Combinations of Neurokinin Receptor Antagonists Reduce Visceral Hyperalgesia

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ABSTRACT

The effect of selective neurokinin receptor (NKR) antagonists for the NK1R (SR140,333), NK2R (SR48,968), and NK3R (SR142,801) on the visceromotor response to noxious colorectal distension (CRD) was examined. NKR antagonists or vehicle were given intrathecally (i.th.) to rats made hyperalgesic by intracolic instillation of zymosan or after intracolic instillation of saline (control). Given alone, the NK1R (up to 3 μg of SR140,333) and NK2R (up to 60 μg of SR48,968) antagonists tested failed to significantly affect responses to the noxious visceral stimulus. However, coadministration of 3 μg of SR140,333 and 60 μg of SR48,968 (both i.th.) significantly reduced responses to noxious CRD (p < 0.05 versus vehicle).

The NK3R antagonist (60 μg of SR142,801) significantly reduced responses to noxious CRD when given alone to either hyperalgesic (zymosan-treated) or normal (saline-treated) rats (p < 0.05 versus vehicle for both groups). Responses of rats receiving the NK3R antagonist in combination with either the NK1R or the NK2R antagonist were not different from rats receiving the NK3R antagonist alone. These results suggest that activation of spinal NK1R and NK2R, presumably by their endogenous ligands (substance P and neurokinin A), maintain visceral hyperalgesia and support the notion that activation of NK3R (presumably by neurokinin B) is pronociceptive.

The neurokinins are a family of endogenous neuropeptides that include substance P (SP), neurokinin A (NKA), and neurokinin B (NKB), agonists at the three known neurokinin receptors (NK1R, NK2R, and NK3R, respectively). These peptides and receptors are present in both the central nervous system and peripheral tissues and mediate a variety of biologic functions, including the transmission of nociceptive information (for review, see Nakanishi, 1991).

There is compelling evidence linking endogenous neurokinin and neurokinin receptors to nociceptive signaling. SP and NKA are costored in large, dense-core vesicles in the central terminals of unmyelinated afferent C-fibers that terminate in lamina I, outer lamina II, and lamina V of the spinal dorsal horn (Ogawa et al., 1985). Accordingly, immunoreactivity for NKRIs is most dense in the superficial dorsal horn (Zerari et al., 1997, 1998; Honoré et al., 1999). NK1R are activated by noxious, but not by non-noxious stimuli (Honoré et al., 1999).

In models of spontaneous pain and hyperalgesia, NK1R, NK3R, preprotachykinin-A (precursor for SP and NKA), and preprotachykinin-B (precursor for NKB) mRNAs are up-regulated in spinal cord (McCarson and Krause, 1994; McCarson, 1999). In rats with selective destruction of spinal NK1R-expressing neurons (by SP-saporin), capsaicin causes a less robust hyperalgesia than in normal rats (Nichols et al., 1999). NK1R antagonists reduce phase II of the formalin test, the development of inflammatory hyperalgesia, and NK1R agonist-induced potentiation of tail-flick latencies and caudally directed scratching and biting (Traub, 1996; Iyengar et al., 1997).

The existence of spinal NK2R has been controversial. Early studies with poorly selective radioligands demonstrated NK2R in the spinal cord (Ninkovic et al., 1984). Subsequent studies with more selective radioligands failed to detect NK2R in the spinal cord (Matuszek et al., 1998). However, this probably relates to the sensitivity of the technique because low levels of NK2R mRNA have been detected in rat spinal cord (Takeda and Krause, 1991). Recently, NK2R have been visualized on astroglial cells in rat spinal cord with the highest concentration in lamina I (Zerari et al., 1998). Behavioral studies supporting the existence of spinal NK2R have shown that intrathecal NK2R antagonists reduce thermal hyperalgesia after burn injury (Lofgren et al., 1999), neuropathic mechanical hyperalgesia (Coudere-Civiale et al., 1998), and both thermal and mechanical allodynia in streptozocin diabetic rats (Coudere-Civiale et al., 2000). In models of visceral pain and hyperalgesia, an NK2R antagonist reduced abdominal contractions induced by intraperitoneal

ABBREVIATIONS: SP, substance P; NKA, neurokinin A; NKB, neurokinin B; NK1R, neurokinin 1 receptor(s); NK2R, neurokinin 2 receptor(s); NK3R, neurokinin 3 receptor(s); NKPR, neurokinin receptor(s); CRD, colorectal distension; EMG, electromyographic; i.th., intrathecal(ly); AUC, area under the curve; ANOVA, analysis of variance; DMSO, dimethyl sulfoxide; MPO, myeloperoxidase; IBS, irritable bowel syndrome.
acetic acid (Julia and Bueno, 1997) and responses to rectal distension (Julia et al., 1994).

Less is known of the role of NKB and NK3R activation in pain and hyperalgesia. In the spinal dorsal horn, the majority of NKB is in intrinsic spinal neurons (Ogawa et al., 1985). NK3R (or its mRNA) have been found in the brain and spinal dorsal horn, but not in dorsal root ganglia or other peripheral tissues (McCarson, 1999). In the spinal dorsal horn, NK3R are found on lamina I and II neurons (Yashpal et al., 1990). NK3R in lamina II have also been localized to nerve terminals (Zerari et al., 1997), including SP-containing primary afferent terminals where their activation enhances SP release (Schmid et al., 1998). In cutaneous models of hyperalgesia, NK3R are up-regulated in the dorsal horn (McCarson and Krause, 1994). In acute visceral pain, an NK3R antagonist reduced both the number of abdominal contractions and responses of pelvic nerve afferents to noxious colonic distension (Julia et al., 1999).

Greater than 80% of visceral afferents contain SP (whereas fewer than 25% of cutaneous afferents do; Perry and Lawson, 1998), suggesting that neurokinins are especially important for visceral pain transmission. The present experiments studied the effects of spinal administration of neurokinin receptor antagonists on visceral hyperalgesia by using the model of colorectal distension (CRD). Selective antagonists for each neurokinin receptor were tested alone and in combination in rats with zymosan-induced visceral hyperalgesia (Coutinho et al., 1996). Because it has been well documented that spinal administration of NK1R antagonists can cause motor deficits, the NK2R and NK3R antagonists were tested for motor effects. Preliminary reports of some of these data have appeared in abstract form (Kamp et al., 2000).

Materials and Methods

Animals

Adult male Sprague-Dawley rats (400–425 g; Harlan Bioproducts for Science, Indianapolis, IN) were used in all experiments. Rats were housed singly with free access to food and water. All experimental procedures adhered to the Institutional procedures and were approved by the Institutional Animal Care and Use Committee at The University of Iowa.

Surgical Preparation

Rats were anesthetized with pentobarbital sodium 50 mg/kg i.p. (Abbott Diagnostics, North Chicago, IL) and electrodes (Teflon-coated stainless steel wire; Cooner Wire Sales, Chatsworth, CA) were sewn into the external oblique abdominal musculature, just above the inguinal ligament, for electromyographic (EMG) recording. At the same time, intrathecal (i.th.) catheters (polyethylene tubing PE-10, 8.5 cm) were placed extending to the lumbar enlargement. The EMG electrodes were subcutaneously guided to the dorsum of the neck and externalized for future access. The wounds were closed in layers with 4-0 silk. After surgery, rats were given 10 ml of 5% dextrose in normal saline (to replace fluids lost during surgery and to provide some nutrition) and allowed to recover for a minimum of 5 days before testing. Any animals with apparent motor defects or that lost significant weight after surgery were excluded. In rats used for motor (rotorod) testing, EMG electrodes were not placed.

Visceral Nociceptive Testing

Behavioral Testing. Contraction of the abdominal musculature during CRD in awake rats was the behavioral response quantified. On the day of testing, a 7-cm-long flaccid latex balloon tied to Tygon tubing was lubricated with Surgilube (E. Fougera and Co., Melville, NY) and inserted intra-anally until the end of the balloon was 1 cm inside the rectum (total insertion distance, 8 cm). The tubing was taped to the base of the tail to prevent displacement and connected to a constant pressure control device (Bioengineering, The University of Iowa, Iowa City, IA) that regulated inflation of the balloon. Materials and methods for CRD and recording and analysis of the EMG signal are fully described elsewhere (Gehart and Sengupta, 1996). Each distension trial lasted 40 s; EMG activity was quantified during the 10 s before distension (baseline), 20 s during distension, and 10 s after distension.

Experimental Protocol. On the day of testing, five phasic distensions (80 mm Hg, 20 s), at 4-min intervals, were given to establish a baseline response magnitude. Rats were then briefly anesthetized with halothane and either zymosan (1 ml, 25 mg/ml in saline; Sigma, St. Louis, MO) or an equal volume of sterile, preservative-free saline (vehicle) was instilled into the distal colon by using a 7-cm-long, 16-gauge stainless steel feeding needle connected to 1-ml syringe. Three hours after instillation of zymosan or saline, five phasic distensions were repeated (as described above) to evaluate the magnitude of colonic hyperalgesia.

After establishing hyperalgesia, drug (or vehicle) was injected into the cerebrospinal fluid surrounding the lumbar enlargement through the chronically implanted i.th. catheter. The externalized end of the i.th. catheter was connected to a 25-μl Hamilton syringe via a length of polyethylene tubing (PE-10) and a 33-gauge injection needle. Five microliters of drug solution (or vehicle) plus a 12-μl saline flush was delivered over 60 s. The progress of the injection was continuously monitored by following the movement of an air bubble in the tubing. The colons were distended immediately (0 min) and 4, 8, 12, 16, 20, 28, 36, 44, 52, and 60 min later. Each rat received only one dose of any drug and dose-response curves were obtained using multiple rats. At the end of each experiment, lidocaine (5 μl, 4% solution; Roxane Laboratories, Columbus, OH) was administered i.th. as described above. Four minutes later, a CRD trial was repeated to confirm proper placement of the i.th. catheter. Rats that did not have a minimum 50% reduction in the response to distension after lidocaine received methylene blue (5 μl i.th.) and the injection site was confirmed after laminectomy. Data from rats with improperly placed i.th. catheters were not included.

Data Analysis. All experimental groups consist of 5 to 12 rats. The visceralomotor response to CRD is represented as percentage of control (% control), where the mean of the prezymosan (naïve) responses is defined as 100%. All 20 s of the distension period was used for analysis. The overall effect of any treatment was determined by using the trapezoidal rule (AUC) of the time-response function for the 4- to 20-min time points, inclusive. Although drug effects were tested for 60 min, inspection of the data revealed that effects of the different drugs tested were reliably maximum during the first 20 min after i.th. drug administration. The AUC was calculated as the sum of the changes in the postdrug response (% control) from the prezymosan (naïve) response (100%) plotted against time by using the trapezoidal rule (AUC = ∫response × 16 min). By using this method, a drug that lowers responses to less than baseline (analgesia) will have a negative AUC. If the drug lowers responses to baseline but not further (antihyperalgesia), the AUC is zero. If the drug has no effect or enhances the responses (pronociceptive), the AUC is positive. The AUC data were analyzed by a one-way ANOVA. The Bonferroni correction for multiple comparisons was used if p < 0.05 for the ANOVA. A t test was used to compare the effect of intracolonic saline and zymosan (SigmaStat; Jandel Scientific, San Rafael, CA). Statistical significance is indicated when p < 0.05.

Motor Function Testing

Experimental Protocol. To determine the effect of neurokinin receptor antagonists on motor function, a rotorod test was used. The rotorod apparatus consisted of an elevated drum (6.5 cm in diameter) with a textured surface that rotated at a constant speed of 4.5 rpm.
The height of the drum from the floor of the test apparatus was 28 cm. Rats were trained on the rotorod until they could walk continuously for 120 s. Rats that could not meet this criterion were excluded. After training, each rat was loosely restrained in a canvas garden glove and a drug was injected through the chronically implanted i.th. catheter (as in CRD tests described above). Rats were tested 8, 30, and 60 min later. Each test consisted of three opportunities (trials) to remain on the apparatus continuously for 60 s. Animals were returned to their cages between trials and until the next time point.

Data Analysis. Experimental groups consisted of three to nine rats. Because only integer values were recorded for each time point (each rat was successful zero, one, two, or three of three trials), nonparametric analysis of the data was used. Calculation of median and percentile values was done by SigmaPlot (Jandel Scientific). Comparisons were done using the Mann-Whitney U test (a nonparametric, unpaired t test) against vehicle (i.th.) (Minitab for Windows, Version 11.12; Minitab Inc., State College, PA). Statistical significance is indicated when p < 0.05.

Drugs

The drugs used in this study were the NK1R antagonist SR140,333; the NK2R antagonist SR48,968; and the NK3R antagonist SR142,801. Also tested was SR142,806, an enantiomer of SR142,801 with significantly reduced NK3R binding affinity. All were generous gifts from Sonofi Research (Montpellier, France). Stock solutions were prepared by dissolving the drugs in 50% DMSO in sterile, preservative-free saline and then diluted as needed. Drug combinations were prepared by dissolving the NK3R antagonist in the desired concentration of either the NK1R antagonist or the NK2R antagonist. The NK1R/NK2R antagonist combination was prepared by mixing stock solutions containing a single antagonist and diluting to the desired concentration. The lack of effect of vehicle (50% DMSO) was determined in preliminary experiments.

Colonic Inflammation

The myeloperoxidase (MPO) assay was used to quantitate colonic inflammation. Rats were killed by an overdose of pentobarbital and the distal colon removed via laparotomy. The fresh tissue was suspended in hexadecyltrimethylammonium bromide (a detergent), minced with scissors, homogenized/sonicated, and freeze-thawed three times. The tissue suspensions were centrifuged and the supernatant assayed for MPO activity spectrophotometrically by measuring the change in absorbance to 460 nm with time. The color change of the supernatant containing 0.0005% hydrogen peroxide and O-dianisidine hydrochloride (a pH-sensitive indicator). The greater the rate of conversion of hydrogen peroxide into acid (by MPO), the more intense the color and the greater MPO present in the tissue.

Rats in these experiments received either no treatment (naive), intracolonic saline (3 or 24 h before testing), or intracolonic zymosan (1 ml, 25 mg/ml, 3 or 24 h before testing). A separate group of rats received five distensions (20 s at 80 mm Hg, 4 min apart). Immediately after the fifth distension, these rats received intracolonic zymosan (as described above). Three hours after zymosan, these rats received another set of five distensions. Colons were removed for the MPO assay immediately after the final distension.

The data were analyzed by a one-way ANOVA. The Bonferroni correction for multiple comparisons was used if p < 0.05 for the ANOVA (SigmaStat; Jandel Scientific). Statistical significance is indicated when p < 0.05.

Results

Intracolonic Zymosan Produces Hyperalgesia. CRD to 80 mm Hg is a noxious stimulus producing robust contractions of the abdominal musculature, termed the visceromotor response. The magnitude of this response is significantly enhanced after intracolonic administration of zymosan (Coutinho et al., 1996). As seen in Fig. 1, responses to noxious CRD were significantly greater in zymosan-treated rats than in saline-treated rats. In this figure and those that follow, an AUC analysis was performed on responses to CRD between the 4- to 20-min time points. As illustrated, prior intracolonic instillation of zymosan produced a visceral (colonic) hyperalgesia.

To further characterize the zymosan model of visceral hyperalgesia, the content of MPO, an enzyme contained primarily in neutrophils and therefore a measure of inflammation, was determined in the colons of rats after intracolonic treatment. The colons assayed from naive animals had 0.24 ± 0.02 MPO units/g of tissue. As expected, intracolonic treatment with saline did not significantly enhance MPO content (0.06 ± 0.01 and 0.32 ± 0.07 MPO units/g of tissue at 3 and 24 h after saline, respectively). In addition, there was no significant increase in MPO activity of colons taken from rats 3 or 24 h after intracolonic zymosan (0.25 ± 0.03 and 0.35 ± 0.10 MPO units/g of tissue, respectively). Repetitive, noxious CRD (as applied in Fig. 1) also did not significantly enhance MPO activity in zymosan-treated rats (0.41 ± 0.02 MPO units/g of tissue).

Motor Function. NK1R antagonists given i.th. produce motor defects in rats (Vaught and Scott, 1987; Traub, 1996), but NK2R and NK3R antagonists have not been similarly studied. We tested the dose-dependent effects of i.th. SR48,968 and SR142,801 on motor function by using the rotorod test. Figure 2 presents the rotorod performance of rats 8 min after i.th. drug (or vehicle) administration.

% control

response to CRD

AUC or salin

response to CRD

intracolonic saline

intracolonic zymosan

0 20 40 60 80 100 120 140 150

region of AUC analysis

saline

zymosan

800

1600

Fig. 1. Effect of treatment with intracolonic zymosan on the visceromotor response to noxious CRD. Responses to CRD (80 mm Hg, 20 s) were recorded from naive rats, 3 h after intracolonic zymosan or saline, and at the indicated times after i.th. vehicle (50% DMSO). Data are represented as mean ± S.E.M., calculated as percentage of control, where the response of each rat before zymosan or saline (baseline, b) is defined as 100%. The inset presents the corresponding AUC of the 4- to 20-min times of the above-described time courses. Responses to CRD were significantly enhanced by intracolonic zymosan relative to saline-treated animals (*p < 0.05; t test; n = 10 for both groups).
Doses of 6 (data not shown), 10, or 30 µg i.th. administered to rats (data not shown) caused no significant reduction in rotorod performance. A greater dose of the NK2R antagonist SR48,968 (300 µg i.th.) caused flaccid paralysis and also significantly decreased rotorod performance in all rats 8 and 30 min after administration, with recovery seen by 80 min (30- and 60-min data not shown). Lesser doses (30 and 60 µg i.th.) of either the NK2R antagonist SR48,968 or the NK3R antagonist SR142,801 produced no obvious deficits in motor function or degradation of rotorod performance at any time tested. In addition, coadministration of the NK1R antagonist and the NK2R antagonist (3 and 60 µg respectively, i.th.) did not reduce rotorod performance. Doses that significantly affected motor function were not considered analgesic/antihyperalgesic.

**Neurokinin Receptor Antagonists Given Singly.** To determine the involvement of spinal NK1R in visceral hyperalgesia, i.th. administration of the NK1R antagonist SR140,333 was tested in intracolonic zymosan-treated (hyperalgesic) rats. Figure 3A shows that 10 and 30 µg of SR140,333 reduced responses to noxious CRD. However, obvious motor deficits were observed in animals that received i.th. doses of 6 (data not shown), 10, or 30 µg (e.g., flaccid paralysis, belly-down posture, delayed/absent righting reflex). Animals given a dose of 3 µg of SR140,333 showed no observable motor impairment; however, there was no significant effect on responses to noxious distension at this i.th. dose. Subcutaneous administration of 3 µg of SR140,333 also did not significantly affect responses to CRD in zymosan-treated rats (data not shown). The AUC evaluation presented in Fig. 3B clearly demonstrates that the only doses with a significant effect on responses to CRD were those doses that also produced significant motor effects (m).

Figure 4A shows the time course of effect of 30, 60, and 100 µg of the NK2R antagonist SR48,968 (i.th.). Again, the AUC was used to evaluate dose-dependent effects. Figure 4B demonstrates that only one dose affected responses to CRD (100 µg); however, motor deficits (m) were demonstrated at this dose in the rotorod test (Fig. 2). Lesser doses (30 and 60 µg) caused no significant motor deficits (Fig. 2) and also had no effect on responses to noxious CRD. In addition, subcutaneous administration of 60 µg of SR48,968 did not significantly affect responses to CRD in zymosan-treated rats (data not shown).

In contrast to the NK1R and NK2R antagonists, the NK3R antagonist SR142,801 significantly reduced zymosan-induced visceral hyperalgesia at a dose that did not also produce motor deficits (60 µg i.th.). Mean responses were reduced from 137.5 ± 9.7% (3 h after zymosan) to 70.6 ± 5.2% of baseline 20 min after i.th. injection (Fig. 5A). A lesser dose (30 µg) of SR142,801 also reduced responses to noxious CRD, but the magnitude of the reduction was not significant at this...
Doses greater than 60 μg may further reduce responses to noxious CRD, but 100 μg of SR142,801 produced significant motor effects in the rotorod test (Fig. 2) and was not tested further. Neither subcutaneous administration of 60 μg of SR142,801 nor i.th. administration of the inactive enantiomer of SR142,801 (60 μg of SR142,806) significantly affected responses to noxious distension in zymosan-treated rats (data not shown), confirming spinal cord and receptor specificity, respectively.

In addition, i.th. SR142,801 (60 μg) significantly reduced responses to noxious distension in animals treated 3 h earlier with intracolonic saline (nonhyperalgesic). The mean response was reduced to 78.6 ± 12.2% of the presaline baseline 20 min after i.th. injection (Fig. 5B). In both normal and hyperalgesic animals, the antinociceptive effect diminished over time with responses returning to baseline within 45 min. A summary of these data (AUC evaluation) is presented in Fig. 5C. At the 60-μg dose, the NK3R antagonist significantly reduced responses to CRD (versus vehicle) in both zymosan-treated (hyperalgesic) and saline-treated (nonhy-

**Fig. 4.** Effect of i.th. administration of the NK2R antagonist SR48,968 on responses to noxious CRD. A, time course of the effect of the NK2R antagonist on zymosan-produced visceral hyperalgesia. Responses to CRD (80 mm Hg, 20 s) were recorded from naive rats, 3 h after intracolonic zymosan (z), and at the indicated times after i.th. drug or vehicle. Data are represented as mean ± S.E.M., calculated as percentage of control where the response of each rat before zymosan (baseline, b) is defined as 100%. B, corresponding area under the curve of the 4- to 20-min points on the above-described time courses for evaluation of dose-dependent effects. The only dose significantly different from vehicle was 100 μg (*p < 0.05 versus vehicle, one-way ANOVA with the Bonferroni correction for multiple comparisons; n = 6–10); this dose also produced significant motor effects (m; Fig. 2).

**Fig. 5.** Effect of i.th. administration of the NK3R antagonist SR142,801 on responses to noxious CRD. A and B, time courses of the effect of the NK3R antagonist in zymosan-treated (hyperalgesic) (A) and saline-treated (nonhyperalgesic) (B) rats. Responses to CRD (80 mm Hg, 20 s) were recorded from naive rats, 3 h after intracolonic zymosan or saline (3 h), and at the indicated times after i.th. drug or vehicle. Data are represented as mean ± S.E.M., calculated as percentage of control where the response of each rat before intracolonic zymosan or saline (baseline, b) is defined as 100%. C, corresponding area under the curve of the 4- to 20-min points on the above-described time courses for evaluation of dose-dependent effects. At the 60-μg dose, the NK3R antagonist significantly reduced responses (versus vehicle, veh) in both hyperalgesic and nonhyperalgesic rats (*p < 0.05 versus vehicle, one-way ANOVA with the Bonferroni correction for multiple comparisons; n = 8–10). Hyperalgesia in zymosan-treated rats is confirmed by the significantly greater AUC (i.th. vehicle) relative to saline-treated rats.

Combinations of Neurokinin Receptor Antagonists. NK1R, NK2R, and NK3R are on different cell populations in the spinal dorsal horn (see Discussion). Presumably then,
each receptor could mediate nociception via a separate mechanism; however, this hypothesis is untested. If indeed separate signaling pathways exist, we would expect that combinations of NK1R, NK2R, and NK3R antagonists would be more efficacious than administration of any single antagonist. The highest dose of each antagonist lacking motor effects was chosen for testing. Motor defects were not observed when antagonists were given in combination (Fig. 2).

All of the antagonist combinations tested significantly reduced responses to noxious CRD in zymosan-treated animals (versus vehicle; Fig. 6A). The AUC was used to generate a summary figure (Fig. 6B), facilitating comparison of the effects of combinations of antagonists to solo administration (replotted from Figs. 3–5). As discussed above, neither the NK1R antagonist (SR140,333, 3 µg i.th.) nor the NK2R (SR48,968, 60 µg i.th.) was efficacious when administered alone. However, simultaneous i.th. administration of the NK1 and NK2 antagonists caused a significant reduction in the response to CRD. The NK3R antagonist was efficacious alone (SR142,801, 60 µg i.th.; Fig. 5). As seen in Fig. 6, coadministration of the NK1R antagonist (SR140,333, 3 µg i.th.) neither reversed nor enhanced the effect of the NK3R antagonist (SR142,801, 60 µg i.th.). We attribute the antihyperalgesic effect of the NK1R/NK3R antagonist combination to the effect of the NK3R antagonist alone. We also tested the effect of coadministration of the NK2R and NK3R antagonists. Like the NK1R antagonist, coadministration of the NK2R antagonist (SR48,968, 60 µg i.th.) neither reversed nor enhanced the antihyperalgesic effect of the NK3R antagonist (SR142,801, 60 µg i.th.) (Fig. 6). Again, the antihyperalgesic

![Figure 6](https://jpet.aspetjournals.org/issue/issue.html) Effect of simultaneous i.th. administration of NKR antagonists on responses to noxious CRD in zymosan-treated rats. A, time course of the effect of simultaneous administration of pairs of neurokinin receptor antagonists: the NK1R antagonist (NK1Ra) SR140,333, the NK2R antagonist (NK2Ra) SR48,968, and the NK3R antagonist (NK3Ra) SR142,801. Responses to CRD (80 mm Hg, 20 s) were recorded from naive rats, 3 h after intracolonic zymosan (zym), and at the indicated times after i.th. drugs or vehicle. Data are represented as mean ± S.E.M., calculated as percentage of control where the response of each rat before zymosan (baseline, b) is defined as 100%. B, corresponding area under the curve of the 4- to 20-min points on the above-described time courses. Single administration data from Figs. 3 to 5 are replotted for comparison. Simultaneous administration of the NK1R and NK2R antagonists reduced responses to CRD at doses that had no effect alone. Simultaneous administration of the NK3Rα with either the NK1Ra or the NK2Ra reduced responses to CRD, but this effect was not greater than the effect of the NK3R antagonist alone. (p < 0.05 versus vehicle; *p < 0.05 versus NK1R antagonist alone; one-way ANOVA with the Bonferroni correction for multiple comparisons; n = 7–10).
effect of the NK2R/NK3R antagonist combination appears to be due to the effect of the NK3R antagonist alone.

**Discussion**

**Intracolonic Zymosan Models Irritable Bowel Syndrome (IBS).** The defining characteristics of IBS are visceral pain accompanied by diarrhea or constipation in the absence of a demonstrable pathology (Thompson et al., 1999). IBS is currently considered a disorder of altered motility and sensation from the small and large intestines. Several studies have shown that IBS patients have decreased visceral pain thresholds and increased areas of referred pain (i.e., visceral hyperalgesia; for review, see Mayer and Gebhart, 1994). This report confirms that intracolonic instillation of zymosan produces a visceral hyperalgesia as determined by increased responses to a noxious intensity of CRD (Coutinho et al., 1996). Because infiltration of neutrophils and inflammation are not associated with intracolonic instillation of zymosan (see Results), the exaggerated responses to CRD model the discomfort and pain that characterize IBS.

**NKR Antagonists Cause Motor Impairment.** That i.th. NK1R antagonists produce motor deficits in rats has been well documented. For example, the NK1R antagonist [d-Pro2, d-Trp7,9]-SP produces flaccid paralysis of the hindlimbs (Vaught and Scott, 1987) and the nonpeptide NK1R antagonist CP 96,345 (i.th.) causes paralysis of the hindlimbs, but at greater doses than required for antinociception (Traub, 1996). However, this motor deficit was not NK1R-specific because the stereoisomer (with no NK1R binding affinity) causes equal motor dysfunction (Yamamoto and Yaksh, 1991). Motor deficits caused by i.th. NK2R or NK3R antagonists have not been previously reported in rats. A few published reports mention that no behavioral changes were noted with i.th. administration (Ishizuka et al., 1995; Coudore-Civiale et al., 1998). In mice, subcutaneous administration of the NK2R antagonist SR48,968 decreases rotordor performance (Seguin et al., 1995). In rats, the NK3R agonist senktide (i.th.) causes transient hindlimb flaccidity, decreased time on the rotordor, and thermal hyperalgesia (Linden and Seybold, 1999). However, at the time of maximal hyperalgesia, there is no motor impairment (Linden and Seybold, 1999). The present study found that both the NK2R antagonist SR48,968 and the NK3R antagonist SR14,281 at doses of 100 μg (i.th.) caused motor impairment. These data suggest that spinal NK2R and NK3R may be involved in motor control. However, the antinociceptive and motor effects may be mediated by different receptors, with the motor effects likely to be due to actions of these compounds on calcium channels.

**An NK3R Antagonist Is Antinociceptive/Antihyperalgesic.** The NK3R antagonist (SR14,281, 60 μg i.th.) significantly reduced responses to CRD in both normal (saline-treated) and hyperalgesic (zymosan-treated) rats, suggesting that the NK3R mediates normal responses to acute visceral pain at the level of the spinal cord. These effects are antinociceptive and are not due to motor impairment. In other models of hyperalgesia, this NK3R (i.th.) antagonist reduced mechanical hyperalgesia in streptozocin-diabetic and mononeuropathic rats (Coudore-Civiale et al., 1998) and blocked NK3R agonist (senktide)-induced thermal hyperalgesia (Linden and Seybold, 1999). In two visceral pain models, systemic administration of this NK3R antagonist reduced both CRD-induced contractions of the abdominal musculature and CRD-induced excitation of pelvic nerve afferents (Julia et al., 1999). Clearly, NK3R modulate normal responses to a noxious stimulus and their activation may be necessary for the maintenance of hyperalgesia.

**NKR Antagonists in Combination.** The present study, and others, noted a significant attenuation, but incomplete suppression of responses to a noxious stimulus by an NK3R antagonist (to ~70% of baseline). It has been shown that some NK3R are located on the central terminals of primary afferents in the spinal dorsal horn (Schmid et al., 1998). NK3R agonists enhance and antagonists inhibit SP release from these central terminals (Schmid et al., 1998). Because SP is the endogenous ligand for the NK1R, we hypothesized that antagonizing spinal NK1R could enhance NK3R antagonist-induced antinociception. On the other hand, several reports have described an antinociceptive effect of spinal NK3R agonists in behavioral tests (Laneuville et al., 1988; Couture et al., 1993). From these reports, one might hypothesize that the addition of an NK1R antagonist could block NK3R antagonist-induced antinociception.

Neither of these hypotheses were supported. The addition of the NK1R antagonist or the NK2R antagonist neither enhanced nor suppressed NK3R antagonist-induced antinociception. Although this interpretation is relevant only for the dose combinations tested, dose selection was limited by confounding motor effects produced by i.th. administration of the NK1R and NK2R antagonists. This result is supported by a study demonstrating that NKB-containing neurons rarely (<5%) synapse with SP-containing neurons. Instead, NKB-containing dendrites are postsynaptic to nonpeptidergic, unmyelinated neurons (McLeod et al., 2000). The present study supports those authors’ suggestion that SP and NKB signal nociception independently.

Other investigators have also found that NK1R and NK2R antagonists fail to modulate NK3R-mediated events. An NK3R agonist causes cardiovascular changes (increased heart rate and mean arterial pressure) and characteristic behaviors (face-washing, grooming, and wet-dog shakes) that were blocked by an NK3R antagonist but not by a cocktail of NK1R and NK2R antagonists (Picard et al., 1994). The NK3R antagonist SR14,281 increases tail-flick latencies, but this antinociception was not blocked by either an NK1R or NK2R antagonist (Couture et al., 2000).

Given alone, the NK1R (up to 3 μg of SR140,333 i.th.) and NK2R (up to 60 μg of SR48,968 i.th.) antagonists had no antihyperalgesic effect. It could be argued that the visceral hyperalgesia produced by zymosan (30–50% increase over baseline responses to CRD) is insufficient to activate NK1R and NK2R systems. However, this is unlikely because zymosan-induced hyperalgesia is sufficient to enhance NK1R activation (as measured by NK1R internalization), presumably by enhancing SP (and therefore NKA) release into the spinal dorsal horn (Honore et al., 2002).

There is other evidence that SP and NKA are neither necessary nor sufficient to nociceptive signaling or the development of hyperalgesia. For example, SP/NKA knockout mice, NK1R knockout mice, and wild-type mice develop the same magnitude of cutaneous hyperalgesia (De Felipe et al., 1998; Basbaum, 1999). Also, rats where spinal NK1R have been knocked down (via antisense oligonucleotides) have nor-
eral behavioral responses to intraplantar formalin (Hua et al., 1998). In addition, neuropeptide receptors may influence cutaneous and visceral hyperalgesia differently. NK1R knockout mice have normal pressor responses to CRD but fail to develop acetic acid-induced visceral hyperalgesia (Laird et al., 2000). However, in these same mice, cutaneous inflammation and hyperalgesia in response to complete Freund's adjuvant is normal (DeFelipe et al., 1998).

SP and NKA are synthesized from the same gene and are colocalized in storage vesicles in the axon terminals of primary afferents (Ogawa et al., 1985). Although both NK1R and NK2R are found in lamina I of the spinal cord, they are expressed on different cell types. Several studies have localized spinal NK1R to neurons in lamina I-VI, around the central canal (lamina X), and the intermediolateral nucleus (for review, see Quartara and Maggi, 1998). In the spinal cord, NK2R are expressed on lamina I astrocytes, not neurons (Zerari et al., 1998). NK1R are not normally expressed on astrocytes, but appear after nerve injury (Mantyh et al., 1989). It is likely that NK1R and NK2R modulate responses to noxious stimuli via different pathways. For these reasons, we simultaneously administered NK1R and NK2R antagonists. Although neither the NK1R antagonist nor the NK2R antagonist was effective in reducing responses to noxious CRD when given alone, simultaneous administration significantly reduced responses to noxious CRD in hyperalgesic rats.

This result is supported by other studies that have investigated the effect of combinations of NK1R and NK2R antagonists. An NK1R antagonist, but not an NK2R antagonist, partially reduces l-dopa-induced bladder hyperactivity and coadministration reduces this hyperactivity more than the NK1R antagonist alone (Ishizuka et al., 1995). Inhibition of NKA causes bronchoconstriction in guinea pigs that is partially reduced by an NK2R antagonist and completely reduced by coadministration of NK1R and NK2R antagonists (Ricciardolo et al., 1999). No other studies have investigated the effect of combinations of NK1R and NK2R receptor antagonists on pain or hyperalgesia.

NK1R, and/or NK2R are elevated in clinical pain states (Lindh et al., 1997; Onouha and Alpar, 1999), including visceral pain states (Mantyh et al., 1988; Renzi et al., 2000). NKA, NKB, and NK3R are likely to be elevated as well. Given the recent failures of NK1R antagonists as analgesics in human pain trials (Goldstein et al., 2000), combinations of antagonists may be necessary to have a significant clinical effect. Combinations of NK1R and NK2R antagonists are likely to be especially important in the management of IBS because NK2R antagonists reduce diarrhea (by slowing intestinal transit) without being constipating (for review, see Scarpignato and Pelosini, 1999).

The principal findings of this study are that an i.th. NK3R antagonist is antihyperalgesic, if not analgesic, and that simultaneous i.th. NK1R and NK2R antagonism is antihyperalgesic, although neither is effective alone. The present results support the notion that visceral hyperalgesia is maintained by actions at spinal NK1R and NK2R, presumably by their endogenous ligands (SP and NKA) released from the central terminals of visceral afferents. In addition, NK3R mediate responses to noxious visceral input. The management of IBS discomfort and pain is a challenging clinical problem. NK3R antagonists and combinations of NK1R and NK2R antagonists may present therapeutic targets for development of novel treatments for visceral hyperalgesia.

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References


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