N-Methyl-D-Aspartate Antagonist Inhibits NR-1 Subunit Phosphorylation of the Spinal N-Methyl-D-Aspartate Receptor Induced by Low Frequency Electroacupuncture

Byeol-Rim Kang and Chang-Beohm Ahn

Department of Acupuncture and Moxibustion, College of Oriental Medicine
Dong-Eui University, Busan 614-052, Korea

Byung-Tae Choi

Department of Anatomy, School of Oriental Medicine
Pusan National University, Busan 609-735, Korea

Abstract: We investigated whether the 2 Hz electroacupuncture (EA) analgesia is associated with phosphorylation of N-methyl-D-aspartate receptor (NMDAR) NR-1 subunits and NMDAR antagonism in the lumbar spinal cord of rats. EA stimulation produced an increase of serine phosphorylation of NMDAR NR-1 subunits in the spinal cord as compared with normal conditions. However, the intrathecal injection of NMDAR antagonist D-2-amino-5-phosphonopentanoic acid significantly prevented serine phosphorylation of NMDAR NR-1 subunits induced by EA stimulation in the dorsal horn of spinal cord. These results indicate that EA analgesia by stimulation of peripheral nerves may be involved in an increase of NR-1 serine phosphorylation in the dorsal horn of the spinal cord.

Keywords: Electroacupuncture; Phosphorylation; NMDA Receptor; D-2-Amino-5-Phosphonopentanoic Acid.

Introduction

Some forms of prolonged analgesia lasting for hours or days are found during the stimulation of peripheral nerves, such as electroacupuncture (EA) analgesia (Melzack et al., 1983). Since the gate control theory cannot satisfactorily explain the long-lasting...
analgesia, the mechanisms of learning and memory in the brain have been introduced into pain research (Sandkühler, 1996; Liu et al., 1998a). The long-term synaptic alterations apparently involve activation of N-methyl-D-aspartate receptors (NMDAR) (Miletic and Miletic, 2000). The NMDAR are known to be a contribution in excitatory synaptic transmission within the spinal cord when evoked by nociceptive primary afferent stimuli in normal animals (Gao et al., 2005).

The ionotropic glutamate receptors are involved in the maintenance of EA analgesia in the spinal dorsal horn of normal animals (Choi et al., 2005). Protein phosphorylation is a major mechanism for the regulation of receptor function. The phosphorylation of NMDAR enhances the response to NMDA in various neurons of the central nervous system (Li and Zhou, 1998; Gao et al., 2005). In the present study, we examined NR-1 phosphorylation and found that an increase in NR-1 serine phosphorylation was associated with EA analgesia and that these phosphorylations were inhibited by the NMDAR antagonist.

**Materials and Methods**

**Animals and Intrathecal (i.t.) Injection**

Male Sprague-Dawley rats, weighing about 120 g on average, were obtained from Hyochang Science Co. in Korea. All experiments conformed to guidelines approved by the National Institute of Health Guide for the Care and Use of Laboratory Animals. The i.t. catheterization was performed according to the methods of Størkson et al. (1996) under 10% chloral hydrate anesthesia (350 mg/kg i.p.).

**EA Stimulation**

Two stainless-steel needles of 0.25 mm diameter were inserted in each hind leg of rat at those acupoints corresponding to Zusanli and Sanyinjiao in human. They were then connected to an electric stimulator (Saechang SM-60, Korea). The NMDA antagonist, D-2-amino-5-phosphonopentanoic acid (AP-5, Sigma Co.) was dissolved in sterile saline and injected intrathecally in a volume of 10 µl via catheter within 1 min and then filled with 8 µl of saline for flushing. The 2 Hz of EA stimulation started 30 min after AP-5 injection.

**SDS-PAGE and Western Blot Analysis**

For Western blot analysis, the L₄-₅ segments of the spinal cords were removed at 60 min after the termination of EA stimulation. The spinal cords were washed in a cold HEPES buffer, and homogenized in 9 volumes of lysis buffer. Equal amounts of proteins were separated by 8–12% SDS-PAGE. The resulting gels were transferred to immobilon-P transfer membranes (Millipore, Bedford, MA) and blocked with 10% skim milk in PBS-T (0.1% Tween-20 in PBS) for 1 hour at room temperature. After rinsing with PBS-T, the membranes were incubated with anti-NR-1 (Sc9058, 1 µg/ml, Santa Cruz
Biotechnology, Santa Cruz, CA) and anti-phospho-NR-1 (S896, 1 µg/ml, Upstate, Lake Placid, NY) for 1–2 hours at room temperature, and then the blots were incubated with horseradish peroxidase-conjugated secondary antibody and the antibody-specific proteins were visualized by the enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL).

**Immunohistochemical Analysis**

The spinal cords were fixed in 4% paraformaldehyde and immersed in 30% sucrose for 48 hours at 4°C for cryoprotection. Frozen 30 µm thick sections were prepared and collected to be processed immunohistochemically as free floating sections. These sections were pre-incubated in 0.3% H$_2$O$_2$ for 15 min, and then incubated in a blocking solution containing 3% normal goat serum and 0.3% Triton-X 100 in PBS for 30 min at room temperature. Sections were incubated for 16 hours at 4°C with a rabbit polyclonal anti-c-fos antibody (1:500, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-NR-1 and anti-phospho-NR-1 (Santa Cruz Biotechnology) diluted in PBS containing 0.3% Triton X-100. After being washed with PBS, sections were incubated with the secondary antibody, biotinylated anti-rabbit IgG for 30 min, followed by washing with PBS. Sections were further incubated with avidin-biotin-peroxidase complex kit (Vector, Burlingame, CA) for 60 min at room temperature. Diaminobenzidine substrate kit (Vector) to detect peroxidase was applied. As controls, treatments with primary and secondary antibodies were omitted.

**Quantification and Data Analysis**

To quantify laminar expression, the dorsal horn of the spinal cord was divided into 3 regions: the superficial laminae (SDH, laminae I and II), the nucleus proprius (NP, laminae III and IV) and the neck of the dorsal horn (NECK, laminae V and VI). The number of c-fos-like immunostained neurons were counted and averaged per section. For the expression of NMDAR, images of dorsal horns were captured at x100 magnification using a digital CCD camera (AxioCam, Carl Zeiss, Jena, Germany). The integrated optical density (IOD) of each entire region of the dorsal horn was measured automatically using Visus Image Analysis software (Foresthill Products Ista-Video TeXt, Foresthill, CA). Calculations of means, standard errors and student’s t-test were made using SigmaPlot. p < 0.05 was considered statistically significant.

**Results**

**Immunohistochemical Analysis for c-fos Expression**

To monitor the activity of neurons in the spinal cord after EA stimulation, we first determined the expression of c-fos in the dorsal horn of rats. The c-fos-immunostained neurons were significantly increased as compared with normal conditions by EA stimulation, but i.t. injection of AP-5 arrested markedly the increase in these neurons (Fig. 1).
Western Blot Analysis for NMDAR NR-1 Subunit

In the Western blot analysis, the higher levels of phospho-NR-1 but not NR-1 of NMDAR in the spinal cord was induced by EA stimulation. The expressions of phospho-NR-1, which may play roles in EA responses, were also inhibited by i.t. injection of AP-5 (Fig. 2).
Immunohistochemical Analysis for NMDAR NR-1 Subunit

As for regional expression of NMDAR NR-1 and phospho-NR-1 in the spinal cord, immunoreactions were observed throughout all laminae of the dorsal horn and more intensive expressions in the SDH region. In each regions of the spinal cord, there was no significant difference in mean IOD for both NR-1 and phospho-NR-1 between EA-treated and normal or AP-5-injected rats. But the mean IOD of phospho-NR-1 in the whole dorsal horn were significantly inhibited by i.t. injection of AP-5 (Figs. 3 and 4).

Figure 3. Effects of EA stimulation with i.t. injection of AP-5 on the mean IOD of NR-1 subunits expression in the dorsal horn of the L4-5 segment (n = 5). A, normal group; B, EA-treated group; C, EA-treated group with AP-5 injection. Scale bar = 200 µm.

Figure 4. Effects of EA stimulation with i.t. injection of AP-5 on the mean IOD of phospho-NR-1 expression in the dorsal horn of the L4-5 segment (n = 5). Note a marked decrease of the mean IOD in dorsal horn by i.t. injection of AP-5 at both concentration of 1.0 and 5.0 µg/ml. *p < 0.05 as compared to EA-treated group. A, normal group; B, EA-treated group; C, EA-treated group with AP-5 injection. Scale bar = 200 µm.
Discussion

Conditioning stimulation of the peripheral nerve can evoke two fundamentally different forms of spinal analgesia. The afferent-induced long-lasting antinociception requires activation of A\(\delta\) fiber. This is different from the antinociception during stimulation of the sensory nerve at A\(\alpha/\beta\) fiber intensity which is short-lived (Liu et al., 1998b). The long-lasting analgesia following EA at a higher A\(\delta\)-fiber intensity is best explained by a long-term depression of synaptic strength in A\(\delta\) and C fiber (Sandkhüler, 1996). The conditioning stimulation of A\(\delta\) fibers induces the release of glutamate from nerve terminals in the spinal dorsal horn that activates NMDAR.

It is known that ionotropic glutamate antagonist causes an attenuation of the state of long-term potentiation in various pain models. EA and NMDAR antagonists have synergetic anti-nociceptive effects against carrageenan-induced hyperalgesia (Zhang et al., 2002). But EA analgesia underlies some involvement of ionotropic receptors in maintenance and is inhibited by the antagonism of NMDA in normal rats (Choi et al., 2005). Furthermore, the long term plastic alterations in synaptic transmission can be regulated by protein phosphorylation such as NMDAR. NMDAR subunits can be phosphorylated by either PKA or PKC. The phosphorylated receptors lead to an increased conductance for Ca\(^{++}\) influx and this influx is necessary for inducing long-term neuronal changes (Hatt, 1999).

Among several NMDAR, NR-1 is required as a subunit forming functional ion channel and is widely distributed in the central nervous system (Cull-Candy et al., 2001). The NR-1 subunits forming a functional ion channel can be activated by phosphorylation and phosphorylated subunits are related to enhancement and synaptic efficacy (Gao et al., 2005). The Serine 897 on NR-1 subunits is phosphorylated by PKA, while serine 890 and 896 are phosphorylated by PKC (Tingley et al., 1997; Gao et al., 2005).

Thus we examined the hypothesis that the development of EA analgesia is associated with NMDAR, especially in enhancement of Ser-896 serine phosphorylation of NMDAR NR-1, in the present study. We found that serine phosphorylation of NMDAR NR-1 was produced by EA treatment. Therefore, we tested NMDA antagonism in EA-treated rats by i.t. injection of AP-5. Interestingly, phosphorylation of NMDAR NR-1 was prevented by i.t. injection of AP-5 in both Western and immunohistochemical analysis.

Recent work has demonstrated that phosphorylation of spinal cord NR-1 subunits of the NMDA receptor is associated with nociception including wind up and central sensitization in pain models (Caudle et al., 2005). The present data agree with the previous study where spinal cord phospho-NR-1, but not NR-1, expression is enhanced in capsaicin-induced central sensitization (Caudle et al., 2005; Gao et al., 2005). These previous studies presented that phosphorylation is closely related to the persistence of peripheral noxious stimulation.

The present data suggests that phosphorylated serine 896 on the NR-1 subunits leads to an increase in NMDAR activity in the spinal cord following peripheral EA stimulation. Thus NR-1 subunits may be involved in EA analgesia in the spinal dorsal horn and share the same process as NMDAR in central nociception. The possible mechanism of low frequency EA in the spinal dorsal horn are presently not known, but low frequency EA may present
analgesic effects at stimulation intensities that produce tolerance nociceptive stimulation (Ward et al., 1996). Consequently, EA analgesia may be produced by peripheral electrical stimulation as one sort of noxious one and NMDAR antagonist prevents EA analgesia by inhibiting serine phosphorylation of NR-1 subunits.

References


