Participation of K_{ATP} Channels in the Antinociceptive Effect of Pregabalin in Rat Formalin Test

[1]Department of Anesthesiology and Pain Medicine, [2]Anesthesia and Pain Research Institute,
Yonsei University College of Medicine, Seoul, Korea

Tae Dong Kweon, MD[1,2], Ji Young Kim, MD[1], Il Won Kwon, MD[1],
Jong Bum Choi, MD[1], and Youn Woo Lee, MD[1,2]

Background:

Pregabalin is an anticonvulsant and analgesic agent that interacts selectively with the voltage-sensitive-Ca^{2+}-channel alpha-2-delta subunit. The aim of this study was to evaluate whether the analgesic action of intrathecal (IT) pregabalin is associated with K_{ATP} channels in the rat formalin test.

Methods:

IT PE-10 catheters were implanted in male Sprague-Dawley rats (250−300 g) under inhalation anesthesia using enflurane. Nociceptive behavior was defined as the number of hind paw flinches during 60 min after formalin injection. Ten min before formalin injection, IT drug treatments were divided into 3 groups: normal saline (NS) 20 μl (CON group); pregabalin 0.3, 1, 3 and 10 μg in NS 10 μl (PGB group); glibenclamide 100 μg in DMSO 5 μl with pregabalin 0.3, 1, 3 and 10 μg in NS 5 μl (GBC group). All the drugs were flushed with NS 10 μl. Immunohistochemistry for the K_{ATP} channel was done with a different set of rats divided into naïve, NS and PGB groups.

Results:

IT pregabalin dose-dependently decreased the flinching number only in phase 2 of formalin test. The log dose response curve of the GBC group shifted to the right with respect to that of the PGB group. Immunohistochemistry for the K_{ATP} channel expression on the spinal cord dorsal horn showed no difference among the groups 1 hr after the formalin test.

Conclusions:

The antinociceptive effect of pregabalin in the rat formalin test was associated with the activation of the K_{ATP} channel. However, pregabalin did not induce K_{ATP} channel expression in the spinal cord dorsal horn. (Korean J Pain 2011; 24: 131-136)

Key Words:

antinociception, formalin test, K_{ATP} channel, pregabalin.

INTRODUCTION

Pregabalin is an anticonvulsant analgesic agent that binds to the alpha-2-delta subunit of voltage sensitive Ca^{2+} channels in the presynaptic area [1-5]. As for the site of action, intrathecal pregabalin reduces presynaptic neurotransmitter release without altering postsynaptic receptors [3]. However, gabapentin and pregabalin reduced the K^+-evoked glutamate release without affecting basal glutamate levels [6]. And the actions of pregabalin may involve both extracellular and intracellular drug target sites and modulation of a variety of neuronal conductances, by direct interactions and through intracellular signaling involving protein kinase A [7].

Opioid receptor agonists open K channels in neurons through the activation of G-proteins. The analgesic effect of morphine can be prevented by the inhibition of K_{ATP} channels and GIRK channels [8]. These 2 types of K channel activations lead to cell membrane hyperpolarization and are involved in opioid induced antinociception [9]. In addition, morphine activates presynaptic opioid G-protein-coupled mu-opioid receptors or inhibits calcium currents, thereby reducing neurotransmitter release [10].

Gabapentin or pregabalin reduce excitatory neurotransmitter release by binding to the alpha-2-delta subunit of voltage sensitive Ca^{2+} channel even though they are not Ca^{2+} blockers [2]. Pregabalin reduces the excitatory properties of cultured DRG neurons by modulating voltage-activated Ca^{2+} and K^+ channels [7]. On the other hand, the anti-allodynic effect of gabapentin was produced by the activation of Ca^{2+} activated and ATP-sensitive K^+ channels [11]. These results led us to postulate that an association between K_{ATP} channels and the analgesic effect of pregabalin may exist. In addition, if K_{ATP} channels are associated with pregabalin action, the evaluation of K_{ATP} channel expression in the spinal cord after intrathecal injection of pregabalin will yield important information on the mechanism of the analgesic effect of pregabalin.

The aim of this study was to evaluate whether the analgesic action of intrathecal pregabalin is associated with K_{ATP} channels in the rat formalin test. In addition, the effect of pregabalin on K_{ATP} channel expression in the spinal cord dorsal horn was also evaluated.

MATERIALS AND METHODS

This study was approved by the Institutional Animal Care Committee. Adult male Sprague-Dawley rats weighing 250-300 g were housed in individual cages on a 12 h night/day cycle at a constant temperature of 20-22°C. Food and water were available *ad libitum*.

1. Catheter implantation

For intrathecal (IT) drug administration, rats were chronically implanted with catheters as previously described [12]. Briefly, under enflurane anesthesia, the rat were placed in a stereotaxic head holder. The occipital muscles were separated and retracted caudal to expose the cisternal membrane at the base of the skull. A polyethylene tube (PE-10 catheter) was passed caudally from the cisterna magna to the level of the lumbar enlargement. After bleeder ligation, the tissues were sutured layer by layer. Rats with evidence of neuromuscular dysfunction were promptly sacrificed. After 5-7 days of the catheterization, rats without neurological deficit and infection were considered for data analysis.

2. Neurobehavioral test

1) Drugs: Forty five rats were divided into 3 groups according to the IT administered drug. In the control (CON) group (N = 5), normal saline 10 μl was administered as a placebo and flushed with normal saline 10 μl. In the pregabalin (PGB) group (N = 20), pregabalin 0.3, 1, 3 or 10 μg dissolved in normal saline 10 μl were administered and flushed with normal saline 10 μl. In the glibenclamide (GBC) group (N = 20), IT injection of glibenclamide 100 μg dissolved in dimethyl sulfoxide (DMSO) 5 μl was followed by pregabalin 0.3, 1, 3, or 10 μg dissolved in normal saline 5 μl and flushed with normal saline 10 μl.

2) Formalin test: After 10 min of IT drug administration, 5% formalin solution 50 μl was injected intradermally to the dorsal surface of the left hind paw. Nociceptive behavior was determined by the number of flinches of the left hind paw during 60 min. Flinching per minute was averaged over 5 min. The initial 10 min was classified as phase 1 and the next 11-60 min as phase 2. Maximum possible effect (%MPE) was calculated as follows:

$$\frac{(\text{No. of flinching in control gr.} - \text{No. of flinching in experimental gr.})}{\text{No. of flinching in control gr.}} \times 100$$

3. Immunohistochemistry

1) Perfusion fixation: Immunohistochemitry was done separately with a different set of 13 rats. In the naïve group (N = 4), rats were deeply anesthetized 1 hr after administration of intrathecal normal saline 20 μl without formalin test. In both the normal saline group (N = 4) and pregabalin group (N = 5), the formalin test was done 10 min after IT administration of normal saline 20 μl or pregabalin 10 μg dissolved in normal saline 10 μl and flushed with normal saline 10 μl. Rats were deeply anesthetized and fixed by vascular perfusion with paraformaldehyde 1 hr after drug injection. The spinal cord was dissected and placed in 4% paraformaldehyde for an additional 2 h. Then the spinal cord was transferred to a 30% sucrose solution and incubated for 1 day at 4°C for cytoprotection. A microtome (Leica Microsystems, Wetzlar, Germany) was used to cut 40 μm floating sections stored in phosphate buffer. Sections were rinsed 3 times in PBS then blocked in 4% goat serum and 0.3% Triton X-100. Antigen was retrieved by the heat induced epitope retrieval method after rehydration. Inactivation of endogenous peroxidase was conducted with 0.3% H_2O_2 incubated for 5 min at room temperature. After 3 more PBS rinses, sections were incubated for 1 hour at room temperature and over-night with the primary antibodies (1 : 100 Kir 6.1 (H-80)) diluted in blocking buffer at 4°C. Sections were rinsed again with PBS and incubated for 20 min at room temperature with secondary antibodies (anti-rabbit IgG, Envision kit, DAKO: Santa Cruz Biotechnology, Inc. Santa Cruz, USA). The slices were counterstained with hematoxylin and dried before they were mounted with media.

2) Imaging: Images were taken using a SP1 confocal microscope and image capturing software (Leica Microsystems, Wetzlar, Germany). Quantifications were done using the Metamorph Imaging System software (Universal Imaging Corporation). Average pixel intensities within a region that approximated the laminae I-II of the ipsilateral spinal cord dorsal horn were calculated and normalized to a region of a comparable area in the contralateral dorsal horn. Threshold intensities were determined for each image above with most of the background noise suppressed. A rectangular box of constant size was placed over the entire width of the dorsal horn and the area of pixels within the box with an intensity value above the threshold was determined for both the injured and uninjured side. The results are expressed as the percentage change in staining ipsilateral to the injury compared to the uninjured side. The calculated values of percent change for each section were then averaged to yield a value for each animal, and a mean and standard error were determined per group. The experimenter was blinded to the treatment group during image collection and quantification.
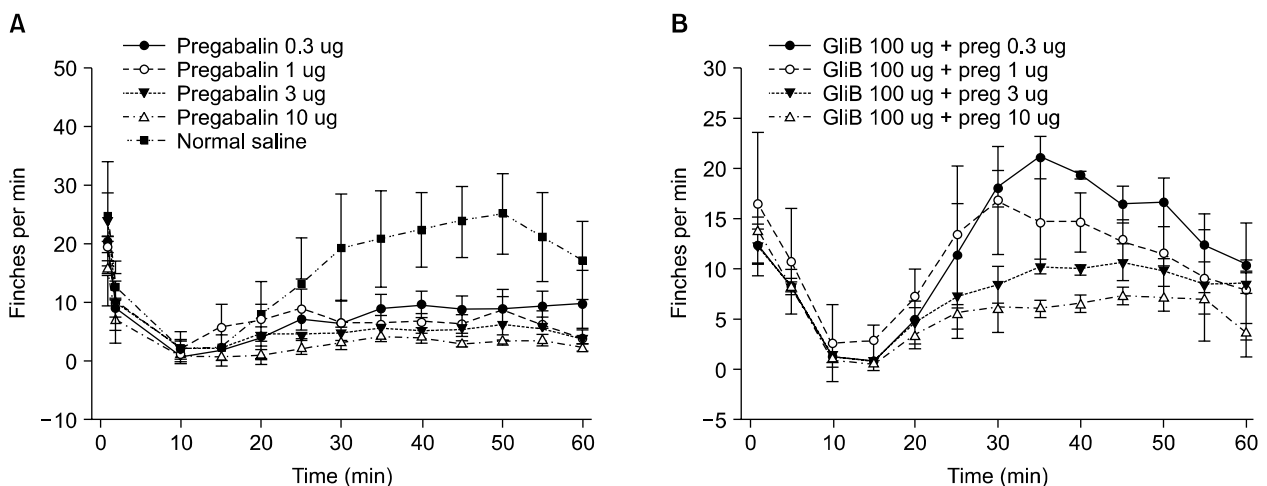

Fig. 1. Time course changes of flinching after intrathecal administration of pregabalin (A) and glibenclamide (GliB) with pregabalin (preg) (B) in the formalin test. Data are presented as the number of flinching. Each line represents the mean ± SEM of bar in 5 rats.

4. Statistical analysis

All of the data were presented as the mean ± SEM. The dose-response data were expressed as %MPE in each phase. The dose response data was analyzed using one-way ANOVA with Scheffe for *post hoc*. The ED_{50} values were analyzed using dose-response analysis and the difference in the ED_{50} between the glibenclamide group and pregabalin group was analyzed with the student's t-test. One-way ANOVA was used for comparison of the K_{ATP} channel expression in the spinal cord among groups. If the
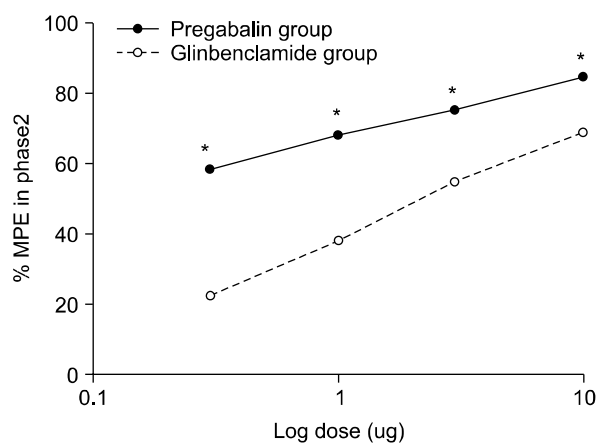
P value was less than 0.05, the results were considered statistically significant.

RESULTS

1. Neurobehavioral test

1) After IT injection of pregabalin and/or glibenclamide, all animals showed no change in motor function such as the placing/stepping reflex and righting reflex tests.

2) A subcutaneous injection of formalin resulted in a highly reliable biphasic display of flinching of the injected paw (Fig. 1A). IT injection of pregabalin decreased the sum of flinches in a dose dependent manner in phase 2 ($P <$ 0.05), but not in phase 1. Additionally, a fixed dose of glibenclamide reversed the analgesic effects of pregabalin (Fig. 1B).

3) The %MPE curve and ED_{50} of pregabalin shifted to the right in a log dose response curve compared to that of the glibenclamide pretreated rats ($P <$ 0.01)(Fig. 2).

2. Immunohistochemistry

The results of the immunohistochemistry on the spinal cord dorsal horn showed no difference among the naïve group (no formalin test), normal saline group (IT normal saline 20 μl 10 min before formalin injection) and pregabalin group (IT pregabalin 10 μg administered 10 min before formalin injection) in pixel intensities for K_{ATP} channels (Fig. 3, Table 1).
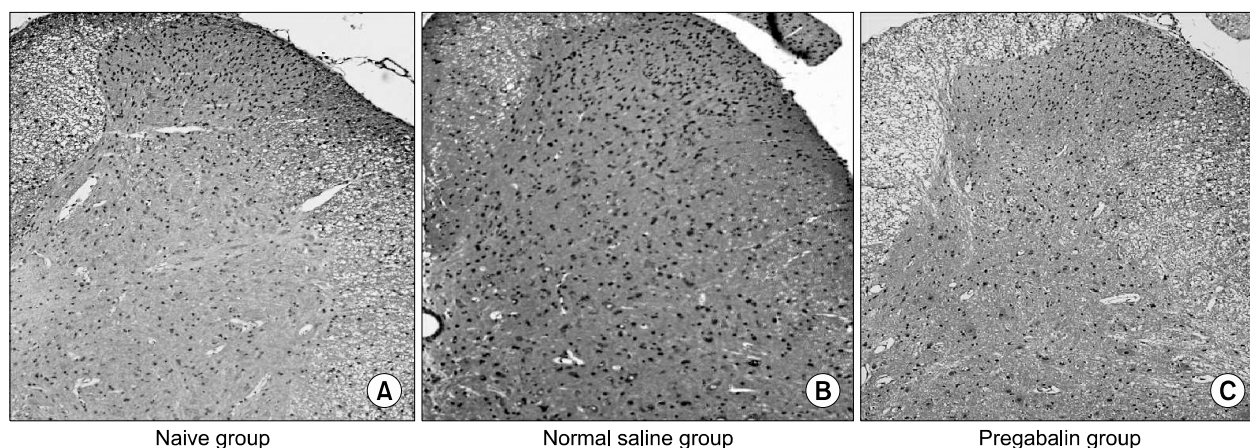

Fig. 2. Log dose response curve of intrathecal pregabalin. Log dose response curve of intrathecal pregabalin (ED_{50} = 0.39 μg) shifted to the right (ED_{50} = 2.34 μg) when glibenclamide was added. *$P <$ 0.01 vs glibenclamide group.

Naive group   Normal saline group   Pregabalin group

Fig. 3. Results of Immunohistochemistry of K_{ATP} channel on the spinal cord dorsal horn (magnification of 10×). The results of immunohistochemistry on the spinal cord dorsal horn shows no difference among the naïve group (A, no formalin test), normal saline group (B, IT normal saline 10 min before formalin injection) and pregabalin group (C, IT pregabalin 10 μg administered 10 min before formalin injection) in pixel intensities of K_{ATP} channel.

Table 1. Comparison of Expression of K_{ATP} Channel in the Spinal Cord Dorsal Horn

Group	N	Area change (%)
Naïve	4	-0.2 ± 0.3
NS	4	-0.3 ± 0.2
PGB	5	-0.3 ± 0.3

Values are expressed as % mean area change $\pm$ SEM. NS: normal saline, PGB: pregabalin.

DISCUSSION

In the present study, IT pregabalin in a dose-dependent manner decreased the number of flinching in phase 2 of the formalin test and the log dose response curve IT pregabalin was shifted to the right when glibenclamide was added. There was no change in K_{ATP} channel expression in the spinal cord dorsal horn after IT pregabailn treatment in the formalin induced pain model.

In this study, the antinociceptive effect of IT pregabalin was inhibited by glibenclamide, which suggests K_{ATP} channel activation as the antinociceptive mechanism of IT pregabalin. This is in agreement with a previous study, which reported that the antiallodynic effect of gabapentin in a spinal nerve ligation model was associated with ATP-sensitive K channels [11]. The K_{ATP} channel is known to exert its antinociceptive effect by regulating the excitability of peripheral neurons and inhibiting neurotransmitters such as substance P in the spinal cord [13–15]. This may explain the preemptive analgesic effect of pregabalin in this study

A recent review demonstrated that although perioperative pregabalin administration did not reduce pain intensity, opioid consumption was significantly decreased during the first 24 h after surgery [16]. Gabapentin and pregabalin modulate the pain pathway by targeting the presynaptic Ca^{2+} channel alpha-2-delta subunit, inhibiting K^+-induced Ca^{2+} influx and reducing the release of presynaptic glutamate and aspartate [17]. It has also been reported that the K_{ATP} channel opening effect of pregabalin is one of the mechanism for reducing neuronal excitability [18]. In Chung's model, an IT K_{ATP} inhibitor such as glibenclamide and a Ca^{2+} activated K^+ channel inhibitor such as charybdotoxin and apamin reduced the antiallodynic effect of gabapentin [11]. A subsequent study showed that this effect was associated with the NO-cyclic GMP-PKG-K^+ channel pathway [19]. Nitric oxide (NO) is synthesized during the pain process and it activates guanylate cyclase, which increases cGMP and opens the K^+ channel [20]. Administration of a (Rp)-analogue of c-AMP prevents an increase in K^+ current induced by pregabalin, which means that the intracellular response of pregabalin is associated with protein kinase A [7]. Another potassium channel suspected to be associated with pain modulation is the big potassium (BK) channel, which is a large conductance Ca^{2+} and voltage activated K^+ channel. Non-inactivating BK channels with low activity can be activated by protein kinase A [21]. BK channel also have an important role in pain modulation and found in the dorsal root ganglion [22]. Therefore, BK channels can be a possible target of pregabalin but further research is needed to clarify the mechanism.

Opening of the K_{ATP} channels results in an efflux of K^+ and occurs by membrane hyperpolarization and reduction in excitability. Therefore, the up-regulation of K_{ATP} channels have a protective effect when ischemia or reperfusion cause tissue injury by Ca^{2+} overload and can be a promising target for pain control. However, our results showed that there is no change in channel expression for K_{ATP} in the spinal cord under the rat formalin test. This result is different from Yin's report that Kir 6.2 mRNA expression was significantly increased after sciatic nerve injury [23]. This may have resulted from the difference between the inflammatory pain model and the neuropathic pain model in terms of K_{ATP} channel involvement in the pain modulation process.

Our study has the following limitations. Animals for the immunohisochemistry analyses were sacrificed 1 hr after the formalin test and there was no difference in gene expression in our study. As with other studies the change in gene expression can be delayed up to 24 hours, and the result such that the expression of the K_{ATP} channels did not change at 1 hour after the formalin test.

The antinociceptive effect of pregabalin in the rat formalin test was indirectly associated with the activation of the K_{ATP} channels. However, pregabalin did not induce K_{ATP} channel expression in the spinal cord dorsal horn 1 hr after the formalin test.

ACKNOWLEDGEMENTS

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