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Anxiolytic-like effects of sanjoinine A isolated from *Zizyphi Spinosi Semen*: Possible involvement of GABAergic transmission

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ABSTRACT

This experiment was performed to investigate the anxiolytic-like effects of sanjoinine A, one of the major alkaloid compounds in *Zizyphi Spinosi Semen* (ZSS), by using experimental paradigms of anxiety in comparison with a known anxiolytic, diazepam. Sanjoinine A (2.0 mg/kg) increased the percentage of time spent on the open arms and the number of open arms entries in the elevated plus-maze test, increased the number of head dips in the hole-board test, and increased the percentage of time spent in the center zone and the center zone locomotor distance in the open field box experiment. However, sanjoinine A (0.5, 1.0, 2.0 mg/kg) had no effect on locomotor activity, while diazepam (2.0 mg/kg) significantly reduced locomotor activity. Sanjoinine A (0.5, 1.0, 2.0 mg/kg) did not influence the grip force in the grip strength meter test either. Molecular experiments showed that sanjoinine A (2.0, 5.0 μM) increased chloride influx in cultured cerebellar granule cells. In addition, sanjoinine A (5.0 μM) treatment resulted in over-expression of α- and γ-subunits of $GABA_A$ receptors and glutamic acid decarboxylase (GAD65/67) in cultured cerebellar granule cells. It is concluded that sanjoinine A may have anxiolytic-like effects in the elevated plus-maze, hole-board test and open field test, and these effects may be mediated by GABAergic transmission.

1. Introduction

Anxiety had been the most prevalent psychiatric disorder among the world population (Rouillon, 1999). Anxiety disorders are common mental diseases of the central nervous system and contribute to ever-increasing health problems worldwide. Anxiety disorders are present in a number of forms, although probably all are dependent upon a number of common neurological circuits (Beck, 1988). Benzodiazepines have been used for the treatment of several forms of anxiety, but these compounds have well-known side effects, such as sedation, muscle relaxation, amnesia and dependence (Gardner et al., 1993; Rickels et al., 2008; Youssef and Rich, 2008). For this reason, many researchers have been evaluating new compounds from herbs with fewer undesirable effects.

It has been suggested that various traditional herbal medicines also possess anxiolytic activity (Une et al., 2001). Some of them, such as St. John's wort and ginseng, have been used for clinical treatment of anxiety (Friede and Freudenstein, 2002). In addition, it has been found that kava-kava (*Piper methysticum* Forst) has an anxiolytic-like effect (Rex et al., 2002). Another study indicated that saiboku-to, an herbal medicine, displayed an anxiolytic-like effect (Yuzurihara et al., 2000). Research has also focused on the development of drugs with fewer side effects, such as sleeping, muscle relaxation and drug dependence (Eisenberg et al., 1998).

Zizyphi Spinosi Semen (ZSS), the dried seed of *Zizyphus jujuba Mill var. spinosa* (Rhamnaceae), is known to contain many pharmacologically active components. It has been used as an analgesic, tranquilizer and anticonvulsant in Oriental countries such as Korea and China for over 2500 years, and it has been prescribed for the treatment of insomnia and anxiety in Asia (Peng and Zhu, 2001). This herbal medicine is rich with pharmacologically active compounds, such as flavones, alkaloids and triterpenes (Lee et al., 1996; Cheng et al., 2000), which may improve cognitive function via anti-cholinesterase activity, protect against NMDA-induced neuronal cell damage (Park et al., 2004), increase the duration of pentobarbital-induced sleep (Adzu et al., 2002), inhibit caffeine-induced excitation and prolong hexobarbital-induced sleep (Chung and Lee, 2002). A recent study reported that the alkaloids and flavonoids of the seeds can produce central inhibitory activity (Park et al., 2004). It has been demonstrated that the ethanol and methanol extracts of ZSS possess an anxiolytic-like effect (Peng et al., 2000; Han et al., 2007). Research has also indicated that the aqueous extracts of Sanjoin-Tang have anxiolytic-like effects in mice (Ahn et al., 2004).

The most ubiquitous mechanisms for anxiolytic effects involve GABA. GABA is the main inhibitory neurotransmitter in the brain (Kaupmann et al., 1997), and it is known to interact with three

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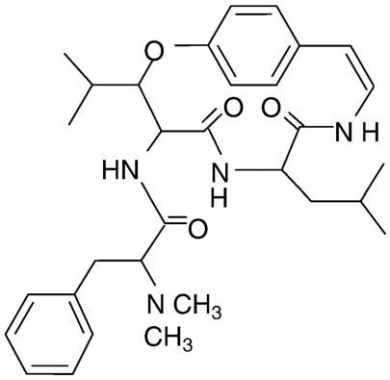

Fig. 1. Chemical structure of sanjoinine A.

receptor types: $GABA_A$ $GABA_B$ and $GABA_C$. The $GABA_A$ receptors are heteromeric ligand-gated chloride ion channels. Seven isoforms of its subunits (α, β, γ, δ, π, ε and θ) containing 16 subtypes (α1–6, β1–3, γ1–3, δ, π, ε and θ) have been discovered (Barnard et al., 1998; Bonnert et al., 1999). Where the subunits meet, there is a channel through which chloride ions can pass. Under normal circumstances, opening of the chloride channel causes the negatively charged chloride ions to flow into the cell, causing hyperpolarization and thus inhibition of cellular activity (Whiting, 1999). $GABA_A$ receptors are important drug targets for a variety of medications used to treat anxiety and insomnia. $GABA_A$ receptors have a rich pharmacology because they have a number of separate binding sites for a variety of drugs that can modulate the activity of GABA (Bateson, 2004). $GABA_A$ receptors possess different binding sites for ligands such as GABA, barbiturate, benzodiazepine and picrotoxin (Brailowsky and Garcia, 1999; Davies et al., 1996). This heterogeneity provides the structural basis for differential targeting by receptor subtype-selective drugs to improve the treatment of insomnia, anxiety, epilepsy and alcohol withdrawal (Korpi et al., 2002).

Benzodiazepines bind to $GABA_A$ receptors. Their binding sites differ from those of GABA. The principal effect is a change in Cl^- conductance. Both the affinity and the efficacy of benzodiazepine are determined by the α and γ subunits of the $GABA_A$ receptor. Benzodiazepines are used to treat a variety of clinical symptoms, including anxiety, convulsions and muscle tension (Whiting, 1999). The anxiolytic effect of benzodiazepines is due to allosteric modulation of $GABA_A$ receptors, leading to GABAergic neurotransmission. Positive allosteric modulators increase the frequency of chloride openings without altering the channel conductance or duration of opening. Therefore, they are used as anxiolytic, anticonvulsant, sedative-hypnotic, and muscle relaxant drugs (Hui et al., 2002).

This study was performed to investigate the anxiolytic-like effects of sanjoinine A (Fig. 1), one of the major alkaloid compounds of ZSS, by using the experimental paradigms of anxiety—including elevated plus-maze, hole-board, open field—for comparison to a known anxiolytic, diazepam. In addition, we measured chloride influx in primary cerebellar neuronal cells, $GABA_A$ receptors' subunits and glutamic acid decarboxylase (GAD65/67) expression in order to understand the possible mechanisms of sanjoinine A.

2. Materials and methods

2.1. Animals

Male ICR mice (SAMTAKO, Korea) weighing 20–25 g, in groups of 10–12, were used for behavioral experiments. Animals were housed in acrylic cages (45×60×25 cm) with water and food available *ad libitum* under an artificial 12-h light/dark cycle (light on at 7:00) and at a constant temperature (22±2 °C). Mice were housed in the departmental room for 1 week before testing to ensure adaptation to the new environment. All of the behavioral experiments were performed between 10:00 and 17:00. All the experiments involving animals were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1985), and the Institutional Animal Care and Use Committee of Chungbuk National University approved the protocol.

2.2. Experimental compounds and drugs

ZSS was purchased from an oriental drug store in Seoul, Korea. ZSS (300 g) was extracted three times with 11 l of hexane in a reflux condenser for 24 h. The residues were extracted with methanol for 24 h, and this methanol extraction was evaporated to dryness by rotary evaporation; the solvent was removed by rotary evaporation and partitioned between 5% hydrochloric acid (60 ml) and ether (50 ml). This aqueous acid solution was extracted three times more with ammonia hydrate (pH 9.0) and chloroform (60 ml×3). The alkaloid fractions in chloroform solution were combined, filtered through Whatman No. 1 filter paper, and concentrated using a rotary vacuum evaporator followed by lyophilization. The yield of the alkaloid fraction was about 0.03% (w/w). Using combined flash column chromatography and preparative thin layer chromatography, 14 alkaloids were isolated in a crystalline state from the alkaloid fraction of ZSS. Alkaloids were labeled as sanjoinine A, sanjoinine B, sanjoinine C, and so on, in order of increasing polarity. The isolation yields were highly varied, ranging from 1.4×10^{-3} to 5×10^{-3}%. The chemical structure of sanjoinine A was established by a combination of chemical correlation methods and spectral analysis. By preliminary analysis on dose– and time–response relationships of anxiolytic effects of diazepam and sanjoinine A, we chose more effective doses and times to pursue further investigation and found the more effective doses and times used in this study. The test sample (sanjoinine A) was dissolved in 0.9% physiological saline with 1% carboxymethylcellulose (CMC) and administered to the mice orally (0.5, 1.0, 2.0 mg/kg, p.o.) 1 h before the behavioral experiments. Diazepam purchased from Myung-In Pharm. Co., Ltd. (Kyunggi-Do, Korea) was dissolved in 0.9% physiological saline and given to the animals orally (0.5/2.0 mg/kg, p.o.) 30 min prior to the experiments. All other chemicals were obtained from Sigma Chemical Co., and the solutions were prepared freshly before the experiments.

2.3. Elevated plus-maze test

The elevated plus-maze apparatus consists of four arms (30×5 cm) elevated 45 cm above the floor, with each arm positioned at 90° relative to the adjacent arms. The two enclosed arms had 30 cm walls, and, to facilitate grip on the open arms, the walls had a raised edge of 0.25 cm. Open and closed arms were connected via a central area (5×5 cm) to form a plus sign. The maze floor was constructed of black Plexiglas, and the wall of the enclosed arms was constructed of clear Plexiglas (Chen et al., 2003). Four 25-W red fluorescent lights arranged as a cross at 100 cm above the maze were used as the source of illumination, and the video camera was suspended above the maze to record movements for analysis. Mice were randomly assigned (with a slight adjustment for matched body weight) to experimental groups. Testing was commenced by placing a mouse on the central platform of the maze, facing an open arm. The number of entries into and the time spent on each of the two types of arms were recorded during the 5-min trial (Klodzinska et al., 2004; Yu et al., 2007). An arm entry was defined as all four paws having crossed the dividing line between an arm and the central area. The plus-maze was thoroughly cleaned with 70% methanol after each trial. Mice were randomly allocated to the following groups: control (normal saline with 1% CMC, p.o.), diazepam (2.0 mg/kg, p.o.), and sanjoinine A (0.5, 1.0 and 2.0 mg/kg, p.o.).

2.4. Hole-board test

The hole-board apparatus (UGO Basile, Italy) consisted of gray Perspex panels (40×40 cm, 2.2 cm thick) with 16 equidistant holes, each 3 cm in diameter, on the floor. Photocells below the surface of the hole measured the number of head dips. The board was positioned 15 cm above a table. Mice were randomly allocated to the following groups: control (normal saline with 1% CMC, p.o.), diazepam (0.5 mg/kg, p.o.), and sanjoinine A (0.5, 1.0 and 2.0 mg/kg, p.o.). Each mouse was individually placed on the center of the board facing away from the observer and allowed to freely roam about the apparatus prior to the testing. Diazepam and sanjoinine A were administered 30 and 60 min before the test, respectively. The number of head dips on the hole-board was counted for 5 min (Takeda et al., 1998; Wei et al., 2007; Silva et al., 2007). After each trial, the floor of the apparatus was wiped with 70% methanol to remove traces of previous paths. The test sessions were recorded with a camera mounted vertically above the hole-board.

2.5. Open field test

The open field test was performed using the ambulatory monitor apparatus (ENV-520 SN 10317). The open field area was made of acrylic, consisting of four transparent walls and a white floor (30×30×15 cm). The mouse's position in the open field was classified into five areas: four corner zones and a central zone. Sanjoinine A and diazepam were administered to the animals 1 h and 30 min before the test, respectively. Each mouse was put in the center of the open field box and tested for 1 h. The time spent and the distances made in the central zone were recorded. After each individual test session, the floor was cleaned using 70% methanol to remove any traces left behind by the animals.

2.6. Locomotor activity

Since the plus-maze experiment was affected by changes in locomotor activity, an additional experiment was carried out with the specific aim of monitoring locomotor activity. Separately from the experiment above, spontaneous locomotor activity was measured automatically with a tilting-type ambulometer (AMB-10, O'Hara, Japan). Each mouse was placed in the activity cage (20 cm in diameter, 18 cm in height), and, after an adaptation period of 10 min, the test compound administration protocol was implemented (Park et al., 2005). Diazepam (2 mg/kg, p.o.) and sanjoinine A (0.5, 1.0 and 2.0 mg/kg, p.o.) were administered to the animals 30 min and 60 min prior to the experiment, respectively, and ambulatory activity was measured for 1 h after the administration of the agents.

2.7. Grip strength test

For each animal, the first 5 sessions of training were limited to handing the animal for 5 min; then, there were 10 training sessions, during which animals were held around the midsection, facing the handle of the grip strength meter (GSM, designed by TSE-Systems and distributed by Scipro, Inc.), and one forearm was manually restrained by the experimenter. As described in a previous study, when the unrestrained forepaw is brought into contact with the handle, the animals reliably grasp the bar, and the animal is then gently pulled away from the device (Kehl et al., 2000). The GSM then measures the maximal force before the animal releases the bar. Each testing session was performed for both forepaws three times 30 min and 60 min after the administration of sanjoinine A (0.5, 1.0 and 2.0 mg/kg, p.o.) and diazepam (2 mg/kg, p.o.) respectively.

2.8. Cell culture

Primary cultured cerebellar neurons enriched in granule cells were prepared from cerebella of 8-day-old Sprague-Dawley rats as previously described (Houston and Smart, 2006). Briefly, cells were plated 1×10^6 cells per 0.2 ml in 96 microplates or 2×10^6 cells per 2.0 ml in 60-mm dishes pre-coated with poly-D-lysine (10 μg/ml) (SIGMA, St. Louis, MO, USA) and cultured in basal Eagle's medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), 2 mM glutamine, gentamicin (100 μg/ml), antibiotic–antimycotic solution (10 ml/l) (SIGMA) and 25 mM KCl; such a high concentration of potassium was necessary to induce persistent depolarization, which promotes the survival of granule cells. The cells were incubated for 6–9 days in a humidified 5% CO_2/95% air atmosphere at 37 °C. Cytosine arabinofuranoside (final concentration, 10 μM) (SIGMA) was added to cultures 18–24 h after plating to inhibit the proliferation of nonneuronal cells. After 7 days, the cultured cells were used for the measurements.

2.9. Measurement of intracellular chloride influx

The intracellular Cl^- concentration ($[Cl^-]i$) in cultured cerebellar granule cells was estimated using the Cl^- sensitive fluorescence probe MQAE according to the method of West and Molloy (1996) with a slight modification. The buffer (pH 7.4) used contained the following: 2.4 mM HPO_4^{2-}, 0.6 mM $H_2PO_4^-$, 10 mM HEPES, 10 mM D-glucose and 1 mM $MgSO_4$. A variety of MQAE-loading conditions were assessed. The cells were incubated overnight in a medium containing 10 mM MQAE (Dojindo, Japan). After loading, the cells were washed three times in the relevant Cl^- containing buffer. The buffer was replaced with buffer with or without the compounds. Repetitive fluorescence measurements were initiated immediately using a FLUOstar (excitation wavelength: 320 nm; emission wavelength: 460 nm; BMG Lab Technology, Germany). The data are presented as the relative fluorescence Fo/F, where Fo is the fluorescence without Cl^- ions and F is the fluorescence as a function of time. The Fo/F values were directly proportional to $[Cl^-]i$.

2.10. $GABA_A$ receptor subunits and GAD 65/67 expression

Primary cultured cells were treated with sanjoinine A or diazepam after 8 days in culture. Sanjoinine A was dissolved in ethanol and diluted sequentially in culture medium to a final concentration of 5.0 μM. Diazepam was diluted in the medium to 10 μM and applied to the cells. For the control group, cells were treated only with culture medium without sanjoinine A. The culture was completely replaced every day. After treatment of sanjoinine A and diazepam for 5 days, cells were harvested and treated with lysis buffer. The extracts were centrifuged at 20,000×g for 20 min. Equal amounts of protein were separated on a SDS/12% polyacrylamide gel, and transferred to a nitrocellulose membrane (Hyboud ECL, Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The blots were blocked overnight at 4 °C with 5% (w/v) non-fat dried milk in a Tris-buffered saline solution (10 mM Tris pH 8.0 and 150 mM NaCl) containing 0.05% Tween-20 (TBST). The membrane was incubated with the specific antibodies, rabbit polyclonal antibodies against $GABA_A$ receptor subunit (1:500) (Santa Cruz Biotechnology Inc.) or GAD65/67, for 4 h at room temperature. The blot was washed in TBST three times and then incubated for 2 h with the corresponding conjugated anti-rabbit immunoglobulin G-horseradish peroxidase (1:2000) (Santa Cruz Biotechnology Inc.). The membrane was washed in TBST three times, and the immunoreactive proteins were detected using the ECL western blotting detection system.

2.11. Statistical analysis

The results are presented as the mean ± S.E.M., and the significance of the effects of the compounds was assessed using analysis of variance (ANOVA). In case of significant variation, the individual

values were compared with Dunnett's test, $p<0.05$ was considered as statistically significant.

3. Results

3.1. Anxiolytic-like effects of sanjoinine A on the elevated plus-maze model

As the positive control, diazepam (2 mg/kg) significantly increased both open arm entries and time spent on open arms compared with the vehicle group ($p<0.005$). The mice treated with sanjoinine A (2.0 mg/kg) also had a greater percentage of open arm entries and more time spent on open arms compared with that of the saline animals ($p<0.05$) (Fig. 2).

3.2. Anxiolytic-like effects of sanjoinine A on the hole-board test and on the open field test

In order to investigate the exploratory and anxiolytic effect, animals treated with diazepam or sanjoinine A were subjected to the hole-board test (Fig. 3A), both diazepam (0.5 mg/kg) and sanjoinine A (2.0 mg/kg) significantly increased the number of head dips ($p<0.01$).

The open field test was performed for 1 h after the administration of sanjoinine A or diazepam. It revealed that both sanjoinine A (1.0 and 2.0 mg/kg) and diazepam (0.5 mg/kg) increased the percentages of central zone locomotor distance and central zone spent time

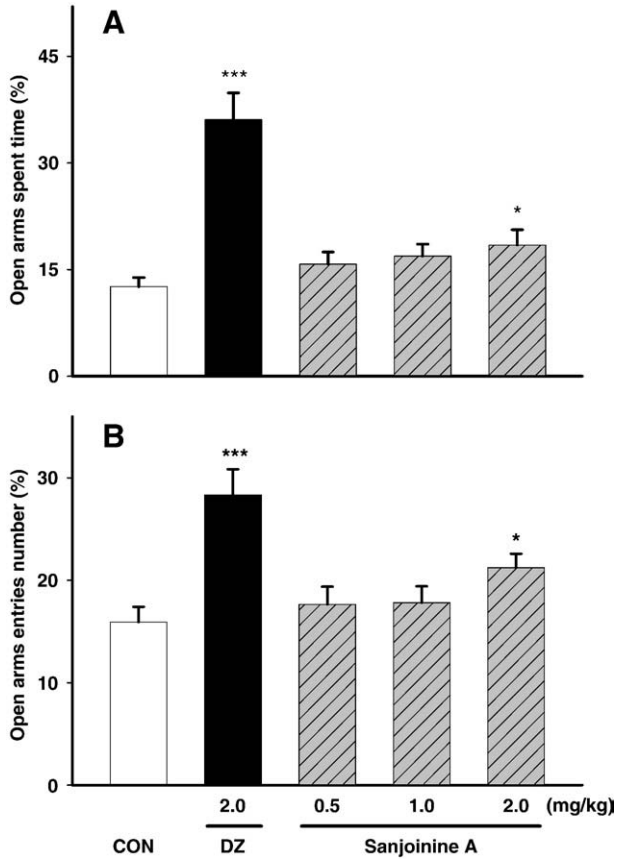

Fig. 2. Effect of sanjoinine A on the percentage of open arm entries and time spent in open arms on the elevated plus-maze in mice. Open arm entries and spent time in open arms in the elevated plus-maze were measured for 5 min, 30 min after oral administration of diazepam and 1 h after the administration of sanjoinine A. A: Open arms spent time; B: Open arms entries. Data are expressed as mean±S.E.M. * $p<0.05$, *** $p<0.001$, compared to the vehicle-treated control group.

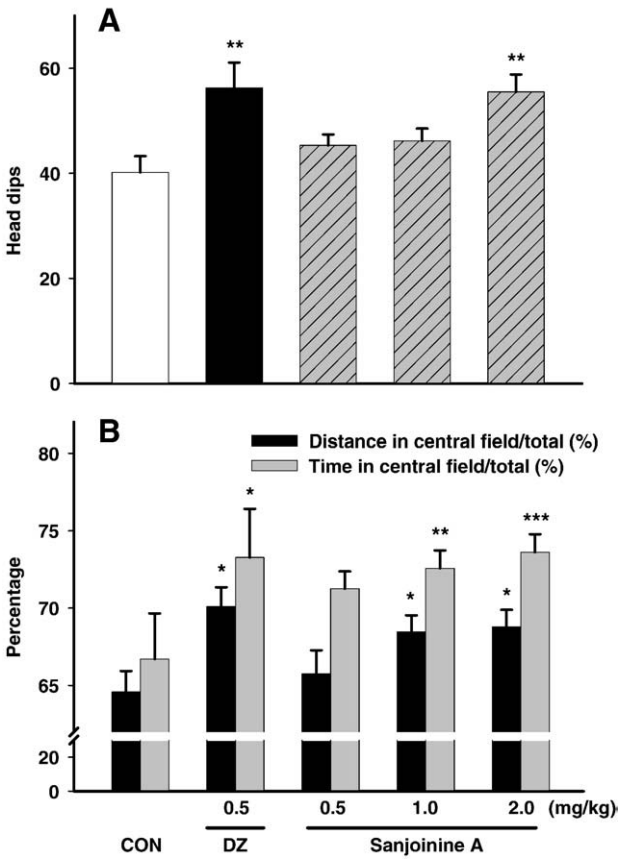

Fig. 3. Effects of sanjoinine A on head dips in hole-board test and in open field test in mice. Head dips in the hole-board test were measured for 5 min, 30 min after oral administration of diazepam and 1 h after sanjoinine A treatment. The open field test was done for 1 h, 30 min after oral administration of diazepam and 1 h after sanjoinine A treatment. A: hole-board test; B: open field test. Data are expressed as mean±S.E.M. * $p<0.05$, ** $p<0.01$, *** $p<0.005$, compared to the control group.

significantly compared with the control group ($p<0.05$ and $p<0.01$) (Fig. 3B).

3.3. Effects of sanjoinine A on locomotor activity and on grip strength

The locomotor activity of mice treated with different dosages of sanjoinine A or diazepam was compared to that of mice treated with vehicle. Locomotor activity was significantly decreased by diazepam (2.0 mg/kg). However, sanjoinine A at doses of 0.5, 1.0 and 2.0 mg/kg did not affect locomotor activity in tested animals ($p>0.05$) (Fig. 4A).

In the grip strength meter test, diazepam (2.0 mg/kg) significantly decreased grip force compared with the control group. In contrast, sanjoinine A had no effect on grip force at the dose of 0.5, 1.0 and 2.0 mg/kg ($p>0.05$) (Fig. 4B).

3.4. Effects of sanjoinine A on intracellular chloride influx

Resting intracellular Cl^- concentrations were calibrated using standard Cl^- solutions of 0, 15, 30, 45, 60 and 75 mM Cl^-. Appropriate amounts of methylsulfate were used to replace Cl^- in these solutions. Thibutyltin chloride (5.0 μM) and nigericin (5.0 μM) were added to artificially facilitate the balance between intracellular Cl^- and extracellular Cl^- concentrations. Resting $[Cl^-]i$ in cultured cerebellar granule cells was 27.30 mM, and treatment of granule cells with 2.0 and 5.0 μM sanjoinine A increased Cl^- influx to 34.12 and 35.94 mM ($p<0.005$). Also, 5.0 μM diazepam increased Cl^- influx to 38.44 mM, compared with the control group ($p<0.005$) (Fig. 5).

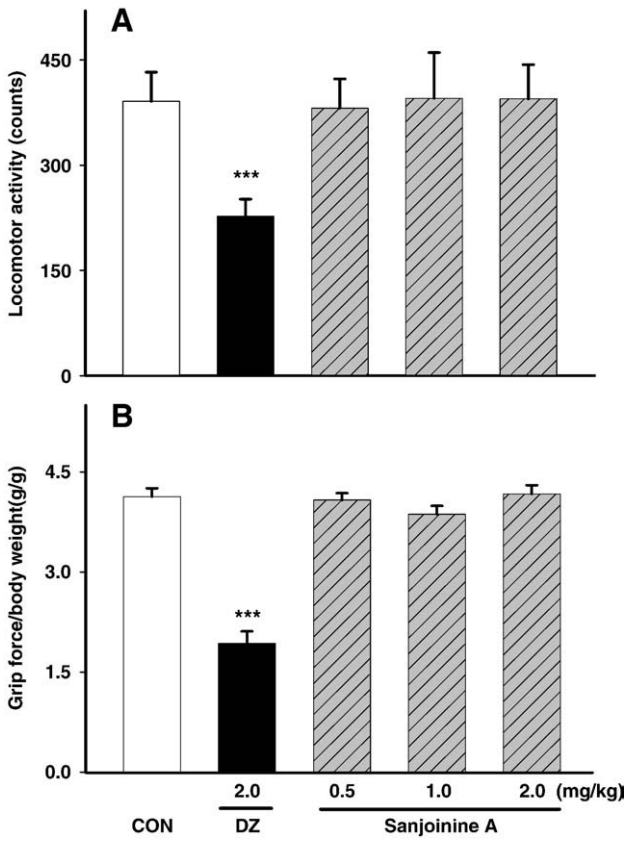

Fig. 4. Effects of sanjoinine A on locomotor activity and grip strength in mice. Ambulatory activity was measured for 1 h, 30 min after oral administration of diazepam and 1 h after the administration of sanjoinine A. Mice were tested 30 min for grip strength after oral administration of diazepam and 1 h after sanjoinine A treatment. A: Locomotor activity; B: Grip strength test. Data are expressed as mean±S.E.M. *** $p<0.001$, compared to the control group.

3.5. Expression of $GABA_A$ receptor subunits and GAD65/67 upon sanjoinine A treatment

$GABA_A$ receptor subunit expression in cerebellar granule cells was examined after treatment with 5.0 μM sanjoinine A and 10.0 μM diazepam for 5 days. Sanjoinine A and diazepam increased α-subunit and γ-subunit expression but had no effect on β-subunit expression ($p<0.05$, $p<0.005$). GAD65/67 expression was examined in cerebellar granule cells after treatment with 5.0 μM sanjoinine A and 10.0 μM diazepam for 5 days. Both treatments enhanced the amount of the GAD65/67 significantly in cultured cerebellar granule cells ($p<0.01$) (Fig. 6).

4. Discussion

In this study, the effects of sanjoinine A, one of the major alkaloid compounds isolated from ZSS, were examined in animal models of anxiety using tests such as the elevated plus-maze, hole-board test, open field teat, locomotor activity and grip strength meter, which are classic models for screening central nervous system activity and providing information about anxiety, myorelaxant activity (Silva et al., 2007; de Melo et al., 2005).

One of the most widely used animal models for screening putative anxiolytics is the elevated plus-maze (Klodzinska et al., 2004; Wei et al., 2007), in which rodents display an avoidance of exposed open areas of the maze, which are presumed to be the most aversive, and a preference for sections enclosed by protective walls (Weiss et al., 1998). The anxiolytic effectiveness of a drug can be demonstrated by a statistically significant increase in rodent activity in the open arms. Conventional anxiety indices in the elevated plus-maze test comprise percent open arm entries and percent time spent in these areas in the maze, with anxiolytics generally increasing and anxiogenics decreasing these measures. In the present study, sanjoinine A (1.0 and 2.0 mg/kg, p.o.) increased the percentages of entries into open arms and time spent in open arms, indicating that sanjoinine A has an anxiolytic-like effect.

The hole-board test provides a simple method for measuring the response of an animal to an unfamiliar environment, and it is widely used to assess emotionality, anxiety and/or responses to stress in animals (Nolan and Parkes, 1973). Head-dipping behavior is sensitive to changes in the emotional state of the animal, and it has been suggested that the expression of an anxiolytic state in animals may be reflected by an increase in head-dipping behavior (Takeda et al., 1998). In our study, sanjoinine A (2.0 mg/kg) increased the number of head dips in the hole-board test, providing further evidence for anxiolytic-like effects of sanjoinine A.

The open field test was used to evaluate the exploratory activity of the animal. This was demonstrated through more central zone visitations by sanjoinine A-treated animals during the 1 h test. From the results of the open field test, we found that the percentages of distance traveled in the central zone and time spent in the central zone were increased by the treatment of sanjoinine A or diazepam, indicating the exploratory effect of sanjoinine A treatment.

Diazepam has been used as a standard anxiolytic and also has been frequently employed in behavioral pharmacology as a reference compound for potentially anxiolytic-acting substances. In our preliminary analysis on anxiolytic effects of sanjoinine A, we found it was more effective in hole board test than plus maze test, so was the effect of diazepam, it seems that the method of hole board test is more sensitive than plus-maze test in evaluation of Anxiolytic effects. At the same time, however, typical GABA/benzodiazepine receptor agonists, such as diazepam, have side effects including muscle relaxation and depressive mood (Hertz et al., 2006). In contrast to diazepam, sanjoinine A showed anxiolytic-like effects without affecting locomotor activity at lower doses (0.5, 1.0 and 2.0 mg/kg), indicating that the extract exerts anxiolytic-like and hypnotic effects at different doses. In the grip strength meter test, sanjoinine A also had no effect in decreasing the strength force, indicating that sanjoinine A has no effect on muscle relaxation.

It is well known that benzodiazepines facilitate the ability of GABA to activate the GABA receptors' intrinsic Cl^- channel and, in turn, to facilitate inhibitory neurotransmission. This is manifested as an increase in the frequency of ion channel opening in response to GABA. Sanjoinine A treatment significantly increased chloride influx of cerebellar granule cells, compared to the control treatment. Therefore,

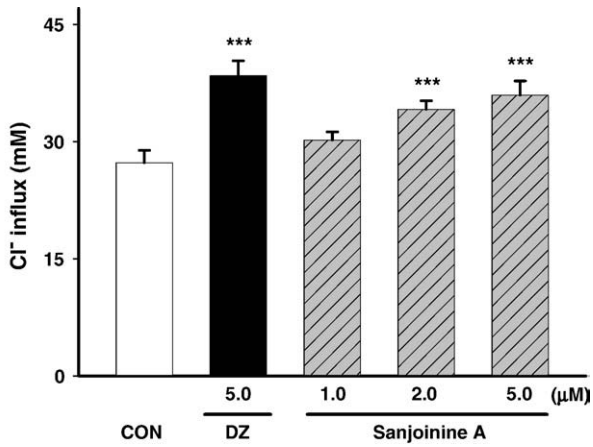

Fig. 5. Effects of sanjoinine A on chloride influx in primary cultured cerebellar granule cells. Each column represents the mean plus S.E.M. *** $p<0.001$, compared to the control group.

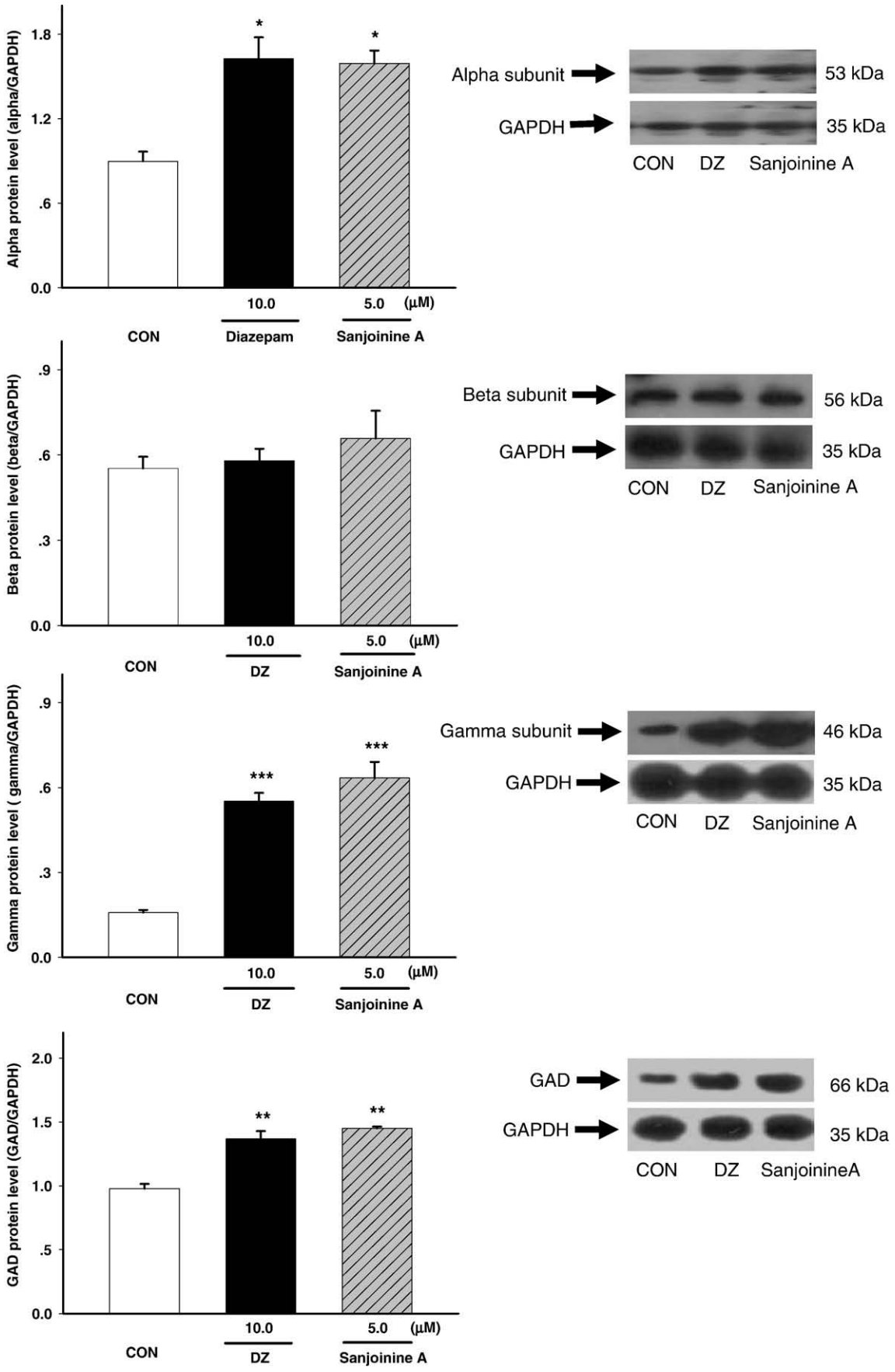

Fig. 6. Effects of sanjoinine A on expression of $GABA_A$ receptor subunits and of GAD65/67. Each column represents the mean plus S.E.M. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared to the control group.

it showed that anxiolytic-like effects of sanjoinine A might be mediated by GABA-benzodiazepine receptors-activated Cl^- channel opening.

$GABA_A$ receptors are heteromeric ligand-gated chloride ion channels that are activated by brief releases of GABA into the synaptic cleft. GABA released from presynaptic interneuronal terminals binds to postsynaptic GABA receptors that gate the chloride ion flux into the postsynaptic cell membrane. This ionic flux typically hyperpolarizes the post-synaptic neuronal membrane, making it less likely that arrival of excitatory neurotransmitters will be able to depolarize the post-synaptic cell sufficiently past the threshold for action potential initiation. Traditional hypnotics and anxiolytic drugs, such as barbiturates and benzodiazepines, enhance GABA transmission via the $GABA_A$ receptor. The α and β subunits provide the binding site for the GABA molecule, while the α and γ subunits are sensitive to benzodiazepine binding (Whiting, 1999; Bateson, 2004). We found that sanjoinine A increased the abundance of the $GABA_A$ receptor α-subunit and γ-subunit but had no effect on the abundance of the β-subunit. This indicates that sanjoinine A might increase responses of GABA receptor to benzodiazepine by influence GABA receptor subunits compositions.

As we know, GABA is synthesized almost exclusively from glutamate, and the critical step in GABA biosynthesis is the decarboxylation of glutamate by GAD65/67, which exists in two different isoforms, GAD65 and GAD67, which are encoded by different genes on separate chromosomes (Bu et al., 1992). So, if the amount of GAD65/67 is changed, GABA transmission may also be influenced. We investigated the effects of sanjoinine A and diazepam on expression of GAD65/67 using western blot technique. We found that sanjoinine A and diazepam treatment increased GAD65/67 expression significantly, suggesting that sanjoinine A might activate GAD65/67 and thus increase GABA transmission.

In our previous research, we found sanjoinine A increased responses of GABA receptor to benzodiazepine by influence GABA receptor subunits compositions (Ma et al., 2007). The pharmacological profile of $GABA_A$ receptors depends upon subunit composition, and distinct $GABA_A$ receptor subtypes, differing in subunit composition (Thompson et al., 1996). The differences in responses of α subunits to sanjoinine A in our experiment and Ma might be induced by different subtype responses of α subunits or the different physiological condition of sleeping behavior and anxiety state (Di Lazzaro et al., 2006; Graham et al., 1996).

To summarize, the data presented here indicate that sanjoinine A induced anxiolytic-like effects in the plus-maze test, hole-board test and open field test, but hypnotic or muscle-relaxant activity at low doses that do not influence locomotor activity and grip force was not induced. These results suggest that sanjoinine A has anxiolytic-like effects, which may be mediated by GABA-benzodiazepine receptor-activated Cl^- channel opening. Sanjoinine A may exert its anxiolytic effect by increasing GABA synthesis via GAD65/67 activation and increasing receptors for benzodiazepine or GABA by influencing GABA receptor subunit compositions. In conclusion, sanjoinine A might be a good compound for treating anxiety. Further investigation is needed to explore sanjoinine A derivatives with strong pharmacological actions and their possible mechanisms.

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