Synergistic antinociceptive effects of N-methyl-D-aspartate receptor antagonist and electroacupuncture in the complete Freund's adjuvant-induced pain model

JI-YEON JANG[1], HA-NEUI KIM[1], SUNG-TAE KOO[1], HWA-KYOUNG SHIN[1], EUN-SANG CHOE[2] and BYUNG-TAE CHOI[1]

[1]Division of Meridian and Structural Medicine, School of Korean Medicine, Pusan National University, Yangsan 626-870; [2]Department of Biology, College of Natural Sciences, Pusan National University, Pusan 609-735, Republic of Korea

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Abstract. This study examined the synergistic antinociceptive effects associated with signaling pathway proteins of the spinal cord in a complete Freund's adjuvant (CFA)-induced pain model when electroacupuncture (EA) and a N-methyl-D-aspartate receptor (NMDAR) antagonist were administered in combination. EA stimulation (2 Hz, 1 mA) was needle-delivered for 20 min once daily at acupoints corresponding to Zusanli and Sanyinjiao with intrathecal injection of the NMDAR antagonist dizocilpine (MK801). Thermal sensitivity of the hindpaw induced by CFA was strongly inhibited by dizocilpine injection and EA stimulation. Co-treatment with EA and dizocilpine showed a synergistic antinociceptive effect against inflammatory pain. On day two of the experiment, we examined the phosphorylation of the NMDAR NR2B subunit, of the extracellular signal-regulated kinase (ERK), p38 and of the cAMP response element-binding protein (CREB) in the ipsilateral dorsal horn of L4-5 segments by Western blot analysis. Phosphorylation of the NMDAR NR2B subunit induced by CFA was markedly inhibited by co-treatment with dizocilpine and EA, but not by dizocilpine or EA treatment alone. CFA-induced phosphorylation of the ERK was inhibited by both dizocilpine and EA, but that of p38 was inhibited by EA only. CFA-induced phosphorylation of CREB was inhibited by dizocilpine, but did not show marked changes. Immunohistochemical analyses confirmed that there was a significant difference in the NMDAR NR2B subunit and ERK phosphorylation. It is possible that the combined treatment with EA and the NMDAR antagonist dizocilpine resulted in synergistic antinociceptive effects in an inflammatory pain model via the inactivation of both the NMDAR NR2B subunit and ERK of the spinal cord.

Correspondence to: Dr Byung-Tae Choi, Division of Meridian and Structural Medicine, School of Korean Medicine, Pusan National University, Yangsan 626-870, Republic of Korea
E-mail: choibt@pusan.ac.kr

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Introduction

N-methyl-D-aspartate receptors (NMDARs) are heteromultimeric complexes that consist of at least two types of subunits, the essential forming subunit NR1 and the modulatory subunit NR2A-D for functional ion channels (1). NMDARs are well established as critical mediators of excitatory synaptic transmission in the spinal cord. Among the NMDAR subunits, NR2B is the most commonly expressed NR2 subunit in the spinal cord and phosphorylation of this subunit contributes to pain conditions including the initiation and development of inflammatory hyperalgesia (2). Inhibition of the phosphorylation of the NR2B subunit suggests that there is potent compensation for enhanced nociceptive activity (3).

Glutamate released by noxious stimulation binds to the corresponding receptor expressed on dorsal horn neurons and regulates gene expression via specific activation of intracellular signaling cascades. Mitogen-activated protein kinases (MAPKs) are the best characterized cascade in dorsal horn cells for generating hypersensitivity (4,5). MAPKs consist of three major members, extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK), which each represent different signaling cascades (6) and play critical roles in inflammatory responses (7,8).

Phosphorylated NMDARs result in increased Ca^{2+} influx, which activates various Ca^{2+}-dependent kinases to initiate the MAPK signaling cascade in the spinal dorsal horn neurons (7,9). Additionally, NMDARs activate ERK via both PKA and PKC, which contributes to central sensitization through the cAMP response element-binding protein (CREB)-mediated transcriptional or non-transcriptional regulation (9,10).

Electroacupuncture (EA) has been widely used in clinical and basic research in Korea as an effective acupuncture technique. It is also well known that EA shows anti-hyperalgesic effects that have been confirmed in previous clinical and animal experimental studies. NMDARs may also be involved in the induction of EA analgesia in the spinal cord of normal animals (11), which indicates that any changes in its activation

may influence EA analgesia. Combined treatment with EA and NMDAR antagonists has a synergistic antinociceptive effect in inflammatory pain models (12,13).

Co-treatment with dizocilpine (MK801) and EA showed synergistic effects against inflammatory nociception in a preliminary experiment conducted in our laboratory. However, the mechanisms underlying the synergistic effects of the EA and NMDAR antagonists are unclear, especially with respect to the spinal NMDAR-related MAPKs pathway. In the present study, we used a complete Freund's adjuvant (CFA) inflammatory pain model to investigate the role of EA and dizocilpine on the NMDAR NR2B subunit and MAPKs activation in the spinal cord. The goal of this study was to investigate the possible role of the spinal NMDAR NR2B subunit, ERK, p38 and CREB signaling pathway on the NMDAR antagonist and EA single or combination treatment following CFA-induced inflammation.

Materials and methods

Animals. Male Sprague-Dawley rats weighing about 200-220 g on average were obtained from the Dooyeol Biotech Co. (Korea). Rats were housed at 22˚C under a 12-h dark-light cycle fed a commercial diet and allowed tap water *ad libitum* starting 1 week before the study, and this was continued throughout the study. All experiments were approved by the Pusan National University Animal Care and Use Committee in accordance with the Council of the International Association for the Study of Pain of December 1982. Each group consisted of 5 or 6 rats and all treatments were administered under isoflurane (Choongwae, Seoul, Korea) anesthesia, which was provided using a calibrated vaporizer (Midmark VIP 3000, Orchad Park, OH, USA).

CFA and intrathecal (i.t.) injection. Rats were injected subcutaneously with 100 μl of CFA (Sigma, St. Louis, MO, USA) into the plantar surface of the left hindpaw under 1% isoflurane anesthesia. Next, i.t. catheterization was performed according to the method described by Størkson *et al* (14). Two days after surgery, only those rats without overt signs of spinal cord or root damage, such as paralysis or lameness, were used for experimentation. The NMDAR antagonist dizocilpine (5 μg, Sigma) was dissolved in sterile saline and injected i.t. at a volume of 10 μl via a catheter within 1 min. The catheter was then filled with 8 μl of saline for flushing. Dizocilpine was administered once daily into the subarachnoid space of the spinal cord 5 min prior to EA stimulation. The vehicle control group for dizocilpine received injections of identical amounts of PBS via an identical method. CFA was injected only one time immediately upon termination of EA stimulation on the first day of the experiment.

EA stimulation. Under light gaseous anesthesia (1.0% isoflurane in air), two stainless-steel needles with a diameter of 0.2 mm were inserted to a depth of approximately 3 mm into each hind leg at the acupoints corresponding to Zusanli (ST36) and Sanyinjiao (SP6) in men and were connected to an electrical stimulator (Pulsemaster Multi-channel Stimulator SYS-A300, World Precision Instruments, Inc., Berlin, Germany). EA with 2 Hz stimulation of 1.0 mA was applied for 20 min once daily for 3 days and gaseous anesthesia was discontinued 3 min prior to termination of the EA treatment. Animals that did not receive EA or dizocilpine treatment were subjected to the same anesthesia as those that received the EA treatment for 3 days.

Measurements of thermal hyperalgesia. The heat paw withdrawal latency of the animals was measured by the plantar test (Ugo-Basile 37370, Comerio, Italy). The rats were placed in six separated cages (17x11.5x14 cm high) for 30 min after EA treatment and nociceptive thresholds of the left and right hind paw were assessed 3 times with a 5 min interval between trials and the mean values were taken as the paw withdrawal latency. The intensity of the infrared generator was adjusted to produce withdrawal latencies of approximately 8-10 sec (infrared intensity of 80) in normal rats. A cut-off period of 15 sec was used. On the first day of the experiments, the basal noxious threshold was simply measured and then treated by i.t. injection of dizocilpine, EA stimulation and CFA injection in regular sequence. On the second and third day of the experiment, latency responses were monitored starting at 30 min after EA stimulation with or without dizocilpine injection. EA non-treated rats were also placed under gaseous anesthesia and the paw withdrawal latency was then measured.

Western blotting. To examine the alterations in the expression of the NMDA NR2B subunit, ERK, p38 and CREB, the L4-5 segments of the spinal cords were removed 60 min after termination of EA stimulation on the third day of the experiment. The ipsilateral half of the spinal cord was excised to the injected side and the dorsal horn part was eventually dissected from it. The spinal cords were then washed in cold HEPES buffer and homogenized in nine volumes of lysis buffer. Equal amounts of proteins were then separated by 8-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), after which the resolved proteins were transferred to a nitrocellulose membrane (Whatman, Dassel, Germany) that was subsequently blocked with 5% nonfat milk in Tris-buffered saline containing 0.4% Tween-20.

The membranes were incubated with anti-NR2B (Millipore, Billerica, MA, USA), anti-phospho-NR2B (pNR2B, ser1303, Upstate, Lake Placid, NY), anti-ERK (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-phospho-ERK (pERK, thr202/tyr204, Cell Signaling Technology, Danvers, MA), anti-CREB (Cell Signaling Technology), anti-phospho-CREB (pCREB, ser133, Cell Signaling Technology), anti-p38 (Santa Cruz Biotechnology, Inc.) and anti-phospho-p38 (pp38, thr180/tyr182, Cell Signaling Technology) for 1-2 h at room temperature, after which the blots were incubated with horseradish peroxidase-conjugated secondary antibody and the antibody-specific proteins were visualized using an enhanced chemiluminescence detection system according to the recommended procedure (Pierce, Rockford, IL, USA). β-actin was used as a loading control for all experiments. Quantification of immunoreactivity corresponding to the total and phosphorylated bands was performed by densitometric analysis using Multi Gauge Version 3.0 (Fujifilm, Tokyo, Japan).

Immunohistochemistry. The L4-5 segments of the spinal cords were fixed in 4% paraformaldehyde and immersed in

30% sucrose for 48 h at 4˚C for cryoprotection on the third day of the experiment. Frozen 14 μm thick sections were then prepared, pre-incubated in 0.3% H_2O_2 for 15 min, and incubated in a blocking solution containing 3% normal goat serum and 0.3% Triton X-100 in PBS for 30 min at room temperature. The sections were then incubated for 24 h at 4˚C with pNR2B and pERK antibody containing 0.3% Triton X-100. After being washed with PBS, the sections were incubated with the secondary antibody, biotinylated anti-rabbit or mouse IgG for 30 min and then washed with PBS. The sections were further incubated with an avidin-biotin-peroxidase complex kit (Vector, Burlingame, CA, USA) for 60 min at room temperature. A diaminobenzidine substrate kit (Vector) was applied using nickel chloride to detect peroxidase. For controls, treatment with primary and secondary antibodies was omitted. The integrated optical density (IOD) for pNR2B in the dorsal horn was measured automatically using the Visus Image Analysis software (Foresthill Products Ista-Video TesT, Foresthill, CA, USA). Immunoreactive cells for pERK were simply counted per regions of the dorsal horn.

Data analysis. Data are expressed as the mean ± SEM. Data were analyzed by a multifactorial analysis of variance (ANOVA) using the SigmaStat statistical program version 11.0 (Systat Software, San Jose, CA, USA). Behavioral and Western blot analysis were performed by a two-way and one-way ANOVA with the Tukey post-hoc test, respectively. Immunohistochemical analysis was performed using a one-way ANOVA with the Dunnett post-hoc test. A P<0.05 was considered to be statistically significant.

Results

EA and dizocilpine treatment on CFA-induced hyperalgesia. Rats usually resumed full activity within 2-5 min of the isoflurane anesthesia being stopped, regardless of whether they received EA stimulation. All heat paw withdrawal latencies were measured after 30 min of anesthesia. For the ipsilateral paw (Fig. 1A), paw withdrawal latency induced by CFA significantly increased from 2.91±0.17 and 4.15±0.10 to 6.55±0.39 and 6.41±0.18 on the second (P<0.001) and third day (P<0.05) of the experiment, respectively, following EA stimulation when compared with the CFA-injected group. Increased paw withdrawal latencies of 4.18±0.40 and 5.37±0.56 were also observed in the dizocilpine-treated rats, but these values did not differ significantly from the CFA-injected group.

Following co-treatment with dizocilpine and EA in CFA-treated rats, a significantly longer latency of 7.23±0.58 was observed in co-treated rats when compared to the 4.18±0.40 latency observed in rats treated with dizocilpine alone on the second day of the experiment (P<0.001). On the third day after CFA injection, the paw withdrawal latency was 8.91±0.57 in co-treated rats, 5.37±0.56 in rats treated with dizocilpine alone and 6.41±0.18 in those treated with EA alone. Rats that were co-treated with dizocilpine and EA showed significant anti-hyperalgesic effects when compared with rats that were treated with dizocilpine (P<0.001) and EA alone (P<0.01) on the third day of the experiment.

In the contralateral paw (Fig. 1B), treatment with dizocilpine or EA alone did not alter the paw withdrawal latency.

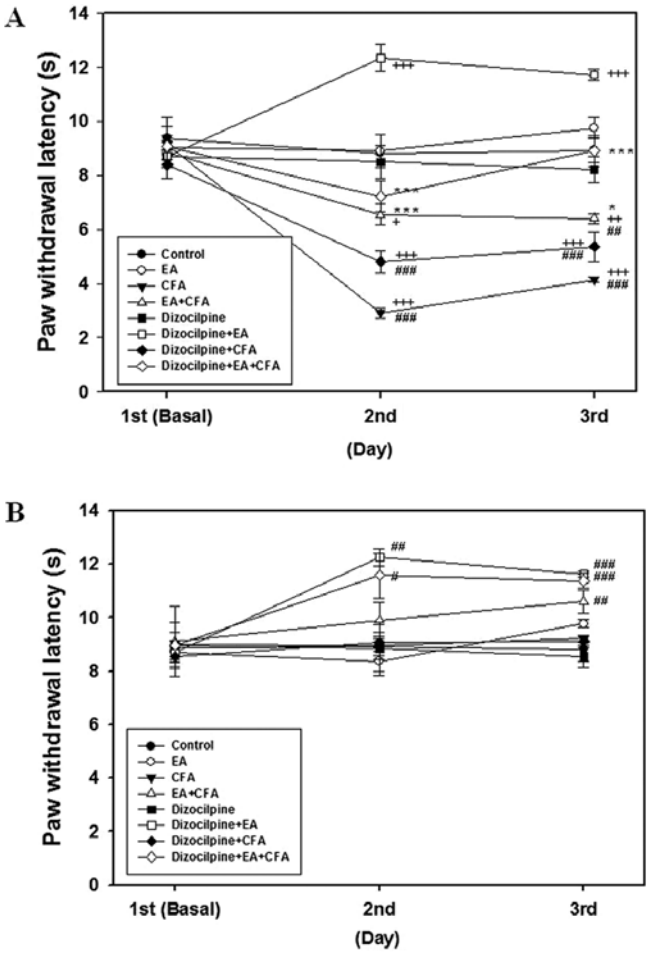

Figure 1. Effect of EA, dizocilpine and a combination of EA and dizocilpine on the paw withdrawal responses to thermal stimuli on the ipsilateral (A) and contralateral (B) sides of CFA-injected rats. Each point indicates the mean ± SEM (n=6). Dizocilpine- and EA-treated rats showed a significant anti-hyperalgesic effect, and dizocilpine and EA co-treated rats produced a synergistic effect. (A) *P<0.05 and ***P<0.001 compared with CFA-injected rats; $^{+}$P<0.05, $^{++}$P<0.01 and $^{+++}$P<0.001 compared with control rats; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 compared with dizocilpine + EA + CFA-treated rats; (B) $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 compared with control rats.

However, combined treatment with dizocilpine and EA led to a marked increase in paw withdrawal latency when compared to control rats.

Western blot analysis for NMDAR NR2B subunit and signaling-related proteins. We first examined the induction of the total and phosphorylated NMDAR NR2B subunit, ERK, p38 and CREB in control and CFA-injected rats that were treated with or without EA. Next, because NMDAR may initiate intracellular signaling cascades, we intrathecally injected the NMDAR blocker, dizocilpine, into a CFA-induced pain model. The ratios of the optical density of the total NR2B, ERK, CREB and p38 bands to the β-actin band were not altered by the EA, dizocilpine or CFA treatments. To determine the phosphorylation or activation of these proteins, we plotted the ratio of the optical density of each band for phosphorylated/total proteins and normalized these values as a percentage of the dizocilpine non-treated control rats (Figs. 2-5).

Two ERK bands for p42 and p44 were measured, regardless of classification, and analyzed in this study. The two

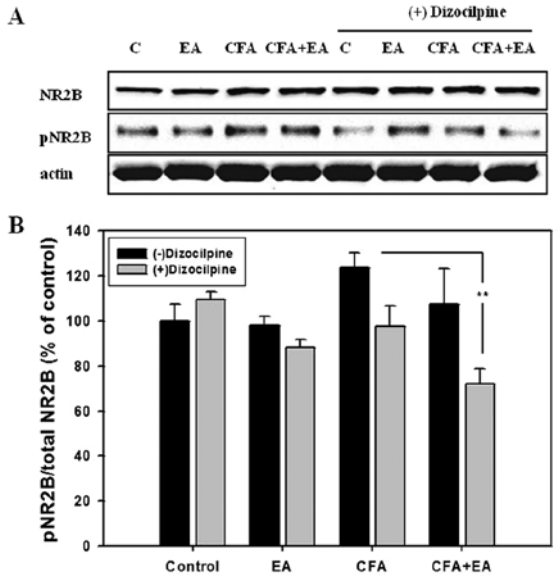

Figure 2. Western blot analysis (A) and its densitometric analysis (B) for NR2B and pNR2B subunits in the ipsilateral dorsal portion of the L4-5 segment of the spinal cord. The level of each protein is expressed as a percentage of the normal control. Each single experiment was conducted on 5 pooled animals. The panel represents a typical result from 3 independent experiments. CFA-induced NR2B subunit activation decreased significantly in response to co-treatment with dizocilpine and EA. $^{**}P<0.01$.

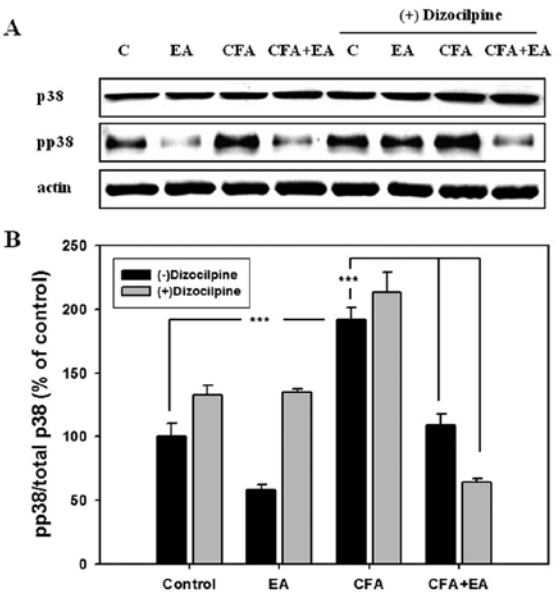

Figure 4. Western blot analysis (A) and its densitometric analysis (B) for the p38 and pp38 in the ipsilateral dorsal portion of the L4-5 segment of the spinal cord. The level of each protein is expressed as a percentage of the normal control. Each single experiment was conducted on 5 pooled animals. The panel represents a typical result from 3 independent experiments. CFA-induced p38 activation was significantly decreased by EA treatment. $^{***}P<0.001$.

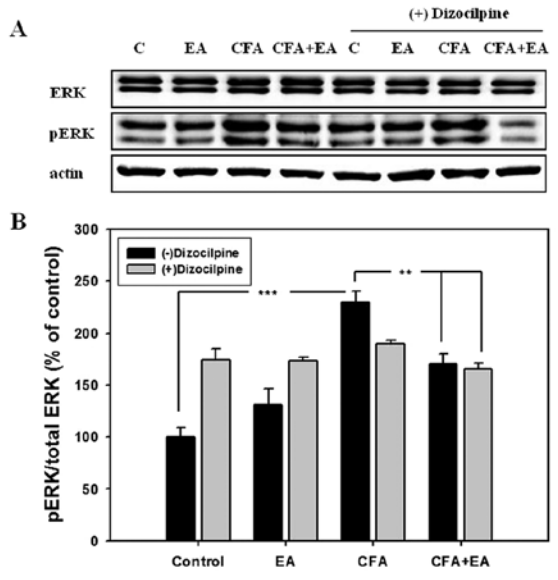

Figure 3. Western blot analysis (A) and its densitometric analysis (B) for ERK and pERK two bands of p42 and p44 in the ipsilateral dorsal portion of the L4-5 segment of the spinal cord. The level of each protein is expressed as a percentage of the normal control. Each single experiment was conducted on 5 pooled animals. The panel represents a typical result from 3 independent experiments. ERK activity induced by CFA was inhibited remarkably in the groups treated with EA alone and both dizocilpine and EA. $^{**}P<0.01$; $^{***}P<0.001$.

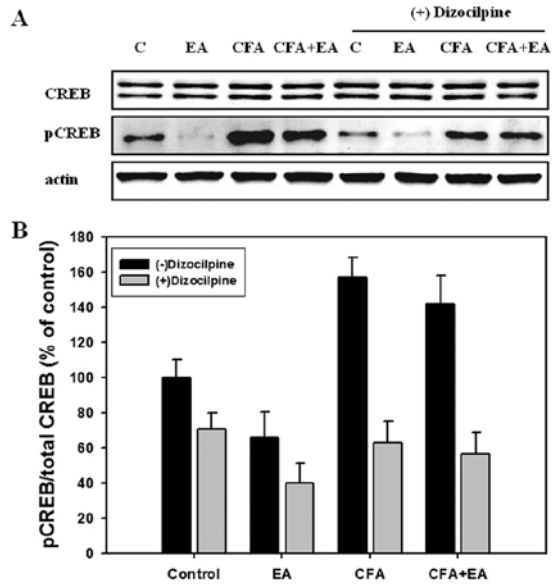

Figure 5. Western blot analysis (A) and its densitometric analysis (B) for representing two band for CREB and pCREB in the ipsilateral dorsal part of the L4-5 segment of the spinal cord. The level of each protein is expressed as a percentage of the normal control. Each single experiment was conducted on 5 pooled animals. The panel represents a typical result from 3 independent experiments.

bands representing CREB were also calculated as the total expression. For activation of the NMDAR NR2B subunit, there was no significant induction by dizocilpine or EA in the CFA non-treated rats. The CFA-induced activation of NR2B was markedly attenuated from 123.6±6.60 to 71.98±6.56% by co-treatment with dizocilpine and EA (P<0.01) (Fig. 2).

For the ERK activation, induction of 229.6±10.9% compared to control rats was induced by CFA (P<0.001). However, CFA-induced activation was significantly modulated to 170.7±9.2 and 165.5±5.4% respectively, in response to EA treatment, when administered alone or in combination with dizocilpine (P<0.01) (Fig. 3). The p38 activation of 191.6±10.9%

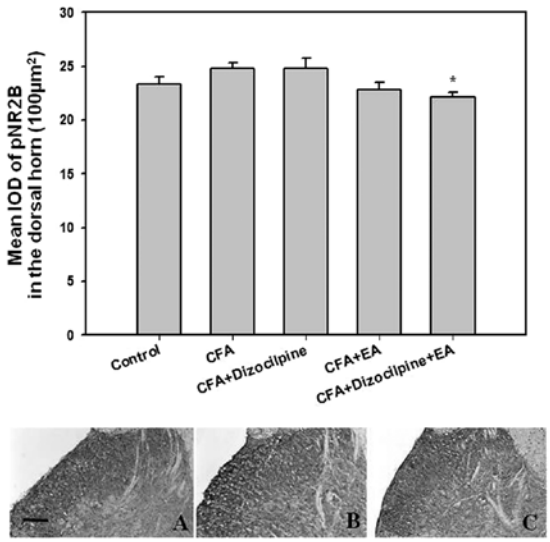

Figure 6. Immunohistochemical analysis for the NMDAR pNR2B subunit in the ipsilateral dorsal horn of the L4-5 segment (n=5). Data are expressed as the mean ± SEM. Dizocilpine and EA co-treated rats showed a significant decrease in mean IOD in the dorsal horn when compared with CFA-injected rats. *P<0.05 compared with CFA-injected rats. Bar, 200 μm. (A) control; (B) CFA; (C) CFA + dizocilpine + EA.

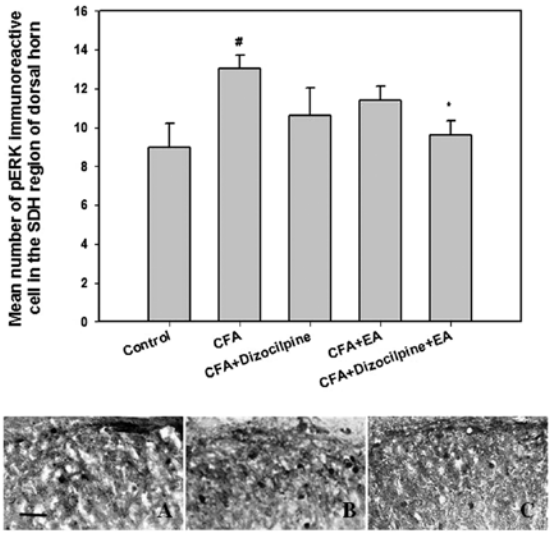

Figure 7. Immunohistochemical analysis for pERK in the SDH region of the ipsilateral dorsal horn of the L4-5 segment (n=5). Data are expressed as the mean ± SEM. A significant decrease in the immunoreative cell number in the dorsal horn of dizocilpine and EA co-treated rats was observed when compared with CFA-injected rats. #P<0.05 and *P<0.05 compared with control and CFA-injected rats, respectively. Bar, 50 μm. (A) control; (B) CFA; (C) CFA + dizocilpine + EA.

compared to control rats was induced by CFA (P<0.001), and this induction was decreased to 108.9±8.8 and 63.9±3.3% by EA stimulation and combined treatment with dizocilpine and EA (P<0.001) (Fig. 4). Activation of the transcription factor CREB revealed no significant difference in rats that were not treated or treated with CFA (Fig. 5).

Immunohistochemical analysis of the NMDAR pNR2B subunit and pERK. NMDAR NR2B and ERK may be involved in the synergistic antinociceptive effects that were observed upon Western blot analysis; therefore, we performed immunohistochemistry to quantify and localize these proteins in the ipsilateral dorsal horn of the spinal cord. The cells that were immunoreactive for the pNR2B subunit and pERK were primarily observed in the superficial dorsal horn (SDH, laminae I and II) of the dorsal horn. The mean IOD in CFA-injected rats was markedly decreased from 24.83±0.45 to 22.16±0.42 by dizocilpine and EA co-treatment (P<0.05) (Fig. 6). The mean number of pERK positive cells also increased significantly in response to CFA injection from 9.0±1.2 to 13.1±0.7 (P<0.05), and this increase were suppressed to 9.6±0.8 by dizocilpine and EA co-treatment (P<0.05) (Fig. 7). There were no significant changes in the contralateral dorsal horn of the spinal cord.

Discussion

Glutamate is a major fast excitatory neurotransmitter that is abundant in nociceptive primary afferent fiber terminals and involved in the process of underlying mechanisms for nociceptive transmission at the spinal level (15). The NMDAR are known to contribute to excitatory synaptic transmission within the spinal cord when evoked by nociceptive primary afferent stimuli (16). EA stimulation markedly reduces inflammatory hyperalgesia by means of inhibitory release of glutamate in the spinal dorsal horn, and NMDAR antagonists show antinociceptive action in an inflammatory pain model (12).

However, EA-induced analgesic effects that occur via the glutamate receptors, especially the synergistic effects of EA and NMDA antagonists, have received relatively little attention. The goal of the present study was to observe alterations in the spinal NMDA NR2B subunit, ERK, p38 and CREB activation in CFA-injected rats due to EA and NMDAR antagonist treatment. The intraplantar administration of CFA has been shown to induce mechanical allodynia and thermal hyperalgesia on the ipsilateral hindpaw and L4-5 nerve roots or inflammation of the dorsal root ganglion (17,18).

Therefore, we examined the inhibitory effects of EA stimulation and dizocilpine injection on heat paw withdrawal latency. CFA-injection resulted in a condition of persistent thermal hyperalgesia throughout the experiments; however, CFA-induced hyperalgesia was partially abolished by treatment with dizocilpine or EA alone in the present study. Moreover, when we co-treated rats with dizocilpine and EA a synergistic anti-hyperalgesic effect was observed (Fig. 1) that was similar to the results observed in a carrageenan-induced pain model (12).

Following peripheral inflammation by CFA, multiple changes in central sensitization occur, leading to hyperactivity in the neurons of the spinal cord and trigeminal dorsal horn (9,19). An increase in calcium influx via calcium permeable ionotropic glutamate receptor, mainly the NMDAR subtype, initiates activation of the MAPK signaling cascade (7,20,21). Additionally, phosphorylation of spinal cord NMDAR subunits leads to enhancement of its function and central sensitization (22). This indicates that glutamate receptors may play an important role in MAPK activation; therefore, we examined the effect of the NMDAR channel blocker, dizocilpine, on the CFA-induced pain model.

Recent studies have suggested that spinal NMDAR NR2B subunit phosphorylation is closely related to the development of inflammatory hyperalgesia, and that suppression of this particular subunit suggests compensation for the enhanced nociceptive activity (3,23). The results of the present study revealed that treatment with EA or dizocilpine alone did not induce significant changes in NR2B phosphorylation on serine residues, but that co-treatment with dizocilpine and EA led to a marked decrease in NR2B activation compared to CFA-injected rats that was correlated with behavioral changes.

Evidence of the contribution of MAPKs to the induction and maintenance of hypersensitivity in the spinal dorsal horn has been obtained under different pain models. Specific inhibitors of MAPK pathways of the neurons and glial cells may lead to new therapies for pain management (9). In addition, ERK phosphorylation by glutamate receptor contributes to central sensitization through post-translational and CREB or Elk-1-mediated transcriptional regulation in dorsal horn neurons (7,10,20).

In the present study, phosphorylation of ERK induced by CFA was arrested by treatment with EA alone and a combination of dizocilpine and EA. In addition, CFA-induced activation of p38 was strongly inhibited by EA stimulation, but not by dizocilpine injection. Although there were no significant changes, the NMDA receptor antagonist dizocilpine suppressed CFA-evoked CREB phosphorylation in the spinal cord. CFA significantly altered the expression of the NR2B subunit, ERK and p38 in the ipsilateral spinal dorsal horn, but not in the contralateral spinal dorsal horn.

These results indicate that both dizocilpine and EA treatment may produce synergistically anti-hyperalgesic effects via inhibition of the phosphorylation of the NR2B subunit and ERK in the ipsilateral spinal dorsal horn. However, CFA-induced phosphorylation of the NR2B subunit and ERK was inhibited by both dizocilpine and EA, and that of p38 and CREB was mainly inhibited by EA alone and dizocilpine alone treatment, respectively. These results also indicate that EA may primarily produce anti-hyperalgesia via p38 inactivation.

Formalin-induced inflammation results in the activation of CREB signaling in the spinal cord through an NMDA receptor-mediated mechanism, and dizocilpine can significantly suppress formalin-evoked CREB phosphorylation in the spinal cord (24). The NMDAR antagonist dizocilpine mainly inhibited the activation of spinal CREB induced by CFA, which was similar to the results of a previous study.

Activation of the transcription factor, CREB, may be initiated via the p38 pathway in the spinal cord. However, activation of this protein leads to the rapid release of inflammatory mediators such as IL-1β, which contributes to central sensitization via post-translational regulation (9,25-27). The p38 phosphorylation in response to CFA injection was not attenuated by dizocilpine alone injection, and was mainly arrested by EA stimulation in our study. These results suggest that anti-hyperalgesia of EA may be produced through post-translational regulation of inflammatory mediators and not by subsequent CREB-mediated transcriptional regulation.

The present study demonstrates that the inflammatory stimulus induced by CFA produced thermal hyperalgesia and involved activation of the NR2B subunit, ERK, p38 and CREB in the ipsilateral dorsal horn of the spinal cord. However, EA and dizocilpine may have produced synergistic antinociceptive effects in an inflammatory pain model via inactivation of both the NMDAR NR2B subunit and ERK. These results were confirmed by immunohistochemical methods. Immunocytochemical analysis in our study revealed that co-treatment of rats with dizocilpine and EA led to a marked decrease in NR2B and ERK phosphorylation of the ipsilateral dorsal horn when compared with CFA-treated rats.

Consequently, hyperalgesia induced by CFA was associated with enhanced phosphorylation of the NR2B subunit, ERK, p38 and CREB in the dorsal horn of the spinal cord. However, combined treatment with NMDA antagonist and EA may produce synergistic anti-hyperalgesia via inhibition of activation of both the NMDA NR2B subunit and ERK.

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References

1. Mori H and Mishina M: Structure and function of the NMDA receptor channel. Neuropharmacology 34: 1219-1237, 1995.
2. Guo W, Wei F, Zou S, Robbins MT, Sugiyo S, Ikeda T, Tu JC, Worley PF, Dubner R and Ren K: Group I metabotropic glutamate receptor NMDA receptor coupling and signaling cascade mediate spinal dorsal horn NMDA receptor 2B tyrosine phosphorylation associated with inflammatory hyperalgesia. J Neurosci 24: 9161-9173, 2004.
3. Pezet S, Marchand F, D'Mello R, Grist J, Clark AK, Malcangio M, Dickenson AH, Williams RJ and McMahon SB: Phosphatidylinositol 3-kinase is a key mediator of central sensitization in painful inflammatory conditions. J Neurosci 28: 4261-4270, 2008.
4. Ji RR, Samad TA, Jin SX, Schmoll R and Woolf CJ: p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. Neuron 36: 57-68, 2002.
5. Galan A, Lopez-Garcia JA, Cervero F and Laird JM: Activation of spinal extracellular signaling-regulated kinase-1 and -2 by intraplantar carrageenan in rodents. Neurosci Lett 322: 37-40, 2002.
6. Johnson GL and Lapadat R: Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 298: 1911-1912, 2002.
7. Wang JQ, Fibuch EE and Mao L: Regulation of mitogen-activated protein kinases by glutamate receptors. J Neurochem 100: 1-11, 2007.
8. Peng HY, Cheng YW, Lee SD, Ho YC, Chou D, Chen GD, Cheng CL, Hsu TH, Tung KC and Lin TB: Glutamate-mediated spinal reflex potentiation involves ERK 1/2 phosphorylation in anesthetized rats. Neuropharmacology 54: 686-698, 2008.
9. Ji RR, Gereau RW IV, Malcangio M and Strichartz GR: MAP kinase and pain. Brain Res Rev 60: 135-148, 2009.
10. Kawasaki Y, Kohno T, Zhuang ZY, Brenner GJ, Wang H, Van Der Meer C, Befort K, Woolf CJ and Ji RR: Ionotropic and metabotropic receptors, protein kinase A, protein kinase C, and Src contribute to C-fiber-induced ERK activation and cAMP response element-binding protein phosphorylation in dorsal horn neurons, leading to central sensitization. J Neurosci 24: 8310-8321, 2004.
11. Choi BT, Lee JH, Wan Y and Han JS: Involvement of ionotropic glutamate receptors in low frequency electroacupuncture analgesia in rats. Neurosci Lett 377: 185-188, 2005.
12. Zhang YQ, Ji GC, Wu GC and Zhao ZQ: Excitatory amino acid receptor antagonists and electroacupuncture synergetically inhibit carrageenan-induced behavioral hyperalgesia and spinal fos expression in rats. Pain 99: 525-535, 2002.
13. Zhang GH, Yoon YW, Lee KS, Min SS, Hong SK, Park JY and Han HC: The glutamatergic N-methyl-D-aspartate and non-N-methyl-D-aspartate receptors in the joint contribute to the induction, but not maintenance, of arthritic pain in rats. Neurosci Lett 351: 177-180, 2003.

14. Størkson RV, Kjørsvik A, Tjølsen A and Hole K: Lumbar catheterization of the spinal subarachnoid space in the rat. J Neurosci Methods 65: 167-172, 1996.
15. Hama AT: Acute activation of the spinal cord metabotropic glutamate subtype-5 receptor leads to cold hypersensitivity in the rat. Neuropharmacology 44: 423-430, 2003.
16. Gao X, Kim HK, Chung JM and Chung K: Enhancement of NMDA receptor phosphorylation of the spinal dorsal horn and nucleus gracilis neurons in neuropathic rats. Pain 116: 62-72, 2005.
17. Obata K, Yamanaka H, Dai Y, Mizushima T, Fukuoka T, Tokunaga A and Noguchi K: Activation of extracellular signal-regulated protein kinase in the dorsal root ganglion following inflammation near the nerve cell body. Neuroscience 126: 1011-1021, 2004.
18. Xu Q, Garraway SM, Weyerbacher AR, Shin SJ and Inturrisi CE: Activation of the neuronal extracellular signal-regulated kinase 2 in the spinal cord dorsal horn is required for complete Freund's adjuvant-induced pain hypersensitivity. J Neurosci 28: 14087-14096, 2008.
19. Iwata K, Tashiro A, Tsuboi Y, Imai T, Sumino R, Morimoto T, Dubner R and Ren K: Medullary dorsal horn neuronal activity in rats with persistent temporomandibular joint and perioral inflammation. J Neurophysiol 82: 1244-1253, 1999.
20. Ji RR, Baba H, Brenner GJ and Woolf CJ: Nociceptive-specific activation of ERK in spinal neurons contributes to pain hypersensitivity. Nat Neurosci 2: 1114-1119, 1999.
21. Agell N, Bachs O, Rocamora N and Villalonga P: Modulation of the Ras/Raf/MEK/ERK pathway by Ca(2+), and calmodulin. Cell Signal 14: 649-654, 2002.
22. Caudle RM, Perez FM, Del Valle-Pinero AY and Iadarola MJ: Spinal cord NR1 serine phosphorylation and NR2B subunit suppression following peripheral inflammation. Mol Pain 1: 25, 2005.
23. Guo W, Zou S, Guan Y, Ikeda T, Tal M, Dubner R and Ren K: Tyrosine phosphorylation of the NR2B subunit of the NMDA receptor in the spinal cord during the development and maintenance of inflammatory hyperalgesia. J Neurosci 22: 6208-6217, 2002.
24. Ji RR and Rupp F: Phosphorylation of transcription factor CREB in rat spinal cord after formalin-induced hyperalgesia: relationship to c-fos induction. J Neurosci 17: 1776-1785, 1997.
25. Clark AK, D'Aquisto F, Gentry C, Marchand F, McMahon SB and Malcangio M: Rapid co-release of interleukin 1beta and caspase 1 in spinal cord inflammation. J Neurochem 99: 868-880, 2006.
26. Ji RR and Suter MR: p38 MAPK, microglial signaling, and neuropathic pain. Mol Pain 3: 33, 2007.
27. Kawasaki Y, Zhang L, Cheng JK and Ji RR: Cytokine mechanisms of central sensitization: distinct and overlapping role of interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha in regulating synaptic and neuronal activity in the superficial spinal cord. J Neurosci 28: 5189-5194, 2008.