

## Pharmacological study on recombinant human GABA-A receptor complex containing $\alpha_5$ (leucine155 to valine) combined with $\beta_3\gamma_{2s}$ subunits

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**KEY WORDS** GABA-A receptor; mutation; recombinant proteins

### ABSTRACT

**AIM:** To investigate the characterization of the pharmacology of recombinant human GABA-A receptor complex containing  $\alpha_5\beta_3\gamma_{2s}$  subunits, and clarify which amino acids are essential for binding affinity. **METHODS:** We constructed chimeric subunit of  $\alpha_5$  (leucine mutated to valine in 155) by site-directed mutation, combined  $\alpha_5$  or  $\alpha_5$  (valine155) with  $\beta_3\gamma_{2s}$  subunits by using the baculovirus-transfected Sf-9 insect cell expressing system. Fifteen commercially available effective compounds for the GABA binding recognition site were determined. **RESULTS:** We found that competitive antagonist SR95531 and inhibitor Thio-4-piol increased  $IC_{50}$  values about 3 folds in  $\alpha_5$  (valine155)  $\beta_3\gamma_{2s}$  compare to  $\alpha_5$  (leucine155)  $\beta_3\gamma_{2s}$  receptor subunit combinations; Other GABA-A receptor ligands have little difference. **CONCLUSION:** The amino acid residues in 155 is important for the binding affinity and efficacy of SR95531 and Thio-4-piol to GABA-A receptor combinations containing an  $\alpha_5$  subunit.

### INTRODUCTION

The GABA-A receptor is a member of the ligand-gated ion channel superfamily and mediates the inhibitory synaptic transmission in the brain<sup>[1]</sup>. Much interest has been focused on the GABA-A receptor function because several clinically important classes of drugs, including anticonvulsants, sedative-hypnotics and general anesthetics allosterically modulate the receptor function<sup>[2,3]</sup>. To date, 19 GABA-A receptor subunits divided into seven

classes have been cloned, namely  $\alpha_{(1-6)}$ ,  $\beta_{(1-4)}$ ,  $\gamma_{(1-3)}$ ,  $\delta_1, \epsilon_1$ ,  $\pi_1$ , and  $\rho_{1-3}$ <sup>[4-7]</sup>. In the present study, we have mainly studied the characterization of the pharmacology of recombinant human GABA-A receptor complexes  $\alpha_5\beta_3\gamma_{2s}$  (including mutant) to clarify the mechanism of drug-receptor interaction.

### MATERIALS AND METHODS

**GABA-A receptor expression in Sf-9 insect cells** Sf-9 insect cells were grown in spinner flask cultures at 27 °C in serum-free medium (Sf 900-II-SFM, Gibco). Batches of 200 mL insect cells at a density of approximately  $1 \times 10^9/L$  were infected with baculovirus containing human cDNA of  $\alpha_5$  (leucine155) or  $\alpha_5$  (valine155) with  $\beta_3\gamma_{2s}$  subunits at multiplicity of infection (MOI) of 1:1:1 in separate experiments, the added amount of virus per cell (MOI value) was optimized for each receptor combination to give maximal <sup>3</sup>H-FNM and <sup>3</sup>H-muscimol receptor binding (a typical  $\alpha \beta \gamma$  MOI value is 1:1:1). Cells were harvested by centrifugation (4000 × g for 10 min) 42-45 h post infection (HPI) and used for receptor binding assay or kept as pellets at -80 °C until use. The amino acid sequences of the subunits of the recombinant human GABA-A receptors used in this study have been published previously<sup>[8]</sup>.

**Homogenized membrane preparations**<sup>[8]</sup> Pellets (Sf-9 cells) containing  $30 \times 10^6$  cells were homogenized in 5 mL Tris-citrate buffer (50 mmol/L, pH 7.1) by an Ultra Turrax homogeniser at 0-4 °C. The homogenate was centrifuged at 15 000 × g for 10 min and the pellet was resuspended in 1.5 mL of Tris-citrate buffer, incubated at 37 °C for 30 min, centrifuged, and followed by two further washes in Tris-citrate.

The final pellet was resuspended in Tris-citrate buffer to give an original cell concentration of  $1.4 \times 10^9/L$  or was kept frozen at -80 °C.

**<sup>3</sup>H-muscimol binding** <sup>3</sup>H-muscimol (640 GBq/

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Received 2000-10-30

Accepted 2001-08-01

mmol, New England Nuclear, Dupont) 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 11731 Semiautomatic Cell Harvester. Non-specific binding was obtained by adding GABA ( $10^{-4}$  mol/L) to separate samples. The radioactivity on the filters was measured in 2.5 mL of scintillation fluid by Liquid Scintillation Counting. Competition experiments were done using  $^3\text{H}$ -muscimol at a concentration of 10 nmol/L. Saturation binding experiments were done using  $^3\text{H}$ -muscimol at a concentration between 0.5 to 20 nmol/L (9 concentration). Drug was diluted into water and added to the binding assay. All assays were done in triplicate at least. GABA receptor agonists and antagonists were purchased from Sigma and RBI. CACA is *cis*-4-amino crotonic acid; TACA is *trans*-4-amino crotonic acid; ZAPA is  $\alpha$ -3-amidiniothio propenoic acid; 3-APS is 3-amino-propyl sulfonic acid; Thip is 4,5,6,7-tetrahydroisoxazolo [4,5-c] pyridin-3-ol; PSA is piperidine-4-sulfonic acid; 4-Piol is 5-(4-piperidyl) isoxazol-3-ol.

**Data analysis** All binding data were analyzed using linear regression and statistic significance was obtained using *t*-test.

## RESULTS AND DISCUSSION

To investigate the characterization of the pharmacology of recombinant human GABA-A receptor complex containing  $\alpha_5\beta_3\gamma_{2s}$  subunits, and clarify which amino acids are essential for binding affinity, we constructed chimeric subunit of  $\alpha_5$  leucine to valine in 155 (because it is near to putative GABA binding site) by site-directed mutation, combined  $\alpha_5$  or  $\alpha_5$ (valine 155) with  $\beta_3\gamma_{2s}$  subunits by using the baculovirus-transfected Sf-9 insect cell expressing system. Fifteen commercially available effective compounds for the GABA binding recognition site were determined. We found that SR95531 [2-(3'-carboxy-2'-propyl)-3-amino-6-*p*-methoxy phenylpyridinium bromide], a competitive antagonist and Thio-4-piol [5-(4-piperidyl) isothiazol-3-ol], an inhibitor increased  $\text{IC}_{50}$  values about 3 folds in  $\alpha_5$ (valine155)  $\beta_3\gamma_{2s}$  compare to  $\alpha_5$ (leucine155)  $\beta_3\gamma_{2s}$  receptor subunit combinations.  $\text{IC}_{50}$  values: SR95531 equal to  $(0.60 \pm 0.13) \mu\text{mol/L}$  ( $\alpha_5\beta_3\gamma_{2s}$ ) and  $(1.7 \pm 0.4) \mu\text{mol/L}$   $\alpha_5$ (valine155)  $\beta_3\gamma_{2s}$ ; Thio-4-piol equal to  $(5.2 \pm 2.2) \mu\text{mol/L}$   $\alpha_5\beta_3\gamma_{2s}$  and  $(16.0 \pm 0.8) \mu\text{mol/L}$   $\alpha_5$ (valine155)  $\beta_3\gamma_{2s}$ , respectively.

Scatchard plots of  $^3\text{H}$ -muscimol binding (9 concentrations; 0.5–20 nmol/L) to membranes from receptor

complexes of  $\alpha_5$ (leucine155)  $\beta_3\gamma_{2s}$  and  $\alpha_5$ (valine155)  $\beta_3\gamma_{2s}$  showed binding affinity constants ( $k_D$ ) values of  $(7 \pm 4)$  nmol/L ( $n = 3$ ),  $B_{\text{max}} = (1130 \pm 110)$  pmol/g protein;  $(9.30 \pm 0.22)$  nmol/L ( $n = 4$ ),  $B_{\text{max}} = (1379 \pm 606)$  pmol/g protein, respectively.

Studies have indicated that the exchange of a single amino acid in a GABA-A receptor subunit could influence the properties of recombinant GABA-A receptor produced with this subunit.

In the present investigation, The inhibition of the GABA-A receptor agonist  $^3\text{H}$ -muscimol binding by GABA receptor antagonist SR95531 and inhibitor Thio-4-piol was decreased in  $\alpha_5$ (valine155)  $\beta_3\gamma_{2s}$  as compared to  $\alpha_5$ (leucine155)  $\beta_3\gamma_{2s}$  complexes (Tab 1). The  $\text{IC}_{50}$  values were increased about 3 folds in  $\alpha_5$ (valine155)  $\beta_3\gamma_{2s}$  as compared to  $\alpha_5$ (leucine155)  $\beta_3\gamma_{2s}$  receptor subunit combination. In contrast, other GABA-A receptor ligands have little difference in their affinity to either  $\alpha_5$ (valine155)  $\beta_3\gamma_{2s}$  or  $\alpha_5$ (leucine155)  $\beta_3\gamma_{2s}$  complexes. It is probable that this amino acid residue is of importance for the binding affinity and efficacy of SR95531 and Thio-4-piol to GABA-A receptor combinations containing a  $\alpha_5$  subunit.

**ACKNOWLEDGEMENTS** This work was finished under directing of Prof Mogens NIELSEN. This research was supported by the Lun Bay Company (Denmark). The author thank Dr WANG Xiao-Mei for discussion and help.

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Tab 1.  $IC_{50}$  values ( $\mu\text{mol/L}$ ) of the investigated compounds for the inhibition of the binding of  $^3\text{H}$ -muscimol (10 nmol/L) to membrane preparations of human recombinant GABA-A receptor complexes containing  $\alpha_5(\text{leucine155})\beta_3\gamma_{2s}$  and  $\alpha_5(\text{valine155})\beta_3\gamma_{2s}$  expressed in Sf-9 cells.  $n = 3 - 5$  determinations.  $\bar{x} \pm s$ .  $^bP < 0.05$  vs  $\alpha_5(\text{leucine155})\beta_3\gamma_{2s}$ . Ratio is  $IC_{50}[\alpha_5(\text{leucine155})\beta_3\gamma_{2s}]/IC_{50}[\alpha_5(\text{valine155})\beta_3\gamma_{2s}]$ .

Compounds	$IC_{50}/\mu\text{mol}\cdot\text{L}^{-1}$		Ratio
	$\alpha_5(\text{leucine155})\beta_3\gamma_{2s}$	$\alpha_5(\text{valine155})\beta_3\gamma_{2s}$	
Muscimol	$0.020 \pm 0.008$	$0.020 \pm 0.008$	1.00
GABA	$0.060 \pm 0.009$	$0.060 \pm 0.003$	0.75
ZAPA	$0.11 \pm 0.04$	$0.130 \pm 0.024$	0.85
TACA	$0.14 \pm 0.06$	$0.160 \pm 0.006$	0.88
Pitrazepine	$0.24 \pm 0.11$	$0.16 \pm 0.05$	1.50
Thio-muscimol	$0.28 \pm 0.05$	$0.340 \pm 0.020$	0.82
3-APS	$0.29 \pm 0.14$	$0.28 \pm 0.03$	1.04
Thip	$0.40 \pm 0.08$	$0.73 \pm 0.20$	0.55
Imidazole-4-aceticacid	$0.53 \pm 0.18$	$0.70 \pm 0.11$	0.76
SR95531	$0.60 \pm 0.13$	$1.6 \pm 0.4^b$	0.35
PSA	$0.7 \pm 0.4$	$1.0 \pm 0.3$	0.64
Thio-4-piol	$5.2 \pm 2.2$	$16.0 \pm 0.8^b$	0.32
Bicuculline methiodide	$27 \pm 4$	$40 \pm 4$	0.67
4-Piol	$36 \pm 9$	$27 \pm 12$	1.30
CACA	$52 \pm 20$	$62 \pm 31$	0.84

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### 重组人的 GABA-A 受体复合物 $\alpha_5(155 \text{ 亮氨酸突变为缬氨酸})\beta_3\gamma_{2s}$ 的药理学特征

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关键词 GABA-A 受体; 突变; 重组蛋白质类

目的: 为了研究重组人的 GABA-A 受体复合物  $\alpha_5\beta_3\gamma_{2s}$  的药理学特征, 阐明哪一个氨基酸是影响亲和性的关键. 方法: 通过定位导向突变, 我们构建了  $\alpha_5(155 \text{ 亮氨酸突变为缬氨酸})$  亚基, 并通过 baculovirus 病毒-Sf-9 昆虫表达系统重组了  $\alpha_5(155 \text{ 亮氨酸突变为缬氨酸})\beta_3\gamma_{2s}$  亚基的 GABA-A 受体复合物, 测定了 15 个 GABA-A 受体 GABA 位点活性化合物. 结果: 发现竞争性拮抗剂 SR95531 和抑制剂 Thio-4-piol 的  $IC_{50}$  值增加了约 3 倍. 结论: 155 位氨基酸很有可能是影响 SR95531 和 Thio-4-piol 对含有  $\alpha_5$  亚基的 GABA-A 受体复合物亲和性和药效的重要位点.

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