Modulation of Amygdalin on Glycine- and Glutamate-induced Ion Currents in Rat Periaqueductal Gray Neurons

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ABSTRACT

Amygdalin is known as vitamin B₁₇, and it was called laetrile. Amygdalin is composed of two molecules of glucose, one molecule of benzaldehyde which induces an analgesic action, and one molecule of hydrocyanic acid which is an anti-neoplastic compound. Amygdalin had been used to treat cancers and relieve pain. In order to evaluate whether the analgesic action of amygdalin is related with descending pain control system, we performed patch clamp study. In the present study, the modulatory effects of amygdalin on glycine- and glutamate-induced ion currents in periaqueductal gray (PAG) neurons were investigated using the nystatin-perforated patch clamp method. Continuous application of lipopolysaccharides (LPS) on PAG neurons resulted in increased glycine-induced ion current, and in decreased glutamate-induced ion current. In contrast, continuous application of amygdalin with LPS resulted in decreased glycine-induced ion current increased by LPS, and increased glutamate-induced ion current decreased by LPS in concentration- and time-dependent fashion. These results demonstrate that amygdalin modulates neuronal activity of PAG by modulation of glycine and glutamate. Based on the present results, it can be suggested that amygdalin participates in the regulation of the descending pain control system in the level of PAG neurons. The present study demonstrated that activation of the descending pain control system is one of the possible analgesic mechanisms of amygdalin.

Key words: cyclooxygenase-2, periaqueductal gray neurons (PAG), glycine, glutamate, patch clamp, amygdalin

INTRODUCTION

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 plants. Amygdalin is known as vitamin B₁₇, and it was called laetrile. Amygdalin is composed of two molecules of glucose, one molecule of benzaldehyde which induces an analgesic action, and one molecule of hydrocyanic acid which is an anti-
neoplastic compound. Amygdalin had been used to treat cancers and relieve pain (Ellison et al., 1978; Fukuda et al., 2003; Chang et al., 2006).

Prostaglandin (PG) biosynthesis in the central nervous system and inflammatory cells is primarily attributed to cyclooxygenase (COX)-2, explaining in part the observed analgesic and anti-inflammatory properties of nonsteroidal anti-inflammatory drugs (NSAIDs) that inhibit COX-2 (Kennedy et al., 1993; Masferrer et al., 1994; Kurumbail et al., 2001). The expression of COX-2 is induced during inflammation by cytokines and bacterial products such as lipopolysaccharides (LPS), and results in the synthesis of PGs that contribute to the pain and swelling of inflammation (O’Sullivan et al., 1992). Studies with COX inhibitors have shown that COX-2 is involved in inflammatory pain and neuropathic pain (Ito et al., 2001). Celecoxib, selective COX-2 inhibitor, suppresses inflammation and alleviates pain and fever with few side effects such as gastric erosion and ulcers associated with inhibition of COX-1 (Hawkey, 1999).

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 (Fields et al., 1991; Kim et al., 1997; Kim et al., 2001). PAG is rich in opioid receptors and opioid peptides and opiates are known to produce analgesia by activating descending pain control pathway, especially at the level of the PAG (Renno et al., 1992; Min et al., 1996; Kim et al., 2001). 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 (Carstens et al., 1990; Renno et al., 1992; Peng et al., 1996; Maione et al., 2000). Glycine is an important inhibitory transmitter in the brainstem and spinal cord. The binding of glycine to its receptor produces a large increase in \( \text{Cl}^- \) conductance, which causes membrane hyperpolarization (Min et al., 1996; Peng et al., 1996). Glutamate is a major excitatory neurotransmitter in the CNS where they participate in a great number of physiological and pathological states (Greenamyre et al., 1984; Carstens et al., 1990).

Although amygdalin is known to be involved in inflammatory pain (Chang et al., 2005), the effect of amygdalin on neuronal activity at the level of PAG has not been reported, yet. In order to elucidate the modulation of amygdalin in the neuronal activity of level PAG, the effect of amygdalin on 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**

**Preparation of the PAG neuron**

PAG neurons were dissociated using a technique described previously (Kim et al., 1997; Kim et al., 2001; Lee et al., 2001). In brief, 10- to 15-day-old Sprague-Dawley rats of both sexes were decapitated under Zoletil 50\(^\circ\)-induced anesthesia (50 mg/kg, i.m.). The brains were removed, and transverse slices (400 \( \mu \)m 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% O\(_2\) and 5% CO\(_2\) 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 \( 40 \sim 60 \) min at 32\( ^\circ\)C and subsequently with thermolysin (protease X, 1 mg/6 ml) for \( 10 \sim 20 \) min at 32\( ^\circ\)C. 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 recording.
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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 7.4 with tris-hydroxymethylaminomethane (Tris-base). The composition of the internal pipette solution for nystatin-perforated patch 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 was prepared and added to the patch pipette solution to reach a final concentration of 200 μg/ml.

Drugs
Pronase, thermolysin, nystatin, glycine, glutamate, LPS and most of the other drugs used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Zoletil 50® was obtained from Virbac (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" (Kim et al., 1997; Kim et al., 2001; Lee et al., 2001). Using this technique, the standard solution surrounding a neuron could be exchanged within 10~20 ms.

Extraction of amygdalin
Armeniacae semen used in this experiment was obtained from the Kyungdong market (Seoul, Korea). Both 500 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. 4.2 g of amygdalin (with the yield rate of 0.84%) was obtained by recrystallizing the extract with ethanol. The amygdalin was used after it had been determined to be over 99.0% of purity using high-pressure liquid chromatography (HPLC; Shiseido, Tokyo).

Electrical measurements
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 MΩ. After the formation of a stable perforated patch, 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 also monitored on a pen recorder (Recti-Horiz-8K, NEC San-ei, Tokyo, Japan). All experiments were performed at room temperature (22~24°C).

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

RESULTS
The 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 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 LPS was not applied. Continuous application of LPS to PAG neurons at a concentration of 10−3 mg/ml did not elicit and ion current in the absence of glycine, while it enhanced the glycine-induced ion current in a time-dependent fashion, 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% of the control level (Fig. 1).

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

**The 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 a 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 fashion, to $0.90\pm0.02$ (n=6, p<0.05) after 6 min, to $0.83\pm0.01$ (n=6, p<0.05) after 12 min, to $0.79\pm0.01$ (n=6, p<0.05) after 18 min, to $0.75\pm0.01$ (n=6, p<0.05) after 24 min, and to $0.69\pm0.04$ (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% of the control level (Fig. 2).

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

**The effect of amygdalin on glycine-induced ion current stimulated by LPS in rat PAG neurons**

Glycine at a concentration of $10^{-5}$ M was applied...
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Fig. 3. Modulation of amygdalin on glycine-induced ion current stimulated by lipopolysaccharides (LPS) in periaqueductal gray (PAG) neurons. Continuous application of 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 concentration-dependent fashion, to $1.00 \pm 0.01$ (n=7, p < 0.05) after 6 min, to $1.12 \pm 0.02$ (n=7, p < 0.05) after 12 min, to $1.25 \pm 0.02$ (n=7, p < 0.05) after 18 min, to $1.29 \pm 0.03$ (n=7, p < 0.05) after 24 min, and to $1.29 \pm 0.03$ (n=7, p < 0.05) after 30 min, with the control value as 1. After washing, the magnitude of the glycine-induced ion current rose, to about $1.01 \pm 0.01$% of the control level.

In the present study, it was shown that continuous application of amygdalin with $10^{-3}$ mg/ml LPS suppresses the glycine-induced ion current by 10% in a time-dependent fashion.

The effect of amygdalin on the 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 fashion, to $0.96 \pm 0.02$ (n=9, p < 0.05) after 6 min, to $0.91 \pm 0.03$ (n=9, p < 0.05) after 12 min, to $0.86 \pm 0.02$ (n=9, p < 0.05) after 18 min, to $0.84 \pm 0.02$ (n=9, p < 0.05) after 24 min, and to $0.82 \pm 0.02$ (n=9, 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 $0.93 \pm 0.01$% of the control level.
Fig. 4. Modulation of amygdalin on glutamate-induced ion current stimulated by lipopolysaccharides (LPS) in periaqueductal gray (PAG) neurons. Continuous application of amygdalin with $10^{-3}$ mg/ml LPS to PAG neurons increased the glutamate-induced ion current decreased by LPS in concentration- and time-dependent fashion. Results are presented as the mean $\pm$ standard error of the mean (S.E.M.). LPS: lipopolysaccharides, amg: amygdalin.

Pain is first response to injury or infection. Injury and infection activates the immune system to produce inflammatory responses (Dickerson et al., 1998). 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 PGE$_2$ 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 (Bennett, 2001; Shin et al., 2003). 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 (Nicol et al., 1997). According Vaughan (1998), 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 (Shin et al., 2003). PAG is rich in opioid receptors and opioid peptides and opiates are known to produce analgesia by activating descending pain control pathway (Fields et al., 1991). The activation of the descending pain control system is influenced by several factors including stress, electroacupuncture, and local electrical stimulation of the PAG (Millan et al., 1987; Lee and Beitz, 1993). 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 (Min et al., 1996; Peng et al., 1996). 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 (Carstens et al., 1990).

We have previously shown that COX-2 modulated...
PAG neuronal activity and involved with the regulation of the descending pain control system in the level of PAG neurons. Based on the results, it is possible that the potentiation of the glycine-induced response and suppressed glutamate-induced responses by LPS is associated with the activation of COX-2 by LPS. These results suggest that COX-2 activity might modulate the responsiveness of PAG neurons to glycine and glutamate. These results also confirmed previous report (Shin et al., 2003).

In this study, continuous application of LPS to PAG region potentiated the ion current induced by glycine and suppressed the ion current induced by glutamate, while continuous 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 fashion.

Amygdalin is one of many nitroisides, which are natural cyanide containing substances abundant in the seeds of the prunasin family such as apricots, almonds, peaches, apples, and other rosaceous plants. Among the prunasin family, Armeniacae semen has been used for the treatment of asthma, bronchitis, emphysema, leprosy, colorectal cancer, and leucoderma in traditional oriental medicine (Pak et al., 1999; Hwang et al., 2003; Chang et al., 2005). Armeniacae semen is divided into the outer husk and an inner part that contains glycoside, amygdaline, starch, and fatty acids. According Chang et al. (2005), Armeniacae semen extract suppressed LPS-induced expressions of COX-2. Among Armeniacae semen, amygdalin is abundant in the seeds of the Prunus genus almond, apricots, and other rosaceous plants. Amygdalin is also known as vitamain B17, which had been used for the treatment of cancers, and vitamain B17 has been named as laetrile (Pak et al., 1999). It has been reported that amygdalin is effective for the relief of the pain of cancer patients (Ellison et al., 1978; Fukuda et al., 2003).

Based on the previous results, it is possible that the suppression of glycine-induced and the potentiation of glutamate-induced response are associated with the inhibition of COX-2 by amygdalin. These results suggest that amygdalin might modulate the responsiveness of PAG neurons to glycine and glutamate.

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, suggestive of increase neuronal excitability in the PAG. Thus, it appears that amygdalin modulates PAG neuronal activity, and it can be suggested that amygdalin is involved in the regulation of the descending pain control system in the level of PAG neurons.

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REFERENCES


