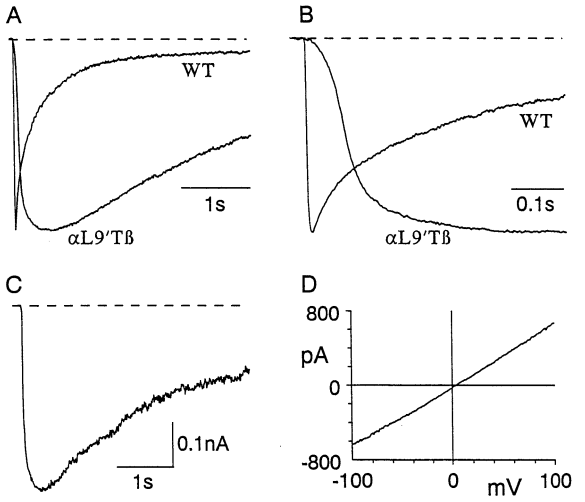

Fig. 1. Chloride currents activated by GABA in cells expressing α(L9′T)β receptors. (*A*) Comparison of the time course of currents elicited by 100 μM GABA in a cell expressing α(L9′T)β receptors and in a cell expressing $\alpha_1\beta_1$ receptors (WT) shown for comparison. The current in the WT cell has been scaled to the same amplitude as in the α(L9′T)β cell to illustrate the difference in time courses. Cells were held at −40 mV during application of the GABA. (*B*) The traces in A are shown on an expanded time base to emphasize the difference in rise times. (*C*) Current elicited in a cell expressing α(L9′T)β receptors by a solution containing 100 μM GABA plus 34 mM Cl^-. The membrane potential was held at 0 mV. (*D*) The current-voltage relationship obtained in one experiment during the decay phase of a current such as that in A by changing the membrane potential in 1-mV steps from −100 to +100 mV in 1 sec.

the rise time of 8.0 ± 1.1 msec for $\alpha_1\beta_1$ receptors in Sf9 cells (Birnir et al., 1995). The decay was also much slower than for $\alpha_1\beta_1$ receptors but was difficult to measure accurately because of a large resting chloride current in these cells (*see below*) that appeared to cause a change in chloride equilibrium potential (E_{Cl}) during the responses to GABA. This would tend to artificially accelerate the decay of the current activated by GABA. Nevertheless, it was quite clear that the decay of the response to GABA was always much slower in α(L9′T)β cells than in cells expressing $\alpha_1\beta_1$ receptors (Fig. 1*A*). In five cells in which the decay of the response to GABA did not appear to be significantly contaminated by a change in E_{Cl}, the current took 1.1 ± 0.18 s to decay by 20% from the peak.

In several experiments, an attempt was made to minimize movements of chloride ions before application of GABA. The membrane potential was held at 0 mV (the equilibrium potential for all the ions in the symmetrical solutions used) and a solution containing a lowered Cl^- concentration ($[Cl^-]$) (gluconate substituted for chloride) together with GABA was rapidly applied to the surface of the cell from a tube positioned close by. Any current which flowed under these conditions would be carried by chloride ions. An example of a current generated in this way in an α(L9′T)β cell is shown in Fig. 1*C*. Again, the time course of the response to GABA was obviously much slower than that of the wild-type $\alpha_1\beta_1$ response: the 10 to 90% rise time of the current in Fig. 1*C* was 90 msec and the time for decay to 80% of the peak was 1.0 sec. The generation of a current by GABA under these conditions shows that the channels activated by GABA in cells expressing the α(L9′T)β receptor were chloride channels. In contrast to the channels formed by $\alpha_1\beta_1$ receptors (Birnir et al., 1995), the channels did not rectify (Fig. 1*D*).

No response to GABA could be elicited with GABA concentrations as high as 10 mM in cells infected with recombinant viruses containing the other heteromeric constructs (αβ(L9′T) and α(L9′T)β(L9′T)), or in cells infected with the single α(L9′T) construct. The lack of response in the α(L9′T) cells indicates that the response to GABA in α(L9′T)β cells was not due to homomeric α(L9′T) receptors that might possibly have formed.

The lack of response of αβ(L9′T), α(L9′T)β(L9′T) and α(L9′T) cells to GABA could have been due to a defect in recombinant protein synthesis, failure to insert subunits in the plasma membrane, or the lack of binding of GABA to a site linked to channel opening. These possibilities were therefore tested.

Detection of α_1 Subunits in the Plasma Membrane of Sf9 Cells

Flow cytometry was used together with a fluorescently labeled antibody to the α_1 subunit to demonstrate expression of α_1 or α(L9′T) subunits at the cell surface. The relative fluorescence levels in cells infected with the different L9′T recombinant viruses were compared with the fluorescence level in cells expressing the wild-type $\alpha_1\beta_1$ receptor. In α(L9′T)β, αβ(L9′T) and α(L9′T)β(L9′T) cells, surface fluorescence was decreased to about 50%, 70% and 40%, respectively, of that seen with the wild type $\alpha_1\beta_1$ receptor (Fig. 2; Table 1). When expressed alone, the α_1 protein could be detected in the insect cell plasma membrane (Table 1) but at about half the level recorded when co-expressed with the β_1 subunit. Even lower levels of protein were detected in cells expressing the α(L9′T) protein alone (Table 1).

The decrease in the amount of α_1 protein at the cell surface could have been due to a reduction in synthesis of α_1 protein within the cell or less efficient insertion of the subunit in the plasma membrane. The relative level of α_1 protein expressed in the cytoplasm was therefore measured by flow cytometry. In cells containing any of the three different L9′T mutant subunit combinations and in cells expressing only the α(L9′T) subunit, intracellular levels of the α_1 subunit were not significantly different from levels recorded in $\alpha_1\beta_1$ cells (Table 1).

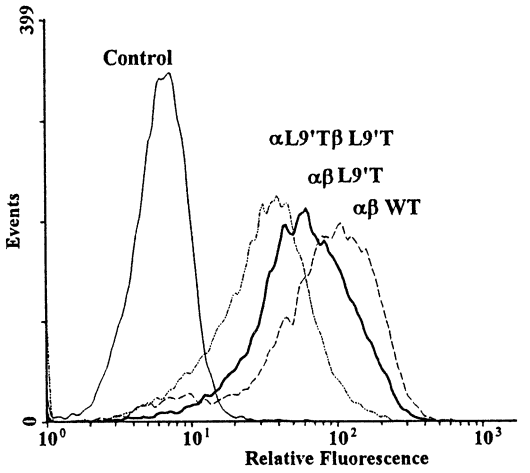

Fig. 2. Flow cytometric analysis of immunofluorescently labeled α_1 protein expressed in Sf9 cells infected with the various L9′T mutant recombinant viruses. Flow cytometry was used to measure the level of immunofluorescently labeled α_1 protein, using the monoclonal antibody bd24, in nonpermeabilized Sf9 cells ~28–30 hpi, as described in experimental procedures. Cells infected with the parent baculovirus, AcNPV, indicate the level of autofluorescence of the Sf9 cells (control). The level of fluorescence detected in cells infected with either the α(L9′T)β or α(L9′T) recombinant viruses was not significantly different from the level of fluorescence in cells infected with the α(L9′T)β(L9′T) recombinant virus (*see* Table 1).

These results reveal a reduced efficiency of insertion of the wild-type or mutated α_1 subunit into the plasma membrane when either or both of the α_1 and β_1 subunits contained the L9′T mutation. The lack of response to GABA in αβ(L9′T), α(L9′T)β(L9′T) and α(L9′T) cells could be due to the absence of the β_1 subunit in the plasma membrane required to form a functional receptor in Sf9 cells (Birnir et al., 1995) or to a defect in receptor function at a step following binding of GABA.

Muscimol binds to the Cells Expressing both α_1 and β_1 Subunits

As binding of muscimol, a high affinity GABA analog, requires the expression of both α_1 and β_1 subunits (Pritchett et al., 1988; Pregenzer et al., 1993; Tierney, 1995), [^{3}H]-muscimol binding was used to test for the presence of a receptor complex able to bind ligand and hence for the assembly of a heteromeric receptor complex. [^{3}H]-muscimol bound to cells containing any of the three combinations of mutated subunits (Fig. 3).

The dissociation constants (K_d) of 11–15 nM (Table 1) measured from the binding curves of the receptors formed from mutated subunits (Fig. 3) were approximately half the K_d measured for the wild-type receptor (29 nM, Table 1). The maximum level of [^{3}H]-muscimol bound (B_{max}) was similar for each of the three mutant combinations but significantly less than for the WT $\alpha_1\beta_1$ receptor. These results indicate that the lack of response to GABA in αβ(L9′T) and α(L9′T)β(L9′T) cells was not due to a failure to form heteromeric receptors at the cell surface.

α(L9′T)β Receptors are Activated by Lower Concentrations of GABA than $\alpha_1\beta_1$ Receptors

The relationship between peak chloride current and GABA concentration is shown for cells expressing α(L9′T)β receptors in Fig. 4. The responses from different cells were normalized to the current generated by 100 μM GABA. The response to the test concentration of GABA was expressed as a fraction of the mean of two responses to 100 μM GABA (recorded before and after the test response), but was accepted only if the two control responses differed by less than 30%.

The fit of the Hill equation to the mean responses gave an EC_{50} of 5.0 μM and a Hill coefficient of 1.2. The superimposed broken line shows the average relationship between peak chloride current and GABA concentration in wild-type $\alpha_1\beta_1$ receptors expressed in Sf9 cells (EC_{50} 11 μM, Hill coefficient 1.3 (Birnir et al., 1995). Given the size of the standard errors of the means, the difference in the EC_{50}s was not considered significant.

Effects of Drugs on the Response of α(L9′T)β Receptors to GABA

The effects of several drugs that modulate the response to GABA in $\alpha_1\beta_1$ receptors in Sf9 cells (Birnir et al., 1995) were tested on the response to GABA in α(L9′T)β cells, the only mutants that responded to GABA. Bicuculline, picrotoxin and penicillin effectively abolished the current generated by GABA. The peak current generated by 100 μM GABA was depressed 93 ± 4.8% (n = 5) by 100 μM bicuculline and 96 ± 4.2% (n = 4) by 100 μM picrotoxin. In two experiments, 10 mM penicillin depressed the response to 100 μM GABA by 97% and 100%. These results are not significantly different from the effects of these drugs on WT $\alpha_1\beta_1$ receptors (Birnir et al., 1995). However, in contrast to $\alpha_1\beta_1$ receptors, the response of α(L9′T)β receptors to 10 μM (n = 6) or 100 μM (n = 2) GABA was not potentiated by 100 μM pentobarbitone.

Cells Expressing Receptors Containing the L9′T Mutation Have a High Resting Conductance

It was noticeable that Sf9 cells expressing α(L9′T)β, αβ(L9′T) and α(L9′T)β(L9′T) subunit combinations

Table 1. Functional properties of the L9′T mutant $GABA_A$ receptors expressed in Sf9 cells

$GABA_A$ Receptor	Flow cytometric analysis		K_d (nM)[b] [^{3}H] Muscimol	B_{max} (fmol per 10^6 cells)
	Plasma membrane Expression (%)[a]	Cytoplasmic Expression (%)[a]		
αβ (WT)	100 (24)	100 (24)	29.0 ± 1.5 (8)	718 ± 67
α (WT)	54 ± 3 (10)	84 ± 6 (10)	NB	
α(L9′T)β	52 ± 9 (2)	79 ± 14 (2)	11.2 ± 0.3 (4)	297 ± 13
αβ(L9′T)	71 ± 5 (7)	97 ± 3 (7)	12.8 ± 0.7 (4)	280 ± 13
α(L9′T)β(L9′T)	42 ± 8 (5)	80 ± 5 (5)	15.1 ± 2.1 (6)	247 ± 8
α(L9′T)	39 ± 10 (5)	78 ± 2 (5)	ND	

[a] Sf9 cells infected with the appropriate recombinant virus were labeled ~28–30 hpi with the α_1-specific monoclonal antibody bd24 and the fluorescence of an FITC-conjugated secondary antibody detected by flow cytometry. The level of plasma membrane and cytoplasmic immunofluorescence is expressed as a percentage of the wild type $\alpha_1\beta_1$ receptor level assayed in parallel. [b] Binding parameters were calculated for a single class of binding sites. All values represent the mean ± the standard error of the mean and the number of determinations is indicated in brackets. ND: not determined. NB: no binding.

had a significantly higher resting conductance than cells expressing $\alpha_1\beta_1$ receptors. Typical currents generated by a voltage step from 0 mV to −40 mV in cells expressing either α(L9′T)β or $\alpha_1\beta_1$ receptors are shown in Fig. 5, *A* and *C*, respectively. The current in the cell expressing α(L9′T)β receptors (Fig. 5*A*) was clearly much larger than in the cell expressing $\alpha_1\beta_1$ receptors (Fig. 5*C*).

The current recorded from the cell expressing α(L9′T)β receptors had an amplitude of about 1 nA. Whole-cell conductance was 27.5 nS and the null (zero current) potential was close to 0 mV (Fig. 5*G*). Sf9 cells expressing the three different combinations of mutant subunits displayed resting conductances of similar magnitude when measured in this manner (Table 2). It appeared that receptors containing the L9′T mutation increased the conductance of the plasma membrane although GABA was not bound to the receptor. The resting conductance was not voltage-dependent as illustrated in the current-voltage (*I–V*) graph in Fig. 5*G*. The slope of the linear relationship gave a conductance of 33.5 nS. The average conductance obtained from similar *I–V* plots in cells expressing each of the mutant receptors was approximately sixfold higher (37–42 nS) than the whole-cell conductance recorded in cells expressing the wild-type $\alpha_1\beta_1$ receptors (5.7 ± 0.6 nS) (Table 2).

The resting conductance in α(L9′T) cells was not higher than normal. The average resting conductance measured from current generated by a −40 mV pulse in Sf9 cells expressing only the α(L9′T) subunit was 8.9 ± 2.2 nS ($n = 15$), not significantly different from the resting conductance in cells expressing wild-type $\alpha_1\beta_1$ receptors. This indicates that the presence of an assembled heteromeric receptor complex in which one or both subunits contained the L9′T mutation was necessary for the increase in the resting conductance.

Mutant Receptors Form Chloride-selective Channels in the Absence of GABA

The increase in conductance of cells containing L9′T mutated subunits could have been due to a decrease in seal resistance, an increase in nonspecific leakiness of the membrane or the presence of new channels formed by mutated subunits open in the absence of GABA. The first two mechanisms would give currents that were not ion-selective. The ion selectivity of the resting conductance in the cells expressing the L9′T mutant subunits was therefore investigated. As before, the membrane potential was held at 0 mV and a solution containing a lowered [Cl^-] was rapidly applied to the surface of the cell from a tube positioned close by. Any current which flowed under these conditions would be carried by chloride ions. The current generated by a step in extracellular [Cl^-] from 184 to 34 mM can be seen in Fig. 5*B* (recorded from the same cell as in Fig. 5*A*). The current amplitude is 590 pA which corresponds to a chloride conductance of about 14 nS (calculated using an E_{Cl} of −42.5 mV). The larger current measured in response to the 40 mV voltage step is presumably due to the resting conductance typically found in Sf9 cells under these conditions (Birnir et al., 1995). The average chloride conductance measured with chloride concentration jumps in cells expressing α(L9′T)β receptors was 21.4 ± 1.3 nS ($n = 64$). The chloride conductances of cells expressing either the αβ(L9′T) or α(L9′T)β(L9′T) receptors were of similar magnitude (Table 2). In contrast, however, in cells expressing only the α(L9′T) subunit, the chloride current generated by dropping $[Cl^-]_o$ from 184 to 34 mM was very small and gave a chloride conductance of 1.0 ± 0.4 nS ($n = 13$).

The channels carrying the chloride current were anion-selective, as illustrated for α(L9′T)β(L9′T) receptors

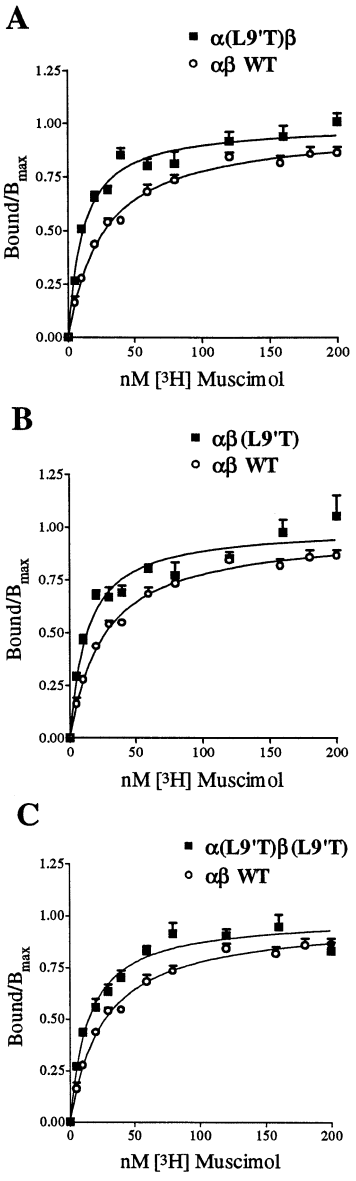

Fig. 3. [^{3}H]-muscimol binding to L9′T mutant $GABA_A$ receptors. Infected Sf9 cells were assayed 42–50 hpi for the binding of [^{3}H]-muscimol to receptors in the insect cell plasma membrane. Cells were incubated in a range of [^{3}H]-muscimol concentrations (5 to 200 nM) for 30 min at 4°C. The data obtained in each experiment were normalized to the B_{max} value obtained by fitting the equation for a single class of binding sites to the data set. Each point represents the mean of 4–6 determinations from 2–5 experiments and the error bars indicate the standard error of the mean. The solid line through the data points represents the theoretical fit of the single binding site equation to the average normalized values.

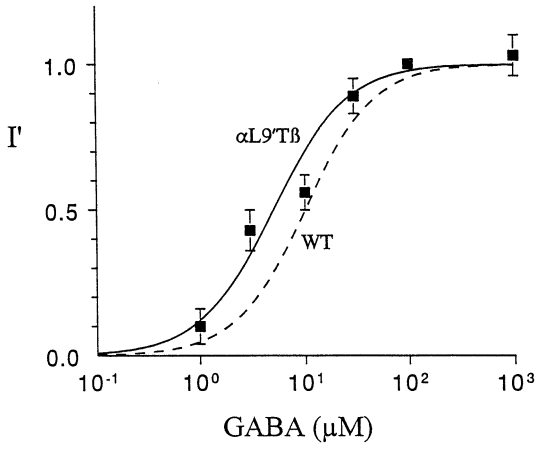

Fig. 4. Relationship between peak amplitude of the chloride current and [GABA] in cells expressing α(L9′T)β receptors. Peak current amplitude was normalized (I', ordinate) to the peak amplitude of the current elicited by 100 μM GABA, and averages obtained in 2–5 cells are shown with vertical lines denoting ± 1SEM. The line through the points shows the least squares fit of the Hill equation ($I' = 1/(1 + (EC_{50}/[GABA])^n)$ to the data points (EC_{50} = 5.0 μM, n = 1.2). The dotted line shows for comparison the fit to data obtained in cells expressing wild-type $\alpha_1\beta_1$ receptors reported previously (Birnir et al., 1995:EC_{50} = 11.0 μM, n = 1.3).

in Fig. 5, *E* and *F*. The chloride current caused by dropping $[Cl^-]_o$ from 184 to 34 mM had a peak amplitude of about 500 pA (Fig. 5*E*) whereas lowering $[Na^+]_o$ from 180 mM to 30 mM (substituting choline for Na^+) gave a current of only about 40 pA (Fig. 5*F*). Similar results were obtained with the other combinations of mutated subunits. These observations indicate that the larger resting current across the membrane of Sf9 cells co-expressing α and β subunits, one or both of which carried the L9′T mutation, was due to the presence of new chloride channels in the plasma membrane of these cells rather than to a decrease in seal resistance which would not be expected to be selective for chloride ions.

Penicillin Blocks the Chloride Channels Formed by Mutant Receptors

If the new chloride channels were formed by the mutated $GABA_A$ subunits, it seemed possible that they might be affected by drugs that normally modulate chloride channels activated by GABA. The drugs tested were bicuculline, picrotoxin, penicillin and pentobarbitone, all of which modulate the response to GABA generated in Sf9 cells expressing the wild-type $\alpha_1\beta_1$ receptors (Birnir et al., 1995). To determine the effect of the drugs on chloride channels selectively, the resting chloride conductance of cells was measured by holding the potential at 0 mV and rapidly lowering $[Cl^-]_o$ as described above. Bicuculline, picrotoxin and pentobarbitone had little effect on the resting conductance or chloride conductance of cells expressing heteromeric mutant receptors (Table 3) in contrast to their effects on chloride channels activated by GABA in cells containing $\alpha_1\beta_1$ receptors (Birnir et al., 1995).

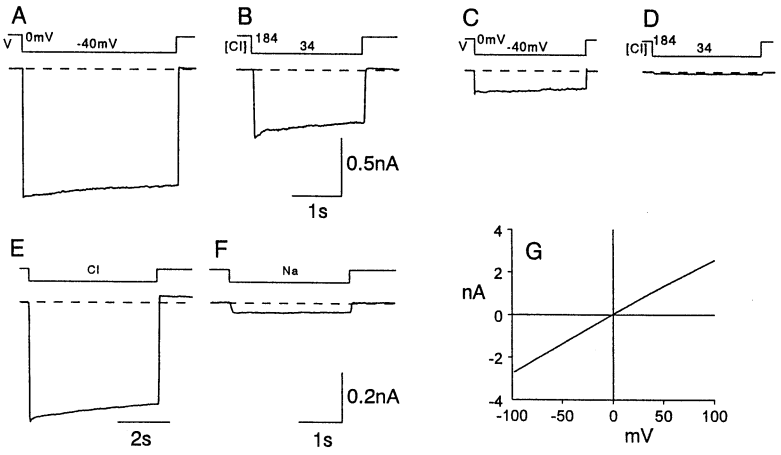

Fig. 5. Characteristics of the resting current in the absence of GABA in cells expressing wild-type and L9′T mutant subunits. (*A* and *B*) Currents generated in a cell expressing α(L9′T)β(L9′T) receptors by changing the membrane potential from 0 to −40 mV (*A*) or $[Cl^-]_o$ from 184 to 34 mM (*B*). (*C* and *D*) Currents generated in a cell expressing wild type $\alpha_1\beta_1$ receptors by changing the membrane potential from 0 to −40 mV (*C*) or $[Cl^-]_o$ from 184 to 34 mM (*D*). (*E* and *F*) Currents generated in a cell expressing α(L9′T)β(L9′T) receptors by changing the membrane potential from 0 to −40 mV (*E*) or $[Na^+]_o$ from 180 to 30 mM (*F*). (*G*) Current (ordinate) generated by a voltage ramp from −100 to +100m V (1 sec) in a cell expressing α(L9′T)β(L9′T) receptors is plotted against membrane potential (abscissa).

Table 2. Resting conductance in Sf9 cells expressing the L9′T receptor mutants

$GABA_A$ Receptor	Resting conductance (nS)[a]	
	Voltage Step (−40 mV)	$[Cl^-]_0$ (−42.5 mV)
αβ (WT)	5.7 ± 0.6 (52)	2.7 ± 0.8 (23)
α(L9′T)β	37.5 ± 2.0 (64)	21.4 ± 1.3 (64)
αβ(L9′T)	42.5 ± 4.3 (17)	22.5 ± 2.9 (17)
α(L9′T)β(L9′T)	41.8 ± 2.0 (38)	23.5 ± 1.3 (38)
α(L9′T)	8.9 ± 2.2 (15)	1.0 ± 0.4 (13)

[a] Infected Sf9 cells were held at 0 mV and a change in the membrane potential was generated by either a voltage step to −40 mV or a rapid drop in extracellular chloride ion concentration equivalent to −42.5 mV (184 mM Cl^- reduced to 34 mM Cl^-).

On the other hand, penicillin (10 mM) caused significant depression of the high resting conductance and the chloride conductance in cells expressing mutant receptors (Table 3). An example of the inhibition by 10 mM penicillin of the current generated by a 40-mV voltage step in a cell expressing α(L9′T)β(L9′T) receptors is shown in Fig. 6*A*. The 1.6 nA current generated by a voltage step from 0 to −40 mV was decreased to 0.4 nA by the penicillin.

This inhibitory effect of penicillin on chloride conductance in a cell expressing α(L9′T)β receptors is shown in Fig. 6*B* and *C*. The chloride current generated by lowering $[Cl^-]_o$ to 34 mM was depressed from a peak amplitude of about 1 nA (Fig. 6*B*) to 0.25 nA by the penicillin (Fig. 6*C*). Penicillin did not depress the small chloride current in Sf9 cells expressing only α(L9′T) subunits.

Discussion

Cells infected with recombinant viruses containing α_1 and β_1 cDNA together, either or both of which contained the L9′T mutation, expressed receptors in the plasma membrane that bound [^{3}H]-muscimol with high affinity. Because it has been shown that both α and β subunits are required to form receptors that bind muscimol (Pritchett et al., 1988; Pregenzer et al., 1993; Tierney, 1995), the binding of muscimol to cells expressing all three combinations of mutated subunits indicates that heteromeric receptors were formed. The B_{max} values for [^{3}H]-muscimol binding were similar for each of the three mutant combinations indicating that the higher level of α_1 protein incorporation in the plasma membrane of αβ(L9′T) cells detected by flow cytometry was due to incorporation of some α_1 protein that did not form a receptor that bound [^{3}H]-muscimol. The high-affinity binding of muscimol suggests that the mutation caused no significant nonlocalized structural perturbation in these receptor complexes. However, the binding (B_{max}) and flow cytometric results indicate that the relative efficiency of insertion of receptor in the plasma membrane was significantly reduced although the level of α_1 protein in the cytoplasm was not reduced. The mutation may reduce the efficiency of the assembly process, perhaps by affecting intersubunit oligomerization or the folding of individual subunits.

The presence of a mutated β_1 subunit in combination with a mutated or wild-type α_1 subunit gave receptors which bound muscimol at one or more high affinity binding sites but no channels were activated by GABA. In cells expressing a mutated α_1 subunit in combination with the wild-type β_1 subunit, GABA did elicit a chloride current but it had a much slower time course than seen with $\alpha_1\beta_1$ receptors. We conclude that the mutation in the β_1 subunit, while not preventing formation of receptors on the cell surface that can bind GABA, results in receptors in which binding of GABA does not convert a closed to an open channel.

Cells expressing any of the three combinations of mutated subunits had a much higher resting chloride conductance than cells expressing wild type $\alpha_1\beta_1$ receptors.

Table 3. Effects of bicuculline (100 μM), penicillin (10 mM), picrotoxin (100 μM) and pentobarbitone (100 μM) on chloride currents (picoamps) in 9 mutant receptors.

Mutant	Control	Bicuculline	*n*	Control	Penicillin	*n*
α(L9′T)β	601 ± 122	543 ± 100	5	778 ± 141	*172 ± 20.0	6
αβ(L9′T)	909 ± 92.2	719 ± 98.1	5	940 ± 144	*280 ± 106	5
α(L9′T)β(L9′T)	852 ± 139	698 ± 121	3	722 ± 115	*130 ± 22.5	5
Mutant	Control	Picrotoxin	*n*	Control	Pentobarb	*n*
α(L9′T)β	624 ± 58.5	481 ± 65.9	9	558 ± 90.0	465 ± 86.6	6
αβ(L9′T)	937 ± 119	612 ± 83.9	4	884 ± 39.0	896 ± 29.9	4
α(L9′T)β(L9′T)	417 ± 47.9	370 ± 26.3	2	899 ± 138	887 ± 136	6

Average current ± SEM in n cells. Asterisks denote a significant change in mean current caused by a drug ($P < 0.01$, Student's *t*-test)

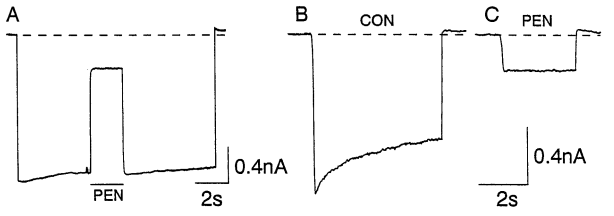

Fig. 6. Penicillin depresses resting current in cells expressing L9′T mutant receptors. (*A*) The current generated in a cell expressing α(L9′T)β(L9′T) receptors by changing the membrane potential from 0 to −40 mV is depressed 75% by 10 mM penicillin (PEN). (*B* and *C*) The current generated in a cell expressing α(L9′T)β receptors by changing $[Cl^-]_o$ from 184 to 34 mM (*B*) is depressed 77% by 10 mM penicillin (*C*).

Because this conductance is blocked by penicillin, a specific blocker of $GABA_A$ chloride channels, it is reasonable to assume that the conductance is due to the L9′T mutation in one or both of the subunits. Sf9 cells expressing only the α(L9′T) subunit incorporated the protein into the insect plasma membrane but the cells did not have a high resting conductance nor was a chloride current activated by GABA. It seems, therefore, that the formation of an open chloride channel in the absence of ligand in receptors containing subunits with the L9′T mutation requires the presence of both α_1 and β_1 subunits. This is analogous to the requirement in the wild-type receptor for the presence of both the α_1 and β_1 subunits to form an agonist-gated chloride channel in Sf9 cells (Birnir et al., 1995). It should be noted that the term ''open channel'' is used to denote a functional rather than a conformational state of the receptor protein: the open channel in mutated receptors in the absence of ligand may be very different from the open channel normally activated by GABA.

A GABA-independent chloride current that is blocked by pictrotoxin has been recorded in toad oocytes expressing $GABA_A$ β_1 subunits (Sigel et al., 1989) but there is evidence that an endogenous oocyte protein is needed in addition to the β_1 subunit for formation of this channel (Sigel et al., 1990). It is unlikely that the GABA-independent chloride current described here was caused by the presence in the plasma membrane of β subunits forming homomeric chloride channels. The current was observed only in cells co-expressing α and β subunits, one or both of which carried the L9′T mutation. It was not seen in cells expressing wild-type α_1 and β_1 subunits and, although blocked by penicillin, it was not blocked by pictrotoxin. Nor was the GABA-independent current due to the formation of homomeric complexes of mutated β subunits as it was seen in cells expressing mutated α with wild-type β subunits.

What appears to remain functionally intact in all three leucine to threonine heteromeric mutant receptors is their ability to conduct chloride ions selectively. The ability of penicillin to block the GABA-activated chloride current in wild-type receptors and the new resting chloride current in cells containing L9′T receptors suggests that at least part of the ion pathway in the mutant receptors may be the same as in wild-type receptor. When the β_1 subunit contained the mutation, the chloride channels formed by unliganded receptors were in an open state but GABA opened no new channels. In these receptors, either the binding of GABA no longer converts closed to open channels or the channels normally activated by GABA are already open and GABA cannot increase their open probability. The observation that the L9′T mutation causes a change in the affinity of receptors for muscimol suggests that there is a linkage between the amino acid at the 9′ position in M2 and the high affinity muscimol binding sites(s) and that this interaction persists in the mutant receptors.

In receptors containing the mutation in the α_1 subunit but not in the β_1 subunit (α(L9′T)β), GABA still elicited a chloride current that was superimposed on the large resting chloride current: the sum of resting and GABA-activated chloride conductances was greater than the resting chloride conductance in αβ(L9′T) or α(L9′T)β(L9′T) cells. This may mean that the resting and GABA-activated chloride channels are distinct. Alternatively, GABA may increase the conductance or open probability of already conducting channels. Indeed, the L9′T mutation in M2 of ACh receptors has been reported to cause an increase in channel open

time (Filatov & White, 1995). Whether the resting chloride channels are formed by the same residues as the GABA-activated channels, the lack of response to GABA in receptors containing the mutation in the β subunit indicates that the β subunit plays a crucial role in linking channel opening to the binding of GABA and that the linkage is broken when threonine replaces leucine at the 9′ position in M2 of the β_1 subunit. The much slower time course of the current in cells expressing α(L9′T)β receptors indicates that there is a change in the linkage between GABA binding and the channel when the 9′ position in M2 of the α_1 subunit is occupied by threonine. This is consistent with the idea that the binding of agonist induces a coordinated conformational change in individual subunits in the receptor complex.

Our results are consistent with a model of the resting (closed) conformation of an $\alpha_1\beta_1$ GABA receptor in which the 9′ leucine in M2 either physically occludes the channel because of its bulky side chain or interrupts a series of sites interacting with chloride ions as they pass through the channel, mechanisms that can be summarized as a ''physicochemical block.'' Replacement of leucine by threonine would relieve a chemical block by providing the polar side chain of threonine for interaction with chloride ions. This is different from the model proposed to explain the effects of mutating the 9′ leucine in the homomeric, cationic α7 ACh receptor (Revah et al., 1991; Bertrand et al., 1992). It was suggested that the L9′T mutation in the α7 receptor converts a desensitized state in the wild-type receptor into a conducting state. This interpretation was based on the appearance of channels of high conductance recorded at low agonist concentrations (<3 μM), together with the observation that antagonists were now able to activate the channel. By contrast, when the polar amino acid serine replaced the 9′ leucine in heteromeric ACh receptors, no higher conductance channels were observed (Labarca et al., 1995): the most dramatic change seen was a tenfold decrease in EC_{50} for each different subunit containing the mutation. It was concluded that each of the five 9′ leucine residues of the nAChR participates independently and symmetrically in the structural transition between closed and open states.

From structural data derived from electron microscopic images of the nAChR, it has been suggested that the 9′ leucine residues align at the position where the proposed M2 helices are seen to 'kink' and point inwards to occlude the conduction pathway (Unwin, 1993). Upon agonist binding the proposed M2 helices rotate, moving the leucine side chains away and thereby ''opening'' the channel (Unwin, 1995). The results from the mutational analysis of the 9′ leucine residues in the α_1 and β_1 subunits of the $GABA_A$ receptor are consistent with this image of the conserved leucine residues blocking the conduction pathway in the resting state of the wild-type receptor. On the other hand, the 9′ leucine residues may not face into the channel in the resting (unliganded) state (*see* Karlin & Akabas, 1995) and substitution of threonine for this leucine may induce a conformational change that gives a constitutively open chloride channel.

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