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Decreased agonist sensitivity of human $GABA_A$ receptors by an amino acid variant, isoleucine to valine, in the α_1 subunit

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Abstract

Recombinant human $GABA_A$ receptors were investigated in vitro by coexpression of cDNAs coding for α_1, β_2, and γ_2 subunits in the baculovirus/Sf-9 insect cell system. We report that a single amino acid exchange (isoleucine 121 to valine 121) in the N-terminal, extracellular part of the α_1 subunit induces a marked decrease in agonist $GABA_A$ receptor ligand sensitivity. The potency of muscimol and GABA to inhibit the binding of the $GABA_A$ receptor antagonist [^{3}H]SR 95531 (2-(3-carboxypropyl)-3-amino-6-(4-methoxyphenyl)pyridazinium bromide) was higher in receptor complexes of α_1(ile 121) $\beta_2\gamma_2$ than in those of α_1(val 121) $\beta_2\gamma_2$ (IC_{50} values were 32-fold and 26-fold lower for muscimol and GABA, respectively). The apparent affinity of the $GABA_A$ receptor antagonist bicuculline methiodide to inhibit the binding of [^{3}H]SR 95531 did not differ between the two receptor complex variants. Electrophysiological measurements of GABA induced whole-cell Cl^- currents showed a ten-fold decrease in the $GABA_A$ receptor sensitivity of α_1 (val 121) $\beta_2\gamma_2$ as compared to α_1(ile 121) $\beta_2\gamma_2$ receptor complexes. Thus, a relatively small change in the primary structure of the α_1 subunit leads to a decrease selective for $GABA_A$ receptor sensitivity to agonist ligands, since no changes were observed in a $GABA_A$ receptor antagonist affinity and benzodiazepine receptor binding. © 1997 Elsevier Science B.V.

Keywords: $GABA_A$ receptor; α_1 subunit; Mutation; Radioligand binding; Electrophysiology

1. Introduction

Cloning of cDNAs for the brain $GABA_A$ receptor has identified a spectrum of subunits which have been grouped into five subclasses, namely α(1–6), β(1–4), γ (1–3), δ and ρ (1,2) (for reference see Dunn et al., 1994; Sieghart, 1995; Tyndale et al., 1995; Mohler et al., 1996). It has been suggested that in the mammalian brain the most common $GABA_A$ receptor complexes have a $\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_3\gamma_2$ or $\alpha_3\beta_3\gamma_2$ subunit composition (McKernan and Whiting, 1996). Expression studies have shown that binding sites for GABA and GABA-regulated Cl^- channels are formed by assembly of different subunits (Levitan et al., 1988; Verdoorn et al., 1990; Atkinson et al., 1992; Carter et al., 1992; Porter et al., 1992).

The exchange of a single amino acid residue in a constitutive $GABA_A$ receptor subunit has been shown to modify the properties of recombinant $GABA_A$ receptors (see Smith and Olsen, 1995). Mutation of phenylalanine 64 to leucine in the rat α_1 subunit, for example, led to a 200-fold decrease in $GABA_A$ receptor sensitivity to GABA when the α_1 subunit was expressed in *Xenopus* oocytes in combination with β_2 and γ_2 subunits; additionally, the affinity of $GABA_A$ receptor antagonists was found to decrease 20–200-fold (Sigel et al., 1992). Also, mutation studies have identified two domains of the rat β_2 subunit to be essential for GABA activation of the Cl^- channel (Amin and Weiss, 1993). Photoaffinity labelling of $GABA_A$ receptors by [^{3}H]muscimol indicated irreversible binding to α and β subunits (Cavalla and Neff, 1985; Bureau and Olsen, 1988). Recently evidence has been presented that [^{3}H]muscimol photoaffinity labels phenylalanine 64 of the α_1 subunit (Smith and Olsen, 1994).

In human cDNA of α_1 subunit a single nucleotide shift (adenosine to guanosine) leads to the change of a single

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amino acid (isoleucine 121 to valine 121). We now report that individual expression of these two subunit variants in combination with β_2 and γ_2 subunits in the baculovirus/Sf-9 insect cell system leads to $GABA_A$ receptors with marked differences in sensitivity to receptor agonists. The affinities of antagonist ligands of the GABA site and benzodiazepine receptor ligands were not changed.

2. Materials and methods

2.1. Construction of expression vectors for the human $GABA_A$ receptors

Standard methods in molecular biology were used for all DNA manipulations (Sambrook et al., 1989). A cDNA clone derived from human fetal brain RNA harbouring the entire α_1 coding sequence was obtained from Prof. P.H. Seeburg, University of Heidelberg (Heidelberg, Germany) (Schofield et al., 1989). The human $GABA_A$ receptor subunits β_2 and γ_{2S} were cloned by using a 1st strand cDNA as template (from human hippocampus and total brain poly-A^+ mRNA, respectively). The mRNAs and the 1st-strand-TM cDNA Synthesis Kit were obtained from Clontech (Palo Alto, CA, USA). Primers that overlap with the start and stop codons were used together with Pfu Polymerase (Stratagene). The primers for the β_2 matched the rat sequence and those for γ_{2S} matched the human sequence. The cDNA genes were directionally cloned into a pBlueBac III transfer vector (Invitrogen) in the *Bgl* II site by subsequent PCR amplifications. Flanking primers (for α_1 cDNA, upper strand primer A: 5′-CCGGAGATCTTATAAATATGAGGAAAAGTCCAGG-TCTGTCTG-3′, lower strand primer B: 5′-CCGG-AGATCTATTGATGTGGTGTGGGGGCT-3′, for β_2 cDNA, upper strand primer C: 5′-CCGGAGATCTTAT-AAATATGTGGAGAGTCCGGAAAAGGGGC-3′, lower strand primer D: 5′-CCGGAGATCTTTTTAGT-TCACATAGTAAAGCCAATA-3′, for γ_{2S} cDNA, upper strand primer E: 5′-CCGGAGATCTTATAAATATGAGT-TCACCAAATATATGG-3′, lower strand primer F: 5′-CCGGAGATCTTCCTCACAGGTAGAGGTAGGAGA-CCCA-3′) were designed to create new cDNA genes with only little leader DNA preceding the initiation codon and to introduce an endonuclease *Bgl* II restriction site at both ends of the genes. The new cDNA constructions are thus suitable for transfer to linearized baculovirus AcNPV (Invitrogen) and expression studies in *Spodotera frugiperda* Sf-9 insect cells (Gibco) by general baculovirus methods (O'Reilly et al., 1992).

Solid-phase DNA sequencing (Dynal) combined with sequenase version 2.0 DNA sequencing kit (USB) of each insert DNA verified that the amino acid sequence of the β_2 subunit was identical to the one previously reported (Hadingham et al., 1993) and that the γ_{2S} subunit varied at amino acid 81 (threonine instead of methionine) and amino acid 142 (threonine instead of serine) compared to the reported amino acid sequences (Pritchett et al., 1989; Mihic et al., 1994).

The amino acid sequence of the investigated α_1 subunit has an isoleucine to valine exchange at amino acid residue 121 compared to the reported amino acid sequence (Schofield et al., 1989). The α_1 subunit with isoleucine as amino acid residue 121 was constructed from α_1 (val 121) by mutagenesis with overlap extension PCR as described (McArn and Readington, 1991) with a G to A change in the first position of the Val 121 codon (GTC to ATC) using the flanking primers A and B in combination with two mutagenic primers 'b': 5′-TGCCATCCT CTGTGATCCGCAGGAGTT TGT-3′ and 'c': 5′-ACAAA CTCCTGCGGATCACAGAGGATGGCA-3′.

2.2. Cell culture and viral infection

Sf-9 insect cells (Gibco) were grown in spinner flask cultures at 27°C in serum-free medium (Sf900-II-SFM, Gibco). Batches of 200 ml insect cells at a density of 10^6 cells/ml were infected with baculovirus containing human cDNA of α_1 (ile 121) or α_1 (val 121) and β_2 and γ_{2S} subunits at multiplicity of infection (MOI) of 1:1:1. Cells were harvested by centrifugation ($4000 \times g$ for 10 min) 42–45 h post infection (HPI) and used for receptor binding assays.

Insect cells for electrophysiological measurements were seeded at a density of 7.5×10^5 cells/35 mm petri dish and pulse infected with recombinant virus at MOI of 1:1:1 for 1 h at room temperature. The infected cells were incubated in a humidified chamber at 27°C for 25 h.

2.3. Membrane preparation and [^{3}H]SR 95531 binding

Pellets containing 4×10^6 cells were homogenised in 5 ml Tris, citrate (50 mM, pH 7.1) by an Ultra Turrax homogeniser at 0–4°C. The homogenate was centrifuged at $30\,000 \times g$ for 10 min and the pellet was resuspended in 2.5 ml Tris, citrate (50 mM, pH 7.1). The membrane suspension was kept at 37°C for 30 min, centrifuged and the pellet washed twice in Tris, citrate buffer. The final pellet was kept frozen at −80°C or directly resuspended in Tris, citrate (50 mM, pH 7.1) to a final concentration of 1.4×10^6 original cells/ml. [^{3}H]SR 95531 (1.4 nM, 49.5 Ci/mmol, NEN) was added to aliquots of 0.5 ml membrane suspension. Following incubation at 0–4°C for 30 min, the samples were rapidly filtered through Whatman GF/C filters. Non-specific binding was obtained by adding GABA (10^{-4} M) to separate samples. Drugs: GABA receptor agonists and antagonists were purchased from Sigma and RBI. SR 95531 is (2-(3-carboxypropyl)-3-amino-6-(4-methoxyphenyl)pyridazinium bromide; CACA

is 4-amino-cis-2-buten-ol; TACA is (*E*)-4-amino-2-butenoic acid and ZAPA is (*Z*)-3-[(aminoiminomethyl)thio]prop-2-enoic acid. 4-PIOL (5-(4-piperidyl)isoxazol-3-ol), Thio-4-Piol 5-(4-piperidyl)isothiazol-3-ol and THIP (4,5,6,7-tetrahydroisoxazolo[4,5-*c*]pyridine-3-ol) were kindly provided by P. Krogsgaard-Larsen (Royal Danish School of Pharmacy, Denmark).

2.4. Electrophysiological measurements

GABA-induced Cl^- currents (see Sirasaki et al., 1992) in the Sf-9 cells were recorded using conventional whole-cell patch mode a room temperature (22–25°C). The resistance between the patch-pipette filled with internal solution and the reference electrode was 4–6 MΩ. The membrane potential was held at −40 mV and measurements were started after stabilization of the GABA response (8–15 min after the establishment of the whole-cell configuration). The ionic composition of the standard external solution was (in mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES and 10 glucose adjusted to pH 7.4 with Tris-base. The internal solution in the patch-pipette was (in mM): 78 $CsCl_2$, 50 $CsMeSO_4$, 6 $MgCl_2$, 5 EGTA, 10 HEPES and 5 K_2ATP adjusted to pH 7.2 with Tris-base.

3. Results

The amino acid residue 121 in the α_1 subunit of the $GABA_A$ receptor complex determines the affinity of agonist $GABA_A$ receptor ligands. $GABA_A$ receptor complexes, α_1(ile 121) $\beta_2\gamma_{2S}$ and α_1(val 121) $\beta_2\gamma_{2S}$ were constructed by expression of recombinant human cDNA in the baculovirus/Sf-9 insect cell system. The inhibition of the $GABA_A$ receptor antagonist [^{3}H]SR 95531 binding by GABA and the GABA receptor agonists muscimol, isoguvacine, *S*- and *R*-dihydroxymuscimol, ZAPA, CACA, isonipecotic acid, THIP and imidazole-4-acetic acid was decreased in α_1(val 121) $\beta_2\gamma_{2S}$ as compared to α_1(ile 121) $\beta_2\gamma_{2S}$ complexes (Table 1). In contrast, the competitive $GABA_A$ receptor antagonist bicuculline methiodide and the partial agonists 4-PIOL (Krogsgaard-Larsen et al., 1994) and Thiol-4-Piol did not differ in their affinity to either α_1(val 121) $\beta_2\gamma_{2S}$ or α_1(ile 121) $\beta_2\gamma_{2S}$ complexes (Table 1). The ability of GABA to induce whole-cell Cl^- currents was decreased by approx. 10-fold in α_1(val 121) $\beta_2\gamma_{2S}$ when compared with α_1(ile 121) $\beta_2\gamma_{2S}$ complexes (Fig. 1). The same difference was observed with α_1(val 121) β_2 and α_1(ile 121) β_2 combinations (data not shown), suggesting that the observed effects of the α_1 amino acid

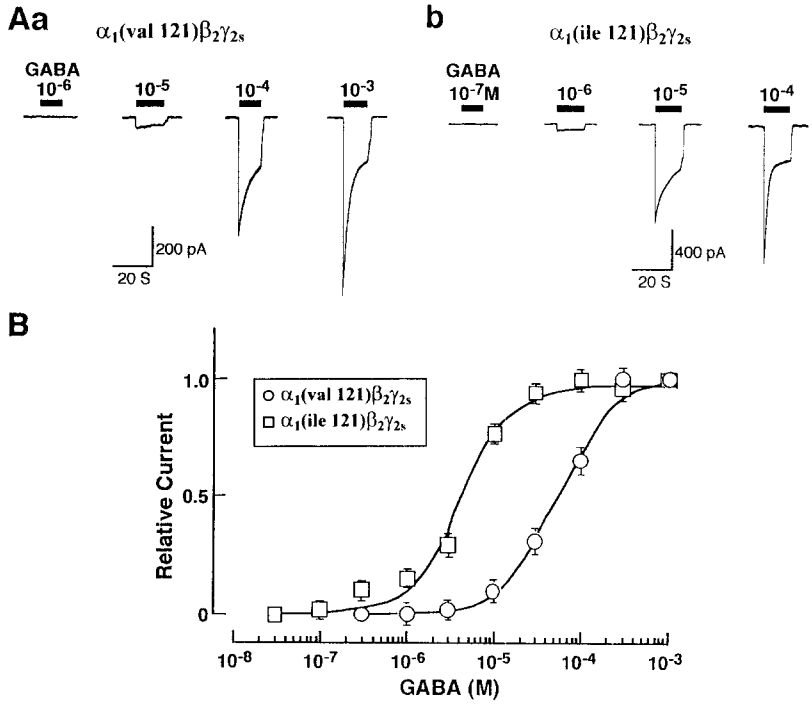

Fig. 1. Concentration-response relationship for GABA-induced currents in Sf-9 transfected with α_1 (val 121) $\beta_2\gamma_{2S}$ or α_1 (ile 121)$\beta_2\gamma_{2S}$ subunits. (A) Current responses to various concentrations of GABA in cells experssing (Aa) α_1 (val 121) $\beta_2\gamma_{2S}$ or (Ab) α_1 (ile 121)$\beta_2\gamma_{2S}$ subunit combinations. Holding potential was −40 mV. (B) Concentration-response relationships for GABA-induced currents. Whole-cell current responses induced by GABA in Sf-9 cells expressing human $GABA_A$ receptors assembled with α_1 (ile 121) (squares) or α_1 (val 121) (circles) together with β_2 and γ_{2S} subunits. All current responses were normalized to the peak current induced by 10^{-3} M GABA in each cell. Each symbol and vertical bar indicates mean ± S.D. of 6–8 cells.

Table 1
Inhibition of [^{3}H]SR 95531 (1.4 nM) binding by various $GABA_A$ receptor ligands in Sf-9 cells expressing α_1(ile 121) $\beta_2\gamma_{2S}$ and α_1(val 121) $\beta_2\gamma_{2S}$ receptor complexes

Substance	K_i, nM		Ratio
	α_1(ile 121) $\beta_2\gamma_{2S}$	α_1(val 121) $\beta_2\gamma_{2S}$	α_1(val)/α_1(ile)
Muscimol	11 ± 4.5	340 ± 47	32
GABA	56 ± 12	1 440 ± 420	26
Isoguvacine	250 ± 70	4 100 ± 190	16
S-Dihydroxy-muscimol	12 ± 2.6	170 ± 37	15
R-Dihydroxy-muscimol	390 ± 80	5 800 ± 3900	15
ZAPA	48 ± 7	710 ± 90	15
TACA	1 180 ± 108	1 180 ± 360	13
CACA	6 900 ± 620	82 000 ± 2 800	12
Isonipecotic acid	1 120 ± 360	12 500 ± 3 500	11
THIP	370 ± 42	3 660 ± 360	10
Imidazole-4-acetic acid	680 ± 250	2 300 ± 750	3.4
SR 95531	10 ± 2.7	14 ± 5.1	1.4
Thiol-4-Piol	2 600 ± 720	2 500 ± 650	1.0
Bicuculline methiodide	7 300 ± 1 200	6 400 ± 1 800	0.8
4-PIOL	8 500 ± 840	5 870 ± 1 700	0.7

All values are means ± S.D. of 3–5 determinations. Scatchard plots of [^{3}H]SR 95531 binding (10 concentrations, 0.5–25 nM) to membranes from receptor complexes of α_1(ile 121) $\beta_2\gamma_{2S}$ and α_1(val 121) $\beta_2\gamma_{2S}$ showed binding affinity constants (K_D) values of 13 ± 5 nM ($n = 7$) and 21 ± 4 nM ($n = 8$), respectively (values as the means ± S.D.). K_i values are calculated according to: $K_i = IC_{50}\ (1/(1 + [L]/K_D)$. Ratio is: K_i (α_1(val 121) $\beta_2\gamma_{2S}$)/K_i (α_1(ile 121) $\beta_2\gamma_{2S}$).

variants are not due to sequence differences between the γ_{2S} subunit used in this study and the published γ_{2S} subunit sequence. We found no differences between α_1(val 121) $\beta_2\gamma_{2S}$ and α_1(ile 121) $\beta_2\gamma_{2S}$ complexes in [^{3}H]flunitrazepam binding to the benzodiazepine receptor (data not shown).

4. Discussion

A number of amino acid residues in the $GABA_A$ receptor subunits have been shown to determine the affinity of $GABA_A$ receptor ligands and the efficacy of $GABA_A$ receptor agonists to gate the Cl^- channel (see Smith and Olsen, 1995): Phe 64 in rat α_1 subunit (Phe 65 in bovine α_1) (Sigel et al., 1992; Smith and Olsen, 1994); Tyr 157, Tyr 205, Thr 160 and Thr 202 in rat β_2 (Amin and Weiss, 1993); Arg 216, Arg 269, Asp 146, Thr 160 and Thr 202 based on molecular modelling investigations (Aprison et al., 1996). In a mutagenesis study, several amino acid residues located within the extracellular disulphide loop have been shown to be inconsequential for GABA binding to the receptor complex (Amin et al., 1994). Interestingly, Birnir et al. (1995) reported that a recombinant human cDNA of the α_1 subunit has an isoleucine to valine mutation in position 121 as well as a isoleucine to phenylalanine mutation in position 317, but both mutations were corrected and thus the influence of valine at position 121 not investigated further. The present data, however, show that a subtle change (isoleucine to valine) in the amino acid sequence of the α_1 subunit induces a decrease selective for the affinity of agonist $GABA_A$ receptor ligands. Since amino acid sequence alignments show that isoleucine 121 is conserved in the α_1 subunit of all species investigated including rat, mouse, bovine and chicken, it is probable that this amino acid residue is of key importance for the binding affinity and efficacy of GABA to $GABA_A$ receptor combinations containing an α_1 subunit. Ongoing experiments are elucidating the nature of this mutation and its possible occurrence in-vivo.

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