ELSEVIER

European Journal of Pharmacology 385 (1999) 283–286

ejp

www.elsevier.nl/locate/ejphar

Short communication

Mutant human $\alpha_1\beta_1$(T262Q) $GABA_A$ receptors are directly activated but not modulated by pentobarbital

Julie E. Dalziel [1], Graeme B. Cox, Peter W. Gage, Bryndis Birnir *

Membrane Biology Program, John Curtin School of Medical Research, Australian National University, Canberra ACT, Australia

Received 21 September 1999; accepted 12 October 1999

Abstract

Pentobarbital activates $GABA_A$ receptors and enhances GABA-activated currents. A threonine residue (262) in the second membrane spanning region at the 12′ position in the β_1 subunit, $\alpha_1\beta_1$(T12′Q), is necessary for the potentiating action of pentobarbital. We examined whether T12′Q-mutated receptors expressed in *Spodoptera frugipedra* (*Sf* 9) cells responded to direct activation by pentobarbital. In both mutant and wild type receptors, pentobarbital (100 μM to 1 mM) evoked a current response. The pentobarbital EC_{50} values were similar; 119 and 158 μM for $\alpha_1\beta_1$ and $\alpha_1\beta_1$(T12′Q) receptors, respectively. The results show it is possible to discriminate between agonistic and potentiating effects of pentobarbital, suggesting these actions involve separate mechanisms.

Keywords: Pentobarbital; Ligand-gated; Anaesthetic; Mutation; $GABA_A$ receptor, human; (*Sf* 9) cell

1. Introduction

Many general anaesthetics act on the $GABA_A$ receptor to reduce neuronal excitability in the central nervous system. Several mutagenesis studies have implicated residues in the second and third membrane-spanning regions in the action of anaesthetic and anticonvulsant drugs on the $GABA_A$ receptors (Wingrove et al., 1994; Belelli et al., 1997; Birnir et al., 1997; Krasowski et al., 1998), the $GABA_C$ receptor (Mihic et al., 1997; Wick et al., 1998; Amin, 1999) and nicotinic receptors (Forman et al., 1995). However, it is not yet known whether anaesthetics bind to a hydrophobic binding pocket that includes these residues or whether the mutations in these regions interfere with transduction mechanisms that occur following drug binding.

Pentobarbital, like some other anaesthetics (Wafford et al., 1996; Belleli et al., 1997; Davies et al., 1997) has agonistic and modulatory effects on $GABA_A$ receptors. It is not yet known whether these functions of pentobarbital involve a common or separate binding site(s). We report here that the mutation, threonine 262 (12′) to glutamine in the β_1 subunit of $\alpha_1\beta_1$ $GABA_A$ receptors, did not adversely affect the agonistic or the inhibitory effect of pentobarbital despite the potentiating effect having been abolished (Birnir et al., 1997).

2. Materials and methods

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, 1997). Site-specific mutations were introduced into single stranded DNA 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.

Techniques for general handling of *Spodoptera frugipedra* (*Sf* 9) cells, production of high titre viral stock and infection procedures have been described previously (Birnir et al., 1995, 1997). 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

* Corresponding author. Tel.: +61-02-6279-8523; fax: +61-02-6249-4761.

E-mail address: bryndis.birnir@anu.edu.au (B. Birnir)

[1] Present address: Department of Molecular and Cellular Physiology, Howard Hughes Medical Institute, Stanford University, Stanford, CA 94305, USA.

0014-2999/99/$ - see front matter
PII: S0014-2999(99)00710-4

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 mosmol l^{-1}). 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, 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 applied to cells by gravity-fed through tubes. Currents were monitored with a current-to-voltage converter (Axopatch-200B, 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 pentobarbital (1 mM) was applied before and after a test concentration of pentobarbital. Results were only used from cells in which the peak current of the two standard pentobarbital concentrations differed by less than 20%. Current responses were calculated as a fraction of the averaged standard responses to 10 mM GABA (fractional peak current).

3. Results

Pentobarbital-activated currents were recorded only in cells infected with recombinant $GABA_A$ viruses. Whole-cell currents in response to 1 mM pentobarbital or 10 mM GABA in one cell are shown in Fig. 1A, for $\alpha_1\beta_1$ receptors and Fig. 1B, for $\alpha_1\beta_1$(T12′Q) receptors. For both receptors, the response to 1 mM pentobarbital was only a fraction of the GABA response. In receptors containing the

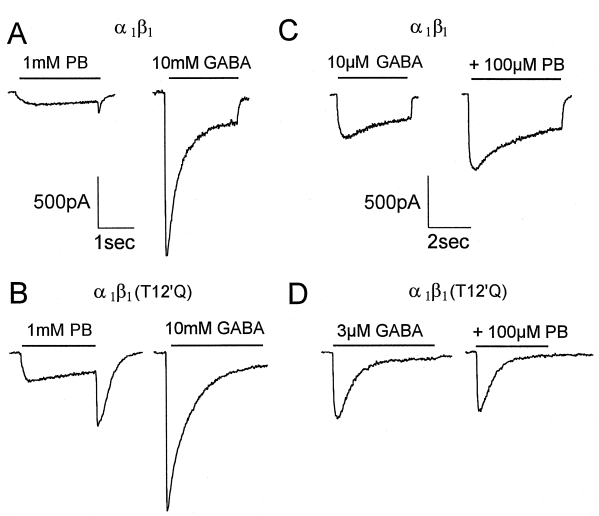

Fig. 1. Direct activation and modulation of $GABA_A$ receptors by pentobarbital. Currents evoked by 1 mM pentobarbital (Pb) or 10 mM GABA in a cell expressing (A) $\alpha_1\beta_1$ wild type or (B) $\alpha_1\beta_1$(T12′Q) mutated $GABA_A$ receptors. Pentobarbital (100 μM) enhanced currents activated by (C) 10 μM GABA in cells expressing wild type receptors but not responses to (D) 3 μM GABA in cells expressing $\alpha_1\beta_1$(T12′Q) receptors.

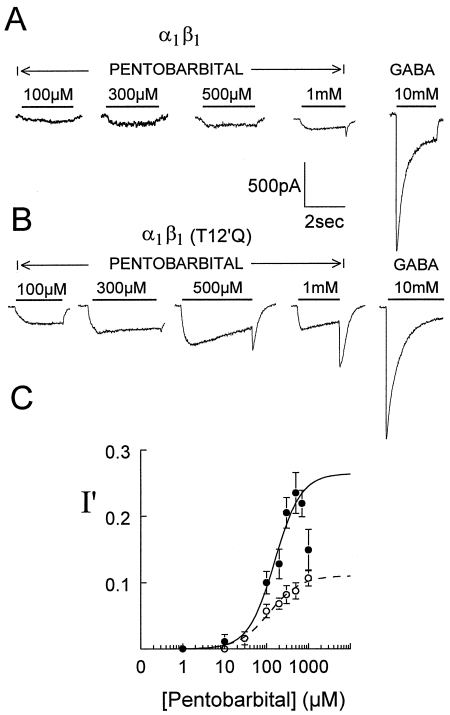

Fig. 2. Direct activation of mutant and wild type receptors by pentobarbital. Currents evoked in a cell by increasing concentrations of pentobarbital are shown for cells expressing: (A) $\alpha_1\beta_1$wild type or (B) $\alpha_1\beta_1$(T12′Q) receptors. Representative currents evoked in response to 10 mM GABA are shown for comparison. (C) The peak pentobarbital induced current represented as a fraction of the 10 mM GABA-activated current (I′) is plotted as a function of the pentobarbital concentration for cells expressing: $\alpha_1\beta_1$wild type (open circles) and $\alpha_1\beta_1$(T12′Q) receptors (filled circles). The vertical bars show ±1S.E.M. in three or more cells. Error bars are not visible if smaller than the symbol. A Hill-type equation (see Results) was fitted to dose–response data for wild type (broken line) and the mutated (solid line) receptors. The data obtained with 1 mM pentobarbital was not included in the fit for the mutant receptors because of the inhibitory effect present.

T12′Q mutation in either the α subunit α_1(T12′Q)$\beta_1$or both subunits α_1(T12′Q)β_1(T12′Q), pentobarbital (500 μM) also activated currents (data not shown). Fig. 1C and D show responses to pentobarbital (100 μM) in the presence of 10 μM (Fig. 1C, $\alpha_1\beta_1$) and 3 μM (Fig. 1D, $\alpha_1\beta_1$(T12′Q)) GABA. Potentiation of the GABA current was recorded in wild type receptors but not in $\alpha_1\beta_1$(T12′Q) receptors, as previously reported (Birnir et al., 1997). As the abolition of the current enhancement by pentobarbital was conferred by the point mutation T12′Q in the β_1 subunit alone, possible changes in direct activation by pentobarbital were examined in the $\alpha_1\beta_1$(T12′Q) receptors and compared with results in wild type receptors.

Direct activation of mutant and wild type receptors by a range of pentobarbital concentrations was examined (Fig. 2). The maximum whole-cell current response was produced at 500 μM pentobarbital in $\alpha_1\beta_1$(T12′Q) receptors (327 ± 52 pA, $n = 4$) but at 1 mM pentobarbital in wild type receptors (137 ± 38 pA, $n = 8$). In the same cells, the

peak current response to 10 mM GABA was 1414 ± 108 ($n = 4$) and 1219 ± 354 pA ($n = 8$), respectively. An ''off current'' developed when application of a high concentration of pentobarbital was stopped. In wild type receptors, millimolar concentrations were required for the ''off current'' to develop whereas in $\alpha_1\beta_1$(T12′Q) receptors an ''off current'' was recorded when 300 μM or higher concentrations of pentobarbital were removed (Fig. 2A and B). The amplitude of the ''off current'' increased as the concentration of pentobarbital was increased. An ''off current'' is thought to develop as inhibition is removed once pentobarbital is washed off (Akaike et al., 1987; Rho et al., 1996).

Pentobarbital-activated whole-cell peak current as a fraction of the 10 mM GABA current in the same cell (I', fractional peak current) is plotted against pentobarbital concentration for cells expressing $\alpha_1\beta_1$(T12′Q) and wild type receptors in Fig. 2C. The lines show the best fit of a Hill-type equation to the data.

$$I' = I'_{\max}[\mathrm{PB}]^h / \left((\mathrm{EC}_{50})^h + [\mathrm{PB}]^h\right)$$

I' is the fractional peak current produced by pentobarbital, $I'_{\max}$ is the value of the estimated maximal or ''saturating'' fractional peak current response, [PB] is the concentration of pentobarbital, h is the Hill coefficient and EC_{50} is the pentobarbital concentration giving half the maximal current response. The pentobarbital EC_{50} for the wild type receptors was 119 ± 30 μM and the Hill coefficient was 1.2 ± 0.3 ($r^2 = 0.98$). In cells expressing αβ(T12′Q) receptors, increasing the pentobarbital concentration from 500 μM to 1 mM reduced the current amplitude. Hence, 1 mM pentobarbital was not included when the Hill-type equation was fitted to the data for $\alpha_1\beta_1$(T12′Q) receptors. In cells expressing $\alpha_1\beta_1$(T12′Q) receptors, the pentobarbital EC_{50} was 158 ± 59 μM and the Hill coefficient was 1.4 ± 0.6 ($r^2 = 0.97$).

The maximum pentobarbital fractional peak current in cells expressing $\alpha_1\beta_1$(T12′Q) receptors was 0.23 ± 0.04 ($n = 4$), or two times greater than for wild type receptors where it was 0.11 ± 0.01 ($n = 8$). The larger response to pentobarbital in cells expressing $\alpha_1\beta_1$(T12′Q) receptors could be due to increased expression of receptors. We have previously reported that the level of expression in *Sf*9 cells can be varied by infecting at different times during the cells' growth curve (see Birnir et al., 1995, 1997). However, the saturating current response to 10 mM GABA in cells optimally expressing $\alpha_1\beta_1$(T12′Q) receptors (2.3 ± 0.3 nA, $n = 11$) was similar to that in wild type receptors (3.4 ± 0.6 nA, $n = 12$). The expression of receptors can be quantitated by radiolabelled muscimol binding (Tierney et al., 1996). Ligand-binding data were fitted by the Henri–Michaelis–Menten equation.

$$B = B_{\max}[\mathrm{muscimol}] / \left(K_\mathrm{d} + [\mathrm{muscimol}]\right)$$

B is the amount of [^{3}H]muscimol bound, $B_{\max}$ is the maximum bound concentration, [muscimol] is the concentration of radioactive muscimol and K_d, the dissociation constant, is the concentration that yields half maximal binding. The saturating level of muscimol binding ($B_{\max}$) and the K_d in cells expressing wild type receptors were 5.6 ± 0.2 pmol/10^6 cells and 34 ± 5 nM ($n = 10$), respectively. In cells expressing $\alpha_1\beta_1$(T12′Q) receptors, the $B_{\max}$ and K_d were 5.9 ± 0.3 pmol/10^6 cells and 16 ± 4 nM ($n = 4$), respectively. The similar $B_{\max}$ values indicate that cells infected with either mutant or wild type recombinant viruses have a similar number of high affinity binding sites.

4. Discussion

The results show that the ability of pentobarbital to activate $\alpha_1\beta_1$ GABA_A receptors was conserved when the (T12′Q) mutation was present in the β_1 subunit although the potentiating effect of pentobarbital on GABA-activated currents was abolished (Birnir et al., 1997). The inhibitory effect of pentobarbital at high agonist concentrations also remained intact as shown by the large off current and the decreased current response in 1 mM pentobarbital. The results are in accord with observations on GABA_A receptors containing the ε subunit, which can be directly activated by pentobarbital whereas potentiation of GABA currents is lost (Davies et al., 1997). Also, pentobarbital does not elicit direct current activation in receptors containing α_4 subunits ($\alpha_4\beta_1\gamma_2$) although the enhancement of GABA-activated current is intact (Wafford et al., 1996). The results from our study demonstrate that it is also possible to discriminate between the agonistic and potentiating effects of pentobarbital by a point mutation in the TM2 region. Together, these findings indicate that the requirements for pentobarbital modulation differ from those of direct activation and inhibition.

In both $\alpha_1\beta_1$(T12′Q) and wild type receptors, the maximal current response to pentobarbital was smaller than the maximal current response to GABA, consistent with results from other studies using $\alpha_1\beta_1$ GABA_A receptors (Malherbe et al., 1990; Horne et al., 1993; Sanna et al., 1995; Cestari et al., 1996). In a previous study (Birnir et al., 1997), no significant current response was detected when 100 μM pentobarbital was applied to human $\alpha_1\beta_1$ wild type receptors (Birnir et al., 1997). In this study, we used a range of pentobarbital concentrations (0.001–1 mM) and optimal expression conditions to examine the effect of pentobarbital. In wild type receptors, 100 μM pentobarbital gave only a very small current response (38 ± 3 pA), which probably explains why it was not seen in the previous study. The greater current response to pentobarbital in $\alpha_1\beta_1$(T12′Q) receptors compared with wild type receptors was probably not due to an increase in the number of heteromeric receptors expressed, as the maximal current response to GABA and the maximal

muscimol binding were similar for both receptor types. It could be due to structural or kinetic changes that affect pentobarbital binding or channel gating. The pentobarbital binding site for direct activation does not overlap with the GABA binding site at $GABA_A$ receptors (Amin and Weiss, 1993). At $\alpha_1\beta_1$ and $\alpha_1\beta_1$ (T12′Q) receptors, there appears to be about 10-fold difference in affinity for the two agonists; GABA $EC_{50} = 11$ and 15 μM (Birnir et al., 1997) and pentobarbital $EC_{50} = 119$ and 158 μM (see Fig. 2B), respectively. It is possible that following pentobarbital binding the second transmembrane region at the level of T12′ is involved in conformational changes that influence gating or coupling between the GABA and pentobarbital binding sites. From the similarity in the receptors' pentobarbital EC_{50} values, it seems unlikely that the 12′ threonine contributes to the pentobarbital agonist binding site(s), but could possibly contribute directly to the binding site(s) for pentobarbital through which its modulatory effects are mediated if the sites are different.

References

Akaike, N., Maruyama, T., Tokutomi, N., 1987. Kinetic properties of the pentobarbitone-gated chloride current in frog sensory neurons. J. Physiol. 394, 85–98.

Amin, J., 1999. A single hydrophobic residue confers barbiturate sensitivity to γ-aminobutyric acid type C receptor. Mol. Pharmacol. 55, 411–423.

Amin, J., Weiss, D.S., 1993. $GABA_A$ receptor needs two homologous domains of the beta-subunit for activation by GABA but not by pentobarbital. Nature 366, 565–569.

Belelli, D., Lambert, J.J., Peters, J.A., Wafford, K., Whiting, P.J., 1997. The interaction of the general anaesthetic etomidate with the γ-aminobutyric acid type A receptor is influenced by a single amino acid. Proc. Natl. Acad. Sci. 94, 11031–11036.

Birnir, B., Tierney, M.L., Pillai, N.P., Cox, G.B., Gage, P.W., 1995. Rapid desensitization of α_1 β_1 $GABA_A$ receptors expressed in (*Sf* 9) cells under optimized conditions. J. Membr. Biol. 148, 193–202.

Birnir, B., Tierney, M.L., Dalziel, J.E., Cox, G.B., Gage, P.W., 1997. A structural determinant of desensitization and allosteric regulation by pentobarbitone of the $GABA_A$ receptor. J. Membr. Biol. 155, 157–166.

Cestari, I.N., Uchida, I., Li, L., Burt, D., Yang, J., 1996. The agonistic action of pentobarbital on $GABA_A$ β-subunit homomeric receptors. Neuroreport 7, 943–947.

Davies, P.A., Hanna, M.C., Hales, T.G., Kirkness, E.F., 1997. Insensitivity to anaesthetic agents conferred by a class of $GABA_A$ receptor subunit. Nature 385, 820–823.

Forman, S.A., Miller, K.W., Yellen, G., 1995. A discrete site for general anesthetics on a postsynaptic receptor. Mol. Pharmacol. 48, 574–581.

Horne, A.L., Hadingham, K.L., Macaulay, A.J., Whiting, P., Kemp, J.A., 1993. The pharmacology of recombinant $GABA_A$ receptors containing bovine $\alpha_1\beta_1\gamma_{2L}$. Br. J. Pharmacol. 107, 732–737.

Krasowski, M.D., Koltchine, V.V., Rick, C.E., Ye, Q., Finn, S.E., Harrison, N.L., 1998. Propofol and other intravenous anesthetics have sites of action on the gamma-aminobutyric acid type A receptor distinct from that for isoflurane. Mol. Pharmacol. 53, 530–538.

Malherbe, P., Sigel, E., Baur, R., Persohn, E., Richards, J.G., Mohler, H., 1990. Functional characteristics and sites of gene expression of the α_1,β_1,γ_2-isoform of the rat $GABA_A$ receptor. J. Neurosci. 10, 2330–2337.

Mihic, S.J., Ye, Q., Wick, M.J., Koltchine, V.V., Krasowski, M.D., Finn, S.E., Mascia, M.P., Valenzuela, C.F., Hanson, K.K., Greenblatt, E.P., Harris, R.A., Harrison, N.L., 1997. Sites of alcohol and volatile anaesthetic action on $GABA_A$ and glycine receptors. Nature 389, 385–389.

Rho, J.M., Donevan, S.D., Rogawski, M.A., 1996. Direct activation of $GABA_A$ receptors by barbiturates in cultured rat hippocampal neurons. J. Physiol. 497, 509–522.

Sanna, E., Garau, F., Harris, R.A., 1995. Novel properties of homomeric β_1 gamma-aminobutyric acid type A receptors: actions of the anesthetics propofol and pentobarbital. Mol. Pharmacol. 47, 213–217.

Tierney, M.L., Birnir, B., Pillai, N.P., Clements, J.D., Howitt, S., Cox, G.B., Gage, P.W., 1996. Effects of mutating leucine to threonine in the M2 segment of α_1 and β_1 subunits of the GABA $\alpha_1\beta_1$ receptors. J. Membr. Biol. 154, 11–21.

Wafford, K.A, Thompson, S.A., Thomas, D., Sikela, J., Wilcox, A.S., Whiting, P.J., 1996. Functional characterization of human gamma-aminobutyric acid A receptors containing the alpha 4 subunit. Mol. Pharmacol. 50, 670–678.

Wick, M.J., Mihic, S.J., Ueno, S., Mascia, M.P., Trudell, J.R., Brozowski, S.J., Ye, Q., Harrison, N.L., Harris, R.A., 1998. Mutations of γ-aminobutyric acid and glycine receptors change alcohol cutoff: evidence for an alcohol receptor?. Proc. Natl. Acad. Sci. 95, 6504–6509.

Wingrove, P.B., Wafford, K.A., Bain, C., Whiting, P.J., 1994. The modulatory action of loreclezole at the γ-aminobutyric acid type A receptor is determined by a single amino acid in the β_2 and β_3 subunit. Proc. Natl. Acad. Sci. 91, 4569–4573.