OPEM
www.opem.org

Oriental Pharmacy and Experimental Medicine 2008 **7(5)**, 549-555
DOI 10.3742/OPEM.2008.7.5.549

KHUP

Modulation of the aqueous extract of *Bupleuri radix* on glycine-induced current in the acutely dissociated rat periaqueductal gray neurons

Yun-Hee Sung[1], Mal-Soon Shin[1], Tae-Soo Kim[1], Sang-Won Lee[2], Youn-Jung Kim[2], Hye-Sook Shin[2], Hong Kim[3] and Chang-Ju Kim[1,*]

[1]Kohwang Medical Research Institute, Department of Physiology, College of Medicine, Kyung Hee University; [2]Department of Nursing Science, College of Nursing, Kyung Hee University, 1 Hoigi-dong, Dongdaemoon-gu, Seoul 130-701, Republic of Korea; [3]Department of Oriental Sports Medicine, College of Herbal Bio-industry, Daegu Haany University, Gyeongsan-si 712-715, Republic of Korea

SUMMARY

Bupleuri radix (Umbelliferae), the dried root of *Bupleurum Chinense* DC, has been clinically used to mitigate pain sensation. The descending pain control system consists of three major components, and modulation of pain in the periaqueductal gray is the most extensively studied descending pain control system. However, the relation of *Bupleuri radix* on the descending pain control system has not been clarified. In the present study, modulation of the aqueous extract of *Bupleuri radix* on glycine-induced ion current in the acutely dissociated periaqueductal gray neurons was investigated by using nystatin-perforated patch-clamp technique under voltage-clamp condition. In the present results, the glycine-induced ion current was significantly suppressed by 0.1 mg/ml *Bupleuri radix*, while treatment with 10^{-5} M naltrexone, opioid receptor antagonist, alleviated *Bupleuri radix*-induced inhibition on glycine-induced ion current. The present study showed that the aqueous extract of *Bupleuri radix* may activate descending pain control system through inhibition on glycine-induced ion current in the periaqueductal gray neurons and this effect is mediated by opioid receptors.

Key words: *Bupleuri radix*; Glycine; Periaqueductal gray; Naltrexone; Patch Clamp

INTRODUCTION

Pain is defined as unpleasant perception of a nociceptive sensation (Verri *et al*., 2006). Nociception or nociceptive sensation is resulted from the various neuronal circuits within 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. Of these, modulation of pain in the PAG is the most extensively studied pain control system (Fields *et al*., 1991). It was reported that electrical stimulation on the PAG produces antinociception and depresses the response of multi-receptive spinal neurons to noxious peripheral stimuli in rats (Millan *et al*., 1987). Microinjection of dipyrone into the PAG inhibited spinal neuronal responses to peripheral noxious stimulation (Vasquez and Vanegas, 2000) and suppressed tail flick reflex (Tortorici and Vanegas, 1994).

Several neurotransmitters in the PAG participate

*Correspondence: Chang-Ju Kim, Department of Physiology, College of Medicine, Kyung Hee University, 1 Hoigi-dong, Dongdaemoon-gu, Seoul 130-701, Republic of Korea. Tel: +8229610407; Fax: +8229642195; E-mail: changju@khu.ac.kr

in the control of nociception. Among them, endogenous opioids, glycine, and glutamate seem to play a crucial role in the processing of pain regulatory signals within this area (O'Sullivan *et al*., 1992; Min *et al*., 1996; Maione *et al*., 2000). Glycine is a simplest amino acid and has diverse metabolic functions within mammalian CNS, and serves as a neurotransmitter at inhibitory synapses (Betz and Laube, 2006). Glycinergic transmission is associated with the processing of motor and sensory information that controls movement, vision, audition, and inflammatory pain sensitization (Lopez-Corcuera *et al*., 2001; Harvey *et al*., 2004). Related to the pain regulation, glycine is known to induce pain by activating the inhibitory interneurons in the PAG (Gurwitz, 2001). Opioid peptides, on the other hand, are known to produce analgesic effects by blocking the inhibitory interneurons and activating the PAG antinociceptive pathway (Basbaum and Fields, 1984). It has been reported that these effects of opiates and opioid peptides are elicited through activating potassium channels (Madison and Nicoll, 1988; Han *et al*., 1999) or inhibiting calcium channels (Rhim and Miller, 1994; Kim *et al*., 1997).

Bupleuri radix (Umbelliferae), the dried root of *Bupleurum Chinense* DC, is one of the most important crude drugs used in traditional medicine (Li *et al*., 2005). *Bupleuri radix* has various pharmaceutical effects such as anti-cancer (Motoo and Sawabu, 1994), anti-viral (Ushio and Abe, 1991), and anti-inflammatory effects (Bermejo Benito *et al*., 1998). Clinically, this herb has been used to treat for inflammation, hepatic injury, fever, immunity, and to mitigate pain sensation (Zhou *et al*., 2006). However, the effect of the aqueous extract of *Bupleuri radix* on the descending pain control system has not been clarified.

In the present study, we investigated the modulation of the aqueous extract of *Bupleuri radix* on glycine-induced ion current in the acutely dissociated PAG neurons by using the nystatin-perforated patch-clamp technique under voltage-clamp conditions.

MATERIALS AND METHODS

Preparation of PAG neurons

The PAG neurons were freshly dissociated using technique as previously described elsewhere (Kim *et al*., 1997; Han *et al*., 1999). In brief, 10- to 15-day-old Sprague-Dawley rats of both sexes were decapitated under Zoletil 50® anesthesia (50 mg/kg; i.m.; Vibac Laboratories, France). The brain was removed and the transverse slices (400 μm thickness) were made with a microslicer (DTK-1000, DSK, Japan). Slices were pre-incubated with 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 - 80 min at 32°C and subsequently with thermolysin (protease X, 1 mg/6 ml) for 10 - 20 min at 32°C. After enzyme treatment, the slices were kept in the enzyme-free incubation solution for 1 h. PAG region was identified in a 60 mm culture dish coated with silicone under a binocular microscope (SZ-ST, Olympus, Japan), and was micropunched out from the slice 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, USA) filled with standard solution. The dissociation procedure was done under an inverted phase-contrast microscope (CK-2, Olympus, Japan). The dissociated neurons usually adhered to the bottom of the dish within 20 min. These cells were remained viable for electrophysiological studies up to 6 h after dissociation.

Solutions

The ionic composition of the incubation solutions was (in mmol/l): NaCl 124, KCl 5, KH_2PO_4 1.2, $MgSO_4$ 1.3, $CaCl_2$ 2.4, glucose 10, and $NaHCO_3$ 24. The pH was adjusted to 7.4 by continuous bubbling with 95% O_2 and 5% CO_2. The composition of the

standard external solution was (in mmol/l): NaCl 150, KCl 5, $MgCl_2$ 1, $CaCl_2$ 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 was prepared and added in a final concentration of 200 μg/ml to the patch pipette solution.

Drugs

Bupleuri radix (Umbelliferae) used in this experiment was obtained from Kyung-Dong Market (Seoul, Republic of Korea). In order to obtain the aqueous extract of *Bupleuri radix*, it was subsequently heat-extracted, pressure-filtered, and concentrated with a rotary evaporator. The resulting 17.92 g of powder (yield of 35.84%) was obtained from 50 g of *Bupleuri radix* through lyophilization for 24 h by a drying machine (Ilsin, Seoul, Republic of Korea).

Most drugs used in this experiment were obtained from Sigma Chemical Co. (St. Louis, USA). Drugs were added to the standard solution at the final concentrations provided in the text and were applied using a rapid application system termed the "Y-tube method" as described elsewhere (Han *et al*., 1999; Kim *et al*, 2001). By this technique, the standard solution surrounding a neuron could be exchanged within 10 - 20 ms.

Electrical measurement

Electrical recording was performed in the nystatin-perforated patch recording mode under voltage-clamp condition. Patch pipette was prepared from glass capillaries with an outer diameter of 1.5 mm on a 2-stage puller (PB-7, Narishige, Japan). 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, currents recording, and filtration of currents (at 2.9 kHz) were obtained with an EPC-7 patch-clamp amplifier (List-Electronic, Germany). The currents and voltage were monitored on a pen recorder. All experiments were conducted at room temperature (22 - 24°C).

Statistical analysis

The results are presented as the mean standard error of the mean (S.E.M.). The data were analyzed by one-way ANOVA followed by Duncan's *post-hoc* test. The differences were considered statistically significant at $P < 0.05$.

RESULTS

Ion currents activated by various concentrations of the aqueous extract of *Bupleuri radix*

In the nystatin-perforated patch-clamp mode, experiments were carried out at a holding potential (V_H) of -50 mV. The aqueous extract of *Bupleuri radix* was applied every 2 min and ion current induced by 0.5 mg/ml *Bupleuri radix* was used as the control value. Inward currents induced by *Bupleuri radix* at various concentrations were recorded. The magnitude of ion currents elicited by *Bupleuri radix* at concentrations of 0.01 mg/ml, 0.05 mg/ml, 0.1 mg/ml, 1 mg/ml, and 3 mg/ml were 0.04 ± 0.01, 0.08 ± 0.04, 0.22 ± 0.01, 1.53 ± 0.12, and 5.31 ± 1.61 of the control value set as 1, respectively. In the present results, the aqueous extract of *Bupleuri radix* elicited ion currents in the PAG neurons as concentration-dependent manner (Fig. 1).

Modulation of the aqueous extract of *Bupleuri radix* on glycine-induced ion current

To determine the modulation of *Bupleuri radix* on glycine-induced ion current, magnitude of ion current elicited by 10^{-5} M glycine was used as the control value. The concentrations of 0.01 mg/ml, 0.05 mg/ml, 0.1 mg/ml, and 0.5 mg/ml *Bupleuri radix* were applied simultaneously with 10^{-5} M glycine. *Bupleuri radix* at concentrations of 0.01

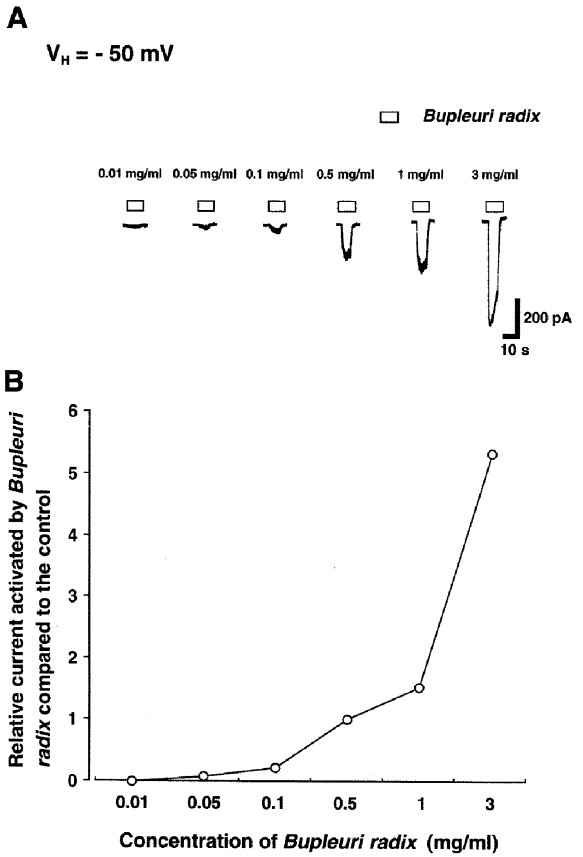

Fig. 1. Ion currents induced by *Bupleuri radix*. Application of the aqueous extract of *Bupleuri radix* elicited ion currents in periaqueductal gray neurons as concentration-dependent manner.

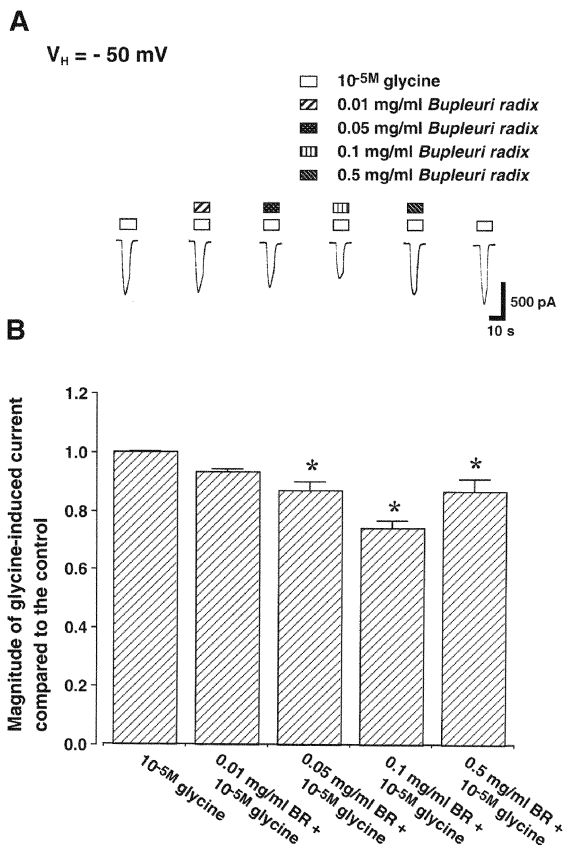

Fig. 2. Modulation of the aqueous extract of *Bupleuri radix* on glycine-induced ion current. The glycine-induced ion current was significantly inhibited by 0.05 mg/ml, 0.1 mg/ml, and 0.5 mg/ml of *Bupleuri radix*. 10^{-5} M glycine was used as a control value. $^{*}P < 0.05$ compared to the control. BR: *Bupleuri radix*.

mg/ml, 0.05 mg/ml, 0.1 mg/ml, and 0.5 mg/ml inhibited glycine-induced ion current as 0.93 ± 0.01, 0.86 ± 0.02, 0.73 ± 0.02, and 0.86 ± 0.01 of the control value set as 1, respectively. In the present results, the glycine-induced ion current was significantly suppressed by the aqueous extract of *Bupleuri radix* at the concentrations of 0.05 mg/ml, 0.1 mg/ml, and 0.5 mg/ml (Fig. 2).

Effect of naltrexone on *Bupleuri radix*-induced inhibition on glycine-induced ion current

To evaluate the involvement of opioid receptor in the *Bupleuri radix*-induced inhibition on glycine-induced ion current in the PAG neurons, naltrexone that is non-selective opioid receptor antagonist, was applied simultaneously with the aqueous extract of *Bupleuri radix*. In the present results, ion current induced by 10^{-5} M glycine was decreased to 0.73 ± 0.02 by 0.1 mg/ml of *Bupleuri radix*, while treatment with 10^{-5} M naltrexone alleviated glycine-induced ion current to 0.94 ± 0.01, with the control value set as 1 (Fig. 3). These results showed that naltrexone alleviated the aqueous extract of *Bupleuri radix*-induced inhibition on glycine-activated ion current.

DISCUSSION

In the present study, the aqueous extract of *Bupleuri radix* at high concentrations (above 0.1

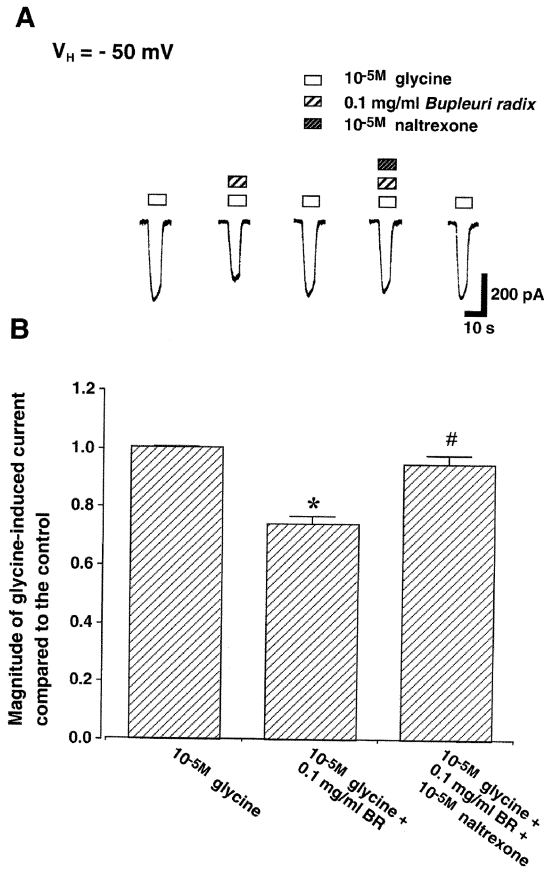

Fig. 3. Effect of naltrexone on *Bupleuri radix*-induced inhibition on glycine-induced ion current. Inhibitory action of *Bupleuri radix* on glycine-induced ion current was abolished by naltrexone application. 10^{-5} M glycine was used as a control value. $^{*}P < 0.05$ compared to the control. BR: *Bupleuri radix*.

mg/ml) elicited ion current in a concentration-dependent manner in the PAG neurons. *Bupleuri radix*, however, did not induced ion current by itself at concentrations below 0.01 mg/ml. For the next experiments, we used *Bupleuri radix* at concentrations of 0.05 mg/ml, 0.1 mg/ml, and 0.5 mg/ml in order to investigate the effect of *Bupleuri radix* at low concentrations on the glycine-induced ion current in acutely dissociated rat PAG neurons.

The major finding of this study is that the glycine-induced ion current in PAG neurons was suppressed by the aqueous extract of *Bupleuri radix* at concentrations of 0.05 mg/ml, 0.1 mg/ml, and 0.5 mg/ml and the most potent inhibitive effect on glycine-induced ion current was shown in the 0.1 mg/ml of *Bupleuri radix* in this study. Previous studies revealed that glycine plays an important role in the modulation of analgesic action at the level of PAG (Sato *et al.*, 1991; Todd *et al.*, 1996; Fujiwara *et al.*, 1998). Maione *et al.* (2000) showed that glycine acts as an inhibitory nociceptive transmitter in PAG. In addition, Gurwitz (2001) reported that inhibition of glycine transporter in the brain and spinal cord may relieve pain. Recently, it was suggested that the analgesic effects of Oriental medicinal herbs such as *Corydalis tuber* and *Chelidonii herba* are closely related to the inhibition on glycine in the PAG neurons (Shin *et al.*, 2003; Cheong *et al.*, 2004). The present results showed the possibility that *Bupleuri radix* may have an analgesic effect by activating the descending pain control system through the inhibition on glycine-induced ion current in PAG neurons.

It is well documented that analgesic action of PAG is mediated by opioid system (Shin *et al.*, 2003; Cheong *et al.*, 2004). Pert and Walter (1976) reported that administration of naloxone, non-selective opioid receptor antagonist, to rats reduced analgesic effect induced by electrical stimulation on the PAG. Wei *et al.* (2003) also showed that central analgesia was blocked by non-selective opioid receptor antagonists such as naloxone, naltrexone, and nalmephene. Recent study demonstrated that microinjection of naltrexone into the ventrolateral PAG significantly attenuated the tolerance to systemic morphine during the pain test such as hot plate and formalin test (Lane *et al.*, 2005). In the present results, *Bupleuri radix*-induced inhibition on the glycine-induced ion current was abolished by naltrexone application. Theses results show that opioid receptors are closely involved in the inhibitory action of *Bupleuri radix* on glycine-induced ion current in the PAG neurons, suggesting that some components of *Bupleuri radix* exert analgesic action through opioid receptors in the

PAG neurons. However, the inhibitory action of *Bupleuri radix* on glycine-induced ion current was not eliminated completely by naltrexone application, suggesting that other components of *Bupleuri radix* may induce analgesic action through direct modulation on glycine-receptors in the PAG neurons.

Bupleuri radix contains a lot of organic compound, and saikosaponins has been suggested as the main pharmaceutical component of this herb (Li *et al*., 2005). Ushio and Abe (1991) showed that saikosaponin increases phagocytosis activity of macrophage. Bermejo Benito *et al*. (1998) reported that saikosaponin exerts anti-inflammatory effect on phorbol myristate acetate- induced ear edema in mice. It can be suspected that saikosaponin is the main component of *Bupleuri radix* responsible for its inhibitory effect on the glycine-induced ion current.

Here in this study, we have shown that the aqueous extract of *Bupleuri radix* may activate descending pain control system through inhibition on glycine-induced ion current in the PAG and this effect is mediated by opioid receptors.

ACKNOWLEDGEMENTS

This research was supported by the MIC (Ministry of Information and Communication), Korea, under the ITRC (Information Technology Research Center) support program supervised by the IITA (Institute of Information Technology Advancement**) (**IITA-2008-(C1090-0801-0002)**.**

REFERENCES

Basbaum AI, Fields HL. (1984) Endogenous pain control systems: brainstem spinal pathways and endorphin circuitry. *Annu. Rev. Neurosci.* **7**, 309-338.

Bermejo Benito P, Abad Martinez MJ, Silvan Sen AM, Sanz Gomez A, Fernandez Matellano L, Sanchez Contreras S, Diaz Lanza AM. (1998) *In vivo* and *in vitro* antiinflammatory activity of saikosaponins. *Life Sci*. **63**, 1147-1156.

Betz H, Laube B. (2006) Glycine receptors: recent insights into their structural organization and functional diversity. *J. Neurochem*. **97**, 1600-1610.

Cheong BS, Choi DY, Cho NH, Lee JD, Chang HK, Shin MC, Shin MS, Kim CJ. (2004) Modulation of *Corydalis tuber* on glycine-induced ion current in acutely dissociated rat periaqueductal gray neurons. *Biol. Pharm. Bull*. **27**, 1207-1211.

Fields HL, Heinricher MM, Mason P. (1991) Neurotransmitters in nociceptive modulatory circuits. *Annu. Rev. Neurosci*. **14**, 219-245.

Fujiwara K, Kaminishi Y, Inoue Y, Yabuuchi M. (1998) Monoclonal antibody monospecific to glycine for brain immunocytochemistry. *Brain Res*. **806**, 210-218.

Gurwitz D. (2001) Profile; glycine transporter GlyT-2 blockers: potential pain-relief and anti-spastic drugs. *Drug Discov. Today* **6**, 1178-1179.

Han SH, Cho YW, Kim CJ, Min BI, Rhee JS, Akaike N. (1999) μ-opioid agonist-induced activation of G-protein-coupled inwardly rectifying potassium current in rat periaqueductal gray neurons. *Neuroscience* **90**, 209-219.

Harvey R, Depner U, Wssle H, Ahmadi S, Heindl C, Reinold H, Smart T, Harvey K, Schtz B, Akbari O, Zimmer A, Poisbeau P, Welzl H, Wolfer DP, Betz H, Zeilhofer U, Mller U. (2004) GlyR α3: an essential target for spinal PGE_2-mediated inflammatory pain sensitization. *Science* **304**, 884-888.

Kim CJ, Rheee JS, Akaike N. (1997) Modulation of high-voltage activated Ca^{2+} channels in the rat periaqueductal gray neurons by μ-type opioid agonist. *J. Neurophysiol*. **77**, 1418-1424.

Kim Y, Shin M, Chung J, Kim E, Koo G, Lee C, Kim C. (2001) Modulation of *Chelidonii herba* on GABA activated chloride currents in rat PAG neurons. *Am. J. Chin. Med*. **29**, 265-279.

Lane DA, Patel PA, Morgan MM. (2005) Evidence for an intrinsic mechanism of antinociceptive tolerance within the ventrolateral periaqueductal gray of rats. *Neuroscience* **135**, 227-234.

Li XQ, Gao QT, Chen XH, Bi KS. (2005) High performance liquid chromatographic assay of saikosaponins from radix Bupleuri in China. *Biol. Pharm. Bull*. **28**, 1736-1742.

Lopez-Corcuera B, Geerlings A, Aragon C. (2001) Glycine neurotransmitter transporters: an update. *Mol. Membr. Biol*. **18**, 13-20.

Madison D, Nicoll RA. (1988) Enkephalin hyperpolarizes interneurones in the rat hippocampus. *J. Physiol*. **398**, 122-130.

Maione S, Marabese I, Rossi F, Berrino L, Palazzo E, Trabace L. (2000) Effects of persistent nociception on periaqueductal gray glycine release. *Neuroscience* **97**, 311-316.

Millan MJ, Czlonkowski A, Millan MH, Herz A. (1987) Activation of periaqueductal gray pools of â-endorphin by analgetic electrical stimulation in freely moving rats. *Brain Res*. **407**, 199-203.

Min BI, Kim CJ, Rhee JS, Akaike N. (1996) Modulation of glycine-induced chloride current in acutely dissociated rat periaqueductal gray neurons by μ-opioid agonist DAGO. *Brain Res*. **734**, 72-78.

Motoo Y, Sawabu N. (1994) Antitumor effects of saikosaponins, baicalin and baicalein on human hepatoma cell lines. *Cancer Lett*. **86**, 91-95.

O'Sullivan MG, Chilton FH, Huggins EM, Jr McCall CE. (1992) Lipopolysaccharide priming of alveolar macrophages for enhanced synthesis of prostanoids involves induction of a novel prostaglandin H synthase. *J. Biol*. *Chem*. **267**, 14547-14550.

Pert A, Walter M. (1976) Comparison between naloxone reversal of morphine and electrical stimulation induced antinociception in the rat mesencephalon. *Life Sci*. **19**, 1023-1032.

Rhim H, Miller RJ. (1994) Opioid receptors modulate diverse types of calcium channels in the nucleus tractus solitarius of the rat. *J. Neurosci*. **14**, 7608-7615.

Sato K, Zhang JH, Saika T, Sato M, Tada K, Tohyama M. (1991) Localization of glycine receptor α_1 subunit mRNA-containing neurons in the rat brain: an analysis using *in situ* hybridization histochemistry. *Neuroscience* **43**, 381-395.

Shin MC, Jang MH, Chang HK, Lim S, Han SM, Park HJ, Shim I, Lee JS, Kim KA, Kim CJ. (2003) Modulation of *Chelidonii herba* on glycine-activated and glutamate-activated ion currents in rat periaqueductal gray neurons. *Clin*. *Chim*. *Acta* **337**, 93-101.

Todd AJ, Watt C, Spike RC, Sieghart W. (1996) Colocalization of GABA, glycine and their receptors at synapses in the rat spinal cord. *J. Neurosci*. **16**, 974-982.

Tortorici V, Vanegas H. (1994) Putative role of medullary off- and on-cells in the antinociception produced by dipyrone (metamizol) administered systemically or microinjected into PAG. *Pain* **57**, 197-205.

Ushio Y, Abe H. (1991) The effects of saikosaponin on macrophage functions and lymphocyte proliferation. *Planta Med*. **57**, 511-514.

Vasquez E, Vanegas H. (2000) The antinociceptive effect of PAG-microinjected dipyrone in rats is mediated by endogenous opioids of the rostral ventromedical medulla. *Brain Res*. **854**, 249-252.

Verri WA, Jr Cunha TM, Parada CA, Poole S, Cunha FQ, Ferreira SH. (2006) Hypernociceptive role of cytokines and chemokines: Targets for analgesic drug development? *Pharmacol*. *Ther*. **112**, 116-138.

Wei G, Moss J, Yuan CS. (2003) Opioid-induced immunosuppression: is it centrally mediated or peripherally mediated? *Biochem*. *Pharmacol*. **65**, 1761-1766.

Zhou C, Xia G, Zhi D, Chen Y. (2006) Genetic characterization of asymmetric somatic hybrids between *Bupleurum scorzonerifolium* Willd and *Triticum aestivum* L.: potential application to the study of the wheat genome. *Planta* **223**, 714-724.