Biochemical and Biophysical Research Communications 413 (2011) 637–642

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Isoliquiritigenin, a chalcone compound, is a positive allosteric modulator of $GABA_A$ receptors and shows hypnotic effects

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ARTICLE INFO

Article history:
Received 22 August 2011
Available online 14 September 2011

Keywords:
Isoliquiritigenin
Chalcones
Sedative–hypnotic activity
$GABA_A$–benzodiazepine receptors
Dorsal raphe nucleus

ABSTRACT

Isoliquiritigenin (ILTG) is a chalcone compound and has valuable pharmacological properties such as antioxidant, anti-inflammatory, anticancer, and antiallergic activities. Recently, the anxiolytic effect of ILTG has been reported; however, its action mechanism and hypnotic activity have not yet been demonstrated. Therefore, we investigated the hypnotic effect and action mechanism of ILTG. ILTG significantly potentiated the pentobarbital-induced sleep in mice at doses of 25 and 50 mg/kg. The hypnotic activity of ILTG was fully inhibited by flumazenil (FLU), a specific gamma-aminobutyric acid type A ($GABA_A$)–benzodiazepine (BZD) receptor antagonist. The binding affinity of ILTG was 0.453 μM and was found to be higher than that of the reference compound, diazepam (DZP, 0.012 μM). ILTG (10^{-5} M) potentiated GABA-evoked currents to 151% of the control level on isolated dorsal raphe neurons. ILTG has 65 times higher affinity for $GABA_A$–BZD receptors than DZP, and the dissociation constant for ILTG was 4.0×10^{-10} M. The effect of ILTG on GABA currents was blocked by 10^{-7} M FLU and ZK-93426. These results suggest that ILTG produces hypnotic effects by positive allosteric modulation of $GABA_A$–BZD receptors.

1. Introduction

Isoliquiritigenin (ILTG, 2′,4′,4′-trihydroxychalcone) is a chalcone compound [1]. Chalcones exhibit a basic structure of two benzene rings linked through an α, β-unsaturated carbonyl group and belong to a group of flavonoids [2]. ILTG is a chalcone that is found in *Glycyrrhiza uralensis* (licorice) [1] and also in *Allium ascalonicum* [3], *Sinofranchetia chinensis* [4], *Dalbergia odorifera* [5], and *Glycine max* L. [6]. There is growing interest on the valuable pharmacological activities of ILTG [7]. ILTG has been found to have antioxidant [8], anti-inflammatory [5], anticancer [9], antiangiogenic [7], and antiallergic [10] activities.

Recently, some neurological activities of ILTG have been reported. For example, ILTG inhibits cocaine-induced dopamine release by modulating gamma-aminobutyric acid type B ($GABA_B$) receptor [11]. ILTG has been found to act as a selective histamine H_2 receptor antagonist [12]. According to Jamal et al. [2], ILTG showed anxiolytic effects in the elevated plus-maze test in mice. Intraperitoneal (i.p.) administration of ILTG (15 or 25 mg/kg) significantly increased the amount of time spent in the open arms and the number of open arm entries. However, the precise mechanism of the anxiolytic activity of ILTG was not demonstrated. Although compounds with anxiolytic activity have the potential to produce hypnotic activity at a higher dose [13,14], the hypnotic activity of ILTG has not been reported. Therefore, we were interested in the hypnotic effects and the action mechanism of ILTG.

In the present study, we investigated the hypnotic activity and the possible *in vivo* mechanism of ILTG using a pentobarbital-induced sleep test. After which, the binding affinity to the $GABA_A$–benzodiazepine (BZD) receptors was evaluated. We also demonstrated the effect of ILTG on GABA-evoked currents in acutely isolated dorsal raphe (DR) neurons. The DR nucleus is one of the arousal centers in

Abbreviations: ACSF, artificial cerebrospinal fluid; BZD, benzodiazepine; CMC, carboxymethyl cellulose; DR, dorsal raphe; DZP, diazepam; FLU, flumazenil; GABA, gamma-aminobutyric acid; ILTG, isoliquiritigenin; ZK, ZK-93426.

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doi:10.1016/j.bbrc.2011.09.026

the brain, and its neuronal activity is depressed during sleep through GABAergic inhibition [15]. In addition, Trulson et al. [16] reported that BZD agonists depressed the activity of DR neurons at a concentration that induces a hypnotic state (>2.5 mg/kg, i.p.). Therefore, the neural mechanism of ILTG-induced sleep was tested with patch-clamp methods using DR neurons.

2. Materials and methods

2.1. Drugs and chemicals

ILTG and flumazenil (FLU, Ro 15-1788) were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). ZK-93426 (ZK) was purchased from Tocris Bioscience. (Eliisville, Mo, USA). Pentobarbital was purchased from Hanlim Pharm. Co. Ltd. (Seoul, Korea). Diazepam (DZP; Myungin Pharm. Co. Ltd., Seoul, Korea), a $GABA_A$–BZD agonist, was used as the reference sedative–hypnotic drug.

2.2. Assay of [^{3}H]-FLU binding to $GABA_A$–BZD receptor

The $GABA_A$–BZD receptor binding assay was a modification of the method described by Risa et al. [17] and Kahnberg et al. [18]. The receptor membrane was prepared from the cerebral cortex of Sprague–Dawley (SD) rats [17]. In a 96-well plate, A membrane suspension (180 μL) was added to 10 μL of a test solution and 10 μL of [^{3}H]-FLU (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) to obtain a final concentration of 1 nM. The solution was mixed and incubated on ice for 40 min. The binding reaction was terminated by rapid filtration with ice-cold 30 mM Tris–HCl buffer. The amount of filter-bound radioactivity was determined by conventional liquid scintillation counting. Total and non-specific bindings were determined using the binding buffer and DZP (1 μM, final concentration), respectively. Binding affinity (K_i) values were determined from the IC_{50} values using the binding affinity constant (K_d = 1.6 nM) for [^{3}H]-FLU binding.

2.3. Pentobarbital-induced sleep test in mice

All the experiments were performed between 1:00 PM and 5:00 PM, and the mice were fasted for 24 h before the experiment. For oral administration, all the samples were suspended in 0.5% (w/v) CMC-saline. The test solutions were administered (p.o.) to the mice 45 min before the pentobarbital injection. The control mice (0.5% CMC-saline, 10 mL/kg) were tested in parallel with the animals receiving the test sample treatment. After the administration of pentobarbital (45 mg/kg, i.p.), mice were placed in individual cages and observed for measurements of sleep latency and duration. The observers were blinded to the individual treatments. The sleep latency was recorded from the time of pentobarbital injection to the time of sleep onset, and sleep duration was defined as the difference in time between the loss and the recovery of the righting reflex. All procedures involving animals were conducted in accordance with the animal care and use guidelines of the Korea Food Research Institutional Animal Care and Use Committee (permission number: KFRI-M-09118).

2.4. Electrical measurements of the ILTG effects on GABA currents

Neurones from the DR nucleus were acutely dissociated from 2- to 3- week-old SD rats of either sex, according to procedures reported elsewhere [19] with some modifications. Briefly, after anesthesia, the brain was resected and placed in ice-cold artificial cerebrospinal fluid (ACSF) composed of the following (in mM concentration): NaCl, 125; KCl, 3; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; dextrose, 10; and $CaCl_2$, 2. The solution was then bubbled with 95% O_2 and 5% CO_2. The area containing the DR was cut into coronal slices (300 μm). These slices were pre-incubated for 2–4 h at 31 °C in well-bubbled ACSF. For dispersion, the brain slices were transferred to a glass-bottomed perfusion chamber filled with standard external solution containing the following (in mM concentration): NaCl, 150; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 2; HEPES, 10; and glucose, 10 (pH 7.4). A fire-polished glass pipette with a 100- to 120-mm tip size was mounted in a custom-made vibrator held by a micromanipulator [20]. Under the stereomicroscope, the oscillating tip was lowered to the surface of the slices within the DR region. The neurons were dissociated from the upper 100 μm of these slices. After removing the slice, the dispersed neurons were allowed to settle and adhere to the bottom of the chamber; the process is completed within 20 min generally. Electrical measurements were performed in the nystatin-perforated [21] patch recordings with modifications. The recording electrodes were filled with a solution composed of the following (in mM concentration): KCl, 50; K gluconate, 100; and HEPES, 10 (pH 7.2). The final concentration of nystatin was 450 μg/mL. The neurons were visualized with phase-contrast equipment on an inverted microscope (I×70; Olympus, Tokyo, Japan) with a 40× objective and a 10× ocular lens. The current was measured with a patch-clamp MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA). All the experiments were performed at room temperature (21–22 °C). The drugs were applied with the Y-tube microperfusion system [22]. With this technique, the external solution surrounding a neuron could be exchanged within 0.1 s.

2.5. Statistical analysis

Comparisons between the data of the two groups were analyzed by the unpaired Student's *t*-test. For multiple comparisons, the data were analyzed using one-way or repeated-measure analysis of variance (ANOVA) (as appropriate) followed by the Dunnett's test. Differences with $p < 0.05$ were considered statistically significant. The significance analysis was performed using Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Hypnotic effects of ILTG

As expected, it was found that a well-known sedative–hypnotic drug, DZP (2 mg/kg, p.o.) significantly potentiated the pentobarbital-induced sleep in mice ($p < 0.01$) as compared to the control group (Fig. 1). ILTG (5–50 mg/kg, p.o.) also caused a dose-dependent decrease in sleep latency (Fig. 1A) and an increase in sleep duration (Fig. 1B).

3.2. Possible in vivo action mechanism of the hypnotic activity of ILTG

To verify the *in vivo* action mechanism of the hypnotic effect of ILTG, we tested the effects of the coadministration of ILTG (50 mg/kg) with FLU (8 mg/kg), a specific $GABA_A$–BZD receptor antagonist. The pretreatment with FLU alone did not affect the pentobarbital-induced sleep latency and sleep duration (Fig. 2). As expected, FLU was found to significantly inhibit the hypnotic activity of DZP, a $GABA_A$–BZD receptor agonist ($p < 0.01$). The hypnotic effect of ILTG was also fully antagonized by FLU ($p < 0.01$).

3.3. Binding affinity of ILTG to the $GABA_A$–BZD receptors

The binding affinity (K_i) value was determined using the $GABA_A$–BZD receptor binding assay. The K_i value of DZP (molecular weight (MW), 284.70), which was used as the reference compound