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Original Article

Effect of Amygdalin from *Armeniacae Semen* on Ion Currents Changed by Lipopolysaccharide in Rat Periaqueductal Gray Neurons

Gil-Jae Lee, Yun-Kyung Song, Hyung-Ho Lim

Department of Oriental Rehabilitation Medicine, College of Oriental Medicine, Kyungwon University

Background : Amygdalin is abundant in *Armeniacae semen*, and it is recently reported to treat cancers and relieve pain. But modus operandi of amygdalin at the level of neuron has not been reported, yet.

Objective : This study aimed to find out the effect of amygdalin on glycine- and glutamate-induced ion currents in periaqueductal gray (PAG) neurons. And it was investigated that amygdalin participates in the regulation of the descending pain control system in the level of PAG neurons.

Method : We investigated that the changes of glycine- and glutamate-induced ion currents in PAG neurons through application of lipopolysaccharides (LPS) and application of amygdalin with LPS by using the nystatin-perforated patch clamp method.

Result : Application of LPS on PAG neurons resulted in increased glycine-induced ion current, and in decreased glutamate-induced ion current. In contrast, application of amygdalin with LPS resulted in decreased glycine-induced ion current increased by LPS, and increased glutamate-induced ion current decreased by LPS.

Conclusion : Amygdalin from *Armeniacae semen* controls glycine- and glutamate-induced ion current by LPS in PAG neurons, and it is suggested that amygdalin participates in the regulation of the descending pain control system in the level of PAG neurons.

Key Words : *Armeniacae semen*, periaqueductal gray(PAG) neurons, glycine, glutamate, patch clamp, amygdalin

Introduction

The transmission of nociceptive information may be altered by various neural circuits within the central nervous system (CNS). The descending pain control system consists of three major components: the periaqueductal gray (PAG) of the midbrain, the rostroventral medulla including the nucleus raphe magnus, and the spinal dorsal horn. Descending modulation of spinal nociceptive neurons by the PAG matter is one of the most extensively studied pain control systems[1-3]. PAG is rich in opioidreceptor and opioid peptide and opiates are known to produce analgesia by activating descending pain control pathway, especially at the level of PAG[3-5]. Several neurotransmitters in the PAG participate in the control of nociception. Among these, endogenous opioids, glycine, and glutamate seem to play a crucial role in the processing of pain-regulatory signals within this area[4,6-8].

Glycine is an important inhibitory transmitter in the brainstem and spinal cord. The binding of

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・Correspondence to : Gil-Jae Lee
Department of Oriental Rehabilitation Medicine, Seoul Oriental Hospital Kyungwon University, 20-8 Songpa-Dong, Songpa-Gu, Seoul Korea
(Tel : +82-2-425-3456 / Fax : +82-2-425-3560
E-mail : leegj21@naver.co)

glycine to its receptor produces a large increase in Cl-conductance, which causes membrane hyperpolarization[5,7]. Glutamate is a major excitatory neurotransmitter in the CNS where they participate in a great number of physiological and pathological states[6,9].

Armeniacae semen is the seed of Prunus armeniaca L. var. ansu MAXIM, which has been classified into Rosaceae. *Armeniacae semen* is known to have many therapeutic effects such as relieving fever, stopping cough, quenching thirst, and so on. In traditional oriental medicine, *Armeniacae semen* has been used for the treatment of asthma, bronchitis, emphysema, constipation, nausea, leprosy[10,11].

Amygdalin is abundant in the seeds of the Prunus genus such asalmond, apricots, and other rosaceous plants. Amygdalin is recently reported to treat cancers and relieve pain[12-18]. Although amygdalin is known to be involved in anti-inflammatory and analgesic effects[19,20], the effect of amygdalin on neuronal activity at the PAG level has not been reported, yet. In order to elucidate the modulation of amygdalin in the neuronal activity of PAG level, glycine- and glutamate-induced ion currents in rat PAG neurons was investigated using the nystatin-perforated patch clamp technique in this study.

Materials and Methods

1. Preparation of the PAG neuron

PAG neurons were dissociated using a technique described elsewhere[2,3,21]. In brief, 10- to 15-day-old Sprague-Dawley rats of both sexes were decapitated under Zoletil 50Ⓡ-induced anesthesia (50 mg/kg, i.m.). The brains were removed, and transverse slices (400 ㎛ in thickness) were made with a microslicer (DTK-1000, DSK, Tokyo, Japan). Slices were preincubated in an incubation solution that had been well saturated though bubbling with 95 % O2 and 5 % CO2 at room temperature for 30 min. Then, the slices were treated with pronase (protease XIV, 1 mg/6 ml of the oxygenated incubation solution) for 40 - 60 min at 32℃ and subsequently with thermolysin (protease X, 1 mg/6 ml) for 10 - 20 min at 32℃ After the enzyme treatment, the slices were incubated in enzyme-free incubation solution for 1 h.

The PAG region was identified under a binocular microscope (SZ-ST, Olympus, Tokyo, Japan) and was micropunched out from the slices with an electrolytically polished injection needle. The punched-out PAG regions were mechanically dissociated with fire-polished fine glass Pasteur pipettes in 35 mm plastic culture dishes (3801, Falcon, Franklin Lakes, NJ, USA) filled with the standard solution. The dissociation procedure was performed under an inverted phase-contrast microscope (CK-2, Olympus, Tokyo, Japan). 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.

2. Solutions

The ionic composition of the incubation solutions was NaCl 124 mM, KCl 5 mM, KH2PO4 1.2 mM, MgSO4 1.3 mM, CaCl2 2.4 mM, glucose 10 mM, and NaHCO3 24 mM. The pH was adjusted to 7.4 by continuous bubbling with 95 % O2 and 5 % CO2. The composition of the standard external solution was NaCl 150 mM, KCl 5 mM, MgCl2 1 mM, CaCl2 2 mM, glucose 10 mM, and N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 10 mM. The pH was adjusted to 7.4 with tris-

hydroxymethylaminomethane (Tris-base). The composition of the internal pipette solution for nystatin-perforated patch recording contained KCl 150 mM and HEPES 10 mM. The pH was adjusted to 7.2 by adding Tris-base. A stock solution containing 10 mg/ml nystatin was prepared and added to the patch pipette solution to reach a final concentration of 200 ㎍/ml.

3. Reagents

Pronase, thermolysin, nystatin, glycine, glutamate, LPS and most of the other drugs used in this experiment were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Zoletil 50 ® was obtained from Virbac Caboratories (Carros, France). Drugs were added to the standard solution to reach the final concentrations provided in the text and were applied using a rapid application system termed the "Y-tube method"[2,3,21]. Using this technique, the standard solution surrounding a neuron could be exchanged within 10 - 20 ms.

4. Extraction of amygdalin

Armeniacae semen used in this experiment was obtained from the Kyungwon university hospital (Seoul, Korea). Both 5oo g of *Armeniacae semen* hatched from the shell and 10 L of 4 % citric acid solution were refluxed for 2 h. After filtering when it was still hot, the filtrate was passed through the column packed with HP-20. The substance absorbed within the column was concentrated after it had been eluted by ethanol. Amygdalin 4.2 g (with the yield rate of 0.84 %) was obtained by recrystallizing the extract with ethanol.

5. Electrical measurement

Electrical recordings were performed in the nystatin-perforated patch recording mode under the voltage clamp condition. Patch pipettes were prepared from glass capillaries with an outer diameter of 1.5 mm on a two-stage puller (PB-7, Narishige, Tokyo, Japan). The resistance between the recording electrode filled with the internal pipette solution and the reference electrode was 6-8 ㏁. After the formation of a stable perforated patch, the series resistance ranged from 16 to 25 ㏁. 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 also monitored on a pen recorder (Recti-Horiz-8K, NEC San-ei, Tokyo, Japan).

Glycine and glutamate at concentration of 10-5 M was applied to PAG neurons every 2 min, and the magnitude of the current induced by glycine and glutamate was used as the control value. The initial amplitudes of the glycine- and glutamate-induced ion current were shown to vary by less than 5 % during the recording period when LPS and amygdalin was not applied.

All experiments were performed at room temperature (22-24℃).

6. Statistical analysis

Results are presented as mean ± standard error mean (S.E.M.), and Student's t-test was used for statistical analysis, withp values less than 0.05 as indicators of statistical significance.

Results

1. Effect of LPS on glycine-induced ion current in rat PAG neurons

Glycine at a concentration of 10-5 M was applied to PAG neurons every 2 min, and the

magnitude of thecurrent induced by glycine alone was used as the control value. The initial amplitudes of the glycine-induced ion current were shown to vary by less than 5 % during the recording period when LPS was not applied. Continuous application of LPS to PAG neuronsat concentration of 10-3 mg/ml did not elicit any ion current in the absence of glycine, while it enhanced the glycine-induced ion current in a time-dependent manner, to 1.11 ± 0.03 ($n = 9$, $p < 0.05$) after 6 min, to 1.14 ± 0.03 ($n = 9$, $p < 0.05$) after 12 min, to 1.29 ± 0.03 ($n = 9$, $p < 0.05$) after 18 min, to 1.37 ±0.03 ($n = 9$, $p < 0.05$) after 24 min, and to 1.39 ± 0.02 ($n = 9$, $p < 0.05$) after 30 min, with the control value as 1. After washing, the magnitude of the glycine-induced ion current declined, to about 107 ± 0.02 % of the control level (Fig. 1).

In the present study, it was shown that application of LPS to PAG neurons potentiated the ion current induced by glycine in a time-dependent manner.

2. Effect of LPS on glutamate-induced ion current in rat PAG neurons

Glutamate at a concentration of 10-5 M was applied to PAG neurons every 2 min, and the magnitude of current induced by glutamate alone was used as the control value. The initial amplitudes of the glutamate-induced ion current were shown to vary by less than 5 % during the recording period when LPS was not applied. Continuous application of LPS to PAG neurons at concentration of 10-3 mg/ml did not elicit any ion current in the absence of glutamate, while it decreased the glutamate-induced ion current in a time-dependent manner, to 0.90 ± 0.02 ($n = 6$, $p < 0.05$) after 6 min, to 0.83 ±0.01 ($n = 6$, $p < 0.05$) after 12 min, to 0.79 ± 0.01 ($n = 6$, $p < 0.05$) after 18 min, to 0.75 ± 0.01 ($n = 6$, $p < 0.05$) after 24 min, and to 0.69 ±0.04 ($n = 6$, $p < 0.05$) after 30 min, with the control value as 1. After washing, the magnitude of the glutamate-induced ion current rose, to about 91 ± 0.01 % of the control level (Fig. 2).

In the present study, it was shown that application of LPS to PAG neurons suppressed the ion current induced by glutamate in a time-dependent manner.

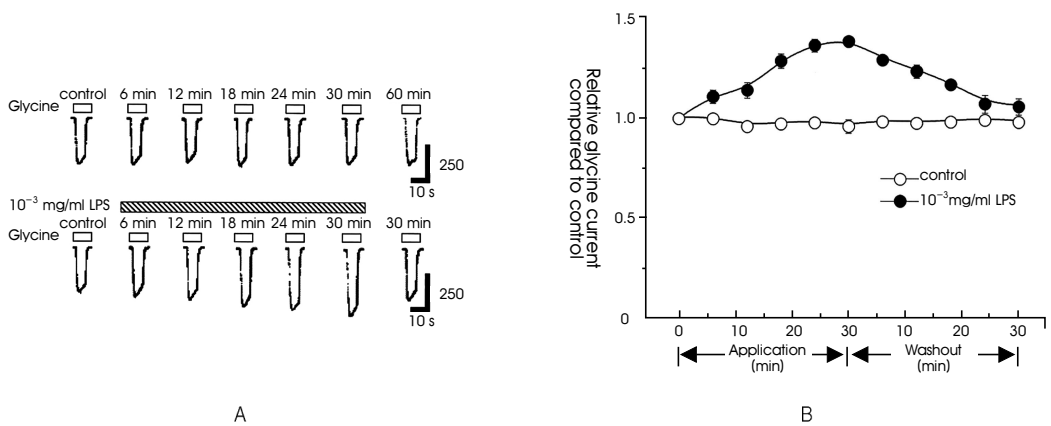

Fig. 1. Effect of LPS on glycine-induced ion current in rat PAG neurons. Application of LPS to PAG neurons potentiated the ion current induced by glycine in a time-dependent manner. LPS, lipopolysaccharides.

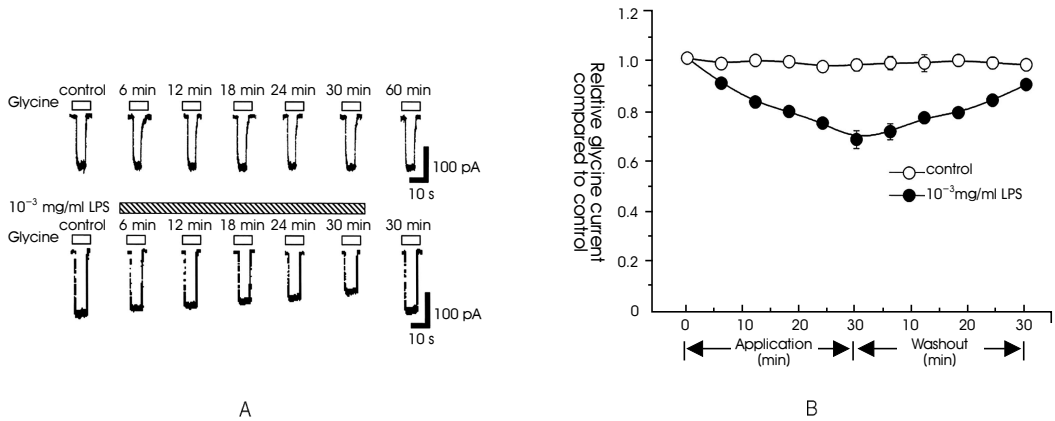

Fig. 2. Effect of LPS on glutamate-induced ion current in rat PAG neurons. Application of LPS to PAG neurons suppressed the ion current induced by glutamate in a time-dependent manner. LPS, lipopolysaccharides.

3. Effects of amygdalin on glycine-induced ion current stimulated by LPS in rat PAG neurons

Glycine at a concentration of 10-5 M was applied to PAG neurons every 2 min, and the magnitude of the current induced by glycine alone was used as the control value. The initial amplitudes of the glycine-induced ion current were shown to vary by less than 5 % during the recording period when amygdalin with LPS was not applied. Continuous application of 0.5 mg/ml amygdalin with 10-3 mg/ml LPS to PAG neurons decreased maximum about 25.97 % of the magnitude of glycine ion current increased by 10-3 mg/ml LPS in a time-dependent manner, to 1.00 ± 0.01 ($n = 7$, $p < 0.05$) after 6 min, to 1.12 ±0.02 ($n = 7$, $p < 0.05$) after 12 min, to 1.25 ± 0.02 ($n = 7$, $p < 0.05$) after 18 min, to 1.29 ± 0.03 ($n = 7$, $p < 0.05$) after 24 min, and to 1.29 ± 0.03 ($n = 7$, $p < 0.05$) after 30 min, with the

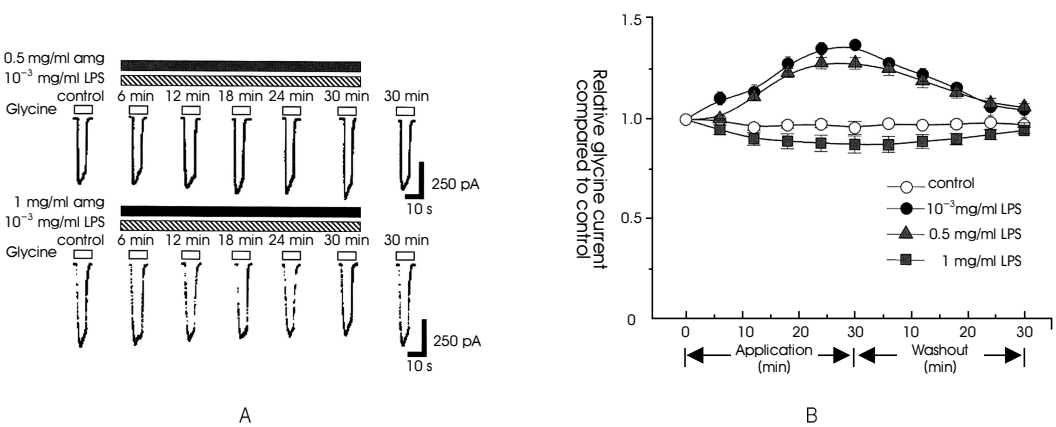

Fig. 3. Effects of amygdalin on glycine-induced ion current stimulated by LPS in rat PAG neurons. Application of amygdalin with LPS suppressed glycine-induced ion current increased by LPS in concentration- and time-dependent manner. LPS, lipopolysaccharides. amg, amygdalin.

control value as 1. After washing, the magnitude of the glutamate-induced ion current rose, to about 108.0 ± 0.02 % of the control level. Also, continuous application of 1 mg/ml amygdalin with 10-3 mg/ml LPS decreased maximum about 131 % of glycine-induced ion current increased by 10-3 mg/ml LPS in a time-dependent manner, to 0.95 ±0.02 ($n = 8$, $p < 0.05$) after 6 min, to 0.90 ± 0.03 ($n = 8$, $p < 0.05$) after 12 min, to 0.89 ± 0.04 ($n = 8$, $p < 0.05$) after 18 min, to 0.88 ± 0.04 ($n = 8$, $p < 0.05$) after 24 min, and to 0.88 ± 0.04 ($n = 8$, $p < 0.05$) after 30 min, with the control value as 1. After washing, the magnitude of glycine-induced ion current rose, to about 96.04 ± 0.02 % of the control level (Fig. 3).

In the present study, it was shown that application of amygdalin with LPS suppressed glycine-induced ion current increased by LPS in concentration- and time-dependent manner.

4. Effects of amygdalin on glutamate-induced ion current stimulated by LPS in rat PAG neurons

Glutamate at a concentration of 10-5 M was applied to PAG neurons every 2 min, and the magnitude of the current induced by glutamate alone was used as the control value. The initial amplitudes of the glutamate-induced ion current were shown to vary by less than 5 % during the recording period when amygdalin with LPS was not applied.Continuous application of 0.5 mg/ml amygdalin with 10-3 mg/ml LPS to PAG neurons increased maximum about 41.94 % of the magnitude of glutamate ion current decreased by 10-3 mg/ml LPS in a time-dependent manner, to 0.96 ± 0.02 ($n = 9$, $p < 0.05$) after 6 min, to 0.91 ± 0.03 ($n = 9$, $p < 0.05$) after 12 min, to 0.86 ± 0.02 ($n = 9$, $p < 0.05$) after 18 min, to 0.84 ± 0.02 ($n = 9$, $p < 0.05$) after 24 min, and to 0.82 ± 0.02 ($n = 9$, $p < 0.05$) after 30 min, with the control value as 1. After washing, the magnitude of glutamate-induced ion current rose, to about 93.3 ± 0.01 % of the control level. Also, continuous application of 1 mg/ml amygdalin with 10-3 mg/ml LPS increased maximum about 71 % of glutamate-induced ion current

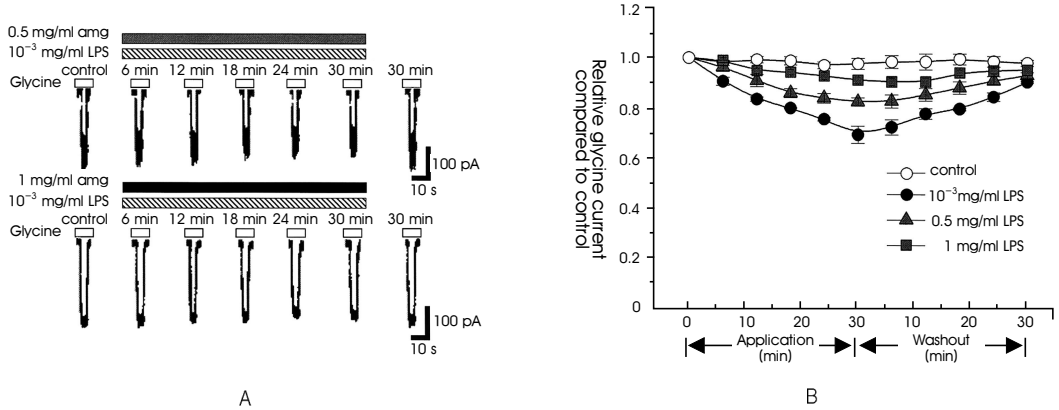

Fig. 4. Effects of amygdalin on glutamate-induced ion current stimulated by LPS in rat PAG neurons. Application of amygdalin with LPS increased glutamate-induced ion current decreased by LPS in concentration- and time-dependent manner. LPS, lipopolysaccharides. amg, amygdalin.

decreased by 10-3 mg/ml LPS in a time-dependent manner, to 0.99 ± 0.01 ($n = 8$, $p < 0.05$) after 6 min, to 0.95 ± 0.01 ($n = 8$, $p < 0.05$) after 12 min, to 0.94 ± 0.01 ($n = 8$, $p < 0.05$) after 18 min, to 0.93 ± 0.01 ($n = 8$, $p < 0.05$) after 24 min, and to 0.91 ± 0.01 ($n = 8$, $p < 0.05$) after 30 min, with the control value as 1. After washing, the magnitude of glutamate-induced ion current rose, to about 95.5 ± 0.01 % of the control level (Fig. 4).

In the present study, it was shown that application of amygdalin with LPS increased glutamate-induced ion current decreased by LPS in concentration- and time-dependent manner.

Discussion

Armeniacae semen has been used for the treatment of asthma, bronchitis, emphysema, and leprosy in traditional oriental medicine. *Armeniacae semen* is also known to have antidiarroheic, antipyretic, antiemetic, and anthelmintic effects[10,11,19]. The anti-inflammatory and analgesic activities of *Armeniacae semen* have also been previously reported[10,22].

Armeniacae semen has traditionally been used as an ingredient of some oriental medicine prescriptions for relieving of pain and inflammation. Xiao Qing Long Tang and Xing Su San are traditional medicines that have *Armeniacae semen* in their formulation, and these medicines are used for the treatment of bronchitis, asthma with fever, headache and pain in limbs. *Armeniacae semen* is also used in the Sang Ju Yin medication which is the prescription for the treatment of influenza, pneumonia, and acute tonsillitis[19].

Armeniacae semen is divided into the outer husk and an inner part that contains glycoside, amygdalin, starch and fatty acids. According Chang et al.[19] *Armeniacae semen* extract suppressed LPS-induced expressions of COX-2.

Among the components of *Armeniacae semen*, amygdalin is one of many nitrilosides, which are natural cyanide containing substances abundant in the seeds of the prunasin family such as apricots, almonds, peaches, apples, and other rosaceous plant. According Shin et al.[23] amygdalin did not show any influence on the synthesis of PGE2 and the expression of COX-2. But Kim et al. reported that amygdalin exert pain control and functional recovery from peripheral nerve injury[24].

Pain is the first response to injury or infection. Injury and infection activate the immune system to produce inflammatory responses[25]. LPS initiates a number of major cellular responses that play vital roles in the pathogenesis of inflammatory responses. COX-2 is responsible for PGs production in the CNS under physiological condition, and enhanced spinal release of PGE2 and induction of COX-2 in the spinal cord are major consequence of peripheral inflammation. Induction of COX-2 by inflammation leads to the release of PGs, which sensitize peripheral nociceptor terminals and produce localized pain hypersensitivity. Peripheral inflammation also generates pain hypersensitivity in adjacent uninjured tissue, through increased neuronal excitability in the spinal cord[26,27]. PGs suppressed outward K+ current in rat DRG neurons, which increased membrane excitability, and then enhanced sensitivity of nociceptors to chemical and mechanical stimuli was observed[28]. Studies with COX inhibitors have shown that COX-2 is involved in inflammatory pain and neuropathic pain[29]. According Vaughan[30], COX inhibitors potentiate the opioid inhibition of GABAergic

synaptic transmission in rat PAG. Also, it was reported that COX-2 modulated PAG neuronal activity and involved with the regulation of the descending pain control system in the level of PAG neurons[27].

PAG is rich in opioid receptors and opioid peptides and opiates are known to produce analgesia by activating descending pain control pathway[1]. The activation of the descending pain control system is influenced by several factors including stress, electroacupuncture, and local electrical stimulation of the PAG[31,32]. It has been proposed that the effect of opioid on the PAG takes place by suppressing the inhibitory influence of glycine on the neurons that form part of the descending pain control pathway[5,7]. In the PAG and the spinal dorsal horn, the relay centers for pain and sensory information, glycine inhibits glutamate-evoked depolarization and represses the firing of neurons. Also, glutamate seems to be involved in PAG-mediated analgesia; microinjections of glutamate and glutamate agonists into the PAG have been shown to induce analgesia[6].

Reports showed that COX-2 modulated PAG neuronal activity and involved with the regulation of the descending pain control system in the level of PAG neurons.

In this study, application of LPS to PAG region potentiated ion current induced by glycine and suppressed ion current induced by glutamate, while application of amygdalin with LPS suppressed glycine-induced ion current increased by LPS and enhanced glutamate-induced ion current decreased by LPS in concentration- and time-dependent manner.

In the present results, activation of COX-2 by LPS in PAG neurons resulted in elevated glycine-induced responses and suppressed glutamate-induced responses, implicative of decrease neuronal excitability in the PAG. However, application of amygdalin with LPS resulted in suppressed glycine-induced response by LPS and elevated glutamate-induced responses by LPS. Thus, it appears that amygdalin modulates PAG neuronal activity, and it issuggested that amygdalin is involved in the regulation of the descending pain control system in the level of PAG neurons.

Conclusion

Based on the present study, we get the following conclusion:

1. Application of LPS to PAG neurons potentiates the ion current induced by glycine in a time-dependent manner.
2. Application of LPS to PAG neurons suppresses the ion current induced by glutamate in a time-dependent manner.
3. Application of amygdalin with LPS suppresses glycine-induced ion current increased by LPS in concentration- and time-dependent manner.
4. Application of amygdalin with LPS increased glutamate-induced ion current decreased by LPS in concentration- and time-dependent manner.

These results indicate that amygdalin controls glycine- and glutamate-induced ion current by LPS in PAG neurons, and it issuggested that amygdalin participates in the regulation of the descending pain control system in the level of PAG neurons.

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