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Pharmacological study on recombinant human GABA-A receptor complex containing α_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 α_5 (leucine mutated to valine in 155) by site-directed mutation, combined α_5 or α_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 α_5 (valine155) $\beta_3\gamma_{2s}$ compare to α_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 α_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[1]. Much interest has been focused on the GABA-A receptor function because several clinically important classes of drugs, including anticonvulants, sedative-hypnotics and general anesthetics allosterically modulate the receptor function[2,3]. To date, 19 GABA-A receptor subunits divided into seven classes have been cloned, namely $\alpha_{(1-6)}$, $\beta_{(1-4)}$, $\gamma_{(1-3)}$, δ_1, ε_1, π_1, and ρ_{1-3}[4-7]. 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.

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MATERIALS AND METHODS

GABA-A receptor expression in Sf-9 insect cells Sf-9 insect cells were grown in spinner flask cultures at 27 ℃ in serum-free medium (Sf 900-Ⅱ-SFM, Gibco). Batches of 200 mL insect cells at a density of approximately 1×10^9/L were infected with baculovirus containing human cDNA of α_5 (leucine155) or α_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 ^{3}H-FNM and ^{3}H-muscimol receptor binding (a typical $\alpha\ \beta\ \gamma$ MOI value is 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 assay or kept as pellets at - 80 ℃ until use. The amino acid sequences of the subunits of the recombinant human GABA-A receptors used in this study have been published previously[8].

Homogenized membrane preparations[8] Pellets (Sf-9 cells) containing 30×10^6 cells were homogenized in 5 mL Tris-citrate buffer (50 mmol/L, pH 7.1) by an Ultra Turrax homogeniser at 0 - 4 ℃. The homogenate was centrifuged at 15 000 $\times g$ for 10 min and the pellet was resuspended in 1.5 mL of Tris-citrate buffer, incubated at 37 ℃ 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 1.4×10^9/L or was kept frozen at - 80 ℃.

^{3}H-muscimol binding ^{3}H-muscimol (640 GBq/

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mmol, New England Nuclear, Dupont) was added to aliquots of 0.5 mL membrane suspension. Following incubation at 0 – 4 ℃ 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}H-muscimol at a concentration of 10 nmol/L. Saturation binding experiments were done using ^{3}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 ware 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 *z*-3-amidinothio propenoic acid; 3-APS is 3-aminopropyl 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 α_5 leucine to valine in 155 (becauce it is near to putive GABA binding site) by site-directed mutation, combined α_5 or α_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 phenylpyridazinium bromide], a competitive antagonist and Thio-4-piol [5-(4-piperidyl) isothiazol-3-ol], an inhibitor increased IC_{50} values about 3 folds in α_5(valine155) $\beta_3\gamma_{2s}$ compare to α_5(leucine155) $\beta_3\gamma_{2s}$ receptor subunit combinations. IC_{50} values: SR95531 equal to (0.60 ± 0.13) μmol/L($\alpha_5\beta_3\gamma_{2s}$) and ($1.7 \pm 0.4$) μmol/L α_5(valine155) $\beta_3\gamma_{2s}$; Thio-4-piol equal to (5.2 ± 2.2) μmol/L α_5 $\beta_3\gamma_{2s}$) and (16.0 ± 0.8) μmol/L α_5(valine155)$\beta_3\gamma_{2s}$, respectively.

Scatchard plots of ^{3}H-muscimol binding (9 concentrations: 0.5 – 20 nmol/L) to membranes from receptor complexes of α_5(leucine155)$\beta_3\gamma_{2s}$ and α_5(valine155)$\beta_3\gamma_{2s}$ showed binding affinity constants (k_D) values of (7 ± 4) nmol/L ($n = 3$), B_{max} = (1130 ± 110) pmol/g protein; (9.30 ± 0.22) nmol/L ($n = 4$), B_{max} = (1379 ± 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}H-mucimol binding by GABA receptor antagonist SR95531 and inhibitor Thio-4-piol was decreased in α_5 (valine155) $\beta_3\gamma_{2s}$ as compared to α_5 (leucine155) $\beta_3\gamma_{2s}$ complexes (Tab 1). The IC_{50} values were increased about 3 folds in α_5(valine155)$\beta_3\gamma_{2s}$ as compared to α_5(leucine155)$\beta_3\gamma_{2s}$ receptor subunit combination. In contrast, other GABA-A receptor ligands have little difference in their affinity to either α_5(valine155)$\beta_3\gamma_{2s}$ or α_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 α_5 subunit.

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Tab 1. IC_{50} values (μmol/L) of the investigated compounds for the inhibition of the binding of ^{3}H-muscimol (10 nmol/L) to membrane preparations of human recombinant GABA-A receptor complexes containing α_5(leucine155)$\beta_3\gamma_{2s}$ and α_5(valine155)$\beta_3\gamma_{2s}$ expressed in Sf-9 cells. $n = 3-5$ determinations. $\bar{x} \pm s$. $^bP < 0.05$ *vs* α_5(leucine155)$\beta_3\gamma_{2s}$. Ratio is IC_{50} [α_5(leucine155) $\beta_3\gamma_{2s}$]/IC_{50}[α_5(valine155) $\beta_3\gamma_{2s}$].

Compounds	IC_{50}/μmol·L^{-1}		Ratio
	α_5(leucine155) $\beta_3\gamma_{2s}$	α_5(valine155) $\beta_3\gamma_{2s}$	
Muscimol	0.020 ± 0.008	0.020 ± 0.008	1.00
GABA	0.060 ± 0.009	0.080 ± 0.003	0.75
ZAPA	0.11 ± 0.04	0.130 ± 0.024	0.85
TACA	0.14 ± 0.06	0.160 ± 0.006	0.88
Pitrazepine	0.24 ± 0.11	0.16 ± 0.05	1.50
Thio-muscimol	0.28 ± 0.05	0.340 ± 0.020	0.82
3-APS	0.29 ± 0.14	0.28 ± 0.03	1.04
Thip	0.40 ± 0.08	0.73 ± 0.20	0.55
Imidazole-4-aceticacid	0.53 ± 0.18	0.70 ± 0.11	0.76
SR95531	0.60 ± 0.13	1.6 ± 0.4^b	0.35
PSA	0.7 ± 0.4	1.0 ± 0.3	0.64
Thio-4-piol	5.2 ± 2.2	16.0 ± 0.8^b	0.32
Bicuculline methiodide	27 ± 4	40 ± 4	0.67
4-Piol	36 ± 9	27 ± 12	1.30
CACA	52 ± 20	62 ± 31	0.84

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重组人的 GABA-A 受体复合物 α_5(155 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合性的关键. **方法**：通过定位导向突变，我们构建了 α_5(155 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 α_5(155 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 α_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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