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A Structural Determinant of Desensitization and Allosteric Regulation by Pentobarbitone of the $GABA_A$ Receptor

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Abstract. Functional properties of the $\alpha_1\beta_1$ $GABA_A$ receptor changes in a subunit-specific manner when a threonine residue in the M2 region at the 12′ position was mutated to glutamine. The rate and extent of desensitization increased in all mutants but the rate of activation was faster in the β_1 mutants. A negligible plateau current and abolition of potentiation by pentobarbitone of the GABA-activated current depended on the Thr 12′ Gln mutation being present in the β_1 subunit. The Hill coefficient of the peak current response to GABA was reduced to less than one also in a β_1 subunit-specific manner. It was concluded that the β_1 subunit dominated conformational changes activated by GABA.

Key words: M2 — Baculovirus — Expression — Ligand gated — Patch clamp — $GABA_A$ subunit

Introduction

$GABA_A$ receptors mediate fast inhibitory transmission in central nervous systems when activated by γ-aminobutyric acid (GABA). The GABA-activated inhibitory postsynaptic chloride conductance rises rapidly to a peak and then decays. The rate of decay of the conductance influences the magnitude of postsynaptic neuronal hyperpolarization. There are varying reports on the rate and degree of desensitization of $GABA_A$ receptors and only recently have structures in ligand-gated receptors been identified which may be involved (for reviews *see* Devillers Thiery et al., 1993 and Karlin & Akabas, 1995). The $GABA_A$ receptor is a member of the ACh superfamily which includes nicotinic acetylcholine (ACh) receptors, glycine receptors and 5-hydroxytryptamine type 3 ($5\text{-}HT_3$) receptors (Karlin & Akabas, 1995). All members show structural similarities and are considered homologous. The most similar amino acid region is the second hydrophobic region termed M2 which is believed to form an α-helix that spans the membrane (Imoto et al., 1986). A leucine residue, conserved in most members of the ACh superfamily, is found in the middle of the M2 region at the 9′ location (Miller, 1989). It has ben shown to influence desensitization and the open time of the channel (Revah et al., 1991; Filatov & White, 1995; Labarca et al., 1995) and when mutated to threonine in the $\alpha_1\beta_1$ $GABA_A$ receptor the GABA-activated current was abolished in a β_1 subunit-specific manner (Tierney et al., 1996). Amino acids about one helical turn on either side of the 9′ leucine (6′,13′) also affect the time course of the ACh-gated current (Devillers Thiery et al., 1993). Together these results imply that the M2 region has a role in the process of receptor activation and desensitization but it is not clear whether it is the nature of the particular amino acid, its location across the membrane or its position on the proposed M2 α-helix that is most important for its influence on these processes. Nor is it known whether all the subunits which form the receptors contribute equally to desensitization.

To address these issues, we have used site-directed mutagenesis to change the 12′ threonine residue to glutamine in the M2 region of the human $\alpha_1\beta_1$ $GABA_A$ receptor. This threonine is on the same face of the helix as the 9′ amino acid leucine and about one helical turn towards the extracellular surface. To allow detection of any subunit-specific effects, $\alpha_1\beta_1$ receptors carried the mutation in either the α_1, or the β_1 or in both subunits. Prior studies with mutated acetylcholine receptors in the

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M2 region have revealed that, in addition to changes in receptor kinetics, receptor pharmacology may also be altered (Leonard et al., 1988; Devillers Thiery et al., 1993; Forman, Miller & Yellen 1995)l. More recently the M2 region has been shown to influence the effects of picrotoxin and loreclezole on GABA receptors (ffrench Constant et al., 1993; Wingrove et al., 1994; Gurley et al., 1995). We therefore examined pharmacological properties as well as the time course of the response using a fast solution exchange system (Birnir et al., 1995). The receptors were expressed using the *Sf9* baculovirus expression system as described previously (Birnir et al., 1995). Our results indicate that the replacement of threonine by glutamine at the 12′ position in the β_1-subunit, but not in the α_1-subunit, affects both the rate of desensitization and allosteric regulation by pentobarbitone. The β_1-subunit also appears to have a dominant role in subunit interactions brought about by GABA binding.

Materials and Methods

Mutagenesis and Isolation of Recombinant Baculoviruses

Human $GABA_A$ α_1 and β_1 cDNA sequences were subcloned into the dual promoter baculovirus transfer vector pAcUW31 (Clontech) as described previously (Birnir et al., 1995). Site-specific mutations were introduced into single-stranded DNA (Taylor, Ott & Eckstein, 1985) and restriction fragments subcloned into compatible cloning sites in the plasmid pAcUW31. All constructs were confirmed by double stranded sequencing across the mutated region of the insert DNAs and restriction mapping of the plasmid. The production, isolation and propagation of recombinant viruses was as described previously (Birnir et al., 1995).

Expression of Receptors

Sf9 cell culture and infection conditions have been described (Birnir et al., 1995). Briefly, *Sf9* cells were maintained in spinner cultures in complete TNM-FH insect media (Sigma) at 25°C. For experiments cells were removed from cultures growing at $1–3 \times 10^6$ cells/ml, seeded into tissue culture plates/flasks, infected at multiplicity of infection (MOI) of more than 10 plaque-forming units per cell and assayed 33 to 48 hr after infection. Largest peak current amplitudes were recorded from cells infected in the plateau phase ($2–3 \times 10^6$ cells/ml) at MOI $\geqslant$ 100.

Electrophysiology

Standard procedures have been described in detail elsewhere (Birnir et al., 1995). Briefly, the whole-cell recording technique originally described by Hamill et al. (1981), was used to voltage clamp *Sf9* insect cells at −40 mV and record currents. Borosilicate glass electrodes containing pipette solution (in mM: 180 NaCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetra-acetic acid), 10 TES (N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid), pH 7.2 and 4 Na_2 ATP (adenosyne 5′-triphosphate) had resistances of 4–6 MΩ after fire polishing. The bath solution contained (in mM): 180 NaCl, 1 $MgCl_2$, 1 $CaCl_2$ and 10 MES (2-[N-morpholino]ethanesulfonic acid), pH 6.2 which is the normal extracellular pH for *Sf9* cells. Currents were monitored with a current-to-voltage converter (Axopatch 200A, Axon Instruments, Foster City, CA), using series resistance compensation (95–100% correction, 5–20 MΩ) and filtered at 1 kHz. Drugs used were dissolved in the bath solution and rapidly (rate of current rise from 10 to 90% was approximately 0.5 msec when bath solution containing 180 mM Cl^- was replaced by 34 mM Cl^-) applied to a voltage-clamped cell through a gravity fed perfusion barrel as described previously (Birnir et al., 1995). Results are presented as mean ± SEM. Some of the blockers had greater effect on the decay of the current response than on the peak current response. To best describe the effectiveness of the blocker the mean current was measured.

Ligand-binding Studies

Binding of [^{3}H] muscimol to infected cells was according to a method described by Kawamoto et al. (1991). The binding buffer consisted of (in mM) 180 NaCl, 1 $MgCl_2$, 1 $CaCl_2$ and 10 MES pH 6.2 and [^{3}H] muscimol concentration was varied from 5 to 200 nM. After 30 min incubation at 40°C, samples were filtrated through 5 μm cellulose-acetate filters using a fast-flow vacuum manifold. Filters were washed with 2 ml of ice-cold binding solution and placed in a scintillation cocktail and counted in a β-counter. Nonspecific binding was determined from [^{3}H] muscimol binding to *Sf9* cells that had been infected with baculovirus (AcNPV) carrying no GABA subunit insert and was subtracted from the total binding values to obtain the specific binding values. Michaelis-Menten binding curves were determined with Prism™ software (version 1.03, San Diego, CA).

Results

Mutational Strategy

Alignment of the M2 regions of the α_1 and β_1 subunits shows high homology between the two subunits (Figure 1; Schofield et al., 1989). Of the 20 amino acids that make up the M2 region, 11 (heavy shading) are identical in the two subunits and four others (light shading) are similar in nature. We examined the role of the threonine residue at the 12′ position within the M2 region. This residue is located about one helical turn more extracellular than the 9′ leucine and the two residues would be on the same face of the α-helix. The threonine is conserved across the $GABA_A$ subunit families with the exception of the ρ subunits where it may be replaced by serine (Cutting et al., 1991). Our strategy was to make the conservative change to glutamine in the hope that the only processes that would be affected by the change were those, if any, where precise interactions of the amino acid side chain within the receptor's microenvironment mattered. Both threonine and glutamine are polar but differ in size (threonine = 116.1 Å^3, glutamine = 143.9 Å^3) and in the location and nature of the polar group on the side chain. Threonine has a hydroxyl group on the β carbon whereas glutamine has an amide group on the δ

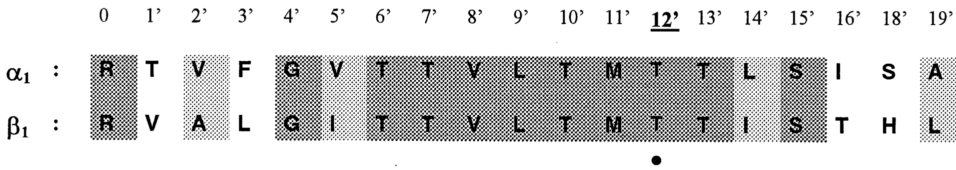

Fig. 1. Amino acid sequences of the M2 region of human α_1 and β_1 subunits. The 0 to 19′ α_1 and β_1 amino acids correspond to residues 255 to 274 and 250 to 269, respectively, in the protein sequence.

carbon. We use the notation α*β to refer to a receptor in which the Thr12′Gln mutation is carried by the α_1 but not the β_1 subunit, αβ* for receptors in which the mutation is carried by the β_1 but not the α_1 subunit and α*β* for receptors in which the mutation is carried by both subunits.

The Rate of Desensitization is Altered when Thr 12′ is Replaced by Gln

Currents activated by GABA in the 12′ mutant receptors all exhibited a time course different from that shown by wild-type receptors. Typical examples of currents generated by 10 mM GABA in cells expressing αβ or mutant receptors are shown in Fig. 2. The holding potential was −40 mV. The decay of the current for each receptor type was complex. It could be fitted with the sum of 3 or 4 exponentials with different time constants (using ''Peakfit,'' Jandel Scientific) according to Eq. 1:

$$I(t) = A_1\exp(-t/\tau_1) + A_2\exp(-t/\tau_2) + A_3\exp(-t/\tau_3) + A_4\exp(-t/\tau_4) + C \quad (1)$$

where A_i (pA) and τ_i (msec) are the initial amplitude and time constant of the ''*i*''th component. *C* is the plateau current. The rise times and the decay time constants of responses to 10 mM GABA at the holding potential of −40 mV are listed in Table 1.

When the Thr12′ Gln mutation was present in the β_1 subunit the current rose about two times faster from 10 to 90% of the peak value in cells expressing the αβ* or α*β* receptors (αβ*, 3.2 ± 0.7 msec; α*β*, 3.8 ± 0.2 msec) as compared to cells expressing the α*β (8.1 ± 1.9 msec) or the wild-type receptors (7.9 ± 1.1 msec). Furthermore, during the application of GABA, the current decayed to baseline in cells expressing αβ* or α*β* receptors and was therefore fitted only up to 1 sec. In cells expressing α*β receptors the current decay was fitted over 2 sec. The current decay in wild-type receptors consisted of three or four exponential components (Table 1) (Birnir et al., 1995). The greatest change between wild-type and the mutated receptors was in the value of the slowest time constant. Inspection of current desensitization in cells expressing the β_1 mutant subunit revealed that these mutant receptors did not exhibit any measurable plateau current (Fig. 2*D* and *E*). This was represented by the absence of any decay time constant in the range of seconds in the β_1 mutants. The slowest time constant for wild-type receptors was on average about 5 sec (Birnir et al., 1995) whereas it was only about 0.5 sec for αβ* and a*β* receptors. In cells expressing α*β receptors the slowest time constant was on average about 1.5 sec and there was a small plateau current (Fig. 2 *B* and *C*). The relative amplitude of the plateau current has been reported to be concentration dependent (Oh & Dichter, 1992). We therefore examined whether the amplitude of the plateau current in the mutants could be correlated with the concentration of GABA applied to the cells. To optimize the signal-to-noise ratio we used highly expressing cells (*see* Materials and Methods) when examining the current generated by low (1 or 0.1 μM) GABA concentrations. This gave peak current amplitudes within the range obtained when 10 mM GABA was applied to the cells commonly used which expressed receptors at a lower level (Fig. 2*D,* 3*B*). Responses to low concentrations of GABA in cells expressing α*β (1 μM GABA), αβ* (0.1 μM GABA) or α*β* (1 μM GABA) receptors are shown in Fig. 3 *A, B* and *C,* respectively. It can be seen that the plateau current in the α*β mutant is a larger fraction of the peak current at low (1 μM, Fig. 3*A*) than at high (10 than at mM, Fig. 2*B*) concentrations of GABA. But, not even at a GABA concentration as low as 0.1 μM did the αβ* mutant maintain a plateau current (Fig. 3*B*) and it can be seen in Fig. 3*C* that there is no plateau current in the α*β* receptor during exposure to 1 μM GABA. This demonstrates that the negligible plateau current in the β_1 mutants during application of 10 mM GABA (Fig. 2 *D* and *E*) is not merely due to increased desensitization at saturating GABA concentrations but rather is an inherent characteristic of the Thr12′Gln β_1 mutated receptor.

The β_1 Mutation Modifies the Dose-Response Curve

Mutations in the M2 region of the acetylcholine receptor which affect the time course of desensitization have often been found to change the EC_{50} of the mutated receptors (Revah et al., 1991; Filatov & White, 1995; Labarca et al., 1995). In the $\alpha_1\beta_1$ $GABA_A$ receptor, mutation of the 9′ leucine to threonine abolished the GABA-activated current in a β_1 subunit dependent manner

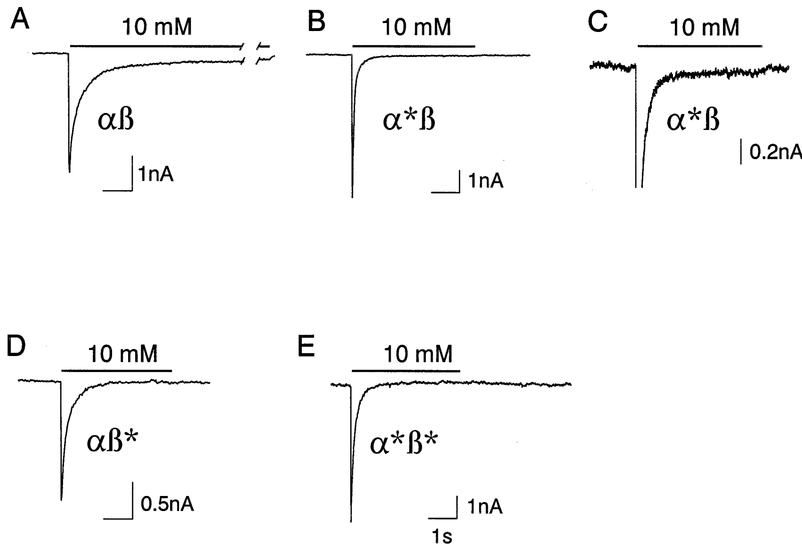

Fig. 2. Time course of whole-cell current activated by 10 mM GABA. GABA was applied during the time period indicated by the bar. In *A,* GABA was applied for 13 sec but only the current response for the first 7 sec and the last 0.5 sec is shown. Cells expressed: (*A*) αβ receptors, (*B*) α*β receptors, (*C*) α*β receptors. The trace is the same as shown in *B* but at higher resolution (*D*) αβ* receptors and (*E*) α*β* receptors. Horizontal calibration bars show 1 sec.

Table 1. Characteristics of whole-cell currents activated by GABA

Mutant	Trace[e] (sec)	Rise time[c] (msec)	C/Ip[a]	A_1/Ip[a]	τ_1 (msec)	A_2/Ip[a]	τ_2 (msec)	A_3/Ip[a]	τ_3 (msec)	A_4/Ip[a]	τ_4 (msec)	n[b]
αβ[d]		7.9 ± 1.1	0.036 ± 0.009	0.16 ± 0.03	23 ± 3	0.29 ± 0.03	158 ± 29	0.50 ± 0.04	550 ± 45	0.12 ± 0.01	5544 ± 1028	11–15[f]
α*β	2	8.1 ± 1.9	0.012 ± 0.006	0.58 ± 0.05	61 ± 6	0.34 ± 0.05	251 ± 34			0.11 ± 0.03	1533 ± 525	8
αβ*	1	3.2 ± 0.7	0.009 ± 0.004	0.41 ± 0.05	75 ± 12	0.43 ± 0.05	240 ± 35	0.21 ± 0.03	487 ± 63			13
α*β*	1	3.8 ± 0.2	0.003 ± 0.001	0.42 ± 0.03	65 ± 3	0.44 ± 0.02	222 ± 12	0.26 ± 0.01	486 ± 21			12

[a] *Ip* is the value of the peak current; [b] n is the number of cells and values are expressed as mean ± SEM; [c] The 10–90% current rise times; [d] Wild type data from Birnir et al. (1995); [e] Shows the length of the trace fitted by equation 1; [f] *n* = 15 in column 3, 4, 9, 10; *n* = 14 in column 7 and 8; n = 13 in column 11 and 12 and n = 11 in column 5 and 6.

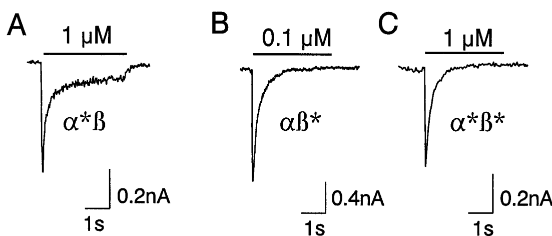

Fig. 3. Time course of whole-cell current activated by low GABA concentrations (1 μM or 0.1 μM). GABA was applied during the time period indicated by the bar. Cells were infected in the plateau phase of growth at high MOI resulting in a high level of expression of receptors. Cells expressed: (*A*) α*β receptors, (*B*) αβ* receptors or (*C*) α*β* receptors.

whereas the GABA EC_{50} (5 μM) and the Hill coefficient (1.2) were similar to wild type when the mutation was present in the alpha subunit only (Tierney et al., 1996). To determine whether the conservative mutation at the 12′ location within the M2 region of the $GABA_A$ receptor also affects the GABA-activation of the receptor, we recorded dose-response curves of the Thr12′Gln mutated receptors. In Fig. 4 (*A, C* and *E*) the peak amplitude of the current activated by GABA is plotted against the GABA concentration. The data could be fitted by a Hill-type equation (Eq 2):

$$I = I_{max} \cdot [GABA]^h/((EC_{50})^h + [GABA]^h) \quad (2)$$

where I_{max} is the value of the estimated ''saturating'' current response, EC_{50} is the GABA concentration that gave the half-maximal current response and h is the Hill coefficient. The solid line through the points is a fit of the Hill equation to the data. The values of the best fit parameters are given in Table 2. Cells expressing α*β receptors had a dose-response relationship similar to that of cells expressing wild-type receptors (Fig. 4*A,* Table 2).

For the β_1 mutant receptors (αβ*, α*β*), the acti-

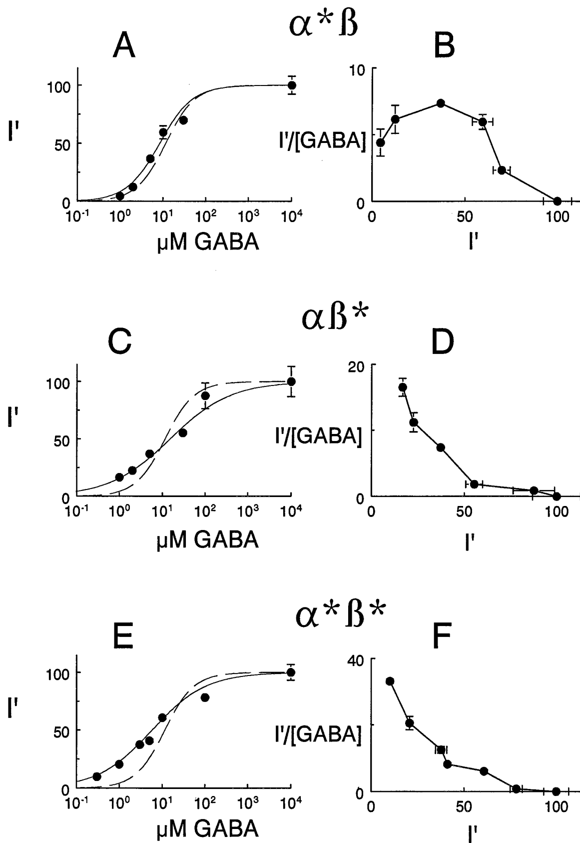

Fig. 4. The influence of GABA concentration on peak whole-cell currents. Dose-response curves of whole-cell currents generated by GABA are shown in *A, C* and *E* and Scatchard plots of the same data as in *A, C* and *E* are shown in *B, D* and *F.* Peak currents elicited in a cell by various concentrations of GABA were normalized to the current elicited in the same cell by 10 μM GABA in cells expressing α*β receptors and to 5 μM GABA in cells expressing αβ* receptors or α*β* receptors. Symbols show the mean normalized current (I') and the vertical bars ± 1 SEM in 3 or more cells. The curves (solid lines) in *A, C* and *E* were calculated by fitting the Hill equation by nonlinear regression analysis to the data. The broken lines show for comparison the wild-type activation curve. The lines connecting data points in *B, D* and *F* are used to emphasize the shape of the plot and have no theoretical significance. The data were gathered from 10 cells in *A* and *B,* 17 cells in *C* and *D* and 21 cells in *E* and *F.*

vation curves were markedly different from wild-type and α*β receptors, although the EC_{50} values were similar (Fig. 4*C* and *E,* Table 2). There were in both instances significant decreases in the slopes of the dose-response relationships resulting in greater sensitivity to lower GABA concentrations. Figure 4 *B, D* and *F* are Scatchard plots of the same data as shown in the dose-response curves in Fig. 4 *A, C* and *E.* The value of h (Table 2) and the shape of the Scatchard plots indicate that wild-type and α*β receptors exhibit positive cooperativity whereas in the β_1 mutant receptors there is either negative cooperativity or dissimilar binding sites.

Muscimol is a potent GABA Receptor agonist and,

Table 2. Dose-response relationship

	EC_{50}[a](μM)	h[b]	n[c]
αβ[d]	11 ± 2	1.3 ± 0.3	12
α*β	8 ± 1	1.1 ± 0.2	10
αβ*	15 ± 7	0.6 ± 0.2	17
α*β*	6 ± 1	0.7 ± 0.1	21

[a] EC_{50} is the GABA concentration that gave half-maximal current response; [b] h is the Hill coefficient; [c] n is the number of cells used to construct the activation curve; [d] Wild-type data from Birnir et al. (1995); Values are mean ± 1 SEM.

in general, alterations in the response to GABA due to mutations in ligand-binding domains have been paralleled by changes in the muscimol response (Amin & Weiss, 1993; Smith & Olsen, 1995). It can therefore be used to examine if the 12′ mutation has caused structural changes in the high affinity ligand-binding domain(s). The binding data could be fitted by the Michaelis-Menten equation. Cells expressing αβ receptors gave a muscimol dissociation constant (K_d) of 29 ± 2 nM (n = 8). In two experiments, cells expressing α*β* receptors had similar K_ds of 21 and 25 nM indicating no significant alterations had occurred in the binding domains due to the 12′ mutations in the M2 regions.

Pentobarbitone Potentiation of the Response to GABA is Abolished in β_1 Mutants

Except for receptors containing the ρ subunit, $GABA_A$ receptors are modulated by anaesthetic barbiturates (Tanelian et al., 1993; Rabow et al., 1996). Barbiturates change the time dourse of the current generated by GABA and there appears to be an increased sensitivity to GABA without a change in the maximum current response (Parker, Gundersen & Miledi, 1986; Rabow, Russek & Farb, 1996). The higher sensitivity to GABA induced by barbiturates at low GABA concentrations is similar to the effects of the Thr12′Gln mutation in the β_1 subunit described above and distinct from the direct activation of some $GABA_A$ receptors by pentobarbitone. This raised the possibility that allosteric regulation by barbiturates of the αβ $GABA_A$ receptor and the Thr 12′ Gln β_1 mutations might be affecting the same mechanism. Furthermore, no significant current response was detected when 100 μM pentobarbitone was applied to cells expressing wild-type receptors at the expression levels used in these experiments, indicating that direct activation of these receptors by pentobarbitone was negligible. Barbiturates have a high oil-water partition coefficient and might well interact with the receptor in the lipid phase of the membrane (Franks & Lieb, 1994). To determine whether the 12′ mutation also affected the modulation by pentobarbitone of the $GABA_A$ response,

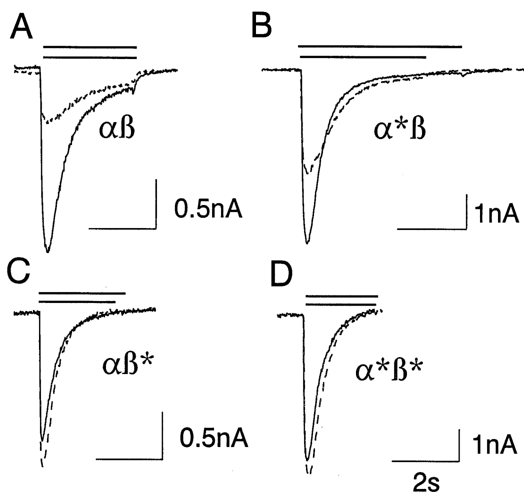

Fig. 5. Modulation of GABA-activated current by 100 μM pentobarbitone. The current response to 3 μM GABA is shown by broken lines. The GABA was applied during the time period indicated by the lower bars. In the same cell the current response to 3 μM GABA + 100 μM pentobarbitone is shown by the solid lines. The 3 μM GABA and 100 μM pentobarbitone were applied during the period indicated by the upper bars. Cells expressed: (*A*) α*β receptors, (*B*) αβ* receptors or (*C*) α*β* receptors. Horizontal calibration bars show 2 sec.

we examined the influence of pentobarbitone (100 μM) on the currents activated by low (3 μM) concentrations of GABA in cells expressing the mutant receptors. Figure 5 and Table 3 demonstrate that pentobarbitone enhanced the response to GABA in cells expressing the wild-type (Fig. 5*A*) or α*β (Fig. 5*B*) receptors whereas potentiation by pentobarbitone was abolished in cells expressing αβ* (Fig. 5*C*) or α*β* (Fig. 5*D*) receptors. Pentobarbitone increased the peak current on average by a factor of 2.5 in wild-type and 2.8 in α*β receptors (Table 3). On the other hand, there was no significant change in the amplitude of the GABA response in the presence of pentobarbitone in cells expressing αβ* or α*β* receptors (Table 3). A decline in the potentiation of the GABA-activated current at high pentobarbitone concentrations (>200 μM) has been reported (Connors, 1981; Parker et al., 1986; Cash & Subbarao, 1988). Why this happens is not clear. At high concentrations, pentobarbitone may act as a channel blocker or it may bind at another site and reverse the potentiation of the current amplitude observed at low concentrations (Willow & Johnston, 1980; Parker et al., 1986). To examine if the effective concentration for barbiturate potentiation had been shifted towards lower concentrations in the mutant receptors, we tested the effects of 10 and 1 μM pentobarbitone on the current evoked by GABA in the β_1 mutants. However, pentobarbitone at these concentrations had no effect on the response to GABA (*data not shown*).

Pentobarbitone Block of the GABA Response in β_1 Mutants

The decreased potentiation of the response to GABA at high pentobarbitone concentrations may indicate that at high concentration pentobarbitone occupies a higher fraction of the binding sites and exerts a different effect on the receptor than when fewer binding sites are occupied. Alternatively, pentobarbitone at high concentrations may bind to other sites or block the channel. To determine whether there was a differential effect of pentobarbitone at a high concentration (1 mM) on the Thr12′Gln mutated receptors, currents were activated with 10 μM GABA in the presence and absence of 1 mM pentobarbitone (Fig. 6, Table 3). Cells expressing wild-type (αβ) or α*β receptors showed no potentiation of the GABA-induced current in the presence of 1 mM concentration of pentobarbitone, and large inward currents were seen following termination of co-application of GABA and pentobarbitone (off-currents). The peak-current amplitudes activated by GABA in the αβ* and α*β mutants in the presence of 1 mM pentobarbitone were reduced to about 0.4, respectively, of the control value (Table 3). Hence, in all receptor constructs, the pentobarbitone potentiation of the current response was abolished. The transient increase in the inward currents on removal of GABA and pentobarbitone indicates that the current inhibition by pentobarbitone was rapidly reversible.

Pharmacology

Some native and reconstituted GABA receptors which are not potentiated by pentobarbitone are not blocked by bicuculline, a specific $GABA_A$ antagonist (Polenzani, Woodward & Miledi, 1991; Rabow et al., 1996). To determine whether bicuculline depressed the αβ* or α*β* receptors, we examined whether the response to 100 μM GABA was inhibited by 100 μM bicuculline. In cells expressing αβ* or α*β* receptors, 100 μM bicuculline reduced the peak current amplitude to 0.35 ± 0.14 ($n = 3$) and 0.29 ± 0.07 ($n = 5$) of the control values, respectively. This is similar to the inhibition reported for wild-type receptors (0.16 ± 0.06, $n = 3$) (Birnir et al., 1995). Hence, the 12′ mutation in the β_1 subunit does not produce bicuculline insensitivity in the mutated receptors. The blockers picrotoxin (100 μM) and penicillin)10 mM) also inhibited the current induced by 100 μM GABA in cells expressing α*β* receptors. Currents integrated over the first second of the response were reduced by picrotoxin to 0.35 ± 0.11 ($n = 5$) of the control response and were completely abolished by penicillin ($n = 7$). This is similar to the inhibition by picrotoxin and penicillin reported for wild-type receptors where the currents

Table 3. Current modulation by 100 μM or 1 mM pentobarbitone

	GABA 3μM + Pentobarb. 100 μM	GABA 10 μM + Pentobarb. 100 μM	GABA 10 μM + Pentobarb. 1 mM
Receptor	Fraction of 3 μM GABA peak current	Fraction of 10 μM GABA peak current	Fraction of 10 μM GABA peak current
αβ	2.47 ± 0.26 (n = 5)	1.62 ± 0.01[a] (n = 8)	1.16 ± 0.13 (n = 4)
α*β	2.75 ± 0.38 (n = 12)	2.08 ± 0.22 (n = 15)	1.01 ± 0.13 (n = 4)
αβ*	0.95 ± 0.04 (n = 5)	0.96 ± 0.04 (n = 8)	0.37 ± 0.04 (n = 3)
α*β*	0.92 ± 0.04 (n = 5)	0.89 ± 0.05 (n = 5)	0.37 ± 0.06 (n = 4)

[a] Wild-type data from Birnir et al. (1995).

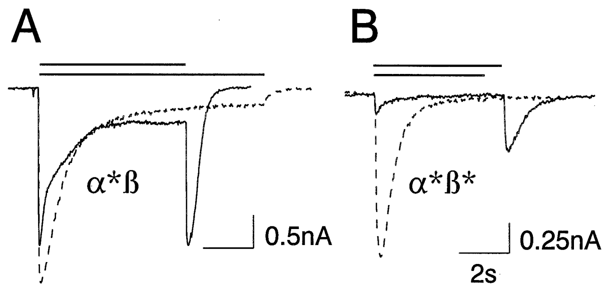

Fig. 6. Inhibition of the current activated by GABA by 1 mM pentobarbitone. Current activated by 10 μM GABA is shown by the broken lines. The GABA was applid during the period indicated by the lower bars. In the same cell the current response to 10 μM GABA + 1 mM pentobarbitone is shown by the solid lines. The 10 μM GABA and 1 mM pentobarbitone were applied during the period of the upper bars. Cells expressed: (*A*) α*β receptors or (*B*) α*β* receptors. Horizontal calibration bars show 2 sec.

were reduced to 0.12 ± 0.02 (n = 8) and 0.20 ± 0.08 (n = 5) of the control response, respectively (Birnir et al., 1995). Interestingly, an off-current was evoked in both wild-type and α*β* receptors when co-application of GABA and penicillin was terminated rapidly. Whether this indicates an open channel block mechanism by pentobarbitone at high concentrations similar to what has been proposed for penicillin (Twyman, Green & MacDonald, 1992) requires further study.

Discussion

In this study, we have examined the effects of replacing a highly conserved threonine residue with a glutamine residue at the 12′ location within the M2 region on the functional characteristics of the $\alpha_1\beta_1$ $GABA_A$ receptor. The effect of the mutation was subunit dependent. When the mutation was carried by the β_1 subunit, it exerted a dominant effect on both the current response evoked by GABA and the modulation of the response by pentobarbitone. Similarly, when the 9′ leucine was mutated to threonine in the $\alpha_1\beta_1$ $GABA_A$ receptor, GABA activated current was abolished in a β_1 subunit dependent manner (Tierney et al., 1996). Together these results imply that some conformational changes that occur within the receptor in response to GABA binding are dominated by the β_1 subunit.

Residues in both the α_1 and β_1 subunits have been shown to contribute to GABA binding and both subunits might therefore be expected to transmit conformational changes that follow binding (Stephenson, 1988; Amin & Weiss, 1993; Smith & Olsen, 1995). Nevertheless, the β_1 mutant subunit appeared to exert a stronger influence on the GABA-activated conformational changes that control the time course of the current than either the mutant or wild-type α_1 subunit. This was manifested in increased sensitivity to low GABA concentrations and also the decreased slope of the activation curve for αβ* and α*β* receptors (Fig. 4). It is possible that the energy barrier for activation has been decreased in the β_1 mutant receptors giving the increased sensitivity to low GABA concentrations. The Hill coefficient (h) and the qualitative shapes of Scatchard plots are good indicators of the nature of transitions within the receptor complex associated with or resulting from GABA binding to its binding sites. A Hill coefficient greater than one and a maximum in the Scatchard plot (convex) are characteristics of a system showing positive cooperativity whereas a Hill coefficient of less than one and a concave Scatchard plot indicate dissimilar sites or negative cooperativity (Dahlquist, 1978). The decrease in h to a value less than 1 in the αβ* and α*β* mutant receptors (0.6 and 0.7, respectively) and the change in shape from convex to concave in Scatchard plots reflect a change in receptor kinetics and suggest a decreased cooperativity of conformational changes as compared with wild-type or α*β receptors. But the data cannot be used to distinguish between negative cooperativity of transitions in the αβ* and the α*β* receptors or the presence of dissimilar sites which do not interact. These results indicate a significant role for the β_1 subunit(s) in transmitting changes to other subunits within the receptor complex upon GABA binding. It is possible that they have a role analogous to that of the α subunits of the

muscle nicotinic ACh receptor (α2βγδ) which have been proposed to be central in the cooperative opening of the channel (Unwin, 1995).

Several studies have shown that when amino acid residues are mutated in the M2 region along an axis normal to the plane of the membrane the current decay is affected. No single site appears to determine the kinetics of desensitization but residues spread across the membrane, 2′, 6′, 9′, 13′ and now 12′, affect the rate of desensitization (Devillers Thiery et al., 1993; Karlin & Akabas, 1995; Tierney et al., 1996). Our finding that homologous M2 amino acids within the α_1 and β_1 subunits which make up the receptor do not influence the rate of desensitization equally is consistent with desensitization resulting from conformational changes involving all subunits but to a different extent. The greater effect of the β_1 subunit mutants on the extent of desensitization suggests that the various phases of desensitization are associated with subunit-specific conformational changes or interactions. Since some ligand-gated receptors are made up of identical subunits it would imply that it is also possible that it is the location of the subunits within the receptor that determines the function of the unit.

The mutant β_1 subunit(s) not only exerts a greater effect on the desensitization properties but also on the potentiation by pentobarbitone. The rate of desensitization and the extent of potentiation by pentobarbitone presumably depend on the ability of the channel structure to remain in an open state(s) (Study & Barker, 1981; macDonald, Rogers & Twyman, 1989; Jones & Westbrook, 1995). Our results are consistent with this conclusion but do not determine whether the phenomena of desensitization and potentiation by pentobarbitone are mechanistically related. Our observation of subunit-specific abolition of pentobarbitone potentiation by a point mutation is the first time that a discrete site which determines modulation by barbiturates of $GABA_A$ receptors has been identified. Pentobarbitone contains an asymmetric carbon atom and it can therefore exist in two enantiomeric forms. In general, the S isomer of barbiturates is more potent than the R isomer (Franks & Lieb, 1994). In light of the effect of the conservative change of Thr 12′ Gln on pentobarbitone modulation of receptor function, the stereoselectivity of pentobarbitone at $GABA_A$ receptors and its lipid solubility it is possible that pentobarbitone interacts with a hydrophobic region in the $GABA_A$ receptor. However, whether the 12′ threonine in the β_1 subunit normally forms part of the pentobarbitone binding site or whether the Thr12′Gln mutation changes the microenvironment in such a way that it renders the pentobarbitone binding site inaccessible, requires further study. It cannot be ruled out either that the binding site may be located away from the M2 region and the presence of the 12′ mutation no longer allows conformational changes necessary for potentiation to take place. The similar GABA EC_{50} values and the similar pharmacological effects of drugs other than pentobarbitone eg muscimol, bicuculline, picrotoxin and penicillin on the β_1 mutants as compared to wild-type indicate that the mutation has not introduced any gross changes in the receptor structure.

Our results suggest that barbiturates regulate the GABA-activated current response by two different mechanisms. Which mechanism dominates the receptor function depends on the barbituate concentration. In the Thr 12′ Gln mutants, the enhancement by pentobarbitone of the GABA response was lost in the β_1 mutants whereas the blocking effect of higher concentrations of pentobarbitone was intact in wild-type and all Thr12′Gln mutated receptors. The 12′ β_1 mutants of the $\alpha_1\beta_1$ receptor may possibly prove useful when designing more specific drugs based on the barbiturate structure since the ability to potentiate (wild-type and $\alpha^*\beta$ receptors) can be separated from the inhibiting action of pentobarbitone ($\alpha\beta^*$ and $\alpha^*\beta^*$ receptors) (Ticku, Rastogi & Thyagavajan, 1985). At inhibitory synapses in the central nervous system where GABA concentrations are high (in the mM range) pentobarbitone affects the time course of current decay and modulates inhibitory postsynaptic currents (Nicoll et al., 1975; Gage & Robertson, 1985). However, $GABA_A$ receptors are also located extrasynaptically on the neuronal soma where they are exposed to low concentrations of GABA. In the hippocampus for example, the steady GABA concentration ranges from 0.2 to 0.8 μM (Tossman, Jonsson & Ungerstadt, 1986). At these extrasynaptic receptors, potentiation by drugs such as barbiturates may significantly modulate the tonic inhibitory system normally provided by the low GABA concentration (Birnir, Everitt & Gage, 1994).

The subunit-specific effects on the different functional properties are somewhat surprising in view of observations on mutated ACh receptors. Progressive changes in EC_{50} values observed when the 9′ leucine residue was mutated in the muscle ACh receptor were interpreted in terms of equal and independent subunits (Labarca et al., 1995; Filatov & White, 1995). However, functional asymmetry of M2 residues has been reported for the 2′ location in the muscle ACh receptor, where the δ subunit was found to be the main determinant of the channel conductance (Villarroel et al., 1992) and for the 9′ location in the $\alpha_1\beta_1$ $GABA_A$ receptor, where the GABA-activated current was abolished when the mutation was present in the β_1 subunit (Tierney et al., 1996). The conservative 12′ amino acid change in the $\alpha_1\beta_1$ $GABA_A$ receptor has further revealed the important role played by the β subunit. It is the β subunit that determines the early conformational changes that follow binding of GABA and the transitions later associated with desensitization of the receptor.

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