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Attenuation of nociception in a model of acute pancreatitis by an NK-1 antagonist

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Abstract

Substance P (SP) acting at the NK-1 neurokinin receptor has a well-documented role in the transmission and maintenance of nociceptive information. SP is found in the majority of fibers innervating the pancreas, and it is up-regulated after pancreatic inflammation. The aim of this study was to investigate the role of the NK-1 receptors in the maintenance of pancreatic nociception. Using a newly developed rat model of acute pancreatic nociception that persists for 1 week, the NK-1 receptor expression in the spinal cord and pancreas was examined using immunohistochemistry and Western blotting procedures. The effects of a specific NK-1 antagonist, CP99,994, on the behavioral manifestations of pancreatic nociception were determined. The antagonist was administered intraperitoneally and intrathecally to differentiate peripheral and central effects. Injection of CP-100,263, the inactive enantiomer of CP-99,994 was used as a control for nonspecific effects of the antagonist. Immunohistochemistry and Western blotting analysis revealed an up-regulation of the NK-1 receptor occurs in the pancreas but not at the spinal cord level. The NK-1 antagonist was able to attenuate the nociceptive behaviors in rats with pancreatitis when applied intraperitoneally with a short duration of effectiveness. Intrathecal application of the antagonist was ineffective. These results suggest the involvement of pancreatic NK-1 receptors in the maintenance of nociception during pancreatic inflammation.

Keywords: Pancreatitis; Pain; Substance P; Hyperalgesia; Inflammation

1. Introduction

Substance P (SP) is a neuropeptide found in unmyelinated somatic and visceral afferent nerve fibers, in enteric sensory neurons and in a number of pathways within the central nervous system (Swain, 1998). The majority of fibers innervating the pancreas express SP (Sharkey et al., 1984, 1987). Intrathecal administration of SP produces a characteristic nociceptive behavioral syndrome, including vocalization, restlessness and escape behaviors, suggesting a noxious stimulus (Cridland and Henry, 1988; Picard et al., 1993; Yasphal et al., 1982). Intrathecal injection of SP also produces visceral pain-related behaviors (Goettl and Larson, 1994). SP acts primarily at high-affinity NK1 tachykinin receptors (Saria, 1999).

Several studies have implicated the NK-1 tachykinin receptor in the induction and maintenance of chronic pain conditions in experimental models (Honore et al., 2000; see review in Willis, 2001). Antagonists for the NK-1 receptor have antinociceptive properties. Ablation of neurons expressing the NK-1 receptor can reduce thermal hyperalgesia and mechanical allodynia associated with persistent pain states (Mantyh et al., 1997; Nichols et al., 1999). The NK-1 receptor also has been implicated in the modulation of visceral pain. Mice with NK-1 tachykinin receptor gene disruption have impaired responses to cyclophosphamide cystitis, no acute reflex responses, or primary hyperalgesia to intracolonic acetic acid (Laird et al., 2000). Expression of NK-1 receptor mRNA is increased in inflammatory bowel disease in humans (Goode et al., 2000), and expression of the NK-1 receptor protein is increased in chronic bladder irritation in rats (Ishigooka et al., 2001). NK-1 receptor antagonists can abrogate visceral pain responses (Julia et al., 1994; McLean et al., 1993).

Pancreatitis is a disease of the pancreas in which abdominal pain is the hallmark symptom (Graham and Bonica, 2001). The pain is usually difficult to treat in the clinical setting. Animal models of pancreatic pain are not

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abundant and usually they are not long lasting in nature, which limits their translational value. Recently, a novel model of persistent pancreatitis pain has been developed in Lewis rats (Vera-Portocarrero et al., 2003). In this model, behavioral signs of visceral nociception persist for 7 days, and alterations of SP expression in the spinal cord are present during the same period. Previous studies in acute models of pancreatitis have revealed that the NK-1 receptor is involved in the pathological processing of pancreatic inflammation since there was a marked reduction of pancreatitis in mice lacking the NK-1 receptor and also in mice lacking the pre-protachykinin gene (Bhatia et al., 1998, 2003). The correlation of increased expression of SP and the increasing behavioral signs of visceral nociception has led us to hypothesize that SP is involved in the maintenance of pancreatic visceral nociception. The present study investigated the expression of the SP receptor, NK-1, during the time period of persistent pancreatic nociception using immunohistochemistry, Western blotting techniques and pharmacological tools to assess the effects of blocking the SP receptor on the behavioral end points.

2. Methods

2.1. Animals

All procedures were approved by our Institution's Animal Care and Use Committee. The experimental subjects were male Lewis rats weighing 150–175 g (Harlan Sprague–Dawley, Houston, TX). Animals were kept two per cage until they underwent surgical procedures, at which time they were kept one per cage. Teklab diet 8626 was provided and the animals were kept under a 12:12 light:dark cycle (lights on at 7 a.m., lights off at 7 p.m.).

2.2. Induction of pancreatitis

The procedures for induction of pancreatitis in Lewis rats have been described elsewhere (Vera-Portocarrero et al., 2003). In brief, dibutyltin dichloride (DBTC, 8 mg/kg body weight) is dissolved in alcohol and injected into the tail vein of experimental animals. Control animals received an injection of vehicle solution. On Days 3, 7 10, 14 and 21 after the injection, blood serum was obtained to confirm the presence of elevated clinical markers of pancreatitis. The injection of DBTC produces inflammation of the pancreas characterized by increased levels of amylase and lipase and histological signs of inflammation (Fig. 1; Vera-Portocarrero et al., 2003).

2.3. Behavioral testing

The procedures for behavioral testing in this model have been described previously (Vera-Portocarrero et al., 2003). In brief, control and experimental animals were evaluated for mechanical and thermal sensitivity. Prior to testing, all animals were acclimated to the testing conditions for 4 h daily for 3 days. During this time animals were housed individually in a Plexiglas cubicle (4 × 4 × 10 in.). Baseline testing took place before any manipulations. Mechanical sensitivity of the abdominal area was quantified by measuring the frequency of withdrawals (either abdominal withdrawal from the von Frey filament or consequent licking of the abdominal area, or whole body withdrawal) in response to normally innocuous von Frey filament stimuli. To perform this test, rats were placed in their test cubicles on an elevated, fine fiberglass screen mesh and acclimated for 60 min before testing. The von Frey filament was applied from underneath though the mesh floor, to the abdominal area. A single trial consisted of 10 applications of the filament applied once every 10 s to allow the animal to cease any response and return to a relatively inactive position. The mean occurrence of withdrawal events in each of the trials was expressed as the number of responses per 10 applications. Three trials were performed in each animal and the withdrawal events were averaged to obtain a single value for each rat (average withdrawal event [AWE]).

Thermal sensitivity was quantified by measuring the latency to withdrawal from a noxious thermal stimuli. Animals were placed in the Plexiglas cubicles on an elevated glass plate through which a high-intensity light beam was shone. The radiant heat stimulus was applied onto the abdominal area as a concentrated beam of light shown through a hole (1 × 1 cm) in the hand-held box surrounding the high-intensity light. The light and timer were immediately stopped when the animal withdrew. A withdrawal event to radiant heat applied to the abdomen was defined as abdominal withdrawal (either abdominal musculature contraction or lifting the abdomen through postural adjustment) accompanied by head turning toward the stimuli and licking of the abdominal area.

2.4. Immunohistochemical procedures

On Days 3 and 7 after tail vein injection of either DBTC or vehicle, animals ($n = 4$ per group per time point) were perfused and the thoracic segments of the spinal cords removed for immunohistochemistry. In brief, rats were perfused transcardially with 50 ml of heparinized saline at 37 °C followed by 500 ml of cold (4 °C) 4% paraformaldehyde solution (pH 7.4). The spinal cords were removed carefully and postfixed in 4% paraformaldehyde solution at room temperature for 4 h before cryoprotection in 30% sucrose/phosphate buffer (PB) overnight. Tissue was cut into 30-μm-thick coronal sections and processed as free-floating sections. The sections were incubated in rabbit anti-NK-1 (1:5000; Novus Biologicals, Littleton, CO) for 24 h after which sections were incubated in goat anti-rabbit IgG (1:200, Vector, Burlingame, CA) for 2 h. After rinsing in PB

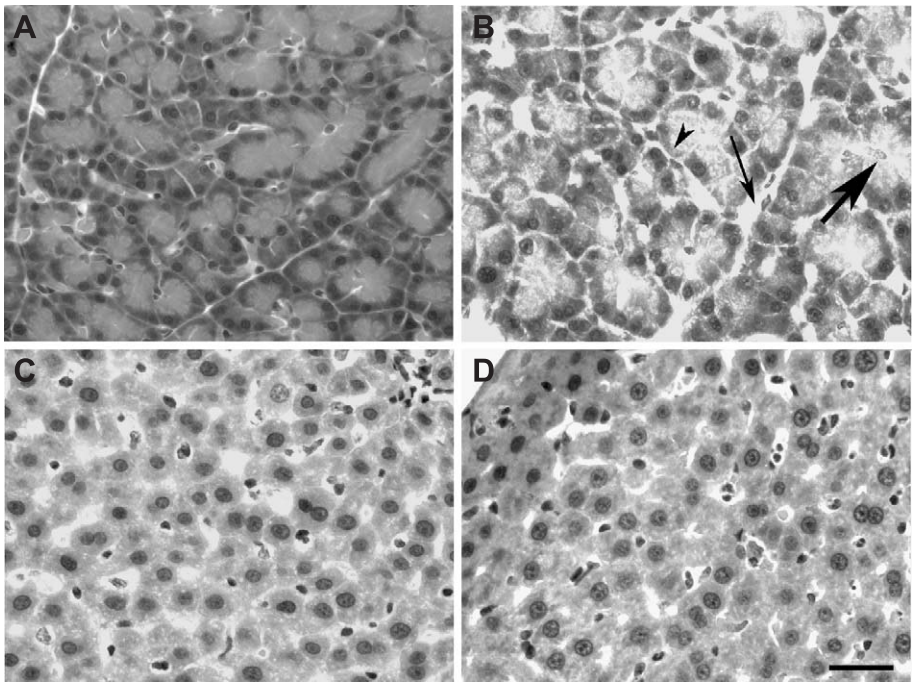

Fig. 1. Paraffin sections of pancreas and liver stained with hematoxylin and eosin from vehicle-injected Lewis rats and rats with an acute pancreatitis produced by injection of DBTC (4-μm sections). (A) The photomicrograph illustrates the histological appearance of normal pancreas. The appearance of the pancreas was similar in both naive and vehicle-injected animals. (B) The pancreas shows evidence of pancreatitis 7 days after DBTC injection in the tail vein (8 mg/kg body weight). The pancreas shows acinar atrophy (arrowhead), stromal proliferation (thick arrow), and edema in the interlobular (thin arrow) and intralobular ducts. (C) Liver tissue section from a control Lewis rat stained with hematoxylin and eosin. (D) Liver section from a Lewis rat injected with DBTC taken 7 days after injection. No signs of inflammation are apparent in the liver. Scale bar: 40 μm.

the sections were incubated in ExtrAvidin Peroxidase/PBS (1:1000; Sigma, St Louis, MO) for 90 min. The stain was visualized with 0.015% 3,3′ -diaminobenzidine containing 0.3% hydrogen peroxide. The sections were mounted onto gelatin-coated slides, air-dried, dehydrated in ethyl alcohol, cleared in xylene and cover slipped. As a negative staining control, sections were processed with the primary antibody deleted, which eliminated staining. Immunoabsorption testing with the corresponding peptide also eliminated staining. All sections from all groups were processed at the same time with the same solutions assuring that all tissues received similar exposure to all reagents.

Pancreatic samples were processed differently since they were taken out before perfusion procedures. The pancreatic tissue was fixed for 24 h in 4% paraformaldehyde and then embedded in paraffin. The resulting paraffin blocks were cut at 6-μm thickness and mounted in gel-coated slides for further processing. The mounted sections were rinsed in PBS six times before immersion in 3% normal goat serum/Triton X-100 (NGST) for 30 min at room temperature. Sections were washed again with PBS and then incubated with anti-NK-1 (1:5000, Novus Biologicals) diluted in 1% NGST for 24 h in a humidified chamber. After incubation, sections were rinsed in 1% NGST twice for 30 min each before being incubated with secondary antibody (Alexa Fluor 594 goat anti-rabbit IgG, 1:200 Molecular Probes, Eugene, OR) for 4 h at room temperature. After incubation the sections were rinsed again with PBS (six times) and the slides were mounted with coverslips using Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA) as a counterstain for cell nuclei. The sections were examined with a Nikon FXA microscope (Nikon, Melville, NY) linked to a Pentium PC though an Optronics DEI-470 digitally enhanced color microscope video camera with built-in digital image processor at $\times 10$ magnification. Controls performed for the immunofluorescence procedures included omission of the primary antibody or preadsortion with the corresponding peptide (Novus Biologicals).

2.5. Western blotting procedures

Samples were homogenized in ice-cold Tris-buffered saline containing 40 mM Tris–HCl (pH 7.5), 2% SDS, protease inhibitor cocktail (in millimolar concentrations: 0.08 aprotinin, 104 AEBSF; 1.4 E-64; 4 bestatin, 1.5 pepstatin-A, and 2 leupeptin; Sigma), 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 1 mM EDTA. Homogenates were centrifuged at $10{,}000 \times g$ for 10 min and the supernatant was collected and stored at -70 °C. Protein concentrations of the homogenate were determined using the BCA protein assay kit (Pierce, Rockford, IL). For gel loading, the homogenate was heated for 4 min at 95 °C in an equal volume of sample buffer (100 mM Tris, pH 6.8, and 2% SDS, 2% 2-mercaptoethanol,

0.001% bromophenol blue, 20% glycerol). The samples were loaded onto 10% SDS-PAGE ready gels (Bio-Rad, Hercules, CA) in equal protein amounts (30 μg per lane). Samples were separated by electrophoresis in Tris–glycine buffer (25 mM Tris, 250 mM glycine, 0.1% SDS) at 100 V for approximately 120 min. Proteins were transferred overnight (12–14 h) to a PVdf membrane at 30 V in transfer buffer containing 20% MeOH, 20 mM Tris, 150 mM glycine, pH 8.0. Membranes were incubated for 1 h at room temperature in blocking buffer containing 5% nonfat powdered milk in TBS–Tween (20 mM Tris, 137 mM NaCl, 0.1% Tween-20), then washed three times for 10 min each in TBS–Tween. Membranes were incubated overnight (12–14 h) with primary antibodies directed against the NK-1 receptor protein (C-terminus, rat NK-1 protein, 393–407, Novus Biologicals) in blocking buffer at a dilution of 1:1000 with β-actin (1:6250, Sigma) used as a loading control. After washing six times for 15 min each with TBS–Tween, membranes were incubated in the appropriate horseradish peroxidase-conjugated secondary antibody, diluted 1:500 in blocking buffer for 2 h, then washed in TBS for 15 min. Peroxidase activity was detected using the Pierce SuperSignal West Femto Maximum Sensitivity Substrate kit (Pierce Chemicals, Rockford, IL) and quantified using LabWorks software (UVP, Upland, CA).

To control for variability in protein concentration between samples and between gels, the density ratio of NK-1/β-actin was compared between control and experimental values. Density levels for β-actin did not change following any of the manipulations, assuring that equal amounts of protein sample were loaded. Exposure times for each Western blot were adjusted so that density readings obtained were in the linear range of the film development process. The amount of protein used per sample (30 μg) was determined by varying the sample protein concentrations and determining the range that yielded a linear relationship between concentration and density readings, thus avoiding excessive loading in the gels and allowing for our antibodies to recognize changes in expression.

2.6. Systemic injection of the NK-1 antagonist

Lewis rats were injected with either DBTC ($n=24$) or vehicle ($n=20$). Also a naïve group receiving no manipulation was used ($n=20$). These original three groups were subdivided into groups receiving an intraperitoneal injection of the NK-1 antagonist CP-99,994 or its inactive enantiomer CP100,263 (generously provided by Pfizer, New York, NY). Doses of 1, 5, 10 and 30 mg/kg were used. In the day of the experiment, animals underwent behavioral testing to confirm the presence of nociceptive behaviors. After the first test, the drug or its enantiomer (dissolved in 0.9% saline) were injected intraperitoneally and behavioral testing was performed again 30 min later and 30 min thereafter up until 90 min after the initial injection.

2.7. Intrathecal injection of the antagonist

Lewis rats ($n=38$) were implanted with an indwelling intrathecal catheter on the same day they received a tail vein injection of either DBTC or vehicle. Rats were anesthetized with methohexital sodium (brevital sodium, 40 mg/kg ip) prior to receiving an intrathecal catheter for administration of drugs according to the method of Yaksh and Rudy (1976). A 4.5-cm intrathecal catheter (32-gauge, ReCathCo, Allison Park, PA) was implanted intrathecally through an opening in the atlanto-occipital membrane to the spinal T6–7 level. The catheter was linked to soft polyethylene tubing (PE10, i.d. 0.28 mm, o.d. 0.61 mm; Clay Adams Brand, Becton Dickinson Primary Care Diagnostics, Becton Dickinson, Franklin, NJ) and then connected to PE20 tubing that was tunneled under the skin where it remained subcutaneously until use. Rats were allowed to recover and after the recovery period, spontaneous normal behavioral activities were observed. The antibiotic gentamycin was applied topically to avoid any infection. Animals were housed in individual cages and monitored closely for the first 24 h.

On Day 3 after the surgery and tail vein injection rats were divided into different groups by doses [1 ($n=4$), 5 ($n=4$), 10 ($n=5$), 15 ($n=3$) and 20 ($n=3$) μg]. Behavioral testing was done before any intrathecal injection took place to assure that animals demonstrated nociceptive behaviors. Control animals included equal numbers of rats with no pancreatitis but with an implanted intrathecal catheter to administer each drug dose. After the first set of behavioral testing, the NK-1 antagonist CP99,994 or its inactive enantiomer CP100,263 was injected through the intrathecal catheter in a volume of 10 μl. A 10-μl ACSF flush was applied immediately after the drug injection. Behavioral testing resumed 30 min after the intrathecal injection. After testing, either morphine or lidocaine (10 μg or 20 mg/ml, respectively) were injected though the intrathecal catheter to confirm the ability of the intrathecal catheter to deliver substances to the subarachnoid space. Other animals received an injection of methylene blue for the same purpose and to check the spread of the intrathecal injection.

2.8. Data analysis and statistical procedures

For immunohistochemical data, stained sections were observed and staining density quantified. The average intensity of staining of lamina I and II in five random sections from each animal was determined by outlining the laminar borders and comparing the density of immunostaining relative to measurements obtained for white matter that was used as a reference point to normalize staining among sections.

For analysis of the Western blotting data, density for control values obtained using the LabWorks software (UVP) was defined as 100%; experimental densities are reported as a percentage of control density. Western blot data were

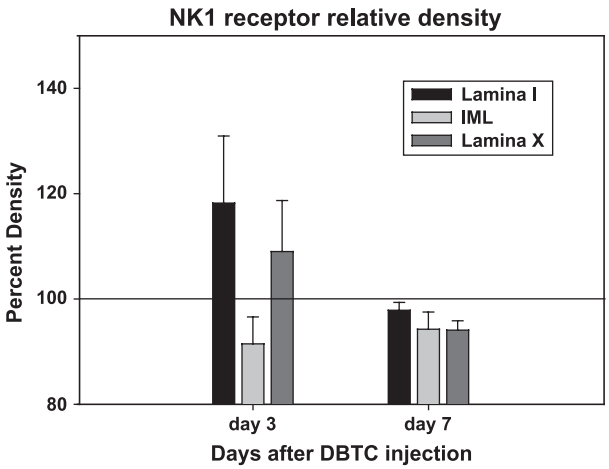

Fig. 2. Bar graph depicting the quantitation of NK-1 receptor staining in lamina I, intermediolateral cell column (IML) and lamina X of the spinal cord. The horizontal line represents the normalized value for staining intensity of control sections (100%). Data are expressed as a percentage of the normal values.

analyzed with one-way ANOVA followed by Fisher's post hoc test when indicated.

Behavioral data are expressed either as ± standard error of the mean or converted to percentages of maximum possible effect (%MPE) using the following formulas:

For mechanical stimulation:

$$\%\text{MPE} = \frac{\text{postdrug response} - \text{predrug response}}{\text{minimum response} - \text{predrug response}} \times 100$$

For thermal stimulation:

$$\%\text{MPE} = \frac{\text{postdrug latency} - \text{predrug latency}}{\text{cutoff time} - \text{predrug latency}} \times 100$$

where the minimum response is zero and the cutoff time is 25 s. The data were statistically evaluated using repeated measures analysis of variance (ANOVA) and differences

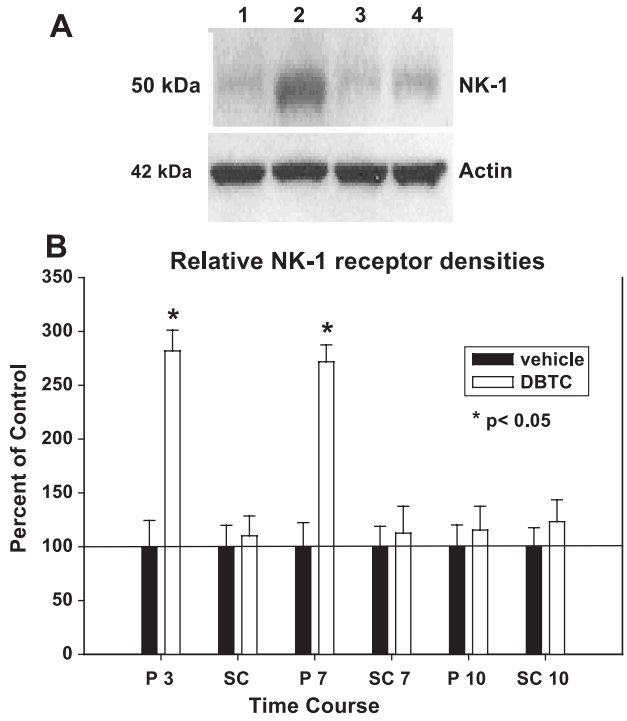

Fig. 4. (A) Representative Western blots of NK-1 protein and β-actin showing control levels in lanes 1 and 3 from vehicle-treated animals. Increased levels of NK-1 protein expressed in DBTC-treated animals with pancreatitis are shown in lanes 2 and 4. (B) The bar graph depicts quantitation of the density of the bands. NK-1 receptor protein in the pancreas (P) and spinal cord (SC) is expressed as a percent of the β-actin control density value.

were determined by post hoc comparison using the Scheffe test. Significance was set at $P < .05$.

3. Results

Staining in the spinal cord for the NK-1 receptor protein using the DAB method of visualization revealed no apparent changes in staining intensity as a result of pancreatitis. Quantitation of the staining demonstrated there were no changes in staining intensity at Days 3 and 7 after tail vein injection of DBTC in either the intermediolateral cell

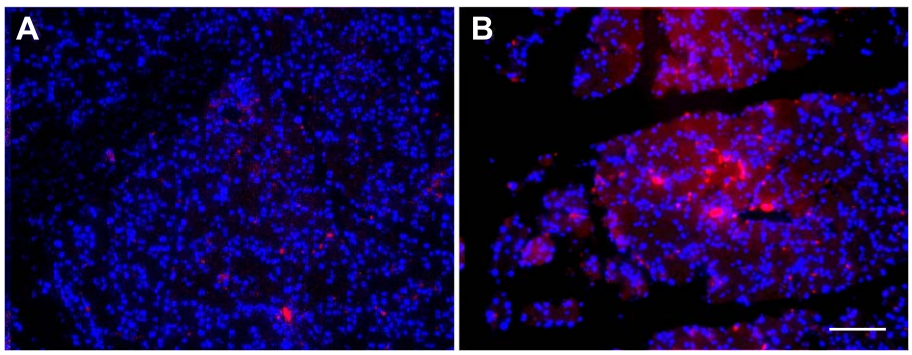

Fig. 3. Photomicrographs of pancreas showing NK-1 staining in the pancreas with CY3-conjugated antibody. Panels A and B are photomicrographs of sections stained with the NK-1 peptide from control and pancreatitis animals, respectively. Scale bar: 80 μm.

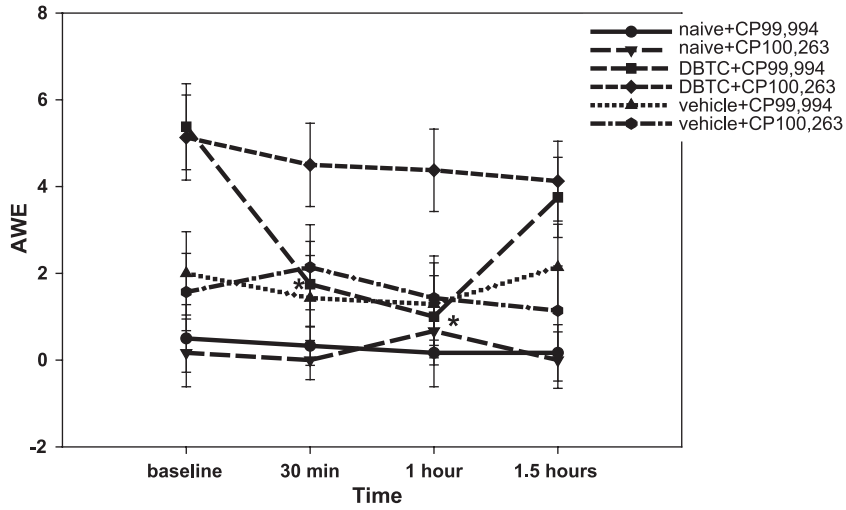

Fig. 5. Time course of the effects of the NK-1 antagonist CP99,994 and its enantiomer CP100,263 on mechanical hypersensitivity on Day 7 after induction of pancreatitis. The time course for the effects of the antagonist in the animals with pancreatitis is represented by the dashed line with squares. Statistical significance (*) was achieved at the 30- and 60-min time points with the antagonist CP99,994. The dose represented in this figure is 10 mg/kg ip. Data is expressed as S.E.M. AWE, average withdrawal events; DBTC, dibutyltin dichloride, used for induction of pancreatitis.

column or lamina X. There was a trend of increased staining density in superficial laminae (laminae I and II) that did not reach statistical significance (Fig. 2).

Immunofluorescent staining for the NK-1 receptor in pancreatic tissue revealed an apparent increase in the presence of immunofluorescence by visual inspection in pancreatic tissue from animals with pancreatitis compared with control tissues (Fig. 3a and b). Preadsorption controls confirmed the specificity of the immunofluorescence observed, as there was no staining present when the preincubation with the peptide preceded staining.

A significant increase in NK-1 receptor protein expression in the pancreas was demonstrated on Days 3 and 7 after tail vein injection of DBTC using Western blotting analysis (Fig. 4). Expression of the NK-1 protein in the spinal cord, however, did not change at any of the time points examined confirming the immunohistochemical results.

Injection of the NK-1 receptor antagonist CP99,994 at the systemic level (ip) attenuated the mechanical and thermal hypersensitivity. On Day 7 after DBTC injection the time course of the effect of CP99,994 was short. Behaviors were monitored for 90 min after injection and the effect for drug was not significant for either behavioral measure (mechanical, Fig. 5; thermal, Fig. 6). Similar results were seen for behavioral testing at other time points after DBTC injection (data not shown). The most effective dose was 10 mg/kg, and it is this dose that is depicted in the time course plot. The 10 and 30 mg/kg doses had the greatest

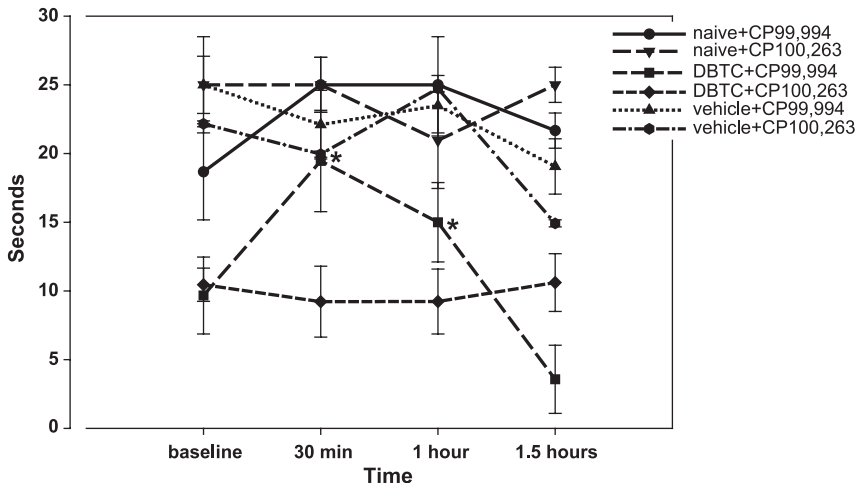

Fig. 6. Time course of the effects of the NK-1 antagonist CP99,994 and its enantiomer CP100,263 on thermal hypersensitivity on Day 7 after induction of pancreatitis. Data is expressed as withdrawal time (seconds) from the thermal stimulus ± S.E.M. applied to the abdomen. Statistical significance (*) was achieved at the 30- and 60-min time points with the antagonist CP99,994. The time course for the effects of the antagonist in the animals with pancreatitis is represented by the dashed line with squares. The dose represented in this figure is 10 mg/kg ip. DBTC, dibutyltin dichloride, used for induction of pancreatitis.

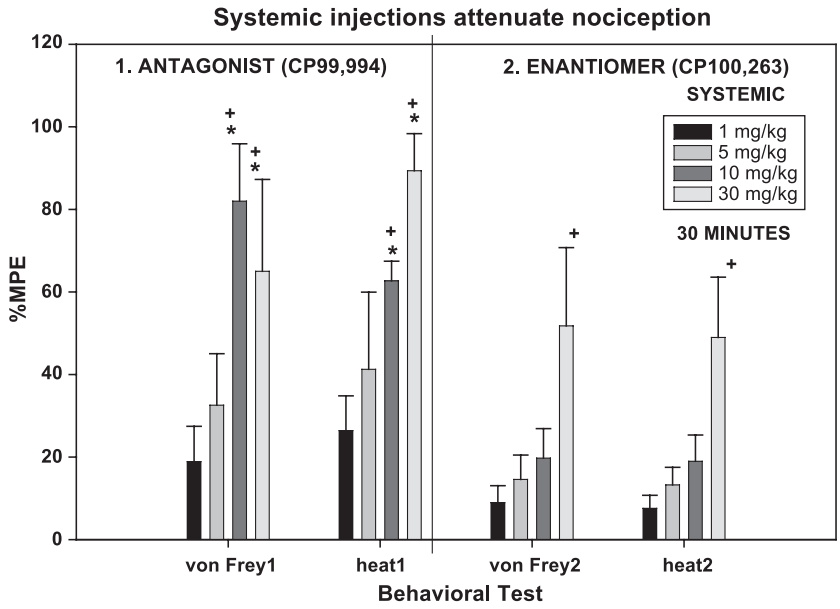

Fig. 7. Bar graph demonstrating the effect of the NK-1 antagonist CP99,994 on referred mechanical and thermal hypersensitivity 30 min after injection in rats with pancreatitis. The effects of the inactive enantiomer are also illustrated at different doses. The injections were made intraperitoneally. MPE, maximum possible effect.* denotes $P < .05$ vs. enantiomer; + denotes $P < .05$ vs. saline controls.

effect for attenuation of mechanical and thermal hypersensitivity. The enantiomer CP100,263 was effective in reducing the mechanical and thermal hypersensitivity only at the 30 mg/kg dose (Fig. 7).

Injection of the NK-1 antagonist at the spinal level (IT) was not effective in attenuating the mechanical and thermal hypersensitivity at any of the doses tested (Fig. 8). The enantiomer likewise did not have attenuating effects. When either morphine or lidocaine was injected at the conclusion of the experiment, both drugs were able to produce effects confirming the ability of the IT fiber to deliver drugs to the spinal cord level. Morphine at the 10-μg dose was able to attenuate the mechanical and thermal hypersensitivity in the few animals that were tested this way (data not shown). Lidocaine given at a dose of 20 mg/ml produced motor deficits in a different group of animals. Methylene blue was injected through the IT fiber to visualize the spread of the drug over the length of the spinal cord. Visual inspection

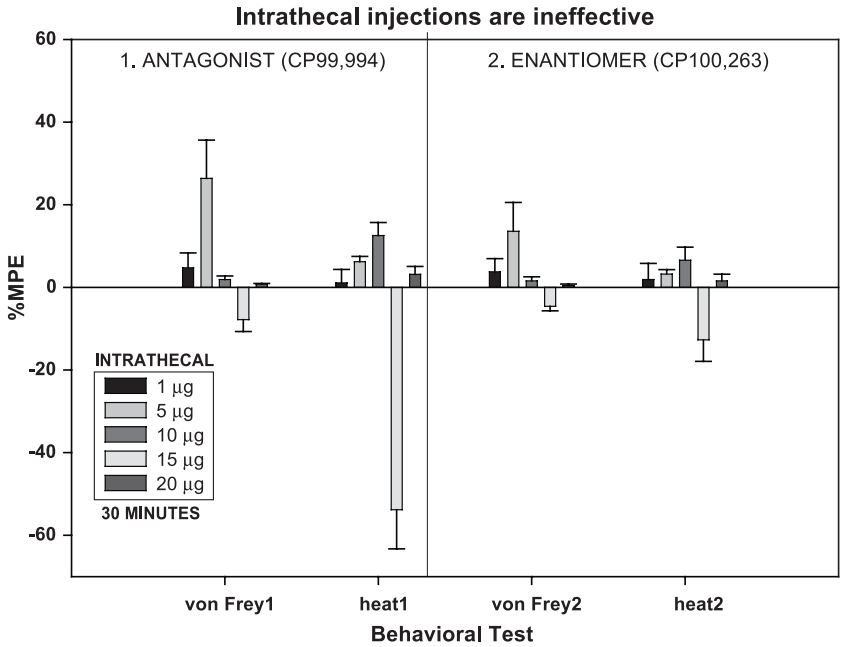

Fig. 8. Bar graph demonstrating the effect of the NK-1 antagonist CP99,994 on referred mechanical and thermal hypersensitivity 30 min after injection. The effects of various doses of the inactive enantiomer are also illustrated. The injections were made intrathecally. MPE, maximum possible effect.

confirmed that substances injected through the IT fiber spread about five segments rostral and caudal to the site where the tip of the catheter was placed.

4. Discussion

In the present study, immunohistochemistry and Western blotting were used to demonstrate increased expression of the NK-1 receptor in the pancreas while no apparent changes were detected in the spinal cord at the time points examined. The time points illustrated were chosen since they correlated with the nociceptive behaviors observed and quantified in a previous study where NK-1 receptor mRNA was up-regulated in the pancreas from patients diagnosed with chronic pancreatitis and this up-regulation correlated with pain scores (Shrikhande et al., 2001). The NK-1 receptor in the clinical study was up-regulated in nerve fibers, ganglia, blood vessels and inflammatory cells. It is important to mention that in chronic pancreatitis a significantly greater diameter and density of pancreatic nerves has been previously described (Bockman et al., 1988). Increased levels of growth-associated protein 43 (GAP-43), a marker of neuroplasticity, has also been reported. Increases in sensory neurotransmitters, such as SP and CGRP, which are present in intrinsic neurons have been demonstrated and are reportedly present in enlarged fibers in pancreatitis (Buchler et al., 1992; Di Sebastiano et al., 1997). These findings suggest that in pancreatitis nerve endings are actively sprouting and may contribute to pain in this condition. In chronic pancreatitis, enlarged pancreatic nerves exhibit increased immunoreactivity for SP (Buchler et al., 1992; Weihe et al., 1991). The findings of the present study that NK-1 receptor expression increases in the pancreas during pancreatic inflammation suggest that the receptor increase is another possible mechanism responsible for the nociceptive behaviors seen in animals with pancreatitis. This hypothesis is further supported by the results obtained in our behavioral study where the NK-1 antagonist CP99,994 was able to attenuate the nociceptive behaviors in animals with pancreatic inflammation. The inactive enantiomer had antinociceptive effects at the highest dose tested (30 mg/kg). Lower doses did not have an effect as expected. The effect at the high dose was likely due to the known effect of both these compounds on calcium channels (McLean et al., 1998). The effects of CP99,994 seen at lower doses are, therefore, specific to blockade of the NK-1 receptors. The site of action of the antagonist is difficult to assess since the antagonist readily crosses the blood–brain barrier (McLean et al., 1993), and therefore effects on the CNS cannot be excluded. The fact that we see an increase of NK-1 protein expression in the pancreas and not in the spinal cord implies that the pancreas is a likely candidate for the site of action of the NK-1 antagonist. The lack of effects by the antagonist when administered at the spinal level also provides further evidence that blockade of pancreatic NK-1 receptors is responsible for the behavioral effects seen in this study. Nonetheless, we cannot dismiss the possible role of NK-1 receptors at supraspinal sites. The doses selected for the intrathecal injections were chosen because they have been used to produce antinociceptive effects in other animal models (Henry et al., 1999; Sakurada et al., 1999; Sluka et al., 1997; Smith et al., 1994; Traub, 1996).

The etiology of pain in pancreatitis is a matter of great discussion. The role of SP in the pancreas is related to the localization of its receptors (NK-1) in the pancreas. There are many possible explanations as to how SP contributes to nociceptive states in pancreatitis. Two hypotheses emerge from the evidence presented here and elsewhere. First, previous studies demonstrated increased NK-1 mRNA in the epineural layers of nerves where the majority of small arteries and arterioles feeding the endoneural vasculature are located (Kummer et al., 1999; Zochodne and Ho, 1991). From these studies it was postulated that localization of NK-1 receptors in this site explained the moderate vasoconstrictor effects of SP in the pancreas. Thus, in view of these findings it was hypothesized that intrapancreatic nerve ischemia, as a result of enhanced NK-1 mediated vasoconstriction, was the likely mechanism involved in generation of pain in pancreatitis. This is consistent with findings in a feline model of pancreatitis where pancreatic blood flow was significantly reduced compared with normal pancreas (Karanjia et al., 1994; Patel et al., 1999). Our demonstration of increased expression of NK-1 receptor and the behavioral consequences of application of the NK-1 antagonist provide contributory evidence that the vasoconstriction induced by NK-1 and the subsequent reduction in blood flow play a role in the generation of increased nociceptive behaviors.

SP in the pancreas can act as both a neurotransmitter and as a contributor to the generation of neurogenic inflammation. SP released in peripheral tissue can produce plasma extravasation through actions on NK-1 receptors (Devor et al., 1989; Lembeck and Holzer, 1979; Saria, 1984). SP can also increase production and release of prostaglandin E_2 (Lotz et al., 1987), release of lysosomal enzymes (Johnson and Erdos, 1973) and release of interleukin 1 and the neutrophil chemoattractant interleukin 6 (Lotz et al., 1988). During inflammation, invading macrophages express NK-1 receptors (Ho et al., 1997; Lai et al., 1998), suggesting that SP in peripheral tissues can perpetuate the inflammatory cascade. Indeed, in previous studies, activation of the NK-1 receptor can produce plasma extravasation in the pancreas (Figini et al., 1997; Grady et al., 2000). Furthermore, it has been shown that the severity of experimental pancreatitis is reduced in NK-1 receptor and pre-protachykinin knockout mice (Bhatia et al., 1998, 2003). This evidence also emphasizes the important role that SP, acting though the NK-1 receptor, plays in the maintenance of pancreatic inflammation. Together with the evidence in the present study, these data establish a link between the role SP and the NK-1 receptor in neurogenic inflammation and pain in pancreatitis.

The conclusions from this study are as follows: (1) The NK-1 antagonist CP99,994 is effective in attenuating nociceptive behavior arising from pancreatic inflammation. (2) These effects occur at peripheral sites and are specific for the NK-1 receptor. (3) The NK-1 receptors in the pancreas have a role in the genesis of pancreatic nociception as demonstrated by their increased expression on the same days we observe increased nociceptive behaviors. The NK-1 antagonist, CP99,994, has been used successfully to reduce acute postoperative pain (Dionne et al., 1998). The possibility exists, therefore, that the NK-1 antagonist could be used to treat postoperative pain of abdominal origin, or more effective NK-1 antagonists could be devised with specificity of action only at peripheral sites to reduce the inflammation as a way to relief pain.

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