Modulation of Corydalis tuber on Glycine-Induced Ion Current in Acutely Dissociated Rat Periaqueductal Gray Neurons

Byung-Shik Cheong, a, b Do-Young Choi, a Nam-Hun Cho, a Jae-Dong Lee, a Hyun-Kyung Chang, b Min-Chul Shin, b Mal-Soon Shin, b and Chang-Ju Kim * a

a Department of Acupuncture and Moxibustion, College of Oriental Medicine, Kyung Hee University; and b Department of Physiology, College of Medicine, Kyung Hee University; #1 Hoigi-dong, Dongdaemoon-gu, Seoul 130-701, Korea.

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Traditionally, Corydalis tuber has been used for the control of pain including headache, stomachache, and neuralgia. In the present study, modulation of the Corydalis tuber on glycine-activated ion current in the acutely dissociated periaqueductal gray (PAG) neurons was studied by a nystatin-perforated patch-clamp technique. High concentrations of Corydalis tuber elicited ion current, which was suppressed by strychnine application, while low concentrations of Corydalis tuber reduced glycine-induced ion current in the PAG neurons. Inhibitory action of Corydalis tuber on glycine-activated ion current was partially abolished by treatment with naloxone, a non-selective opioid antagonist. Application of N-methylmaleimide (NEM), a sulfhydryl alkylating agent, also partially abolished the inhibitory action of Corydalis tuber on glycine-activated ion current in the PAG neurons. These results suggest that the inhibitory effect of Corydalis tuber on glycine-activated ion current in the PAG neurons is one of the analgesic mechanisms of the Corydalis tuber.

Key words Corydalis tuber; glycine; periaqueductal gray neurons; opioid; GTP-binding protein

Corydalis tuber is the root of Corydalis yahusuo W. T. Wang classified as Papaveraceae. Aqueous extract from Corydalis tuber is known to stimulate blood circulation and relieve pain. It has traditionally been used for the treatment of neuralgia, dysmenorrhea, and gastrointestinal spasm. 1) The analgesic and spasmylic properties of the Corydalis tuber are essentially ascribed to several alkaloids: dl-tetraphthalalmine (THP), d-cotydaline, and corydalis H, J, K, and L. The alkaloids exhibited analgesic, antiarrhythmic, antithrombosis, antiinflammatory, antitumor, antihypertensive, and antiallergic activities. 2) Among others, THP is a very effective monoamine depletor in the brain and also possesses analgesic, sedative, hypnotic, and antihypertensive actions. 3) The transmission of nociceptive information may be altered by various neuronal circuits within the central nervous system (CNS). One of them is a descending pain control system, which consists of three major components: the periaqueductal gray (PAG) of the midbrain, the rostroventral medulla (RM) including the nucleus raphe magnus, and the spinal dorsal horn. Of these, modulation of pain in the PAG matter is the most extensively studied pain control system. 4, 5) PAG is known as a major target of analgesic action of the opioid in the CNS. 6) Stimulation within the midbrain PAG produces an opioid receptor-mediated analgesia. 7)

Opioid peptides and opiates produce analgesia by activating the descending pain modulatory pathways, especially at the level of the PAG. 8, 9) They also regulate the nociceptive transmission in part by inhibiting the release of transmitters. 8, 10) The effects of opiates and opioid peptides also have been reported to activate potassium channels 11,12) or to inhibit calcium channels. 13,14) It is proposed that endogenous opioid peptides can activate PAG output neurons by inhibiting inhibitory interneurons. 5)

The amino acid glycine is a major inhibitory neurotransmitter in the brain and spinal cord. The inhibitory action of glycine is mediated by a strychnine-sensitive glycine receptor and a glycine-gated chloride ion channel. Inhibitory glycine synapses in the brainstem and spinal cord are closely implicated in the transmission of nociception, in which glycine inhibits neurotransmission and relieves pain. In addition, the glycine-mediated inhibitory effect induces muscle relaxation, whereas inhibition of glycine receptors by strychnine induces convulsion. 5,16) The analgesic mechanism of Corydalis tuber in the context of the descending pain control system has not yet been clarified. In the present study, the modulation of Corydalis tuber on glycine-activated ion current in the acutely dissociated PAG neurons was investigated using a nystatin-perforated patch-clamp technique under voltage-clamp conditions.

MATERIALS AND METHODS

Preparation of PAG Neurons The PAG neurons were freshly dissociated using a technique described elsewhere. 9,13) In brief, 10- to 15-d-old Sprague–Dawley rats of both sexes were decapitated under Zoletil 50® anesthesia (50 mg/kg; i.m.). The brain was removed and transverse slices (400 μm thick) were made with a microslicer (DTK-1000, DSK, Tokyo). Slices were preincubated at room temperature for 30 min in an incubation solution that had been well saturated with 95% O2 and 5% CO2. Then, the slices were treated with pronase (protease XIV, 1 mg/ml of the oxygenated incubation solution) for 40—80 min at 32°C and subsequently with thermolysin (protease X, 1 mg/ml) for 10—20 min at 32°C. After enzyme treatment, the slices were kept in the enzyme free incubation solution for 1 h. The PAG region was identified in a 60 mm culture dish coated with silicone under a binocular microscope (SZ-ST, Olympus, Tokyo), and was micropunched out from the slices with an electrolytically polished injection needle. The micropunched PAG regions were mechanically dissociated in a different dish with fire-polished fine glass Pasteur pipettes in 35 mm plastic culture dishes (3801, Falcon, Franklin Lakes, NJ, U.S.A.) filled with standard solution. The dissociation procedure was done...
under an inverted phase-contrast microscope (CK-2, Olympus, Tokyo). The dissociated neurons usually adhered to the bottom of the dish within 20 min. These cells remained viable for electrophysiological studies up to 6 h after dissociation.

**Solutions**  The ionic composition of the incubation solutions was (in mM): NaCl 124, KCl 5, KH₂PO₄ 1.2, MgSO₄ 1.3, CaCl₂ 2.4, glucose 10, and NaHCO₃ 24. The pH was adjusted to 7.4 by continuous bubbling with 95% O₂ and 5% CO₂. The composition of the standard external solution was (in mM): NaCl 150, KCl 5, MgCl₂ 1, CaCl₂ 2, glucose 10, and N-2-hydroxyethylpiperazine-N°-2-ethanesulphonic acid (HEPES) 10. The pH was adjusted to the 7.4 with tris-hydroxymethylaminomethane (Tris-base). The composition of the internal pipette solution for nystatin perforated recording contained (in mM): KCl 150 and HEPES 10. The pH was adjusted to 7.2 by adding Tris-base. A stock solution containing 10 mg/ml nystatin in methanol was prepared and added at a final concentration of 200 μ/ml to the patch pipette solution.

**Drugs**  *Corydalis tuber* used in this experiment was obtained from the Kyungdong market (Seoul, Korea). After washing, *Corydalis tuber* was immersed in cold water for 12 h. In order to obtain aqueous extracts of *Corydalis tuber*, it was subsequently heat-extracted, pressure-filtered, and concentrated with a rotary evaporator. The resulting 3.2 g of powder (yield of 10.6%) was obtained from 30 g of *Corydalis tuber* through lyophilization by a drying machine for 24 h.

**Electrical Measurements**  Electrical recordings were performed in the nystatin-perforated patch recording mode under voltage-clamp conditions. Patch pipettes were prepared from glass capillaries with an outer diameter of 1.5 mm on a two-stage puller (PB-7, Narishige, Tokyo). The resistance between the recording electrode filled with the internal pipette solution and the reference electrode was 6—8 MΩ. After stable perforated patch formation, the series resistance ranged from 16 to 25 MΩ.

Electrical stimulation, current recordings, and filtration of currents (at 2.9 kHz) were obtained with an EPC-7 patch-clamp amplifier (List-Electronic, Darmstadt/Eberstadt, Germany). The current and voltage were monitored on a pen recorder (Recti-Horiz-8K, NEC San-ei, Tokyo). All experiments were performed at room temperature (22—24 °C).

**Data Analysis**  Results are presented as mean±standard error mean (S.E.M.) and Student’s t-test was used for statistical analysis and p value less than 0.05 was considered significant.

**RESULTS**

**Ion Current Activated by *Corydalis tuber***  In the nystatin-perforated patch-clamp mode, experiments were carried out at a holding potential (V_H) of −50 mV. *Corydalis tuber* was applied every 2 min and ion current-activated by 1 mg/ml *Corydalis tuber* used as the control value. Inward currents were recorded by application of *Corydalis tuber* at various concentrations. The concentration of 0.05 mg/ml of *Corydalis tuber* did not elicit ion current, while application of 0.1 mg/ml, 0.5 mg/ml, 3 mg/ml, and 5 mg/ml of *Corydalis tuber* elicited ion current 1.82±1.84%, 57.91±2.82%, 158.19±2.93%, and 243.52±4.44% of the control value. The present results showed that *Corydalis tuber* elicited ion current in a concentration-dependent manner in the PAG neurons (Fig. 1).

**Effect of Strychnine on *Corydalis tuber*-Activated Ion Current**  To evaluate the pharmacological properties of the ion current activated by *Corydalis tuber*, the magnitude of ion current elicited by 1 mg/ml *Corydalis tuber* was used as the control value, and strychnine, a glycine receptor antagonist, at concentrations of 10⁻⁷ M, 10⁻⁶ M, 10⁻⁵ M, and 10⁻⁴ M was applied simultaneously with 1 mg/ml *Corydalis tuber*. The ion current induced by 1 mg/ml *Corydalis tuber* was not changed by 10⁻⁷ M strychnine application and treatment with 10⁻⁷ M strychnine inhibited the ion current about 14.00±7.74% with no statistical significance. Strychnine at concentrations of 10⁻⁵ M and 10⁻⁴ M inhibited ion current induced by 1 mg/ml *Corydalis tuber* 24.75±5.94% and 74.25±6.98% with statistical significance. These results showed that ion current activated by 1 mg/ml *Corydalis tuber* was suppressed by strychnine in a concentration-dependent manner (Fig. 2).

**Modulation of *Corydalis tuber* on Glycine-Induced Ion Current**  To determine the modulation of *Corydalis tuber*...
on the glycine-induced ion current, the magnitude of ion current elicited by 10^{-5} \text{M} glycine was used as the control value and 0.01 mg/ml, 0.05 mg/ml, 0.1 mg/ml, 0.5 mg/ml, and 1 mg/ml Corydalis tuber was applied simultaneously with 10^{-5} \text{M} glycine. Corydalis tuber at concentrations of 0.01 mg/ml, 0.05 mg/ml, 0.1 mg/ml, and 0.5 mg/ml suppressed glycine-induced ion current \(1.23 \pm 0.62\%\), \(3.24 \pm 1.53\%\), \(14.83 \pm 1.61\%\), and \(45.74 \pm 1.52\%\) of the control value, respectively. In contrast, 1.0 mg/ml Corydalis tuber potentiated glycine-induced ion current \(1.82 \pm 2.72\%\) of the control value. In the present results, the most potent suppression on the glycine-induced ion current was thus observed at 0.5 mg/ml of Corydalis tuber (Fig. 3).

Effect of Naltrexone on Corydalis tuber-Induced Inhibition on Glycine-Activated Ion Current To evaluate the involvement of opioid receptor on Corydalis tuber-induced inhibition on glycine-activated ion current in the PAG neurons, naltrexone which is an opioid antagonist and stable naloxone analogue, was applied simultaneously with 10^{-5} \text{M} glycine. Corydalis tuber inhibited glycine-induced ion current \(44.39 \pm 4.65\%\) of the control value, but this inhibitory action was significantly alleviated to \(29.65 \pm 4.77\%\) by 10^{-5} \text{M} naltrexone application (Fig. 4).

Effect of N-Ethylmaleimide (NEM) on Corydalis tuber-Induced Inhibition on Glycine-Activated Ion Current In order to elucidate the involvement of GTP-binding proteins (G-proteins) in Corydalis tuber-induced inhibition on glycine-activated ion current, the effect of NEM, a sulfhydryl alkylating agent, to the inhibition of Corydalis tuber on glycine-induced ion current was investigated. After perfusion...
with the standard solution containing NEM at a concentration of 5×10^{-5} M for 2 min, the inhibitory action of *Corydalis tuber* on glycine-induced chloride current was significantly abolished: 0.5 mg/ml of *Corydalis tuber* inhibited glycine-induced chloride ion current 44.39±4.65% compared to control but this was decreased to 25.21±9.82% after NEM perfusion (Fig. 5).

**DISCUSSION**

In the present study, high concentrations (above 1 mg/ml) of *Corydalis tuber* elicited ion current in the PAG neurons in a dose-dependent manner (Fig. 1), and *Corydalis tuber*-induced ion current was inhibited by application of a glycine receptor antagonist, strychnine. (Fig. 2) From this experiment, it can be suggested that high concentrations of *Corydalis tuber* activate glycine receptors in the PAG neurons.

However, the major finding of this study is that low concentrations (0.1 mg/ml and 0.5 mg/ml) of *Corydalis tuber* reduced the glycine-induced ion current in the PAG neurons. Antagonists of glycine receptors as well as those of γ-aminobutyric acid (GABA) receptors are known to induce convulsion by suppressing the inhibitory pathways in the CNS. It has been proposed that the analgesic effect of opioids on the PAG acts by suppressing the inhibitory influence of the neurotransmitters on the neurons that form part of a descending antinociceptive pathway. Based on the present results, it is possible that the inhibitory action of *Corydalis tuber* on glycine-induced ion current may activate the descending pain control system.

Naltrexone and naloxone are clinically prescribed as opioid antagonists and cross the blood–brain barrier. These non-selective opioid receptor antagonists block both central analgesia and the adverse effects of opioid medications. Stimulation of the PAG matter produces a kind of analgesia which is mediated by the release of endogenous opioids and blocked by pretreatment with naltrexone. In the present study, naltrexone application partially abolished *Corydalis tuber*-induced inhibition on glycine-activated ion current in the PAG neurons. These results show that the opioid receptors are partly involved in the inhibitory action of *Corydalis tuber* on glycine-activated ion current in the PAG neurons, suggesting that some components of *Corydalis tuber* exert analgesic action through opioid receptors in the PAG neurons. However, the inhibitory action of *Corydalis tuber* on glycine-activated ion current was not eliminated completely by naltrexone application, suggesting that other components of *Corydalis tuber* may induce analgesic action through direct modulation on glycine-receptors in the PAG neurons.

Neurotransmitters acting through G-proteins coupled receptors change the electrical excitability of neurons. Activation of receptors can affect the voltage dependence, the speed of gating, and probability of the opening of various ion channels, thus changing the computational state and outputs of a neuron. The G-proteins under consideration are heterotrimeric molecules with α-, β-, and γ-subunits. The α-subunits can be classified into three families, depending on whether they are targets for pertussis toxin (PTX), cholera toxin, or neither. In neurons, the most widespread modulatory signaling pathway is characterized by sensitivity to PTX, which indicates that the receptors couple to G-proteins of the Gi family, such as G\textsubscript{i} or G\textsubscript{ai}.

NEM has been used to block PTX-sensitive G-protein action. It is a sulfhydryl alkylating agent that can selectively inhibit PTX-sensitive G-protein-mediated effects in the central, peripheral, and invertebrate neurons. The advantage of using NEM is that it allows us to examine PTX-sensitive G-protein-mediated action before and after inhibition of PTX-sensitive G-protein within a given recording. In this study, the inhibitory action of *Corydalis tuber* on glycine-induced ion current was partially abolished by NEM pretreatment for 2 min (Fig. 5). These results show that G-proteins are partly implicated in the inhibitory action of *Corydalis tuber* on glycine-activated ion current in the PAG neurons, suggesting that some components of *Corydalis tuber* may induce analgesic action through G-proteins in the PAG neurons. However, the inhibitory action of *Corydalis tuber* on glycine-activated ion current was not eliminated completely by NEM pretreatment. It means that some components of *Corydalis tuber* may induce analgesic action without involving G-proteins in the PAG neurons, suggesting directly affect glycine-receptors in the PAG neurons.

The descending pain control system consists of three major components: the PAG of midbrain, the RM which is divided into rostral ventromedial medulla (RVM) and rostral ventrolateral medulla (RVLM), and the spinal dorsal horn. Nucleus reticularis paragigantocellularis (NRPG) and nucleus reticularis gigantocellularis (NRGC) belong to the RVM and RVLM, respectively. NRPG/NRGC neurons are also known to regulate the descending pain control system. Activation of the PAG neurons subsequently
activates NRPG/NRGC neurons. The antinociceptive effect induced by excitation of PAG neurons is mediated by an excitatory innervation from the PAG to the RVM, innervating the dorsal horn of the spinal cord.4)

Traditionally, aqueous extract from *Corydalis tuber* has been widely used for pain control. The PAG region of the brain is known to be involved heavily with nociception. The present results suggest that inhibitory modulation of *Corydalis tuber* on the glycine-activated ion current is one of the analgesic mechanisms of the *Corydalis tuber*, which may activate the descending pain control system in PAG neurons.

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**REFERENCES**