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Role of Prostaglandin Receptor EP_1 in the Spinal Dorsal Horn in Carrageenan-induced Inflammatory Pain

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Background: **Prostaglandin E_2 (PGE_2) and the receptor for PGE_2 (EP receptor) are key factors contributing to the generation of hyperalgesia caused by inflammation. The current study was designed to investigate the roles of PGE_2 and EP_1 receptors in the spinal cord in the development and maintenance of inflammatory pain, using behavioral, microdialysis, and intracellular calcium ion concentration ($[Ca^{2+}]_i$) assays.**

Methods: **Inflammation was induced by an injection of carrageenan into the plantar surface of the rat hind paw. The effects of inflammation were evaluated at the time points of 3 h (early phase) and 15 h (late phase) after carrageenan injection. In behavioral assays, withdrawal thresholds to mechanical stimuli were evaluated. The effect of an intrathecally administered selective EP_1 antagonist, ONO-8711, on the carrageenan-induced hyperalgesia was examined. Using a spinal microdialysis method, PGE_2 concentration in the spinal dorsal horn was measured. In $[Ca^{2+}]_i$ assays, we measured $[Ca^{2+}]_i$ in the spinal dorsal horn in transverse spinal slices and examined the effects of pretreatment with ONO-8711. Sensitivities of the changes in $[Ca^{2+}]_i$ to PGE_2 perfusion were also assessed.**

Results: **Mechanical hyperalgesia and paw edema were observed in both the early and late phases. The hyperalgesia was inhibited by intrathecal ONO-8711 in the late, but not early, phase. The concentration of PGE_2 in the spinal dorsal horn increased in the late phase. The $[Ca^{2+}]_i$ in the dorsal horn increased on the ipsilateral side to the inflammation in the late, but not early phase. This increase was suppressed by the pretreatment with ONO-8711. Magnitude of the increase in $[Ca^{2+}]_i$ on the ipsilateral side in response to PGE_2 perfusion was greater in the late phase than in the early phase.**

Conclusion: **The results suggested that activation of spinal EP_1 receptors was crucial in the carrageenan-induced mechanical hyperalgesia in the late phase. It seems that some of the mechanisms underlying inflammation-induced plastic changes are mediated by time-dependent increase in PGE_2 concentration, activation of EP_1 receptors, and increase in $[Ca^{2+}]_i$ in the spinal dorsal horn.**

PERIPHERAL tissue damage and inflammation elicit pain-related behaviors such as spontaneous pain, hyperalgesia, and allodynia. At the site of inflammation, prostaglandins synthesized by the inducible isoform of cyclo-oxygenase (cyclooxygenase-2) sensitize peripheral nociceptors through the activation of receptors for prostaglandin E_2 (PGE_2), EP receptors, on peripheral nerve terminals.[1,2] Recent evidence indicates that PGE_2 is also produced in the spinal cord after tissue injury.[3,4] Moreover, behavioral and electrophysiologic studies have suggested that PGE_2 facilitates nociceptive transmission in the spinal cord,[5,6] contributing to central sensitization, an increase in excitability of spinal dorsal horn neurons. Since ongoing inputs from the damaged peripheral sites persist and affect central regions, spinal EP receptors may be repeatedly activated. The repetitive activation of EP receptors may initiate intracellular cascades in the dorsal horn neurons, resulting in induction and maintenance of central sensitization after inflammation.

It is known that PGE_2 activates different second messenger pathways. The receptors for PGE_2 are subdivided into four subtypes (EP_1, EP_2, EP_3, and EP_4) on the basis of the distinct genes and signal transduction pathways.[7] The activation of EP_1 receptors initiates an influx of calcium ions, resulting in an increase in intracellular calcium concentration ($[Ca^{2+}]_i$). The EP_2 and EP_4 receptors are essentially coupled to stimulation of adenylate cyclase, which leads to an elevation of intracellular cyclic adenosine monophosphate. The EP_3 receptors mediate an inhibition of adenylate cyclase, resulting in decrease of intracellular cyclic adenosine monophosphate.

The carrageenan model for inflammatory pain, which is characterized by a time-dependent increase in paw edema and by development of thermal and mechanical hyperalgesia, has been well established.[8–11] Although behavioral studies have shown that rats exhibit hyperalgesia in both the early (2–6 h) and the late (15–24 h) phases of carrageenan-induced inflammation,[12,13] recent studies have demonstrated that excitabilities in the spinal dorsal horn neurons are different and greater in the late phase than in the early phase.[14,15] These findings suggest that the behavioral hyperalgesia following carrageenan injection observed in the early phase is due to peripheral sensitization and that central sensitization contributes to maintenance of hyperalgesia in the late phase.

Recently, a novel selective EP_1 receptor subtype antagonist, 6-[(2S,3S)-3-(4-chloro-2-methylphenylsulfonylaminomethyl)-bicyclo[2.2.2]octan-2-yl]-5Z-hexenoic acid (ONO-8711), has been chemically synthesized.[16] ONO-8711 is the most selective antagonist for EP_1 receptors currently available. The Ki values of this compound in Chinese hamster ovary cell lines are 1.7 and 0.6 nM for mouse and human EP_1 receptors, respectively. Its Ki values for other receptors, including mouse DP, mouse EP_2, mouse EP_4, mouse FP, and human IP receptors, are greater than 1,000 nM.[16] In the current study, using ONO-8711, we conducted behavioral, microdialysis, and $[Ca^{2+}]_i$ assays to determine whether activation of EP_1

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receptors contributes to time-dependent plastic changes in the spinal cord in a carrageenan-induced inflammation.

Materials and Methods

The protocol for this study was approved by the Sapporo Medical University Animal Care and Use Committee. The animals used were male Sprague-Dawley rats (weighing 150–250 g; Japan SLC, Hamamatsu, Japan) that were housed individually in a temperature-controlled (21 ± 1°C) room with a 12-h light–dark cycle and given free access to food and water.

The current study consisted of three experiments: behavioral, microdialysis, and $[Ca^{2+}]_i$ measurement experiments.

Animal Model

In this study, we used a rat model of carrageenan-induced inflammatory pain. Unilateral peripheral inflammation was induced by an intraplantar injection of 2 mg carrageenan in a volume of 100 μl into the left hind paw. Experiments were performed with normal rats and rats that had been injected with carrageenan. Carrageenan-injected rats were used in the experiments 3 h (early phase) or 15 h (late phase) after the injection.

Behavioral Study

Animal Preparation. During general anesthesia (3% isoflurane in oxygen), a polyethylene intrathecal catheter (PE-10; Becton Dickinson, Sparks, MD) was inserted into the lumbar subarachnoid space at the L4–L5 intervertebrae with the tip of the catheter located near the lumbar enlargement of the spinal cord, using a method described previously.[17] Only animals that showed normal behavior and motor function were used in the experiments.

Evaluation of Hyperalgesia. To evaluate the mechanical hyperalgesia, withdrawal threshold to mechanical stimulation was determined using calibrated von Frey filaments (0.0045–75.8580 g in bending force; Stoelting, Wood Dale, IL) that were applied from underneath the cage through openings in the wire mesh floor to the plantar surface of the hind paw on the carrageenan-injected side and to the same area on the contralateral side. Each filament was applied once starting with 0.0045 g and continuing until a withdrawal response occurred. A withdrawal response was considered to be complete lifting of the hind paw off the surface of the cage or flinching. The test was repeated three times at each time point. The minimum force that produced a response to at least one of three applications was considered as the withdrawal threshold.

Effects of Intrathecal ONO-8711 on Mechanical Hyperalgesia. The effects of the EP_1 antagonist ONO-8711 were examined in normal and carrageenan-treated rats (n = 48). Before the carrageenan injection, withdrawal thresholds were measured as control values on each side of hind paw. The carrageenan-treated rats were used in the experiment in the early (3 h) or late (15 h) phase after carrageenan injection. Baseline values of the threshold were determined in the early and late phases. ONO-8711 (1, 10, or 100 μg) or saline in a volume of 10 μl was administered intrathecally. The thresholds were assessed for up to 120 min on both sides of the hind paw.

Evaluation of Paw Thickness. The magnitudes of inflammatory response to carrageenan were evaluated by measuring the thickness of the dorsal–ventral paw using a vernier micrometer.

Microdialysis Study

Construction of Microdialysis Probe and Implantation. A spinal cord dialysis probe was made according to our modification of the method described by Skilling *et al.*[18] The probe was constructed from a 1-cm-long dialysis fiber (ID of 200 μm, OD of 220 μm, and 50-kd molecular weight cutoff; DM-22; Eicom, Kyoto, Japan) that had been coated with an epoxy glue (Devcon, Danvers, MA) along the whole length except for a 2-mm region in the middle. Each end of the fiber was attached to polyethylene catheters (PE-10), and each end of the polyethylene catheter was then attached to a Teflon tube (JT-10, Eicom).

Rats were anesthetized with pentobarbital (50 mg/kg administered intraperitoneally), and an incision was made along the dorsal midline from T2 to L2. The lateral surfaces of vertebra L1 were exposed, and bilateral holes were carefully made through the bone, exposing the spinal cord laterally at the level of the dorsal horn. A dialysis tube was placed through the holes, passing transversely through the dorsal spinal cord. The two distal ends of the probe were tunneled subcutaneously and externalized through the skin in the neck region.

Microdialysis in the Spinal Cord and Measurement of Prostaglandin E_2 in Dialysate. The experiments were performed 24 h after implantation of the dialysis probe. Only animals that showed normal behavior were used (n = 6). The animals were allowed to move freely in a plastic cage during the experiments. The dialysis probe was perfused with artificial cerebrospinal fluid (140 mM NaCl, 4.0 mM KCl, 1.26 mM $CaCl_2$, 1.15 mM $MgCl_2$, 2.0 mM Na_2HPO_4, 0.5 mM NaH_2PO_4, and pH 7.4) at a constant flow rate of 4 μl/min. Collected samples were frozen at −80°C until used for analysis. The samples were collected as 30-min fractions. After obtaining the three consecutive samples for determination of basal level, carrageenan or saline was injected. Dialysate samples were collected for 15 h after carrageenan or saline injection, and the samples collected at the time points of 3, 8, and 15 h were used.

After each experiment, methylene blue dye was perfused through the dialysis probe to verify the position of

the dialysis fiber. The data used for analysis were from rats in which dye remained in the dorsal half area of the dorsal horn.

The concentration of PGE_2 in dialysate was measured using a commercially available PGE_2 enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI). The limit of quantification was 20 pg/ml.

Measurement of Intracellular Calcium Ion Concentration in the Dorsal Horn of Spinal Cord Slices.

Preparation. To measure $[Ca^{2+}]_i$ in the dorsal horn of the spinal cord, transverse spinal cord slices were prepared according to our modification of the method described previously.[19,20] Briefly, during urethane anesthesia (1.5 g/kg administered intraperitoneally), thoracolumbar laminectomy was performed, and the lumbar spinal cord was excised. The spinal cord was mounted on a Vibratome, and two or three 400-μm-thick transverse slices at the level of the L4 root entry were obtained. The slices were mounted in a chamber and were perfused (25 ml/min) continuously with preoxygenated Krebs solution. The slices were then incubated with Krebs solution (37°C) containing 10 μM fura-2/AM (Dojindo, Kumamoto, Japan) for 45 min. Thereafter, slices were perfused with Krebs solution for 5 min and then placed in a recording chamber mounted on an inverted fluorescence microscope (TE300; Nikon, Tokyo, Japan). The recording chamber was perfused (12–15 ml/min) with oxygenated Krebs solution (37°C) for 30 min before starting the study. The composition of Krebs solution was as follows: 117 mM NaCl, 3.6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM glucose.

Imaging and Calculation of Intracellular Calcium Ion Concentration in the Spinal Cord Slices. Images were viewed through an objective lens (Plan fluor 4×/0.13; Nikon). Light from a Xenon lamp was filtered through either of two different band-pass filters (340 or 380 nm) in the excitation path and conducted to the specimen on the microscope stage through a diachronic mirror. The excitation wavelength was constantly switched between 340 and 380 nm. The fluorescence emitted from the slice was passed through a band-pass filter (510 nm). Video images, passed through a TV lens (C-0.45×; Nikon), were obtained using CCD video camera (C6790–81; Hamamatsu Photonics, Hamamatsu, Japan). The whole area of the spinal cord slice image captured by CCD camera was created by a computerized imaging system (ARGUS-50 HiSCA; Hamamatsu Photonics). Fura-2 fluorescence ratio images (340/380 nm) were calculated in real time (fig. 1A). $[Ca^{2+}]_i$ in the dorsal horn of the spinal cord slice was calculated according to a previously described method.[20,21] Briefly, calibration at high and low $[Ca^{2+}]_i$ was made after treating the slices with 0.01% Triton X-100 in Krebs solution followed by exchange with calcium-free medium containing 1 mM EGTA and 2 mM $MnCl_2$ in Krebs solution.

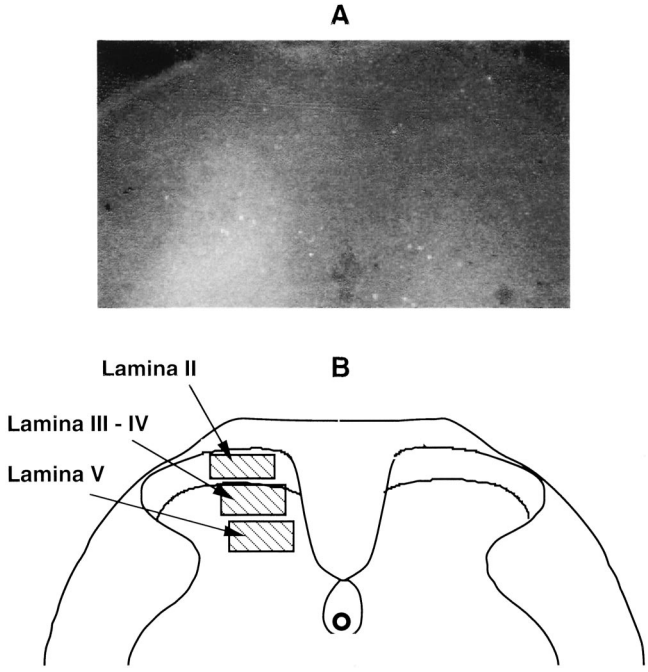

Fig. 1. Fura-2 fluorescence ratio image (340/380 nm) for $[Ca^{2+}]_i$ (*A*) and sampling area for measuring $[Ca^{2+}]_i$ (*B*) in the spinal dorsal horn.

This produced calcium signals equivalent to the bath concentration (saturated calcium) and to zero calcium. Calculation of $[Ca^{2+}]_i$ was performed using the following equation[20,22]:

$$[Ca^{2+}]_i = KD[R - Rmin]/(Rmax - R)](F_0/F_1)$$

where KD is the dissociation constant of fura-2 (224 nM), R is the ratio of fura-2 fluorescence intensity at 510 nm elicited by excitation at 340 and 380 nm, Rmin is the value in the presence of saturating $[Ca^{2+}]_i$, and F_0/F_1 is the ratio of the 380-nm excitation intensity at zero $[Ca^{2+}]_i$ to that at saturated $[Ca^{2+}]_i$ levels. Thus, $[Ca^{2+}]_i$ was imaged and calculated in 400 μm × 200 μm areas of laminae II, III-IV, and V in the spinal dorsal horn (fig. 1B).

Measurement of Intracellular Calcium Ion Concentration in the Dorsal Horn. Intracellular calcium ion concentration in the spinal dorsal horn in normal rats (n = 6) and in carrageenan-treated rats with inflammation in the early phase (3 h; n = 6) and late phase (15 h; n = 6) were measured, and the concentrations on both sides of the dorsal horn were compared. In some rats (n = 6), 100 μg of ONO-8711 was administered *via* an intrathecal catheter that had been implanted. Thirty minutes after the administration of ONO-8711, the spinal cord was removed, and $[Ca^{2+}]_i$ in the spinal dorsal horn was also measured.

Effects of Prostaglandin E_2 and ONO-8711 on Intracellular Calcium Ion Concentration in the Dorsal Horn. To assess the effects of PGE_2 on $[Ca^{2+}]_i$ in the normal and carrageenan-treated rats and the effects of ONO-8711, PGE_2 alone (n = 18) or a combination of

PGE_2 and ONO-8711 (n = 18) were applied by changing the perfusion solution. After determination of control $[Ca^{2+}]_i$ on both sides in dorsal horn, 10 μM PGE_2 was perfused for 7 min, and $[Ca^{2+}]_i$ was measured. Using slices from other rats, changes in $[Ca^{2+}]_i$ were assessed following perfusion with 10 μM ONO-8711 for 2 min and then perfusion with a combination of PGE_2 (10 μM) and ONO-8711 (10 μM) for 7 min. After exposure to the drugs, each slice was rinsed with Krebs solution to verify whether $[Ca^{2+}]_i$ would return to the initial values in each study.

Chemicals

Prostaglandin E_2 and ONO-8711 were supplied by Ono Pharmaceutical (Osaka, Japan). ONO-8711 was dissolved in 1 N of NaOH and then diluted to the desired concentration in saline or Krebs solution immediately before use. All other chemicals and solvents were purchased from Sigma Chemical (St. Louis, MO).

Statistical Analyses

The withdrawal threshold was expressed as a median for ordinal data and was analyzed by the Kruskal-Wallis test followed by Dunn test. Changes in PGE_2 concentrations were presented as means ± SD of the percentages of basal levels. Changes in PGE_2 concentrations and changes in paw thickness were analyzed by a one- and two-way analysis of variance, respectively, followed by Bonferroni correction. Other data in the current study are shown as means ± SD, and statistical comparisons of values were performed by paired or unpaired two-tailed Student *t* tests. $P < 0.05$ was considered statistically significant.

Results

Paw Thickness

Intraplantar injection of carrageenan resulted in formations of edema and erythema. Before the carrageenan injection, the paw thickness was 5.0 ± 0.4 mm. Three hours after the injection (early phase), the thickness on the ipsilateral side significantly ($P < 0.05$) increased (11.2 ± 0.6 mm), and this increase lasted for at least 15 h (late phase, 10.6 ± 0.4 mm). The thickness on the contralateral side did not show any significant changes in either the early or late phase.

Behavioral Assessments

Withdrawal thresholds to mechanical stimulation significantly ($P < 0.05$) decreased in both the early and late phases of inflammation on the ipsilateral side to the injection (figs. 2A and B). In the early phase of carrageenan-induced inflammation, intrathecal ONO-8711 did not show any effects on hyperalgesia (fig. 2A). However, in the late phase, 10 and 100 μg ONO-8711, but not 1 μg of ONO-8711 or saline, significantly ($P < 0.05$) increased the withdrawal threshold (fig. 2B). The thresholds on the contralateral hind paw did not show any changes after ONO-8711 administration at all doses administered in both the early and late phases of carrageenan-induced inflammation (figs. 2C and D, respectively). In the normal rats, intrathecal ONO-8711 at the dose of 100 μg did not show any effects on the paw withdrawal threshold to mechanical stimulation (data not shown).

Prostaglandin E_2 Concentration

Our preliminary study showed that a dialysis equilibrium was obtained within 120 min after the start of artificial cerebrospinal fluid perfusion at a constant flow rate of 4 μl/min and that basal values were stable for at least 15 h (data not shown). The mean basal value of PGE_2 concentration in the dorsal horn was 105.78 ± 20.78 pg/ml.

Three hours after carrageenan injection to the hind paw (in the early phase), PGE_2 concentration in the dialysates increased, but not significantly (fig. 3). A significant ($P < 0.05$) increase was observed 15 h after the carrageenan (in the late phase) compared with the baseline concentration of PGE_2 (fig. 3). The concentrations of PGE_2 in the dialysates did not show significant changes after intraplantar saline injection (data not shown).

The position of the probe after each experiment was verified, and the level of the spinal cord dialysized was L4 in all rats.

Intracellular Calcium Ion Concentration in the Dorsal Horn of Spinal Cord Slice

Figure 4 shows the $[Ca^{2+}]_i$ in the spinal dorsal horn of normal rats and rats treated with carrageenan. In the normal rats, $[Ca^{2+}]_i$ on the ipsilateral and contralateral sides of the dorsal horn were comparable (fig. 4A). There was also no difference of $[Ca^{2+}]_i$ between on the ipsilateral and contralateral sides to the carrageenan injection in the rats with early-phase inflammation (fig. 4B). However, in the rats with late-phase inflammation, significantly ($P < 0.05$) higher $[Ca^{2+}]_i$ in laminae II and V were observed on the ipsilateral side compared with those on the contralateral side (fig. 4C).

Figure 5 shows the effects of pretreatment with intrathecal ONO-8711 on changes in $[Ca^{2+}]_i$ in the carrageenan-treated rats with late-phase inflammation. In contrast to the significant ($P < 0.05$) increases in $[Ca^{2+}]_i$ on the ipsilateral side of the dorsal horn to the carrageenan in the rats that had not received ONO-8711 (fig. 5A), no significant increases in $[Ca^{2+}]_i$ on the ipsilateral side were observed in the rats that had been pretreated with intrathecal ONO-8711 (fig. 5B).

The changes in $[Ca^{2+}]_i$ in the dorsal horn following PGE_2 perfusion are shown in figure 6. The $[Ca^{2+}]_i$ in

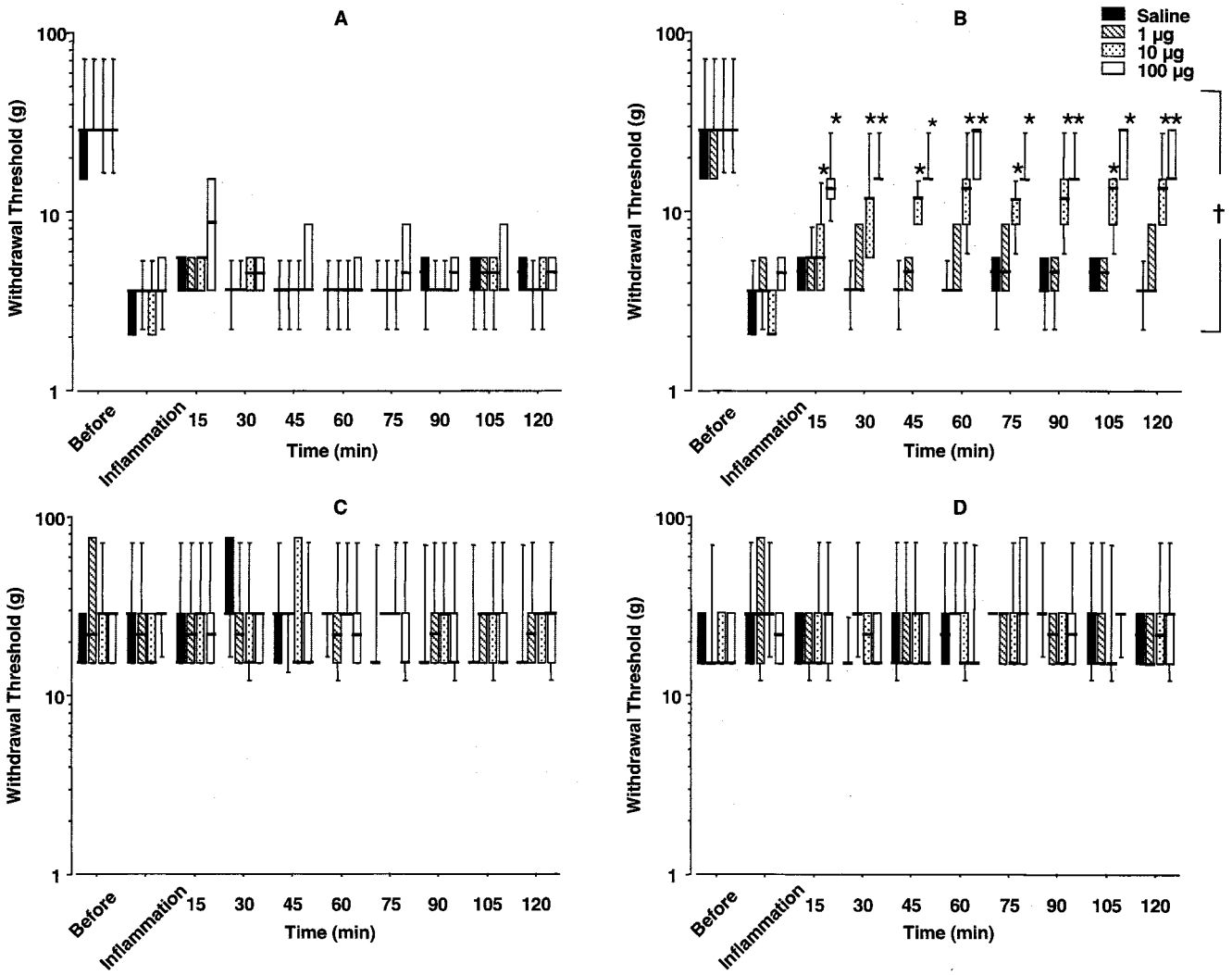

Fig. 2. Effects of intrathecal administration of ONO-8711 (1, 10, and 100 μg) and saline on withdrawal threshold to mechanical stimulation on ipsilateral (*A* and *B*) and contralateral (*C* and *D*) sides of the hind paw 3 h (early phase; *A* and *C*) and 15 h (late phase; *B* and *D*) after carrageenan injection. Data are expressed as medians (horizontal lines) with first and third quartiles (boxes) and 10th and 90th percentiles (vertical lines). N = 6 in each group. *$P < 0.05$ compared with inflammation, †$P < 0.05$ among the four doses.

laminae II, III-IV, and V were increased by perfusion with 10 μM PGE_2 in normal and carrageenan-treated rats. In the normal rats and in the rats with early-phase inflammation, there were no differences between the increase in $[Ca^{2+}]_i$ on the two sides of the dorsal horn (figs. 6A and B). However, the increases in $[Ca^{2+}]_i$ were significantly ($P < 0.05$) greater in laminae II and V on the ipsilateral side than on the contralateral side in the rats with late-phase inflammation (fig. 6C).

While the magnitudes of PGE_2-induced changes in $[Ca^{2+}]_i$ were comparable on ipsilateral and contralateral sides in normal rats and in rats with early-phase inflammation, the increase in $[Ca^{2+}]_i$ in rats with late-phase inflammation were significantly ($P < 0.05$) greater on the ipsilateral side than on the contralateral side in laminae II and V of the spinal dorsal horn. The increase in $[Ca^{2+}]_i$ on the ipsilateral side in the rats with late-phase inflammation was significantly ($P < 0.05$) greater than that on the referred side in lamina II in both the normal rats and rats with early-phase inflammation. Perfusion with ONO-8711 significantly ($P < 0.05$) suppressed the PGE_2-induced elevations in $[Ca^{2+}]_i$ in normal rats and in rats with early-phase inflammation. In the rats with late-phase inflammation, ONO-8711 significantly ($P < 0.05$) suppressed the increase in $[Ca^{2+}]_i$ on both sides of the dorsal horn; the greater elevation in the $[Ca^{2+}]_i$ in response to PGE_2 on the ipsilateral side in rats with late-phase inflammation were also suppressed by perfusion with ONO-8711.

Discussion

Prostaglandin E_2 Release and Effects of EP_1 Receptor Antagonist in Inflammation

We previously reported that peripherally administered ONO-8711 inhibited incision-induced mechanical hyper-

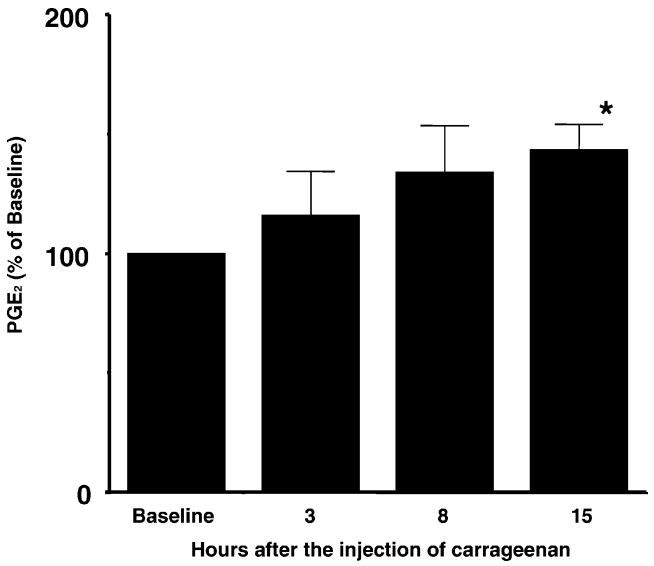

Fig. 3. Time course of changes in prostaglandin E_2 (PGE_2) concentration in the spinal dorsal horn before and after carrageenan injection (n = 6). *$P < 0.05$ compared with baseline.

algesia,[2] suggesting that EP_1 receptor-mediated peripheral sensitization of sensory nerve fibers contributes to the generation of mechanical hyperalgesia produced by tissue damage. The findings in the current study suggest that ONO-8711 suppressed inflammation-induced mechanical hyperalgesia at the level of the spinal cord. Taken together, these findings indicate that peripherally and centrally released PGE_2 following tissue damage and inflammation activates the EP_1 receptors at peripheral and spinal sites, respectively.

Recent behavioral studies have demonstrated that pretreatment with intrathecal ibuprofen and cyclooxygenase-2 inhibitors prevented carrageenan-induced thermal hyperalgesia.[23,24] However, at 3 h after carrageenan injection, ibuprofen and cyclooxygenase-2 inhibitor were ineffective.[24] The results of these studies suggest that spinal prostaglandins are important in the development of thermal hyperalgesia when inflammation begins to develop. In the current study, intrathecal EP_1 antagonist had no effect on the mechanical hyperalgesia at 3 h after the carrageenan injection but was effective at 15 h after the carrageenan. Furthermore, a remarkable release of PGE_2 was observed 15 h after the carrageenan injection. There have been other studies on spinal PGE_2 release after inflammation. An injection of complete Freund's adjuvant caused an elevation of PGE_2 content in lumbar spinal cord homogenates with an early peak at 8 h after injection.[25] Guhring *et al.*[26] reported an elevation of PGE_2 release induced by zymosan injection into a mouse hind paw, with a peak at 8 h. In addition, in an inflammation model by intraarticular injection of kaolin and carrageenan, a pronounced enhancement of release of intraspinal PGE_2 was observed 430–530 min after the injection.[27] Thus, it seems that there is a discrepancy in the time courses of previously reported effects of cyclooxygenase inhibitors in behavioral assays and our results, including effects of an EP_1 antagonist and PGE_2 release in the spinal cord. There are several possible explanations for this discrepancy. First, the different time courses of mechanical and thermal hyperalgesia may reflect the different mechanisms that are thought to underlie these two phenomena. Hedo *et al.*[14] reported that both thermal hyperalgesia and mechanical hyperalgesia were induced by carrageenan but that the time courses of the effects were different. The thermal hyperalgesia was maximally developed at 45 min after injection but had returned to baseline levels within 20 h after the injection, whereas significant mechanical hyperalgesia was observed 3 h after the treatment and was still present 6 days after the treatment. Second, the inhibitory effects of intrathecal cyclooxygenase inhibitors on thermal hyper-

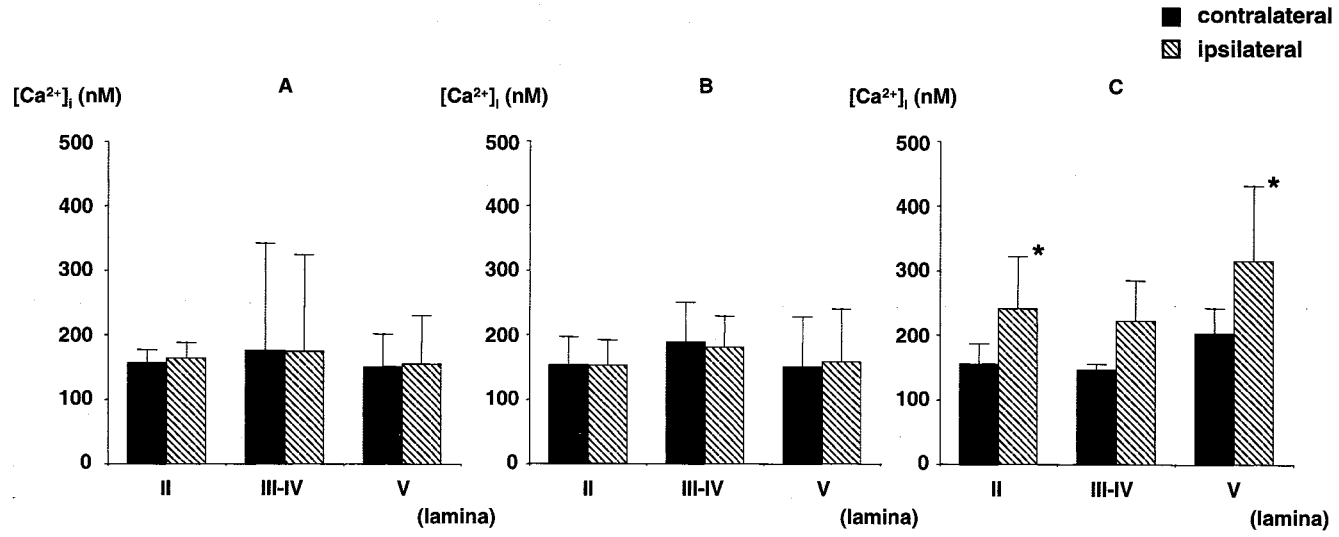

Fig. 4. $[Ca^{2+}]_i$ in the dorsal horn of spinal cord slices from normal rats (*A*) and carrageenan-treated rats with inflammation in the early (3 h; *B*) and late phase (15 h; *C*). N = 6 in each group. *$P < 0.05$ compared with the contralateral side.

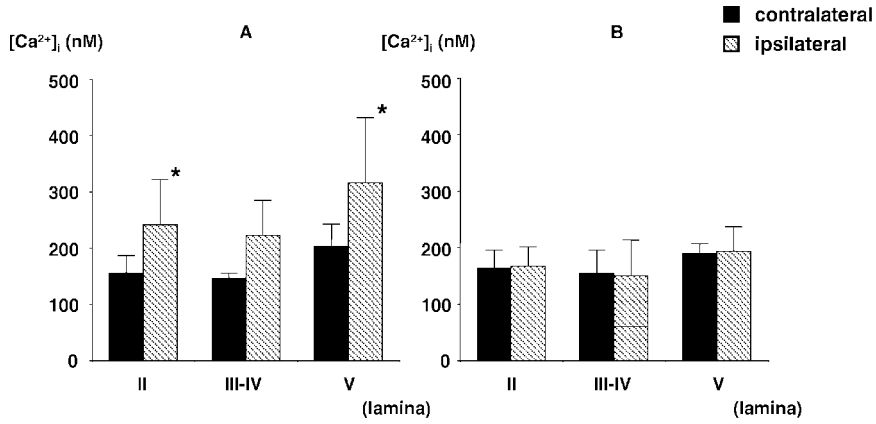

Fig. 5. $[Ca^{2+}]_i$ in the dorsal horn of spinal cord slices from carrageenan-treated rats with inflammation in the late phase (15 h). (*A*) No pretreatment (n = 6); (*B*) pretreated with intrathecal ONO-8711 (n = 6). *$P < 0.05$ compared with the contralateral side.

algesia seen within 3 h after the carrageenan injection might have been mediated by the suppression of production of prostanoids other than PGE_2, such as PGD_2, that also cause hyperalgesia when administered spinally.

Inflammation-induced Intracellular Calcium Ion Concentration Changes in the Spinal Dorsal Horn

In the late phase of inflammation, increases in $[Ca^{2+}]_i$ on the ipsilateral side to the inflammation were observed in spinal slices from which the dorsal root had been removed. This finding suggests that the increase in $[Ca^{2+}]_i$ persists for a long time relatively independent of primary afferent inputs once $[Ca^{2+}]_i$ has been elevated. Repetitive stimulation of spinal neurons may lead to long-term or sustained increases in $[Ca^{2+}]_i$. Therefore, these increases in $[Ca^{2+}]_i$ may have been involved in plastic changes in the spinal neurons of the rats with peripheral inflammation. These changes were also observed in a peripheral neuropathic pain model of rats.[20] In the current study, an increase in $[Ca^{2+}]_i$ was not observed in the early phase of inflammation, suggesting that central changes induced by carrageenan develop increasingly during the period of inflammation and are different and probably more significant in the late phase than in the early phase, as was also suggested by the results of our behavioral study.

We also showed that the magnitudes of changes in $[Ca^{2+}]_i$ induced by PGE_2 perfusion were greater on the ipsilateral side to inflammation in slices obtained from the rats with late-phase inflammation. The increase in $[Ca^{2+}]_i$ induced by PGE_2 perfusion was inhibited by coperfusion with an EP_1 antagonist. Thus, the sensitivity of $[Ca^{2+}]_i$ in the spinal dorsal horn neurons to PGE_2 increased in the late phase of carrageenan-induced inflammation, and this increase in the responsiveness is likely mediated by the activation of spinal EP_1 receptors.

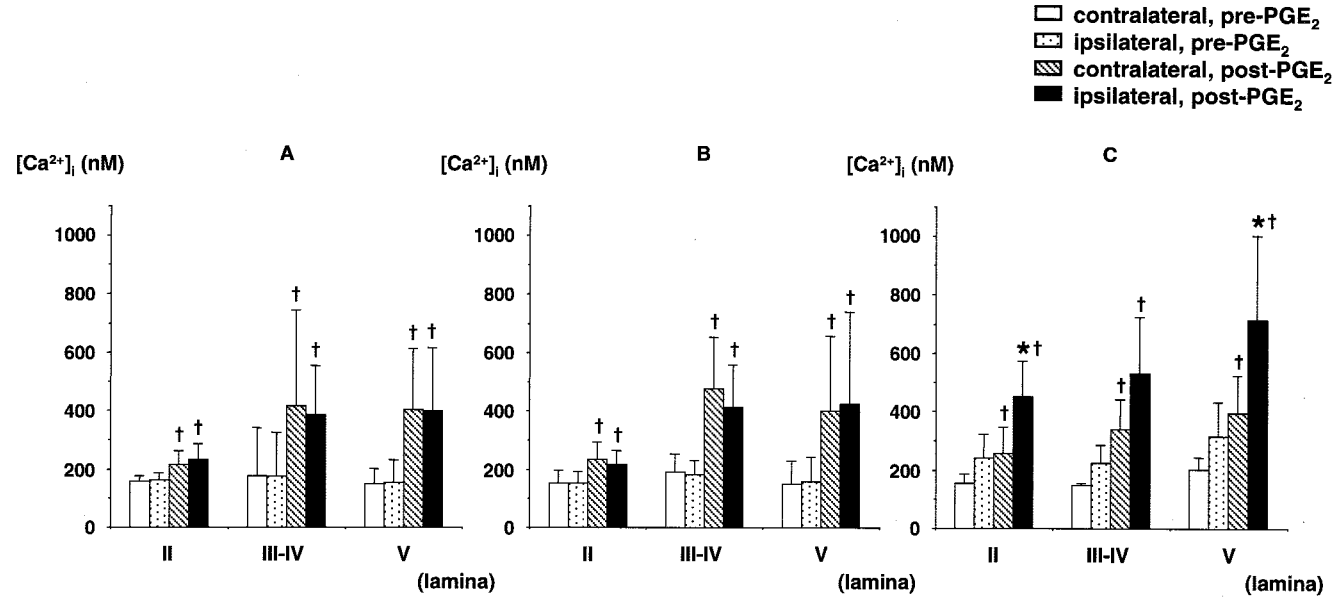

Fig. 6. Effects of prostaglandin E_2 (PGE_2) perfusion on $[Ca^{2+}]_i$ in the dorsal horn of spinal cord slices from normal rats (*A*) and carrageenan-treated rats with inflammation in the early phase (3 h; *B*) and late phase (15 h; *C*). N = 6 in each group. *$P < 0.05$ compared with post-PGE_2 perfusion on the contralateral side; †$P < 0.05$ compared with pre-PGE_2 perfusion on the referred side.

Autoradiographic studies have indicated that the highest density of spinal PGE_2 binding sites is in substantia gelatinosa (lamina II). Matsumura *et al.* reported that dorsal rhizotomy reduced, but did not eliminate, PGE_2 binding in lamina II,[28] suggesting that receptor sites are located on both presynaptic terminals of nociceptive fibers and postsynaptic dorsal horn neurons. Therefore, PGE_2 may act presynaptically to facilitate neurotransmitter release[29–31] and postsynaptically to directly excite dorsal horn neurons.[32] Although we did not examine the PGE_2 binding sites, especially EP_1 receptor sites, in the current study, the superficial and deep layers would contain many PGE_2-responsive elements of the spinal cord. The current study showed that $[Ca^{2+}]_i$ was increased not only in lamina II but also in lamina V of the dorsal horn by PGE_2 perfusion. This might be related to the finding that PGE_2-induced activation of EP_1 receptors in both superficial and deep layers increases $[Ca^{2+}]_i$.

Central Sensitization and EP_1-mediated Cascade in Inflammation

The results of the current study suggest that the late phase of carrageenan-induced inflammation increases the production and release of PGE_2 and enhances the function of spinal EP_1 receptors involved in nociceptive transmission. Thus, it is likely that the activation of EP_1 receptors is important for development and maintenance of central sensitization caused by peripheral inflammation. Hedo and colleagues[14] reported that, in an isolated spinal cord preparation, nociceptive reflexes were augmented more in the late phase (20 h) than in the early phase (3–6 h) of carrageenan-induced inflammation. A study using *in vivo* electrophysiologic assays showed that the effects of a spinally administered *N*-methyl-D-aspartate (NMDA) receptor agonist and antagonist on spinal nociceptive responses were greater in the late phase than in the early phase of carrageenan-induced inflammation.[15] The results of these studies and those of the current study therefore suggest that the hyperalgesia observed during the early phase may be due to the activation of the mechanisms other than spinal EP_1 activation, such as glutamate or NK_1 receptors, and peripheral sensitization, and that some of the changes in release of PGE_2, activation of EP_1 receptors, and subsequent elevation of $[Ca^{2+}]_i$ in the spinal dorsal horn in the late phase may contribute to maintenance of hyperalgesia, independent of ongoing peripheral input.

The current study demonstrated that intrathecal treatment with ONO-8711 suppressed not only the increases in $[Ca^{2+}]_i$, but also behavioral hyperalgesia in the rats with late-phase inflammation. These findings suggest that $[Ca^{2+}]_i$ is maintained at a high level by activation of spinal EP_1 receptors during maintenance of central sensitization. The phase-dependent changes in the contribution of EP_1 receptor activation to central plasticity may be explained by one or more of the following possibilities: generation of more EP_1 receptors, increased affinity for EP_1, increased response to PGE_2 binding, or an up-regulation of factors triggered by EP_1 receptor activation. Regarding generation of EP_1 receptors, Donaldson *et al.*[33] found, by using semiquantitative reverse transcription polymerase chain reaction, that EP_1 receptor mRNA concentrations in the DRG and spinal cord were unaffected by inflammation following the injection of Freund's complete adjuvant. Further study on the expressions and locations of EP_1 receptors in DRG neurons and the spinal dorsal horn is needed to clarify the regulation of EP_1 receptors (up-regulated or down-regulated) during peripheral inflammation.

Following peripheral inflammation, excitatory neurotransmitters such as glutamate are released from primary afferents.[34,35] The subsequent glutamate-mediated NMDA receptor activation leads to a Ca^{2+} influx into the neurons and initiates an enzymatic cascade that finally evokes the release of nitric oxide and PGE_2.[36–38] Nitric oxide and PGE_2, in turn, have been hypothesized to further increase glutamate release,[30,31,36] leading to an ongoing activity in primary afferents and increased sensitivity of dorsal horn neurons. PGE_2 also directly excites dorsal horn neurons.[32] These effects of PGE_2 contribute, finally, to the development of central sensitization. Thus, it is thought that there exists a positive feedback cascade mediated through PGE_2 in which increased primary afferent activity with peripheral inflammation sensitizes spinal neurons and increases synaptic glutamate release from primary afferent terminals. In this feedback cascade, glutamate acts on the NMDA receptors on the postsynaptic neurons to evoke an increase in $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ activates phospholipase A_2, leading to increases in production of arachidonic acid and prostanoids. The released PGE_2 may interact with EP_1 receptors on the presynaptic terminals and postsynaptic neurons, resulting in $[Ca^{2+}]_i$ elevation, which facilitates the release of glutamate from primary afferent terminals and depolarization of dorsal horn neurons. The fact that an EP_1 antagonist suppressed the increase in $[Ca^{2+}]_i$ in the spinal dorsal horn suggests that the increase in $[Ca^{2+}]_i$ resulting from the activation of NMDA receptors and EP_1 receptors in inflammation can be suppressed by blocking this cascade.

In summary, plastic changes in the spinal dorsal horn mediated by the activation of EP_1 receptors were observed in the late phase of carrageenan-induced inflammation. Mechanisms underlying inflammation-induced hyperalgesia may be mediated by neuronal plasticity in the central nervous system resulting from long-term or sustained increases in PGE_2 concentrations, activation of EP_1 receptors, and increases in $[Ca^{2+}]_i$ in the spinal dorsal horn. It is likely that an EP_1 antagonist can block the positive feedback cascade mediated by PGE_2, resulting in inhibition of carrageenan-induced hyperlagesia. Thus, a selective EP_1 receptor antagonist is a potential analgesic for persistent inflammatory pain.

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