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NMR structure of an intracellular third loop peptide of human $GABA_B$ receptor

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Abstract

$GABA_B$ receptor is a G protein-coupled receptor for GABA and drug target for neurological and psychiatric disorders. From the analysis of GTPγS binding assay, we found that a synthesized peptide (GABAb: ETKSVSTEKINDHR) corresponding to the intracellular third loop region of metabotropic $GABA_B$ receptor could activate G_i protein α subunit directly. The three dimensional molecular structure of the peptide in SDS-d_{25} micelles was determined by 2D ^{1}H-NMR spectroscopy. GABAb peptide formed an α helical structure and a positive charge cluster at the C-terminal site. These structural features were also found in several other G protein activating peptides. From the comparison among these peptides, we found that peptides with high helical content show the high activity.

Keywords: $GABA_B$ receptor; NMR; Peptide structure; Intracellular loop peptide

GABA (γ-amino-butyric acid) is an inhibitory neurotransmitter in the synapses of the central nervous system. It activates metabotropic and ionotropic receptors. Metabotropic GABA type B ($GABA_B$) receptor belongs to G protein-coupled receptor (GPCR) superfamily [1] and interacts with G_i protein and inhibits cAMP formation. GPCRs play a critical role in intracellular signal transduction [2], and most of GPCRs are expected to possess seven transmembrane domains [3]. The $GABA_B$ receptor is the first GPCR shown to require heterodimerization for its function and be composed of two related seven-transmembrane domain receptor subunits: $GABA_{B1a}$ or $GABA_{B1b}$ and $GABA_{B2}$ [4].

Peptides corresponding to the intracellular third loop (IC3) region of human β2-adrenergic receptor [5], prostaglandin EP3α receptor [6], and APG1 receptor derived from human homologous *methuselah* gene [7] could activate G protein directly *in vitro*. Therefore investigation of the IC3 peptide of GPCR will be the important key for resolving the molecular mechanism of G protein activation. In the previous studies of an IC3 peptide of prostaglandin EP3α receptor and APG1 receptor, these receptor peptides have formed helical structures and positive charge clusters in SDS micelles [6,7]. In this work we investigated a peptide (GABAb: ETKSVSTEKINDHR) corresponding to the IC3 region of $GABA_B$ receptor protein (Fig. 1), to clarify the G_i protein activation mechanism and the solution structure.

Materials and methods

Sample preparation. A peptide (GABAb: ETKSVSTEKINDHR) corresponding to the IC3 region of human $GABA_B$ receptor 1a and a

Abbreviations: COSY, correlated spectroscopy; G protein, guanine nucleotide-binding protein; GABA, γ-amino-butyric acid; NOE, nuclear overhauser effect, also used for NOESY cross peak; NOESY, NOE spectroscopy; ppm, parts per million; SDS-d_{25}, sodium dodecyl-d_{25} sulfate.

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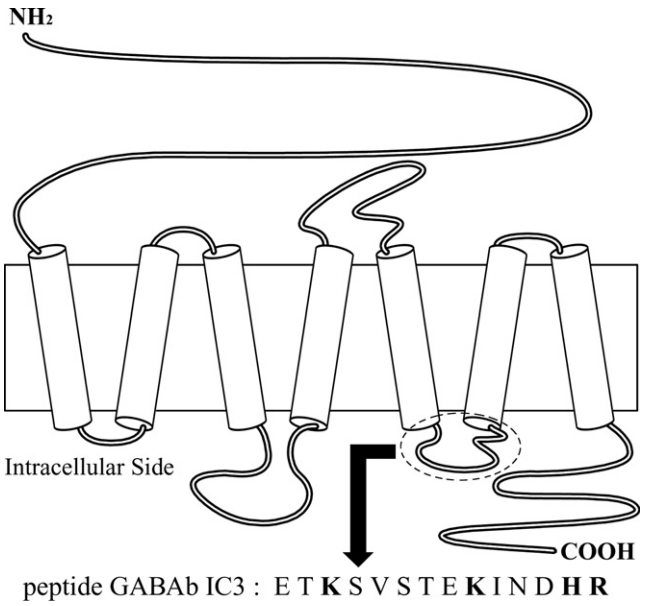

Fig. 1. Schematic drawing of human $GABA_B$ receptor. Circle region represents the third intracellular loop (IC3) of GABAb receptor.

peptide (GABAb IC2: HTGFTKKEEKKEWRKTLEPWK) corresponding to the intracellular second loop (IC2) of human $GABA_B$ receptor were synthesized by standard solid phase reaction procedures. The used protected amino acid derivatives, piperidine, and *N*-methyl pyrrolidone, were purchased from Wako Pure Chemical Industries. The crude reaction products were purified by the reverse phase HPLC. Purity and molecular weight of the peptide were confirmed by ion-spray mass spectrometry on a Perkin-Elmer SCIEX API III mass spectrometer.

GTPγS binding assay. GTPγS binding to 10 nM G_i proteins was measured in 20 mM Tris buffer (pH 7.5) containing 1 mM EDTA, 1 mM DTT, 25 mM $MgCl_2$, 1 μM ^{35}S-GTPγS, and peptides at 30 °C. The total amount of G_i protein was determined to be 10 nM, based on the measurement of maximal GTPγS binding in the presence of 1 μM GTPγS and 25 mM $MgCl_2$ at 30 °C [8].

NMR spectroscopy. NMR spectra of 2 mM peptide at 298 K were measured on a Bruker AM-600 (600 MHz) and Varian INOVA 600 spectrometers. The peptide was dissolved in 100 mM SDS-d_{25} micelles in 90% H_2O, 10% D_2O (v/v), and 50 mM phosphate buffer (pH 6.2). Two-dimensional COSY [9], TOCSY [10], and NOESY [11] spectra were recorded. The NOESY spectra were observed in the standard pulse schemes with mixing time of 150 ms and 500 ms. The mixing times for TOCSY with MLEV-17 spin-lock pulse were 25 ms and 50 ms. In all experiments, water peaks were suppressed by presaturation. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as an external reference of ^{1}H chemical shifts. Typical data size for all experiments was 2048×512. All the NMR data were processed with NMR Pipe [12] and analyzed with the PIPP program [13].

Restrained molecular dynamics calculation. NOE restraints were classified into three categories: strong, medium, and weak corresponding to the distance restraints of 1.8–3.0 Å, 1.8–4.0 Å, and 1.8–5.0 Å, respectively. Distance Geometry calculations were carried out with CNS program (version 1.1) [14]. The calculations were performed in three divided stages. In the first stage, 4000 random peptide structures were generated. In the second stage, high-temperature dynamics were carried out to correct residual distance violation. In the third stage, the temperature was decreased from 3000 K to 100 K through 20 K steps, and the structure was energy-minimized under the NOE constraints. The final 20 lowest energy structures were analyzed by using the program MOLMOL [15].

Results

We examined $GTP_\gamma S$ binding activity of G_i protein caused by GABAb peptide directly *in vitro*. Fig. 2 shows the $GTP_\gamma S$ binding activity of G_i protein by GABAb peptide, APG1 peptide derived from IC3 region of human APG1 receptor [7], and EP3a peptide derived from IC3 region of prostaglandin EP3α receptor [6]. Positive control is Mastoparan-X [16], which is a wasp venom peptide and negative control is a peptide corresponding to the IC2 region of human $GABA_B$ receptor, GABAb IC2. As shown in Fig. 2, the GABAb IC3 peptide could activate G_i protein and increase the GTPγS binding activity of G_i protein as well as the IC3 peptides of prostaglandin EP3α receptor and APG1 receptor derived from human homologous *methuselah* gene.

In order to examine the structural requirement for G_i protein activation by GABAb peptide, we performed NMR experiments with GABAb peptide in SDS micelles solution. There were a few NOE cross peaks at the N-terminal part, while (i, $i+3$) and (i, $i+4$) NOE cross peaks, which indicate the existence of a helical structure, were observed at the central and C-terminal part of GABAb peptide. We executed CNS calculation using these NOE data and determined the lowest-energy structure of GABAb peptide (Fig. 3A1). The structural statistics for the 20 lowest-energy structures of 4000 calculated structures are shown in Table 1. The RMSD values were also calculated by using the program CNS. An α-helix structure was formed in the C-terminal part of GABAb peptide. The basic amino acid residues (K3, K9, H13, and R14) were located on one side of the molecules. Electrostatic potential surface of GABAb peptide calculated by the program MOLMOL indicated that the positive charges were concentrated on one side of the molecular surface (Fig. 3A2 and A3).

Discussion

Our present results of GTPγS binding assay indicated that the peptide corresponding to the IC3 region of human

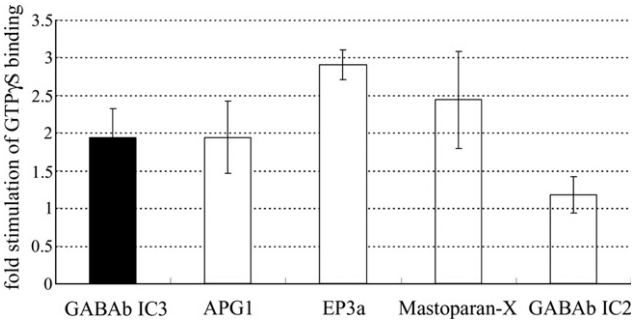

Fig. 2. The effects of specific receptor segments on G_i protein activation of GTPγS binding activity. The GTPγS binding activity was expressed as a ratio of the control buffer that contained 10 nM G_i protein in the absence of sample peptides. GTPγS binding activity of G_i protein in the presence of each 10 μM peptides (positive control: Mastoparan-X, negative control: GABAb IC2). The data represent the means ± SE of three experiments.