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Restoration of spontaneous exploratory behaviors with an intrathecal NMDA receptor antagonist or a PKC inhibitor in rats with acute pancreatitis

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Abstract

The aim of this study was to determine the influence of a glutamate receptor antagonist or a protein kinase C (PKC) inhibitor on the central visceral nociceptive amplification process present in an experimental pancreatitis model. The acute pancreatitis model was produced by combining intraductal infusion of an irritative bile salt, glycodeoxycholic acid (GDOC), with intraperitoneal injection of a CCK analogue, caerulein, in male Sprague–Dawley rats. Exploratory activities were measured with an automated photobeam activity system and compared among different treatment groups. To confirm the inflammation, the pancreas was weighed and compared histologically with those taken from naive rats.

Exploratory activity changed significantly in rats with experimental pancreatitis (i.e., rearing events, rearing time, active time, distance traveled, and total activity all were decreased; whereas resting time was increased). The inflamed pancreatic tissues were edematous, with moderate to marked acinar atrophy and inflammatory infiltrate. Intrathecal administration (at the T7–T9 spinal levels) of an NMDA receptor antagonist (D-AP5, 1 μg) or a selective PKC inhibitor (GF109203X, 0.15 μg) significantly reversed the changes in exploratory activity when compared with the vehicle-treated group of rats with experimental pancreatitis.

Our results demonstrate that pancreatitis pain is the result of central pain processes that play a role in the amplification of responses to peripheral visceral input through NMDA receptor activation and PKC phosphorylation signaling pathways.

Keywords: Nociception; Pain; Exploratory activity; Inflammation; Signal transduction

1. Introduction

Visceral pain causes suffering and dysfunction in malignant and inflammatory states. Less is known about the mechanisms and neuronal systems involved in visceral pain than those involved in cutaneous pain. Visceral inflammatory pain causes central sensitization by mechanisms that in part differ from those responsible for the central sensitization produced by noxious somatic sensation. The neurotransmitters and modulators involved, nonetheless, are likely to include amino acids and peptides.

It is well known that receptors for the excitatory amino acid glutamate (non-NMDA, NMDA, or mGlu) play a role in the induction and maintenance of central mechanisms associated with hyperalgesia following peripheral injury (Haley et al., 1990; Ren et al., 1992; Sher and Mitchell, 1990; Willis, 2001; Woolf and Thompson, 1991). Non-NMDA or NMDA receptor antagonists produce antinociceptive effects in other visceral pain models in animals (Al-Chaer et al., 1996; Coutinho et al., 1996; Ide et al., 1997; Kolhekar and Gebhart, 1994; Zhang et al., 2000).

Many recent studies focus on central sensitization and the activation of several second messenger cascades [protein kinase C (PKC), PKA, and NO/PKG signal transduction pathways] in neuropathic or inflammatory conditions (Coderre, 1992; Mao et al., 1994; Souza et al., 2002). Little is known about second messengers, such as PKC, in central sensitization as a result of visceral pain. The aim of this study is to determine if a glutamate receptor antagonist or a PKC inhibitor can influence the central visceral nociceptive amplification processes in an acute pancreatitis model in rats.

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Visceral pain sensation is a complex experience with multiple facets in human. Unlike cutaneous pain, visceral pain is usually a diffuse, not "clearly" distinguishable, deep pain accompanied by discomfort and decreased movement. It is difficult to get a direct stimulation–reaction relationship in animal experiments. To assess visceral nociception in an acute pancreatitis animal model, an automated photobeam activity system was used to measure animal exploratory activity. An assessment of exploratory activity as a measure of spontaneous pain has been used in other studies such as formalin-induced pain models (Aloisi et al., 1995), arthritic models of chronic pain (Larsen and Arnt, 1985), chronic central pain following spinal cord injury (Mills et al., 2001), and in visceral pain models (Houghton et al., 1997; Palecek et al., 2002).

2. Materials and methods

These experiments were approved by the University Animal Care and Use Committee and were consistent with the ethical guidelines for animal experimentation of the National Institutes of Health and the International Association for the Study of Pain.

Forty-seven male Sprague–Dawley rats, weighing between 250 and 280 g, were used in the study. The animals were obtained from Harlan Sprague–Dawley (Houston, USA) and housed with a 12:12-h light/dark cycle where the light cycle begins at 7:00 a.m. and the dark cycle begins at 7:00 p.m. The animals were given a standard rat chow and water. Animals were divided into six groups: pancreatitis + intrathecal catheter implantation ($n=6$), pancreatitis + intrathecal vehicle ($n=6$), pancreatitis + intrathecal AP5 ($n=11$); pancreatitis + intrathecal GF109203X ($n=12$); intrathecal drug only control ($n=9$), and sham operation control ($n=3$).

2.1. Intrathecal catheter implantation

The intrathecal catheter was 16 cm in length and made by joining three polyethylene tubes of different diameters. The smallest diameter PE32 tubing (5 cm in length) (Micor, PA, USA) was inserted into the subarachnoid space. The other end was connected with PE10 (3 cm) and then PE20 (8 cm) (Becton Dickson, MD, USA) for step-down connection to a Hamilton syringe. Each joint of the catheter was annealed with epoxy glue. Before insertion, the catheter was dried, sterilized by immersion in 70% ethanol and fully flushed with sterile saline prior to use. A length of stainless steel wire, whose diameter just fits into the PE32 tubing, was used to guide insertion.

The rats were anesthetized with sodium pentobarbital (50 mg/kg ip). A midline incision was made beginning at the occipital crest and extending caudally about 2 cm on the back of the neck. The superficial neck muscles were separated along the midline to expose the underlying layers of muscle by blunt dissection. A small bone scrapper (or 18-gauge disposable needle) was used to free the muscles from their point of insertion on the occipital crest of the skull for about 0.5 cm on either side of midline. The neck musculature was gently removed with a curved retractor. When the back of the skull was visible, minor retraction was used to remove the fascial layer covering the cisterna magnum. A small slit was then made in the midline of the atlanto-occipital membrane using the tip of a sterile 26-gauge disposable needle (BD, Becton Dickson, NJ, USA) as a cutting edge. As the dural sac was opened, the clear cerebrospinal fluid could be seen flowing out of the small slit.

To initiate the catheter insertion, the rat's head was rotated, nose downward, while holding the curved retractor flat against the musculature until the head was held approximately at 90° to its body. This position facilitates insertion of the catheter parallel to the dorsal aspect of the cord. The catheter was then carefully advanced in a caudal direction while gently rotating it between the thumb and forefinger until the whole length of PE32 (5 cm) was fitted into the subarachnoid space. The guide wire was then removed from the catheter. The free end of the catheter was heat sealed and secured with the muscle incision closure. The exposed portion of the catheter was embedded under the skin at the nape of the neck at closure. In the sham operation, rats underwent the same operation procedures but the intrathecal catheter was not inserted. The wound was treated with triple antibiotic ointment (Clay-Park Labs, NY). After surgery, rats were given an antibiotic (Gentamicin, 2 mg im, Elkins-Sinn NJ, USA) and allowed to recover for 4–5 days in their normal environment. At this point, the tip of the catheter was located at a point somewhere between the T7 and T9 spinal levels of the freely moving rats (body weight at 250–300 g).

2.2. Induction of acute pancreatitis

The acute pancreatitis was induced by intraductal infusion of a bile salt, glycodeoxycholic acid (GDOC) (Sigma, St. Louis, MO). In order to speed up the contraction of the bile system, caerulein (Sigma), a CCK analogue, was injected intraperitoneally (Houghton et al., 1997). Rats were anesthetized with sodium pentobarbital (50 mg/kg ip). The abdomen was opened with a midline incision. The common duct (bile and pancreatic) was identified at the pancreatic and duodenal junction. The duodenal end of the duct was tightly ligated and the common duct wall was punctured with small iris scissors. A soft polythene tubing cannula (PE10, Becton Dickson) was advanced about 5 mm into the common biliopancreatic duct. A ligature was placed around the duct and the ductal system was allowed to empty of bile and pancreatic fluid. To prevent misdirected flow of the chemical into the liver, the end of the duct was ligated just below the liver. The GDOC in glycylglycine–NaOH-buffered solution (pH 8.0), 10 mM, 0.5 ml was then infused into the pancreas. After infusion, the catheter was removed, the

duct was tightly ligated, and the abdomen was sutured closed. The rats then received caerulein 10 μg/100 μl in normal saline, one injection each hour, injected intraperitoneally over a 6-h period (total volume of 60 μg). In sham pancreatitis rats, the common bile and pancreas duct was identified and cleared as in the rats in which pancreatitis was induced. A loose ligature was placed around the duct but the duct was not cannulated nor was any substance infused. After surgery, rats were returned back to their own cage overnight before behavioral testing.

2.3. Drugs and injection

D-(−)-2-Amino-5-phosphonopentanoic acid (D-AP5) (Tocris, Ellisville, MO), a widely used competitive NMDA receptor antagonist, was dissolved in normal saline. The GF109203X (Bisindolylmaleimide I) (Tocris), a potent selective inhibitor of PKC, was dissolved in 10% dimethyl sulfoxide (DMSO) in saline. Drugs for intrathecal injection were dissolved in a 10-μl volume of the vehicle containing the desired concentration of the agent. All drugs were injected over a 1- to 2-min time period. A 10-μl injected volume has been shown to spread 2 cm rostral and caudally within a 10-min period (Yaksh and Rudy, 1976). After intrathecal drug injection, the catheter was flushed with a subsequent 10-μl injection of vehicle. A Hamilton microinjection syringe (10 μl, Hamilton Reno, NV) was used for all injections. All drugs were applied as a single dose after induction of pancreatitis, i.e., posttreatment, and behavioral testing begins 30 min later in the present study.

2.4. Spontaneous visceral pain behavioral measurements

Behavioral testing was carried out before and 5 days after intrathecal catheter implantation, as well as on the day after induction of pancreatitis. Exploratory activity of the animals was monitored using the Flexfield Animal Activity System (San Diego Instruments, San Diego, CA) with Photobeam Activity System software (PAS) coupled to a Compaq 486 computer (Hewlett Packard, Palo Alto, CA). The activity

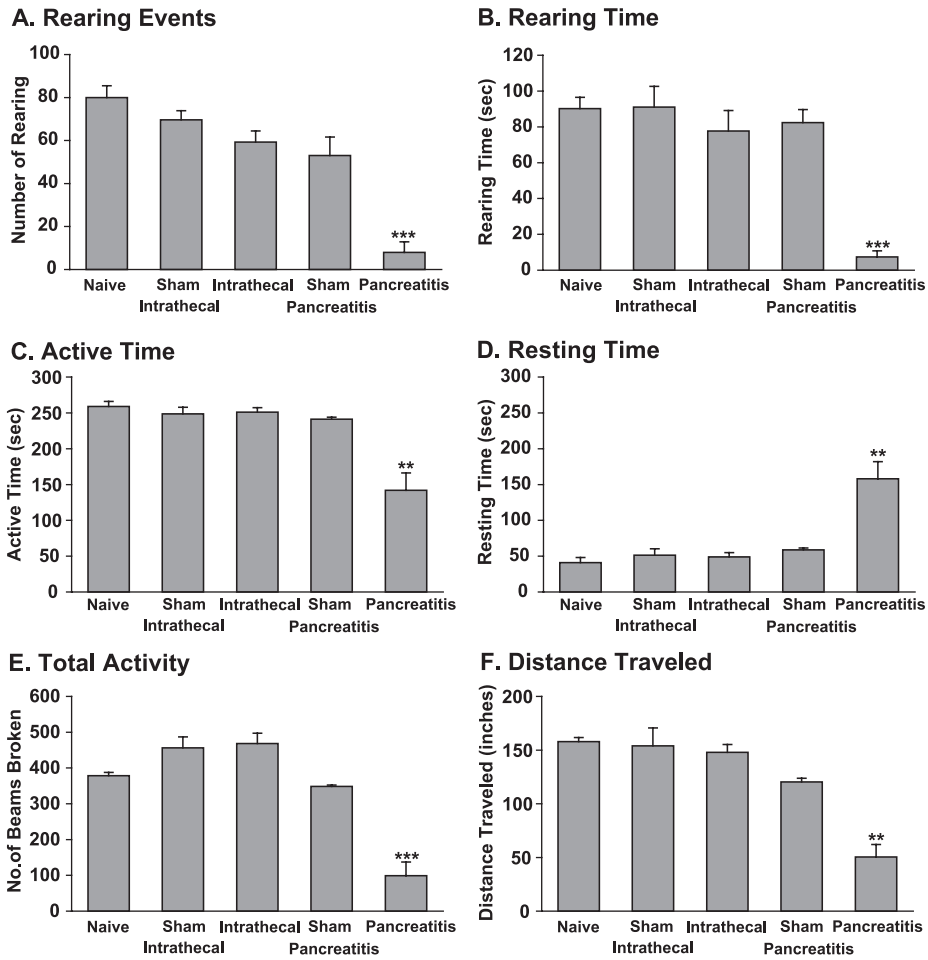

Fig. 1. Exploratory activities in the first 5-min interval after introduction of the rat into the novel cage. Compared to naïve rats, sham-operated rats do not have significant alterations in exploratory activity. Rats with GDOC-induced acute pancreatitis displayed decreased exploratory activity, including decreased number of rearing events (A); shortened rearing time (B); decreased active time (C); increased resting time (D); decreased number of photobeams broken representing a decrease in total activity (E); and decreased distance traveled (F). The pancreatitis group was compared to all other groups. ***P<.001. **P<.01.

cage rack enclosure includes two transparent Plexiglas chambers (40 × 40 × 40 cm) equipped with infrared photobeam sensors, 16 beams on each axis (total 32), arranged 4 cm above the chamber floor. Obstruction of these photobeams constitutes movements in the *x* and *y* plane. Another set of 16 beams is located 12 cm above the chamber floor to record movements along the *z*-axis (rearing events and time). Data were collected in 5-min intervals each phase. Each activity chamber was cleaned with Cavicide between tests to eliminate urine and other olfactory cues from previous subjects.

The experiment was carried out in an isolated, sound-attenuated, temperature-controlled (22 ± 2 °C) room. When the tests start, the observer exits the room leaving the subjects undisturbed. Animals were tested at the same time (7–9 a.m.) of the day. To avoid acclimation of animals to the environment, repeat testing of the same animal occurs at least 24 h after the last test (Palecek et al., 2002).

Six main parameters were measured in two 5-min intervals: rearing events and rearing time; active time and resting time; total activity, i.e., number of photobeams broken (includes the number of different zone entries and stationary fine movement, e.g., grooming); and distance traveled. Resting time is defined as a period when the animal remains in place for 1 s or longer. This test is based on the natural exploratory or investigatory behaviors of rodents in a novel "open field" environment. All of the six parameters are important for evaluation of spontaneous pain and for comparison of the effects of drug treatment. Changes in activity may not be reflected by a single parameter; therefore, each of the six parameters was evaluated. Since most activities occurred within the first 5 min following introduction of the rat into the novel environment, changes in activity were evaluated during the first 5-min interval and compared among different groups (Houghton et al., 1997; Mills et al., 2001).

2.5. Histopathological assessment of pancreatitis

After behavioral testing, animals were deeply anesthetized (50 mg/kg ip) and the pancreas were removed, weighed, washed with phosphate-buffered saline (PBS), and then immediately fixed in 10% formaldehyde for 24 h. The liver and the lungs were removed for histological study as well. Standard paraffin embedding procedures are carried out to prepare the tissue blocks. The tissue sections were cut at 3-μm thickness with a microtome (Leitz 1512, San Marcos, CA) and stained with hematoxylin and eosin for pathological assessment of pancreatitis. The pancreas, liver, and lungs from sham rats served as control in the histological study.

2.6. Statistical analysis

One-way ANOVA was performed to assess behavioral changes followed by post hoc comparisons using Newman–Keuls multiple comparisons test. All treatment groups with pancreatitis were compared to vehicle-treated rats with pancreatitis. All six activity measures were compared during the first 5-min interval. Unpaired *t* tests were used to compare pancreas tissue weight in naïve rats with that of rats with acute pancreatitis. Data were expressed as mean ± S.E. A *P* value of less than .05 was considered significant.

3. Results

3.1. The effect of intrathecal catheter implantation on exploratory behavior

Animal motor function and the six behavioral parameters were evaluated before and after surgery (Fig. 1). No animals were excluded from the study for failure to maintain their weight relative to the naïve group (within 20%), nor did any develop neurological sequelae after catheter implantation. Due to the catheter insertion at the nape of the neck, however, rearing events and rearing time were adversely affected after surgery (Fig. 1a–b).

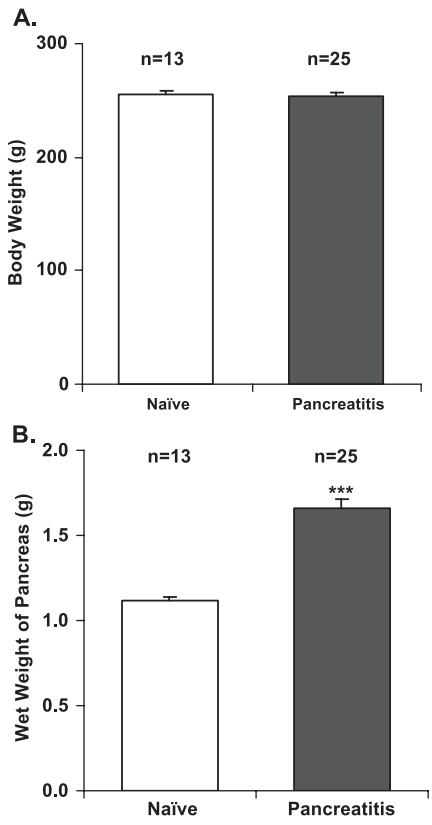

Fig. 2. Body and pancreas weights. (A) The naïve rats ($n = 13$) and the rats with GDOC-induced acute pancreatitis ($n = 25$) maintained the same body weight range throughout the study. (B) The wet weight of the pancreas of rats with GDOC-induced acute pancreatitis was much higher than that of naïve rats (* * * $P < .001$).

For example, the average rearing events and rearing time in naïve rats were 80 ± 5.4/5 min and 90.2 ± 6.3 s/5 min but decreased to 59.3 ± 5.1/5 min and 77.65 ± 11.4 s/5 min, respectively. General exploratory behaviors, such as the average length of active time (251.13 ± 6.11 s/5 min), resting time (48.87 ± 6.11 s/5 min), distance traveled (147.98 ± 7.12 in./5 min), and total activity: 468.33 ± 28.5/5 min, of rats with intrathecal implantation ($n = 11$) were not significantly different during the first 5-min interval testing (Fig. 1c–f). For comparison, before implantation surgery, the average length of active time was 258.97 ± 7.15 s/5 min, resting time was 41.03 ± 7.13 s/5 min, distance traveled was 157.94 ± 3.72 in./5 min, and total activity was 378.67 ± 9.17/5 min ($n = 11$, Fig. 1).

3.2. GDOC-induced pancreatitis changes exploratory activity

When combined intraductal injection of GDOC (10 mM, 0.5 ml) and intraperitoneal injection of caerulein (60 μg) was administered to rats, a moderately severe acute pancreatitis was developed after 12 h. The average wet weight of the pancreas was 1.122 ± 0.02 g from naïve rats with an average body weight of 254 ± 2.9 g ($n = 13$). On the other hand, the average wet weight of the pancreas in rats with acute pancreatitis (1.656 ± 0.06 g; body weight of 252 ± 3 g; $n = 18$; $P < .001$; unpaired t test, Fig. 2) was increased significantly due to extravasation. Histological comparisons were made to pancreatic tissue from sham rats in which the glandular architecture is entirely normal (Fig. 3a). Histo-

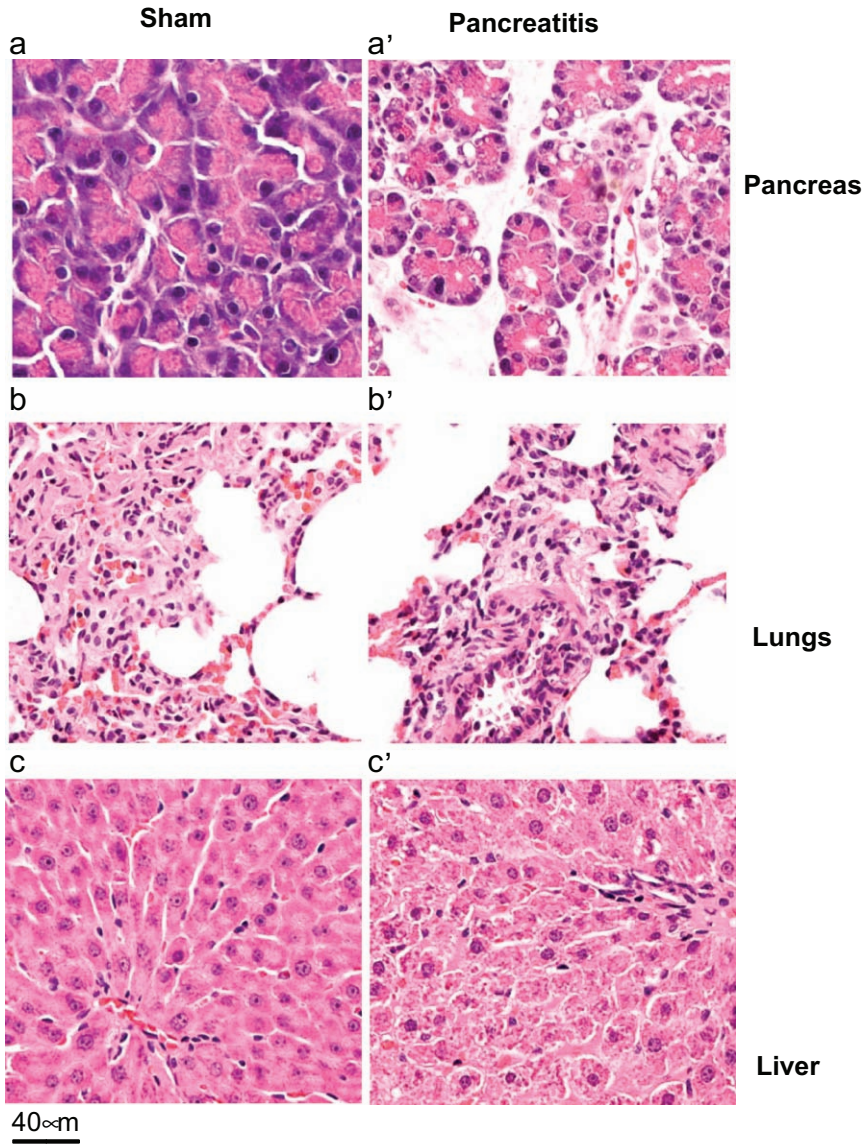

Fig. 3. Histology of pancreas, lungs, and liver. Photographs were taken from histological sections of the pancreas, the lungs, and the liver of a sham rat (a, b, and c) and a rat with GDOC-induced acute pancreatitis (a′ , b′ , and c′). The inflamed pancreas (a′) was edematous, with moderate acinar atrophy. The lungs (b′) and the liver (c′) showed a slight edema. The paraffin sections were cut at a thickness of 3 μm and stained with H & E (scale bar is 40 μm).

pathological features of the pancreatic tissue taken from rats with pancreatitis included moderate interstitial edema, substantial inflammatory infiltration, and moderate hemorrhage, as well as significant cellular necrosis (Fig. 3a′). The lungs and the liver from pancreatitis rats showed a mild edema and hemorrhage (Fig. 3b′ and c′).

Spontaneous exploratory activity in rats with GDOC-induced pancreatitis was greatly affected compared to the sham controls (Fig. 1). Rearing events decreased significantly from 53 ± 8.62/5 min to 8.0 + 2.19/5 min and rearing time decreased from 82.37 ± 7.3 s/5 min to 7.36 ± 3.46 s/5 min (Fig. 1A and B). Active time decreased from 241.3 ± 2.65 s/5 min to 142.1 ± 24.18 s/5 min and resting time increased from 58.7 ± 2.65 s/5 min to 157.9 ± 24.18 s/5 min (Fig. 1C and D). Total activity significantly decreased from 348.33 ± 4.09/5 min to 99.2 ± 37.76/5 min compared to sham surgical controls (Fig. 1E). Consequently, distance traveled decreased significantly from 120.25 ± 3.44 in./5 min down to 50.48 ± 11.67 in./5 min (Fig. 1F). The differences in each case were highly significant ($P < .001$, one-way ANOVA, Newman–Keuls multiple comparison test).

3.3. Intrathecal NMDA receptor antagonist, D-AP5, restores the exploratory activity in rats with GDOC-induced pancreatitis

Twelve hours after intraductal injection of GDOC, 11 rats with acute pancreatitis received intrathecal posttreatment

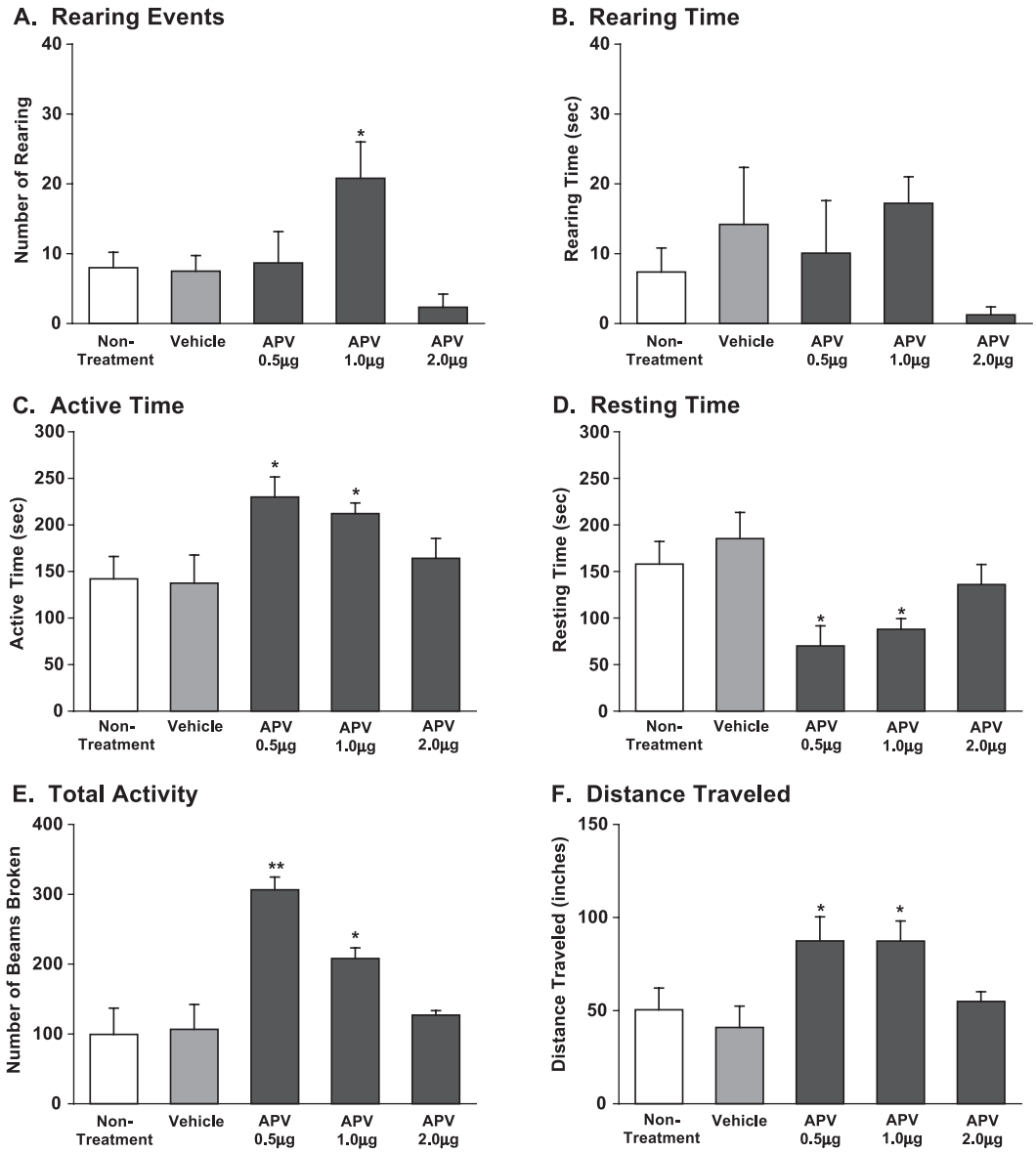

Fig. 4. Dose response to AP5. In rats with GDOC-induced acute pancreatitis, posttreatment with intrathecal NMDA receptor antagonist, AP5 (APV), at the dose of 0.5 or 1 µg significantly restored spontaneous exploratory activities compared to rats with acute pancreatitis treated only with the vehicle. The AP5 treatment increased (A) rearing events, (C) active time, (E) total activity, and (F) distance traveled as well as shortened the (D) resting time. Rats appeared to have locomotor abnormalities with the 2-µg dose of AP5. $*P < .05$; $**P < .01$.

with NMDA antagonist, D-AP5. Thirty minutes after intrathecal injection of D-AP5, the rats were introduced into the cage rack enclosures. The exploratory activities were recorded for the first 5-min interval in the novel environment (Fig. 4). Intrathecal D-AP5, at the dose of 1 μg, significantly restored the following exploratory activities: rearing events 20.8 ± 5.2/5 min, active time 212.06 ± 11.48 s/5 min, resting time 87.94 ± 11.48 s/5 min, total activity 207.8 ± 15.4/5 min, and distance traveled 87.382 ± 10.72 in./5 min. These exploratory activities were also significantly different compared to the rats with pancreatitis in the vehicle treatment group (rearing events 7.5 ± 2.21/5 min, active time 137.375 ± 30.185 s/5 min, resting time 162.625 ± 30.185 s/5 min, total activity 106.75 ± 35.3/5 min, and distance traveled 40.99 ± 11.48 in./5 min; $P < .05$, one-way ANOVA, Newman–Keuls multiple comparisons test). The results showed that the effective dose of D-AP5 was between 0.5 and 1.0 μg. Most rats had locomotor abnormalities in the 2-μg treatment group (Fig. 4).

3.4. Intrathecal GF109203X, a potent selective PKC inhibitor, restores exploratory activity in rats with GDOC-induced pancreatitis

Posttreatment with intrathecal GF 109203X produced a potent effect (Fig. 5). In 12 rats, GF 109203X, at the dose of 0.15 μg, restored the spontaneous exploratory activities in rats with GDOC-induced pancreatitis. There was an increased number of rearing events (Fig. 5A); increased rearing time (Fig. 5B); prolonged active time (Fig. 5C), as well as a shortened resting time (Fig. 5D); and increased total activity and distance traveled (Fig. 5E and F). Rearing

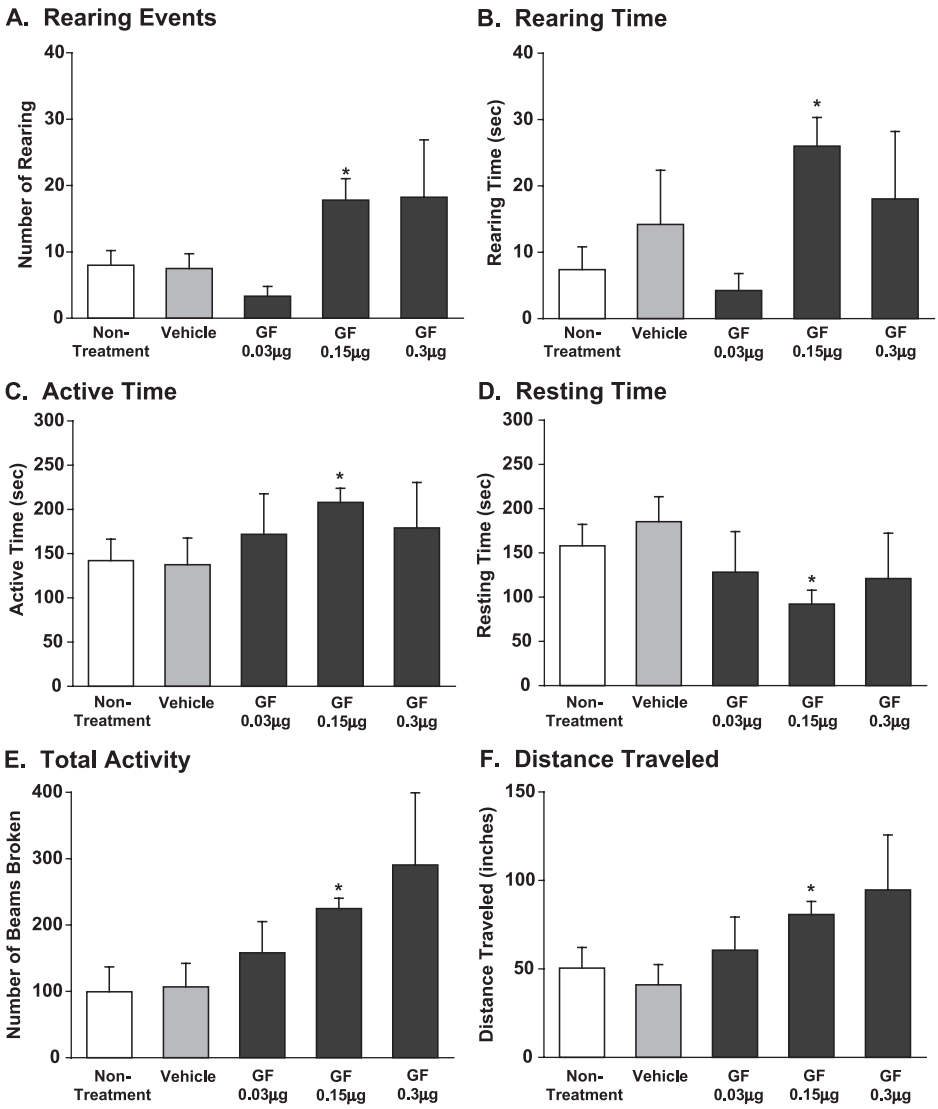

Fig. 5. Dose response to GF 109203X. In rats with GDOC-induced acute pancreatitis, posttreatment with intrathecal administration of a potent PKC inhibitor, GF109203X (0.15 μg), significantly restored the spontaneous exploratory activity compared to the vehicle-treated group. The rats treated with PKC inhibitor displayed (A) a greater number of rearing events and increased (B) rearing time, (C) active time, (E) total activity, and (F) distance traveled. Resting time (D) was shortened. * $P < .05$.

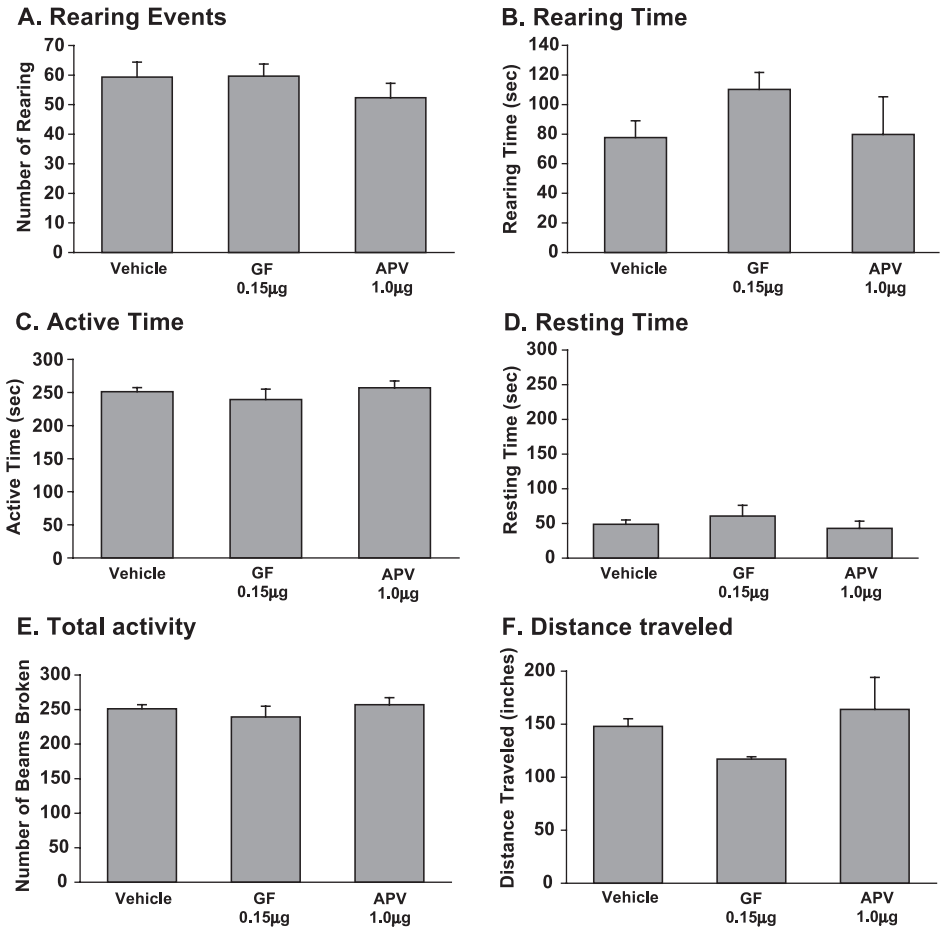

Fig. 6. Intrathecal drug controls. Naïve rats were treated intrathecally with GF 109203X, AP5, or with the same volume of vehicle solution. The doses used were 0.15 and 1 μg, respectively. There were no signs of drug effects in naïve rats observed with the dose effective in restoring exploratory activity in rats with acute pancreatitis. For the six parameters (A–F) of exploratory activities, no differences were observed among these three control groups.

events occurred 17.8 ± 3.22/5 min, rearing time 25.98 ± 4.33 s/5 min, active time 207.98 ± 15.74 s/5 min, resting time 92.02 ± 15.74 s/5 min, total activity 224.8 ± 15.4/5 min, and distance traveled 80.73 ± 7.4 in./5 min. The exploratory activities were significantly different from those of animals with pancreatitis in the vehicle-treated group, $P < .05$ (one-way ANOVA, Newman–Keuls multiple comparisons test). Some evidence of restoration was noted with doses of 0.03 or 0.3 μg.

In order to determine the effects of the drugs alone on normal animal exploratory activity, a group of naïve rats were treated with intrathecal GF 109203X or AP5 or vehicle at the effective dose of 0.15 μg, 1 μg, or same volume, respectively. There was no significant difference in the drug control group when compared to the vehicle-treated rats for the drug concentration tested (Fig. 6).

4. Discussion

In the present study, an acute pancreatitis model was produced in rats with intraductal infusion of GDOC and intraperitoneal caerulein. An automated photobeam activity system widely used for testing rodent exploratory activity was used to assess spontaneous visceral pain. The activity system was a useful tool for measurement of the exploratory activity in comparisons of naïve rats and rats with pancreatitis (with or without pharmacological treatments).

During measurement of exploratory activity, rats with GDOC-induced acute pancreatitis displayed a decreased number of rearing events and total activity; shortened active time, rearing time, and distance traveled; and prolonged resting time. Posttreatment with intrathecal NMDA receptor antagonist AP5 or PKC inhibitor GF 109203X effectively restored the exploratory activity in animals with GDOC-induced acute pancreatitis.

The present data demonstrate that visceral inflammation induces a central sensitization, amplifying noxious peripheral input at the spinal cord level. The discomfort, decreased exploratory activity, and increased spontaneous pain-related behaviors following inflammation of the pancreas (or other visceral organs) reflect secondary hyperalgesia resulting from the positive feedback of central visceral nociceptive

amplification processes (Coutinho et al., 1996; Houghton et al., 1997; Mayer and Gebhart, 1994).

The NMDA receptor antagonist, AP5, in our experiments produced an antihyperalgesic effect on rats with GDOC-induced acute pancreatitis when applied intrathecally. This demonstrates that central sensitization depends on the action of excitatory amino acids at NMDA receptors. These results are well supported by previous reports. For example, increased concentration of amino acids was measured in the spinal cord in response to topical application of bradykinin onto the surface of the pancreas (Zhang et al., 2000); spinal AP5 blocked the effect of turpentine sensitization on visceromotor response to CRD (Ide et al., 1997); and MK-801 attenuated the enhanced response to CRD during colonic inflammation (Coutinho et al., 1996).

The PKC inhibitor, GF 109203X, also produced a significant antinociceptive effect in our visceral pain experimental model, as evidenced by restored exploratory activities in rats with acute pancreatitis when it was spinally applied at a very low dose. The inhibition of PKC by GF 109203X is reported to be highly selective. It has not been shown to alter other protein kinases, such as PKA, even with concentrations up to 200- to 300-fold higher (Toullec et al., 1991; Wajima et al., 2000). Several PKC isozymes, including α, βI, βII, and γ, are identified in spinal neurons and GF 109203X inhibits the activity of the four PKC subtypes with a similar potency (Mori et al., 1990; Toullec et al., 1991). The experiments presented here provide strong evidence for the contribution of PKC to central hyperalgesia induced by noxious visceral input. We propose that pancreatic inflammation leads to a sensitization of spinal cord dorsal horn neurons by phosphorylation of NMDA receptors through the activation of PKC.

It is noteworthy that AP5 and GF 109203X were employed as a posttreatment in all of these experiments and showed a significant antihyperalgesic effect with the spinal level administration. This suggests that the NMDA receptor and PKC are involved not only in initiation but also in the maintenance of central sensitization during visceral inflammation and may be explored as effective pharmacological tools in the treatment of visceral pain.

Acknowledgements

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