대한경락경혈학회지 Vol.20, No.4, pp.17~29, 2003
The Korean Journal of Meridian & Acupoint

Modulation of *Harpagophytum procumbens* on ion channels in acutely dissociated periaqueductal gray neurons of rats

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천수근이 중심회백질 신경세포에서 이온통로 조절작용에 미치는 영향

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Abstract

목적 : 천수근은 염증성 관절염과 염증성 장 질환 등에 사용되어왔다. 천수근이 중심회백질 신경세포에서 하행성 진통작용에 미치는 영향을 연구하였다.

방법 : 중심회백질 신경세포를 분리하여 전위고정하에서 nystatin-perforated patch-clamp technique을 시행하였다.

결과 : 천수근에 의하여 유발되는 이온전류는 GABA, glycine, 그리고 glutamate 수용체를 모두 활성화시켰다.

Key words : *Harpagophytum procumben;* Patch clamp; Periaqueductal gray; GABA; glycine; glutamate

Ⅰ. Introduction

Harpagophytum procumbens (generally known as Devil's claw) has been used for the treatment of inflammatory arthritis, and inflammatory bowel disorders[1,2]. The anti-inflammatory and analgesic properties of the *Harpagophytum procumbens* are essentially ascribed to the sum of the three phenolic glycosides; acteoside, isoacteoside, and bioside[3]. There are many reports on the phytochemical[4] and biological aspects[5,6] of this plant. Pharmacological studies have shown that *Harpagophytum procumbens* possesses analgesic, antiphlogistic, and antiinflammatory actions and it improves motility and reduces sensitivity to pain[7].

The transmission of nociceptive information may be altered by various neuronal circuits within the central nervous system

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(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[8]. Several neurotransmitters in the PAG participate in the control of nociception. Among these, endogenous opioids, GABA, glycine, and glutamate seem to play a crucial role in the processing of pain regulatory signals within this area[9,10]. γ -Aminobutyric acid (GABA) and glycine are a key inhibitory transmitters in the brainstem and the spinal cord. In the PAG and the spinal dorsal horn, the relay centers for pain and sensory information, GABA and glycine inhibits glutamate-evoked depolarization and represses the firing of neurons. The binding of GABA and glycine to its receptor produces a large increase in Cl^- conductance, which causes membrane hyperpolarization[11].

Glutamate is excitatory neurotransmitters in the brain and abundant glutamate binding sites have been localized in the dorsolateral subdivision of the PAG[12]. Glutamate seems to be involved in PAG mediated analgesia. Microinjection of glutamate or glutamate agonists into the PAG neurons induced analgesic effect[13-15].

In this study, modulatory action of *Harpagophytum procumbens* on inhibitory neurotransmitter-activated ion currents (GABA- and glycine-activated ion currents) and excitatory neurotransmitter-activated ion currents (glutamate-activated ion current) in acutely dissociated PAG neurons was investigated using nystatin-perforated patch-clamp technique under voltage-clamp condition.

Ⅱ. Materials and methods

2.1. Preparation of the PAG neuron

The PAG neurons were freshly dissociated using technique described previously elsewhere[16,17]. In brief, 10- to 15-day-old Sprague-Dawley rats of both sexes were decapitated under Zoletil 50 anesthesia (50 mg/kg i.m.).

The brain was removed and the transverse slices (400 ㎛ thickness) were made with a microslicer (DTK-1000, DSK, Tokyo, Japan). Slices were preincubated in the incubation solution that had been well saturated 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 40-80 min at 32 ℃ and subsequently with thermolysin (protease X, 1 mg/6 ml) for 10-20 min at 32 ℃. 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, Tokyo, Japan), 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, USA) filled with standard solution. The dissociation procedure was done 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 were remained viable for electrophysiological studies up to 6 h after dissociation.

2.2. Solutions

The ionic composition of the incubation solutions was (in mM): 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 mM): 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 ㎍/ml to the patch pipette solution.

2.3. Drugs

Harpagophytum procumbens used in this experiment was obtained from Kyungdong market (Seoul, Korea). After washing, *Harpagophytum procumbens* was immersed in cold water for 12 h. To obtain an aqueous extract of *Harpagophytum procumbens,* 300 g of *Harpagophytum procumbens* was added to distilled water, heat extracted, concentrated with a rotary evaporator, and lyophilized. The resulting powder, weighing 35 g, was diluted with saline solution.

Most drugs used in this experiment were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The following drugs were used: nystatin, tubocurarine, bicuculine, strychnine, 6-cyano-7-nitroquinoxaline-2-3-dione (CNQX), D-2-amino-5-phosphonopentanoic acid (D-AP 5), GABA, glycine, glutamate. 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 [16,18]. By this technique, the standard solution surrounding a neuron could be exchanged within 10 - 20 ms.

2.4. Electrical measurements

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 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 stable perforated patchformation, 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/Eberstat, Germany). The current and voltage were monitored on a pen recorder (Recti-Horiz-8K, NEC San-ei, Tokyo, Japan). All experiments were performed at room temperature (22 - 24 ℃).

2.5. Statistical analysis

Results are presented as mean standard error mean (S.E.M.) and Student's *t*-test was used for statistical analysis and *P*-values less than 0.01 were considered significant.

Ⅲ. Results

3.1 Ion current activated by Harpagophytum procumbens

In the nystatin-perforated patch-clamp mode, experiments were carried out at a holding potential (VH) of 50 mV. *Harpagophytum procumbens* was applied every 2 min and ion current activated by 1 mg/ml *Harpagophytum procumbens* used as a control. Inward current was recorded by *Harpagophytum procumbens* at various concentrations (Figure 1). The concentration of 0.05 mg/ml of *Harpagophytum procumbens* did not elicit ion current (n = 4, P < 0.01). *Harpagophytum procumbens* at concentration of 0.1 mg/ml, 0.5 mg/ml, 3 mg/ml, and 5 mg/ml was elicited ion current about 1.94 ± 1.21 pA (n = 5, P < 0.01), 42.47 ± 2.18 pA (n = 5, P < 0.01), 257.81 ± 21.7 pA (n = 7, P < 0.01), and 466.97 ± 44.1 pA (n = 6, P < 0.01), respectively.

In this study, *Harpagophytum procumbens* was shown to elicit ion current as concentration-dependent manner in PAG neuron.

3.2. Effect of antagonists on Harpagophytum procumbens-activated ion current

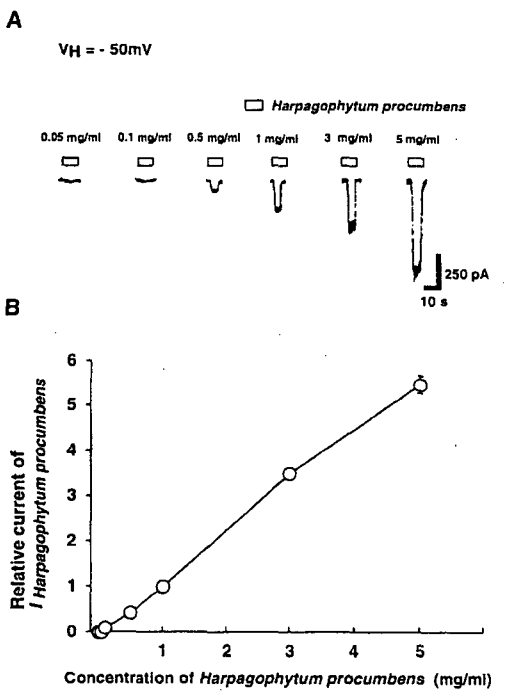

Fig. 1. Ion current activated by *Harpagophytum procumbens*. Nystatin-perforated patch-clamp under voltage clamp condition (VH = 50 mV) was performed on acutely dissociated periaqueductal gray neuron. *Harpagophytum procumbens* was applied every 2 min. Inward current was recorded by *Harpagophytum procumbens* at various concentrations. In this study, *Harpagophytum procumbens*elicited ion current in concentration dependent manner in PAG neuron.

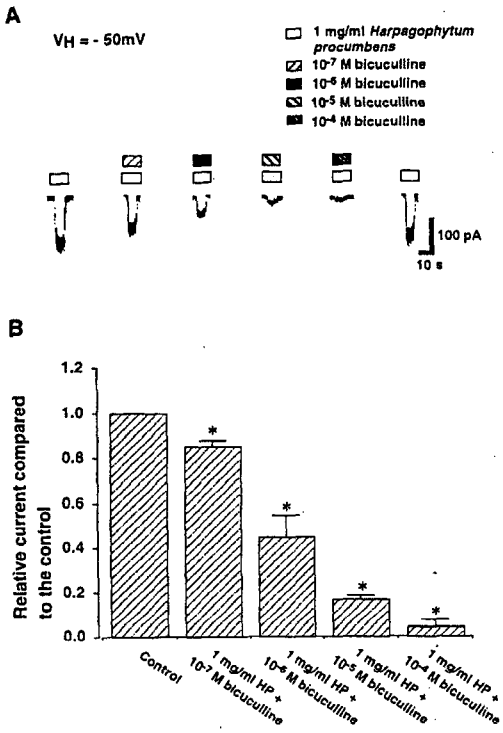

Fig. 2. Effect of bicuculline on the *Harpagophytum procumbens*-activated ion current. *Harpagophytum procumbens* was applied every 2 min and ion current activated by 1 mg/ml *Harpagophytum procumbens*used as a control, ion current activated by *Harpagophytum procumbens*was suppressed by bicuculline in a concentration dependent manner. * represents $P < 0.01$ compared to the control. HP: *Harpagophytum procumbens*.

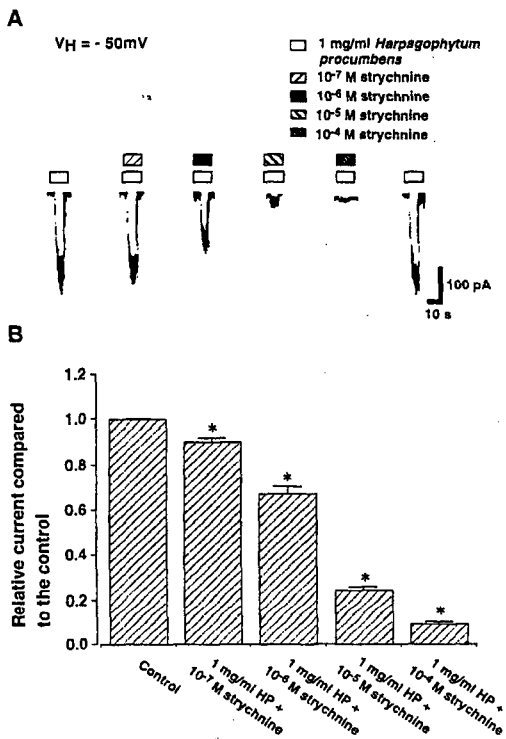

Fig. 3. Effect of strychnine on the *Harpagophytum procumbens*-activated ion current. *Harpagophytum procumbens* was applied every 2 min and ion current activated by 1 mg/ml *Harpagophytum procumbens*-used as a control, ion current activated by *Harpagophytum procumbens* was suppressed by strychnine in a concentration dependent manner. * represents $P < 0.01$ compared to the control. HP: *Harpagophytum procumbens* .

In order to evaluate pharmacological properties of the ion current activated by *Harpagophytum procumbens,* the effects of bicuculline (a $GABA_A$ receptor antagonist), strychnine (a glycine receptor antagonist), tubocurarine (a nicotinic acetylcholine receptor antagonist), D-AP5 (an NMDA sensitive glutamate receptor antagonist), and CNQX (an AMPA/kainite sensitive glutamate receptor antagonist) were examined.

3.2.1. Effect of bicuculline on Harpagophytum procumbens-activated ion current

*Harpagophytum procumbens*was applied every 2 min and ion current activated by 1 mg/ml *Harpagophytum procumbens*used as a

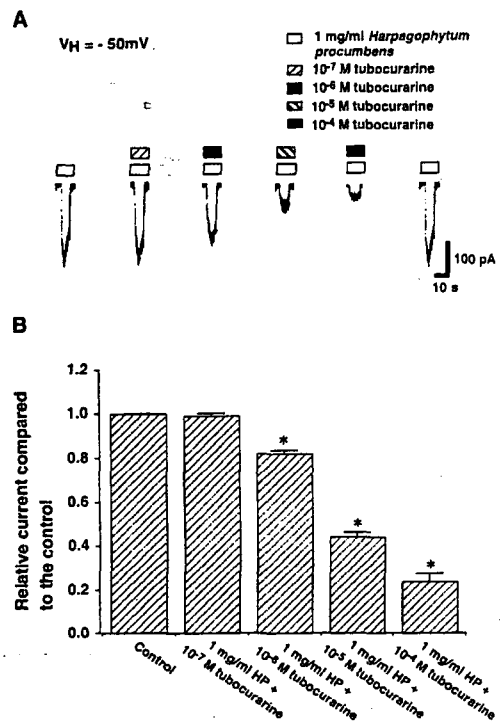

Fig. 4. Effect of tubocurarine on the *Harpagophytum procumbens*-activated ion current. *Harpagophytum procumbens* was applied every 2 min and ion current activated by 1 mg/ml *Harpagophytum procumbens*-used as a control, ion current activated by Harpagophytum procumbens was suppressed by tubocurarine in a concentration dependent manner. * represents $P < 0.01$ compared to the control. HP: *Harpagophytum procumbens* .

control. Ion current activated by *Harpagophytum procumbens* was suppressed by bicuculline in a concentration-dependent manner (Figure 2). Bicuculline at concentrations of 10^{-7} M, 10^{-6} M, 10^{-5} M, and 10^{-4} M inhibited *Harpagophytum procumbens*-activated ion current about 15.30 ± 2.80 % (n = 4, P < 0.01), 55.00 ± 9.09 % (n = 5, $P < 0.01$), 83.30 ± 1.40 % (n = 4, $P < 0.01$), and 95.40 ± 2.67 (n = 4, $P < 0.01$) of the control value, respectively.

3.2.2. Effect of strychnine on the Harpagophytum procumbens-activated ion current

Ion current activated by 1 mg/ml *Harpagophytum procumbens* used as a control. Ion current activated by *Harpagophytum procumbens* was suppressed by strychnine in a concentration-dependent manner (Figure 3). Strychnine at concentrationsof 10^{-7} M, 10^{-6} M, 10^{-5} M, and 10^{-4} M inhibited *Harpagophytum procumbens*-activated ion current about 9.90 ± 1.74 % (n = 4, $P < 0.01$), 32.8 ± 3.29 % (n = 4, $P < 0.01$), 75.5 ± 1.42 % (n = 4, $P < 0.01$), and 90.6 ± 0.98 % (n = 5, $P < 0.01$) of the control value, respectively.

3.2.3. Effect of tubocurarine on Harpagophytum procumbens-activated ion current

Ion current activated by 1 mg/ml *Harpagophytum procumbens* used as a control. Ion current activated by 1 mg/ml *Harpagophytum procumbens* was suppressed by tubocurarine in a concentration-dependent manner (Figure 4). Tubocurarineat concentrations of 10^{-7} M, 10^{-6} M, 10^{-5} M, and 10^{-4} M inhibited *Harpagophytum procumbens*-activated ion current about 1.00 ± 1.00 % (n = 4, $P < 0.01$), 18.2 ± 1.49 % (n = 4, $P < 0.01$), 56.00 ± 1.95 % (n = 4, $P < 0.01$), and 76.00 ± 3.47 % (n = 4, $P< 0.01$) of the control value, respectively.

3.2.4. Effect of CNQX on the Harpagophytum procumbens-activated ion current

Ion current activated by 1 mg/ml *Harpagophytum procumbens* used as a control.

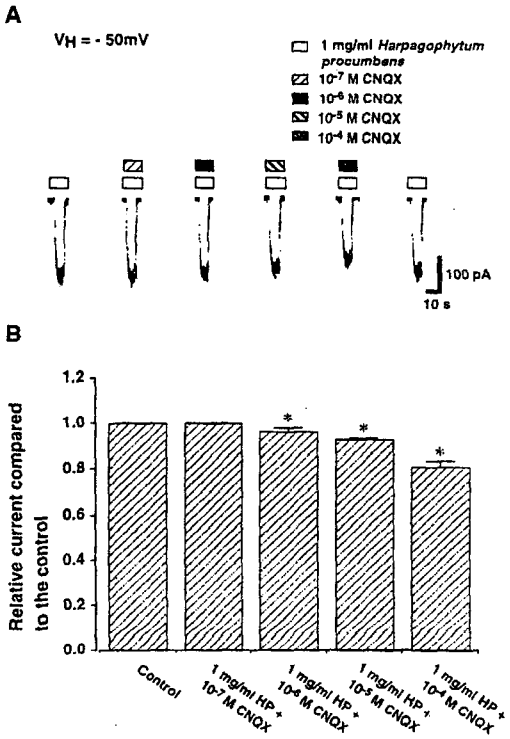

Fig. 5. Effect of CNQX on the *Harpagophytum procumbens*-activated ion current. *Harpagophytum procumbens* was applied every 2 min and ion current activated by 1 mg/ml *Harpagophytum procumbens* used as a control, ion current activated by Harpagophytum procumbens was suppressed by CNQX in a concentration dependent manner. * represents $P < 0.01$ compared to the control. HP: *Harpagophytum procumbens* .

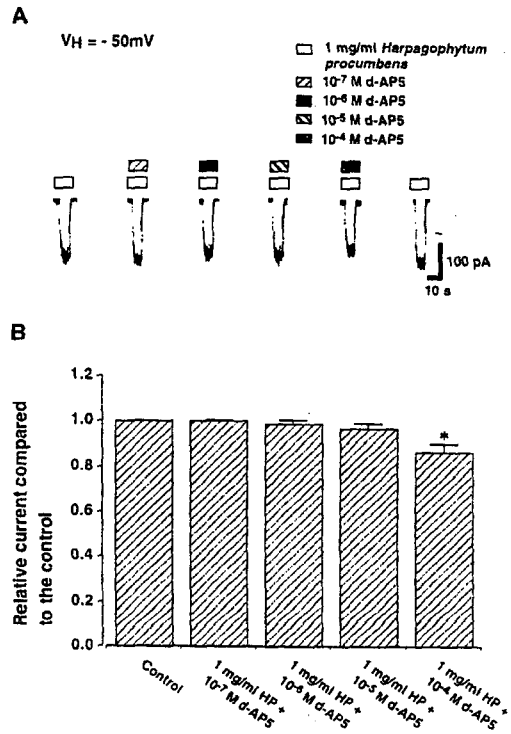

Fig. 6. Effect of D-AP5 on the *Harpagophytum procumbens*-activated ion current. *Harpagophytum procumbens* was applied every 2 min and ion current activated by 1 mg/ml *Harpagophytum procumbens* used as a control, ion current activated by Harpagophytum procumbens was suppressed by concentration of 10^{-4} M D-AP5. * represents $P < 0.01$ compared to the control. HP: *Harpagophytum procumbens* .

Ion current activated by *Harpagophytum procumbens* was suppressed by CNQX in a concentration- dependent manner (Figure 5). CNQX at 10^{-7} M did not affect *Harpagophytum procumbens* activated ion current (n = 4, P< 0.01). CNQX at concentrations of 10^{-6} M, 10^{-5} M, and 10^{-4} M inhibited *Harpagophytum procumbens*-activated ion current about 3.20 ± 1.34 % (n = 4, $P < 0.01$), 7.10 ± 0.48 % (n = 4, $P < 0.01$), and 18.8 ± 2.44 % (n = 5, $P <$ 0.01) of the control value, respectively.

3.2.5. Effect of D-AP5 on the Harpagophytum procumbens-activated ion current

Ion current activated by 1 mg/ml *Harpagophytum procumbens* used as a control.

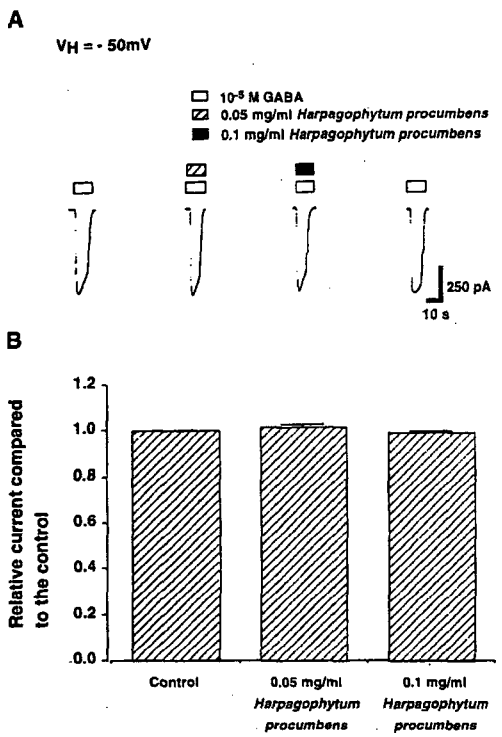

Fig. 7. Modulation by *Harpagophytum procumbens* of GABA-activated ion current. Low concentration of *Harpagophytum procumbens* which does not elicit ion current itself, suppressed GABA-activated ion current and this inhibition was reversible. But this inhibition was no meaning.

Ion current activated by *Harpagophytum procumbens* was suppressed by D-AP5 in a concentration-dependent manner (Figure 6). D-AP5 at 10^{-7} M did not affect *Harpagophytum procumbens* activated ion current (n = 4, P< 0.01). D-AP5 at concentrations of 10^{-6} M, 10^{-5} M, and 10^{-4} M inhibited *Harpagophytum procumbens*-activated ion current about 1.30 ± 1.32 % (n = 4, P < 0.01), 3.50 ± 2.03 % (n = 4, P < 0.01), and 13.70 ± 3.52 % (n = 4, P < 0.01) of the control value, respectively.

3.3. Modulation of neurotransmitters-activated ion currents by Harpagophytum procumbens

In order to evaluate modulatory action of *Harpagophytum procumbens* on neurotransmitters-activated ion currents were investigated.

3.3.1 Modulation of Harpagophytum procumbens on GABA-activated ion current

The concentrations of 0.05 mg/ml and 0.1 mg/ml of *Harpagophytum procumbens* did not elicit ion current by itself when applied to PAG neuron externally. To investigate the modulatory action of *Harpagophytum procumbens* on GABA-activated ion current, magnitude of ion current activated by 10^{-5} M GABA used as the control value, and 0.05 mg/ml or 0.1 mg/ml of *Harpagophytum procumbens* was applied at the same time with GABA. In the present results, 0.05 mg/ml of *Harpagophytum procumbens* did not affect GABA-activated ion current (n = 6, P < 0.01), while 0.1 mg/ml of *Harpagophytum procumbens* inhibited GABA-activated ion current about 1.04 ± 0.68 % (n = 6, P < 0.01) (Figure 7).

3.3.2 Modulation of Harpagophytum procumbens on glycine-activated ion current

To investigate the modulatory action of *Harpagophytum procumbens* on glycine-activated ion current, magnitude of ion current activated by 10^{-5} M glycine used as the control value, and 0.05 mg/ml or 0.1 mg/ml of *Harpagophytum procumbens* was applied at the same time with glycine. In the present results, 0.05 mg/ml of *Harpagophytum*

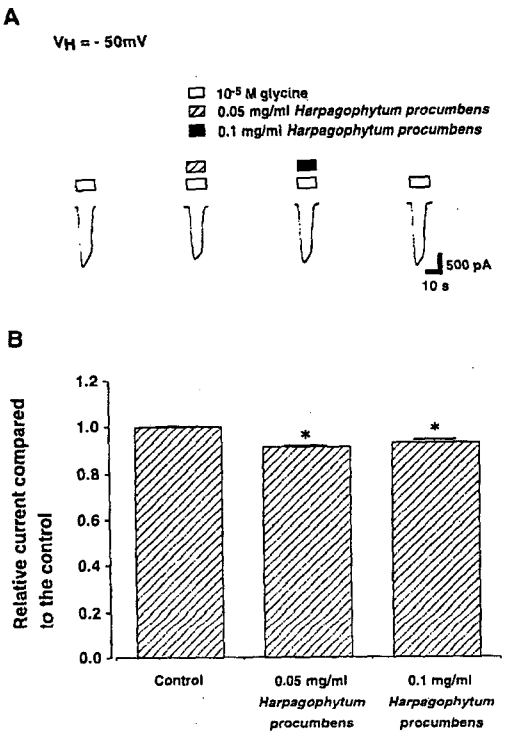

Fig. 8. Modulation by *Harpagophytum procumbens* of glycine-activated ion current To investigate the modulatory action of *Harpagophytum procumbens* on glycine-activated ion current, magnitude of ion current activated by 10^{-5} M glycine used as the control value, and 0.05 mg/ml and 0.1 mg/ml of *Harpagophytum procumbens* was applied at the same time with glycine. In the present result, low concentration of *Harpagophytum procumbens* suppressed glycine-activated ion current and this inhibition was reversible. * represents $P < 0.01$ compared to the control.

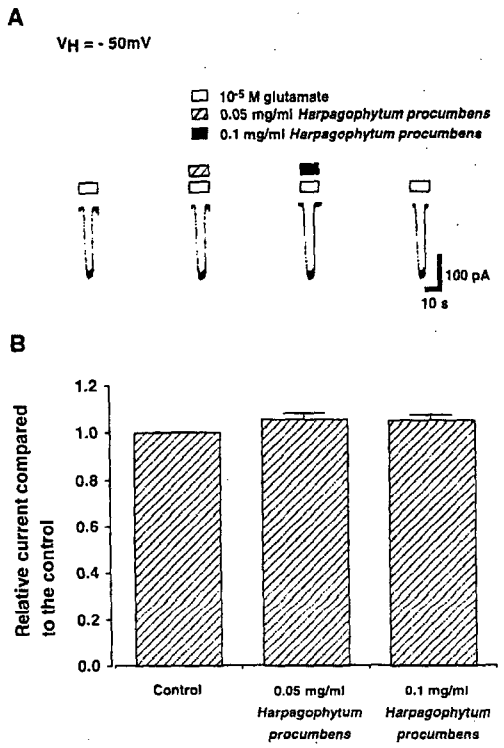

Fig. 9. Modulation by *Harpagophytum procumbens* of glutamate-activated ion current To investigate the modulatory action of *Harpagophytum procumbens* on glutamate-activated ion current, magnitude of ion current activated by 10^{-5} M glutamate used as the control value, and 0.05 mg/ml and 0.1 mg/ml of *Harpagophytum procumbens*was applied at the same time with glutamate. In the present result, low concentration of *Harpagophytum procumbens* increased glutamate-activated ion current and this inhibition.

*procumbens*inhibited glycine-activated ion current about 8.52 ± 0.48 % (n = 6, $P < 0.01$), and 0.1 mg/ml of *Harpagophytum procumbens* inhibited glycine-activated ion current about 6.73 ± 1.04 % (n = 6, $P < 0.01$). This inhibition was reversible (Figure 8).

3.3.3 Modulation of Harpagophytum procumbens on glutamate-activated ion current

To investigate the modulatory action of *Harpagophytum procumbens* on glutamate-

activated ion current, magnitude of ion current activated by 10^{-5} M glutamate used as the control value, and 0.05 mg/ml or 0.1 mg/ml of *Harpagophytum procumbens* was applied at the same time with glutamate. In the present results, 0.05 mg/ml of *Harpagophytum procumbens* increased glutamate-activated ion current about 5.80 ± 0.02 % (n = 6, P < 0.01), and 0.1 mg/ml of *Harpagophytum procumbens*increased glutamate-activated ion current about 5.01 ± 0.02 % (n = 6, P < 0.01). This increment was reversible (Figure 9).

Ⅳ. Discussion

Harpagophytum procumbens has been commercially used as remedies for the inflammation and arthritis [1,2]. In 1987, acteoside, iridoid glycoside, iridoid glycoside, and bioside were isolated from *Harpagophytum procumbens*[3]. The aqueous extract of *Harpagophytum procumbens* have been evaluated for anti-inflammatory action in mice and rats[19]. Pharmacological studies showed reduction of inflammatory pain sensation of this agent[7].

In the present study, in order to evaluate pharmacological properties of the ion current activated by *Harpagophytum procumbens,* the effects of bicuculline, strychnine, tubocurarine, D-AP5, and CNQX were examined. In the results, ion current was elicited by applying *Harpagophytum procumbens*, and this current was almost suppressed by 10^{-4} M bicuculline and 10^{-4} M strychnine. Bicuculline reduces $GABA_A$-activated chloride current by decreasing open frequency and mean duration[20], and produces a competitive antagonistic action on $GABA_A$ receptor current by competing with GABA. Strychnine is a highly selective and extremely potent competitive antagonist on glycine receptor and reduces glycine-activated chloride current[21]. In contrast, this current was less suppressed by 10^{-4} M CNQX and 10^{-4} M D-AP5. CNQX is widely used as an inhibitor on non-NMDA receptor-mediated synaptic transmission[22-24].This competitive antagonist acts on both AMPA and kainite receptors, respectively. D-AP5 is used as an inhibitor on NMDA receptor-mediated synaptic transmission[25] and this competitive antagonist acts on NMDA receptor. In this study, the ion current evoked by *Harpagophytum procumbens* was suppressed by bicuculline, and strychnine, and tubocurarine, indicatingthat ion current induced by *Harpagophytum procumbens* at high concentration (10^{-4} M in this study) directly activates $GABA_A$, and glycine receptors. The ion current evoked by *Harpagophytum procumbens* was lesssuppressed by CNQX, and D-AP5, indicating that ion current induced by *Harpagophytum procumbens* at high concentration exerts less effect onglutamate receptors both of the NMDA-sensitive and the non-NMDA-sensitive subtypes.

Descending modulation of spinal nociceptive neurons by the PAG matter is one of the most extensively studied pain control systems[8]. Several neurotransmitters, such as GABA, glycine, and glutamate, in the PAG participate in the control of nociception[26-28]. In this study, modulatory action of *Harpago-*

phytum procumbens on these neurotransmitters was also investigated in acutely dissociated PAG neuron. The low concentrations of 0.05 mg/ml and 0.1 mg/ml of *Harpagophytum procumbens* did not elicit ion current by itself when applied to PAG neuron externally. To investigate modulation of these low concentrations of *Harpagophytum procumbens* on GABA-, glycine-, and glutamate-activated ion current were applied at the same time with each neurotransmitter, respectively. In the present study, concurrent application of low concentration of *Harpagophytum procumbens* with GABA or glycine or glutamate exerted no significant effect on these neurotransmitters-activated ion currents in PAG neuron.

In this study, it was shown that ion current induced by *Harpagophytum procumbens* at high concentration directly activates GABA, glycine, and glutamate receptors of both the NMDA-sensitive and the non-NMDA-sensitive subtypes. These results account for one of the analgesic mechanism of *Harpagophytum procumbens* in the level of PAG neurons.

Acknowledgements

This work was supported by a grant from the Chungbuk Provincial Government at the year of 2002.

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