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Short communication

A threonine residue in the M2 region of the β_1 subunit is needed for expression of functional $\alpha_1\beta_1$ $GABA_A$ receptors

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Abstract

Although there is a high degree of homology in the M2 transmembrane segments of α_1 and β_1 subunits, subunit-specific effects were observed in $\alpha_1\beta_1$ $GABA_A$ receptors expressed in *Spodoptera frugipedra* (*Sf*9) cells when the conserved 13′ threonine residue in the M2 transmembrane region was mutated to alanine. When threonine 263 (13′) was mutated to alanine in the β_1 subunit, high-affinity muscimol binding and the response to GABA were abolished. This did not occur when the threonine 263 (13′) was mutated to alanine in the α_1 subunit, but the rate of desensitisation increased and the effect of bicuculline, a competitive inhibitor, was reduced. The results show differential effects of subunits on receptor function and support a role for M2 in desensitisation. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: M2 transmembrane segment; Ligand-gated; $GABA_A$ receptor, human; *Sf*9 cell; Desensitization; Receptor assembly

1. Introduction

γ-Aminobutyric acid (GABA) binds to $GABA_A$ receptors and activates a chloride-selective ion channel to mediate inhibitory transmission in central nervous systems. $GABA_A$ receptors belong to the nicotinic acetylcholine family of ligand-gated ion channels (Karlin and Akabas, 1995). $GABA_A$ receptor subunits are grouped into six families, α_{1-6}, β_{1-4}, γ_{1-3}, δ, ε and ρ_{1-3}, which form pentamers (Rabow et al., 1995; Sigel and Buhr, 1997). Each subunit contains four membrane-spanning regions (M1–M4). M2 is most highly conserved among $GABA_A$ receptor subunits and is thought to line the pore of the channel. Using the numbering system devised by Miller (1989) for the M2 region, 1′ is located at the intracellular end and 19′ at the extracellular end. Xu and Akabas (1993, 1996) identified nine residues in the M2 region of the α_1 subunit of the $GABA_A$ receptor that are exposed in the channel. Eight of these align along one side of M2 when modeled as an α helix. Exposed residues capable of providing solvation of ions could facilitate ion permeation. Threonine residues present at 6′, 7′, 10′ and 13′ positions in M2 are all potential candidates. T13′ is conserved among $GABA_A$ subunits. The homologous residue in α_7 neuronal nicotinic acetylcholine receptors, valine (position 251), has been implicated in desensitisation and ion selectivity (Galzi et al., 1992).

When threonine residues in both subunits of the human $\alpha_1\beta_1$ $GABA_A$ receptor at the 13′ position were mutated to alanine, no GABA-activated current could be recorded and no muscimol binding was detected (Tierney et al., 1998). Due to the high degree of sequence homology between subunits in the M2 region and the symmetrical arrangement of subunits around a central ion pore, we investigated whether T13′ residues from each subunit have similar roles in channel function. In order to examine whether the loss of response to GABA in α(T13′A)β(T13′A) receptors was due to the mutation being present in a specific subunit, mutations were carried out in either the α_1 subunit (position 268), α(T13′A)β, or the β_1 subunit (position 263), αβ(T13′A), of $\alpha_1\beta_1$ $GABA_A$ receptors. Functional properties of mutant receptors were examined, including activation by GABA and modulation by other pharmacological agents and muscimol binding.

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2. Materials and methods

Techniques for general handling of *Spodoptera frugipedra* (*Sf*9) cells, production of high titre viral stock, infection procedures and generation of mutants have been described (Birnir et al., 1995). Muscimol binding was performed as described by Tierney et al. (1996). Whole-cell currents were recorded from voltage-clamped cells at a holding potential of −40 mV. Cells were perfused with bath solution (14 ml/min) containing (mM): 180 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 MES (2-[*N*-morpholino]ethanesulfonic acid) adjusted to pH 6.2 with NaOH (330 mmol/l). Pipettes made from borosilicate glass with resistances of 3–10 mΩ were filled with a solution containing (mM): 178 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 EGTA ($[Ca^{2+}] < 5 \times 10^{-8}$ M), 4 mM ATP and 10 TES (*N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid) adjusted to pH 7.2 with NaOH. Drugs were dissolved in bath solution and rapidly applied to cells by gravity feed through tubes (Birnir et al., 1995). Currents were monitored with a current-to-voltage converter (Axopatch-1D, Axon Instruments, Foster City, CA) using series resistance compensation.

To account for rundown of whole-cell currents over successive drug applications, a control concentration of GABA was applied before and after a test concentration of GABA or GABA plus drug. Results were only used from cells in which the two standard concentrations gave currents that differed by $< 20\%$ in amplitude. Responses were calculated as a fraction of the averaged control responses to GABA. Concentration–response data were averaged for each concentration and fitted with a Hill-type equation using Slidewrite™ 4.0 software. Results are presented as mean ± S.E.M. and comparisons were made using a two-tailed *t*-test, $P \leq 0.05$.

3. Results

3.1. α(T13′A)β receptors

Cells expressing α(T13′A)β receptors gave currents in response to 10 mM GABA that were similar in amplitude to currents in cells expressing wild-type receptors, but decayed more rapidly than normal (Fig. 1A; Table 1). The average half-decay time in 14 cells expressing α(T13′A)β receptors was significantly faster (62 ± 24 ms) than in wild-type $\alpha_1\beta_1$ receptors (223 ± 43 ms, $n = 6$), but the 10–90% rise time of the current was unchanged. There was no significant change in the relationship between GABA concentration and peak amplitude of the whole-cell current in cells expressing α(T13′A)β receptors compared to wild-type receptors (Fig. 1B). The solid lines show the best fit (non-linear least squares) of the Hill-type equation:

$$I = I_{max}[\mathrm{GABA}]^{n_H} / \left((EC_{50})^{n_H} + [\mathrm{GABA}]^{n_H}\right),$$

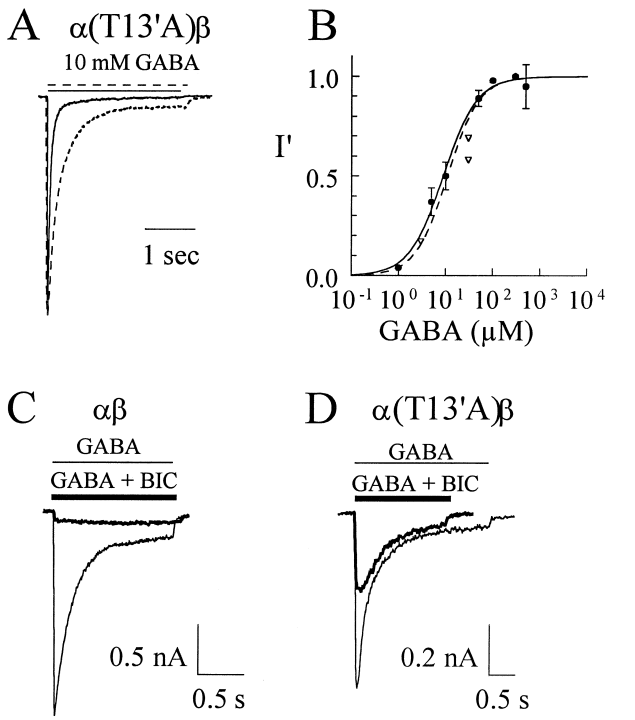

Fig. 1. Whole-cell current response to GABA. (A) Whole-cell current decay in response to 10 mM GABA in a cell expressing αβ (broken line) and α(T13′A)β receptors (solid line). Currents are normalised to the peak current amplitude in the cell expressing wild-type receptors (3.2 nA). The duration of GABA application to wild-type receptors (broken bar) and to α(T13′A)β receptors (solid bar) are shown. (B) Relationship between GABA concentration and whole-cell peak current. All GABA concentrations were applied to a single cell where possible. Otherwise, currents are relative to the response to 100 μM GABA in the same cell. Solid circles show the mean normalised current (I') and the vertical bars ± S.E.M. in three or more cells expressing α(T13′A)β receptors. The triangles represent individual cells. The solid curve is a fit of the Hill equation to the data. The broken line shows the concentration–response curve for wild-type receptors (Birnir et al., 1995). Whole-cell currents elicited in response to 100 μM GABA (thin line) and in the presence of 100 μM bicuculline (bold line) for cells expressing (C) wild-type receptors and (D) α(T13′A)β receptors. Drugs were applied for the period indicated by the bars.

where I is the peak current (pA) produced following application of GABA, I_{max} is the value of the estimated maximal or 'saturating' peak current response, [GABA] is concentration of GABA, n_H is the Hill coefficient, and EC_{50} is the GABA concentration giving half the maximal current response. The EC_{50} and Hill coefficient in α(T13′A)β receptors were 9 ± 1 μM and 1.2 ± 0.1 ($n = 16$), respectively, not significantly different from values obtained in wild-type receptors (11 ± 2 μM and 1.3 ± 0.3, respectively, $n = 12$) (Birnir et al., 1995).

Mutation of residues in M2 have been found to alter the effects of drugs that affect $GABA_A$ receptor function (Wingrove et al., 1994; Zhang et al., 1994; Gurley et al., 1995; Belelli et al., 1997; Birnir et al., 1997a; Mihic et al., 1997; Krasowski et al., 1998). The inhibitory effect of bicuculline (competitive antagonist), picrotoxin (non-com-

Table 1
Properties of $GABA_A$ receptors in *Sf*9 cells

$GABA_A$ receptor	T_{50} (ms)	Bicuculline (100 μM)	Picrotoxin (100 μM)	Penicillin (10 mM)	Pentobarbital (100 μM)	K_d (nM)	B_{max}
		I_p	I_m	I_m	I_m		
αβ (WT)	223 ± 43 (6)	0.16 ± 0.06 (3)[a]	0.12 ± 0.02 (8)[a]	0 (3)	2.25 ± 0.24 (5)	37 ± 5 (9)	5.5 ± 0.2 (9)
α[b]	ND	–	–	–	–	ND	–
α(T13′A)β	62 ± 24 (6)*	0.45 ± 0.03 (5)*	0.26 ± 0.04 (5)*	0.08 ± 0.03 (4)	4.50 ± 0.89 (7)*	21 ± 5 (3)	3.4 ± 0.1 (3)*
αβ(T13′A)	ND	–	–	–	–	ND (4)	–
α(T13′A)β(T13′A)[c]	ND	–	–	–	–	ND	–

Characteristics of whole-cell currents activated by 10 mM GABA are summarised for cells expressing either T13′A mutated or wild-type (WT) receptors. T_{50} is the time taken for the peak current to decay by 50%. Binding parameters were calculated for a single binding site. K_d is the muscimol dissociation constant and B_{max} is maximal binding (pmol/10^6 cells). The number of experiments carried out is indicated in parentheses. The degree of inhibition or potentiation by different drugs are given as a fraction of the peak current amplitude ($I_{p,pA}$) or of the mean current measured over 1 s ($I_{m,pA}$), in response to GABA obtained in the presence of a drug. Control concentrations of GABA were 100 μM for the inhibitory drugs and 10 μM for pentobarbital. Data show the mean ± S.E.M. The number of cells used for each experiment is indicated in parentheses.
[a] Birnir et al. (1995); [b] Tierney et al. (1996); [c] Tierney et al. (1998).
ND, not detected.
* Significant difference between mutant and wild-type receptors, two-tailed *t*-test, $P \leq 0.05$.

petitive antagonist) and penicillin (open channel blocker) and the potentiating effect of pentobarbital upon the GABA response in α(T13′A)β receptors were therefore examined. Results are shown in Table 1. As some drugs had a greater effect upon the decay of the current response than on the peak current response, measuring the mean current best described the effects upon the GABA-induced current. Inhibition of the response to GABA in α(T13′A)β receptors by bicuculline (Fig. 1C and D) and picrotoxin was about 50% less than the inhibition produced in wild-type receptors, whereas inhibition by penicillin was similar. Potentiation of the GABA response by pentobarbital was two times greater than that produced in wild-type receptors.

3.2. αβ(T13′A) receptors

No current was detected in response to 10 mM GABA in cells expressing αβ(T13′A) receptors ($n = 27$). Application of 100 μM pentobarbital (with 10 mM GABA), which potentiates the response to GABA in wild-type receptors, still did not elicit a response ($n = 5$). The lack of response to GABA in αβ(T13′A) receptors could be due to changes in the allosteric properties of the receptor and may result in altered agonist binding. This was assessed using a radiolabeled-muscimol-binding assay. Data were fitted by the Henri–Michaelis–Menten equation (non-linear least squares).

$$B = B_{max}[\text{muscimol}]/(K_d + [\text{muscimol}]),$$

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 radioactive muscimol and K_d is the concentration that yields half maximal binding, the dissociation constant. In cells expressing α(T13′A)β receptors, the K_d was not significantly different from that in wild-type receptors, but the B_{max} was reduced (Table 1). This indicates that the binding affinity of muscimol was unchanged but that the total number of binding sites was less than in wild-type receptors. In αβ(T13′A) receptors, no specific muscimol binding was detected. Changes in the structure of the agonist binding site could explain why receptors do not respond to GABA. When the α_1 subunit is expressed alone, however, receptors do not bind muscimol (Tierney et al., 1996). Both α_1 and β_1 subunits must be present in order for specific muscimol binding to be detected. Therefore, it is possible that muscimol binding cannot be detected because α and β(T13′A) subunits are not forming heteromeric receptors.

4. Discussion

The increase in the rate of desensitisation observed in α(T13′A)β receptors is consistent with results obtained when the homologous mutation was carried out in the α_6 subunit of rat $\alpha_6\beta_2\gamma_2$ receptors (Im et al., 1995). Reduced inhibition in response to bicuculline and picrotoxin and increased potentiation by pentobarbital, but similar degree of block by penicillin, is of interest because these drugs bind to different sites to alter the response to GABA. Pentobarbital and picrotoxin have allosteric actions and recent evidence suggests that bicuculline can also act as an allosteric inhibitor, whereas penicillin acts by an open channel block mechanism (Rabow et al., 1995; Ueno et al., 1997). The effects observed may be due to the mutation altering the conformational changes involved in desensitisation and allosteric modulation of receptor function. The reduction in the number of high affinity muscimol binding sites in α(T13′A)β receptors, as compared to wild-type, indicates a reduction in the number of heteromeric receptors, possibly due to changes in subunit structure that affect receptor assembly.

The lack of response to GABA and loss of muscimol binding in $\alpha\beta$(T13′A) receptors show that the T13′ residue in the β_1 subunit is essential for receptor function. Whether this is due to an effect upon the agonist binding site or to effects upon receptor assembly is unclear. However, the observations are consistent with GABA and muscimol sharing a common agonist binding site involving the β subunit (Casalotti et al., 1986; Amin and Weiss, 1993). Mutations in the M2 region of $\alpha_1\beta_1$ receptors at positions 5′, 9′, 10′, 12′ and now 13′ have been found to affect the rate of current decay (Tierney et al., 1996, 1998; Birnir et al., 1997a,b). Three of these have been further examined (9′, 12′ and 13′) and the modification of receptor function was found to be more dramatic when the mutation was in the β_1 subunit (Tierney et al., 1996; Birnir et al., 1997a). It appears that the β_1 subunit in the $\alpha_1\beta_1$ receptor has a distinct functional role from the α_1 subunit and that the β_1 M2 segment has a central role in the molecular mechanism of $GABA_A$ receptor operation.

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