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1

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# SINOMENINE PRODUCES PERIPHERAL ANALGESIC EFFECTS VIA INHIBITION OF VOLTAGE-GATED SODIUM CURRENTS

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**Abstract—*Sinomenium acutum* has been used in traditional medicine to treat a painful disease such as rheumatic arthritis and neuralgia. Sinomenine, which is a main bioactive ingredient in *Sinomenium acutum,* has been reported to have an analgesic effect in diverse pain animal models. However little is known about the detailed mechanisms underlying peripheral analgesic effect of sinomenine. In the present study, we aimed to elucidate its cellular mechanism by using formalin-induced acute inflammatory pain model in mice. We found that intraperitoneal (i.p.) administration of sinomenine (50 mg/kg) suppressed formalin-induced paw licking behavior in both the first and the second phase. Formalin-induced c-Fos protein expression was also suppressed by sinomenine (50 mg/kg i.p.) in the superficial dorsal horn of spinal cord. Whole-cell patch-clamp recordings from small-sized dorsal root ganglion (DRG) neurons revealed that sinomenine reversibly increased the spike threshold and the threshold current intensity for evoking a single spike and decreased firing frequency of action potentials evoked in response to a long current pulse. Voltage-gated sodium currents ($I_{Na}$) were also significantly reduced by sinomenine in a dose-dependent manner ($IC_{50}$ = 2.3 ± 0.2 mM). Finally, we confirmed that intraplantar application of sinomenine suppressed formalin-induced pain behavior only in the first phase, but not the second phase. Taken together, our results suggest that sinomenine has a peripheral analgesic effect by inhibiting $I_{Na}$.** **© 2017 IBRO. Published by Elsevier Ltd. All rights reserved.**

**Key words: pain, analgesia, sinomenine, formalin test, c-Fos, voltage-gated sodium channel.**

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*Abbreviations:* APs, action potentials; DRG, dorsal root ganglion; $I_{Na}$, voltage-gated sodium currents; VGSCs, voltage-gated sodium channels.

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## INTRODUCTION

*Sinomenium acutum* has been traditionally used as a herbal medicine to treat rheumatic arthritis, arrhythmia and neuralgia (Zhao et al., 2012). Sinomenine is its main bioactive ingredient in *Sinomenium acutum*, which is well known to have immunosuppressive and anti-inflammatory effects (Wang and Li, 2011). Interestingly, recent studies have also demonstrated analgesic effects of sinomenine. Sinomenine has been reported to have analgesic effects on neuropathic pain models as well as inflammatory pain models (Gao et al., 2013). Sinomenine-induced analgesia is reversed by a $GABA_A$ receptor antagonist in neuropathic pain models (Zhu et al., 2014). Apart from the pathological animal pain models, tail flick test shows that sinomenine has analgesic effects by activating opioid μ-receptor (Wang et al., 2008). While these studies have found potential cellular targets for sinomenine in the central nervous system (CNS) (Zhu et al., 2016), little is known about the cellular mechanism or sinomenine-induced peripheral analgesia.

Primary afferent nociceptive neurons such as Aδ and C-fibers convey pain signals from peripheral injury sites to the CNS. Voltage-gated sodium channels (VGSCs) are the main ion channels for generating action potentials, thereby responsible for transmitting pain signals in the nociceptive neurons (Mathie, 2010; Waxman and Zamponi, 2014; McEntire et al., 2016). Thus, VGSCs in primary afferent neurons are the key molecular target for diverse analgesics including local anesthetics such as lidocaine (Inan et al., 2009). In line with this, we previously showed that eugenol, the main component in clove plant, has local anesthetic actions like lidocaine in periphery by inhibiting voltage-gated sodium currents ($I_{Na}$), thereby providing a pharmacological mode of action for their wide use in dental clinic to alleviate dental pain (Park et al., 2009). It is also possible to selectively block pain signals by blocking VGSCs with the permanently charged lidocaine derivative QX-314 that enters through large-pore ion channels selectively expressed in nociceptive neurons (Binshtok et al., 2009; Kim et al., 2010; Puopolo et al., 2013).

In this study, we sought to explore the analgesic effect of sinomenine by using formalin-induced acute pain model and further focused its peripheral mechanisms by examining its effects on the excitability of nociceptive neurons. We found that sinomenine can produce peripheral analgesic effect by reducing cellular excitability of nociceptive neurons and the inhibition of $I_{Na}$ is likely to contribute to its action.

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## EXPERIMENTAL PROCEDURES

### Animals

Male C57BL/6 mice weighing 20–28 g were used for the experiment. They were housed 4–6 per cage at a temperature-controlled room (23 ± 1 °C, 12 h/12 h light/dark cycle) and maintained with pellet diet and tap water ad *libitum*. All surgical and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Seoul National University.

### Formalin test

Mice were acclimated in cage at least for a week and then adapted in an acrylic observation chamber (size ranges 12 × 12 × 12 cm) before the experiment at least three times. A mirror was located at 45° angle below the chamber to observe the paws. Formalin test was performed by intraplantar (i.pl.) injection of 1% formalin as previously described (Cho et al., 2006). On the day of test, mice were acclimatized for 30 minutes in an acrylic chamber and then 20 μl of 1% formalin was injected subcutaneously into the plantar surface of the right hind paw with a 31-gauge needle of 0.3-ml insulin syringe. Following formalin injection, the animals were immediately placed in a test chamber and recorded using a video camera for a period of 40 min. The time mice spent licking or biting was measured during each 5 min by an observer who was blinded to the treatment. Formalin-induced pain behaviors during 0–10 min after formalin injection represented the first phase and during 10–40 min after formalin injection represented the second phase.

### c-Fos immunohistochemistry

All procedures were prepared as previously described (Badral et al., 2013). Animals were sacrificed 2 h after formalin injection for immunohistochemical analysis of c-Fos proteins. Animals were anesthetized by intraperitoneal (i.p.) injection of pentobarbital (60 mg/kg) and transcardially perfused with 0.01 M phosphate-buffered saline (PBS) including 500 U/L heparin followed by 4% paraformaldehyde (PFA) in 0.01 M PBS. The spinal cord was post-fixed by 4% PFA overnight and transferred to 30% sucrose in 0.01 M PBS for 2–3 weeks. Frozen specimen was transversely sectioned into 30 μm with cryotome and sections were stored in cryoprotectant at −20 °C. Free floating sections were washed with 0.01 M PBS and incubated in 0.3% $H_2O_2$ (in distilled water) for 30 min at room temperature (RT). After elimination of endogenous peroxidase, sections were washed with 0.01 M PBS and Pre blocked with 5% normal goat serum (NGS) in PBS with 0.3% triton × (PBST) for 1 h at RT. Sections were incubated in 1st antibody (PC38, Calbiochem, USA; 1:1000 in 1% NGS (in 0.3% PBST)) for 48 h at 4 °C, washed with 0.01 M PBS to remove 1st antibody and incubated in biotinylated goat anti rabbit (BA1000, Vector laboratories, USA; 1:400 in 0.01 M PBS) for 2 h at RT. Sections were processed with ABC kit (PK-6100, Vectastain ABC kit, Vector laboratories, USA), visualized with DAB kit (DAB substrate kit for peroxidase, Vector laboratories, USA) and then mounted on slide glass. After air drying, all sections were cover slipped with mountant (H-1000, Vectashield mounting medium, Vector laboratories, USA).

### Cell counting and image analysis

For the quantification of c-Fos expression, we confirmed that most c-Fos expression induced by injection of 1% formalin (i.pl.) localized in Lumbar (L) 4–5 segment. Four sections with highest expression of c-Fos per animal were chosen and superficial dorsal horn (lamina I-II) of L4-5 segments was selected for analysis. The number of c-Fos-positive neurons was counted blindedly and the mean value was used as representative counts. Using image J, the image of selected sections was converted to gray scale, background subtracted, enhanced and sharpened. Intensity threshold was adjusted and then analyzed.

### Dorsal root ganglion (DRG) culture

DRG neurons were prepared as previously described (Kim et al., 2009). Mice were sacrificed by exposure to isoflurane and decapitated. DRG were isolated in cold HBSS (Welgene, Korea) and incubated in 3-ml HBSS containing 1 mg/ml collagenase (Roche, USA) / 2.4 U/ml dispase II (Roche, USA) for 1 h at 37 °C. Then DRG were digested by 0.25% trypsin in PBS for 7 min at 37 °C, inactivated by 2-ml 0.25% trypsin inhibitor (Sigma, USA) and 2-ml DMEM containing 10% fetal bovine serum (Gibco, USA)/1% pen strep (Gibco, USA) and washed by 4-ml DMEM (FBS/pen strep). DRG neurons were triturated using fire-polished pasteur pipette to separate cells. The cells were resuspended in neurobasal medium (Gibco, USA) containing B-27 supplement (Gibco, USA), 1% pen strep, L-glutamine (1 mM), placed on cover slips coated with poly-D-lysine (Sigma, USA). After 1 h cells were fed with fresh neurobasal medium (B-27 supplement / pen strep / L-glutamine) and maintained in a 5% $CO_2$ – 95% $O_2$ incubator at 37 °C.

### Electrophysiological recordings

Whole-cell current- and voltage-clamp recordings were performed as previously described (Park et al., 2009) in small-sized DRG neurons ( < 20 μm diameter) at RT with an Axopatch 200B amplifier to record action potentials (APs) and $I_{Na}$. Data were sampled at 10 kHz. The patch pipettes were pulled from borosilicate capillaries. When filled with following pipette solutions, their resistances were 2–6 MΩ. A −10 mV liquid junction potential was corrected. The extracellular solution was driven by gravity and continuously perfused (1–2 ml/min). The pipette solution for current clamp contained (in mM): K-gluconate 140, $CaCl_2$ 1, $MgCl_2$ 2, EGTA 10, $K_2ATP$ 5, HEPES 10, adjusted to pH 7.4 with KOH, osmolarity 300 mOsm. Extracellular solution for current clamp contained (in mM): NaCl 140, HEPES 10, $CaCl_2$ 2, $MgCl_2$ 1, glucose 10, KCl 5, adjusted to pH 7.4 with NaOH, osmolarity 300 mOsm. Single APs were evoked by 5-ms depolarizing current pulses. The spike threshold was measured

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