Effects of Kappa Opioid Receptor-Selective Agonists on Responses of Pelvic Nerve Afferents to Noxious Colorectal Distension

X. Su, J. N. Sengupta, and G. F. Gebhart
Department of Pharmacology, College of Medicine, The University of Iowa, Iowa City, Iowa 52242

Su, X., J. N. Sengupta, and G. F. Gebhart. Effects of kappa opioid receptor-selective agonists on responses of pelvic nerve afferents to noxious colorectal distension. J. Neurophysiol. 78: 1003–1012, 1997. The aim of this study was to examine the effects of κ-opioid receptor selective agonists on responses of mechanosensitive afferent fibers in the pelvic nerve. Single-fiber recordings were made from pelvic nerve afferents in the decentralized S1 dorsal root of the rat. A total of 572 afferent fibers in the S1 dorsal root were identified by electrical stimulation of the pelvic nerve; 252 (44%) responded to noxious colorectal distension (CRD; 80 mmHg). Of these 252 fibers that responded to CRD, 100 were studied further. All 100 fibers gave monotonic increases in firing to increasing pressures of CRD. Eighty-eight fibers had low thresholds for response (mean: 3 mmHg) and 12 fibers had high-thresholds for response (mean: 28 mmHg). Responses of 17 fibers also were tested after instillation of 5% mustard oil (MO) into the colon. The resting activity of 16/17 fibers significantly increased after MO instillation; 13 (77%) also exhibited sensitization of responses to graded CRD when tested 30 min after intracolonic MO instillation. The effects of κ₁-opioid receptor preferring agonists (U50,488H, U69,593 and U62,066), the κ₂-opioid receptor preferring agonist bremazocine, and the κ₉-opioid receptor preferring agonist naloxone benzoxyhydrzone (nalBzoH) were tested on responses of 64 mechanosensitive afferent fibers to noxious CRD. All five agonists dose-dependently inhibited afferent fiber responses to noxious CRD. Doses producing inhibition to 50% of the control response to CRD did not differ among the five agonists, ranging from ~4 to 15 mg/kg. The effects of κ₁, κ₂, and κ₉ receptor agonists were attenuated by naloxone; two κ-opioid receptor-selective antagonists were ineffective. There were no differences in the dose-response relationships of these drugs for fibers recorded from untreated and irritant-treated colons. Conduction velocities of the fibers remained unaffected after high doses of all tested agonists. In an in vitro study, U50,488 (10⁻⁴ M) did not produce any significant change in the tension of colonic smooth muscle. These results document that responses of mechanosensitive pelvic nerve afferent fibers innervating the colon are inhibited by κ-opioid receptor agonists having varying affinities for putative κ-opioid receptor subtypes. The inhibitory effects of these drugs likely are mediated by an action at receptors associated with the afferent fibers. The receptor at which these effects are produced is κ-opioid-like but clearly different from the κ-opioid receptor characterized in the CNS and is perhaps an orphan receptor.

INTRODUCTION

Discomfort and pain are common components of functional bowel disorders such as the irritable bowel syndrome (Mayer and Gebhart 1994), for which pharmacological treatments have been largely unsuccessful. Recent studies suggest that κ-opioid receptor agonists (κ-ORAs) may represent a useful therapeutic approach. In a model of visceral nociception in the rat, intrathecal administration of μ- and δ-opioid receptor agonists, but not the κ-ORAs U50,488, attenuate pseudoadverse visceromotor and cardiovascular responses to noxious colorectal distension (CRD) (Danzerbrink et al. 1995; Harada et al. 1995a,b). Systemic administration of U50,488, however, attenuates these pseudoadverse responses to noxious CRD, suggesting that κ-ORAs can attenuate visceral nociceptive responses by a peripheral action. Recent electrophysiological studies support this interpretation. We have found that κ-opioid (U50,488 and fedotozine), but not either μ-opioid (morphine and fentanyl) or δ-opioid [D-pen², D-pen⁵-énkaphalin (DPDPE) and SNC-80], receptor agonists dose-dependently inhibit responses of mechanosensitive pelvic nerve afferent fibers to noxious CRD in the rat (Sengupta et al. 1996a). In a subsequent study, we similarly found that κ-opioid (U50,488, U69,593, and U62,066), but not μ- or δ-opioid receptor agonists dose-dependently inhibited responses of mechanosensitive pelvic nerve afferent fibers innervating the urinary bladder of the rat (Su et al. 1997).

Thus κ-ORAs clearly have peripheral antinociceptive actions that may be useful in the control of pelvic visceral pain. Several features of our previous results, however, require amplification. First, evidence from in vitro binding studies indicates that different κ-ORAs show different degrees of selectivity for κ-ORA subtypes (Attali et al. 1982; Devlin and Shoemaker 1990; Nock et al. 1990; Zukin et al. 1988; see Rothman 1994 for review); we have to date studied only κ-ORAs with greatest affinity for the κ₁ receptor. Second, although only a limited number of κ-ORAs have been studied, the calculated effective doses were unexpectedly similar. Finally, whereas the effects of the κ-ORAs were antagonized by the nonselective opioid receptor antagonist naloxone, the κ-opioid receptor-selective antagonist nor-BNI was ineffective (Sengupta et al. 1996a; Su et al. 1997), suggesting that the receptor in the periphery at which κ-ORAs act may be different from the κ-opioid receptor characterized in the CNS.

Accordingly, the objectives of this study were fourfold: to examine the effects of κ-ORAs with relative selectivity for different subtypes of the κ-opioid receptor on responses of mechanosensitive pelvic nerve afferent fibers to noxious CRD; to evaluate the effect of acute inflammation of the colon on these mechanosensitive afferent fibers; to examine the relative potency of κ-ORAs in the presence of acute colonic inflammation; and to determine whether the effects of the κ-ORAs tested are due to an effect of conduction of the axons or on the compliance of the colon. Some of these data have been presented previously in abstract form (Su et al. 1995).
METHODS

General procedures

Male Sprague-Dawley rats weighing 410–530 gm (Harlan, Indianapolis, IN) were used in this study. Food, but not water, was withheld for 24 h before the experiment. Rats were anesthetized initially with sodium pentobarbital (40–45 mg/kg i.p.; Nembutal, Abbott Laboratories, North Chicago, IL) and were maintained thereafter with supplementary intravenous doses of 5–10 mg·kg⁻¹·h⁻¹. The trachea was cannulated to permit artificial ventilation with room air. For intra-arterial drug administration, a catheter was passed to the descending aorta via the left common carotid artery. The femoral artery and vein were catheterized for measurement of arterial pressure and administration of sodium pentobarbital, respectively. The rat was paralyzed with pancuronium bromide (0.3 mg/kg iv) and subsequently ventilated with room air with a positive pressure pump (55–60 strokes/min and 3 ± 4 ml stroke volume). Supplemental doses of pancuronium bromide (0.2–0.3 mg·kg⁻¹·h⁻¹) were given to maintain paralysis during the course of the experiment. Mean arterial blood pressure was monitored continuously and was maintained >80 mmHg with intravenous 5% dextrose in saline given in a bolus of 1–1.5 ml as required. Core body temperature was maintained at 36°C by a hot-water-circulating heating pad and an overhead feedback-controlled heat lamp (thermoprobe inserted into the thoracic esophagus). At the end of experiments, rats were killed by an overdose of intravenous pentobarbital. The experimental protocol was approved by the Institutional Animal Care and Use Committee of The University of Iowa.

Surgical procedures

The lower abdomen was exposed by a 3- to 4-cm-long incision laterally at the left flank. The urinary bladder was emptied and catheterized (PE-100) through the fundus. The urethra was ligated close to its entry to the penis and urine was evacuated constantly via the fundic catheter. The urinary bladder was distended (80 mmHg) to estimate the proportion of pelvic nerve innervation to this organ.

For CRD, a 6- to 7-cm long, 2- to 3-cm diam flaccid, flexible latex balloon was inserted via the anus into the descending colon and rectum. The outside diameter of the balloon when inflated was greater than the intraluminal diameter of the colon of the rat. Therefore, the pressure measured during distension reflected actual intracolonic pressure. The balloon catheter was connected to a distension control device via a low-volume pressure transducer (see Gebhart and Sengupta 1995 for details).

The left testis, vas deferens, and seminal vesicle were tied and removed. The prostate was reflected laterally to access the major pelvic ganglion and pelvic nerve. The pelvic nerve was isolated from the surrounding fatty tissues and a pair of Teflon-coated stainless steel wires stripped at the tips were wrapped around the pelvic nerve and sealed with nonreactive Wacker gel (Wacker Silicone, Adrian, MI). The hypogastric, pudendal, and femoral nerves were isolated and transected. The sciatic nerve was approached through the ischiatric notch and transected. The lateral tail nerve was approached at the root of the tail and transected and the abdomen was closed with silk sutures.

The lumbarosacral spinal cord was exposed by laminectomy (T₃–S₃), and the rat was suspended in a stereotactic frame by thoracic vertebra and ischia clamps. The dorsal skin was reflected laterally and tied to make a pool for mineral oil. The dura was removed carefully, and the spinal cord was covered with warm (37°C) mineral oil.

Recording of afferent nerve action potentials

The S1 dorsal root was identified and decentralized close to its entry to the spinal cord. Recordings were made from the distal cut end of the central processes of primary afferent fibers. A length of nerve fiber was draped over a black micro-base plate immersed in warm (37°C) mineral oil. The dorsal rootlet was split into thin bundles, and a fine filament was isolated from the bundle to obtain a single unit. Electrical activity of the single unit was recorded by placing the teased fiber over one arm of a bipolar silver-silver chloride electrode; a fine strand of connective tissue was placed across the other pole of the electrode. Action potentials were monitored continuously by analogue delay and displayed on a storage oscilloscope after initial amplification through a low-noise AC differential amplifier. The action potentials were processed through a window discriminator and frequency of impulses was counted (1-s binwidth) on-line using the spike2/CED 1401 data acquisition program. Peristimulus time histograms, intracolonic pressure, and blood pressure were displayed on-line continuously.

Experimental protocol

Pelvic nerve input to the S₁ dorsal root was identified first by electrical stimulation of the pelvic nerve (single 0.5-ms square-wave pulse at 3–8 mA). Fibers were classified on the basis of their conduction velocities; those with conduction velocities <2.5 m/s were considered unmyelinated C fibers, and those with conduction velocities >2.5 m/s were considered thinly myelinated Aβ-fibers. The organ innervated was identified by response to phasic CRD (80 mmHg, 2–3 s), phasic UBD (80 mmHg, 2–3 s), or mechanical probing of the anal mucosa. If a fiber responded to CRD, a stimulus-response function to phasic distending pressures of 5, 10, 20, 30, 40, 60, 80, and 100 mmHg, 30 s at 4-min intervals was determined.

For acute inflammation of the colon, the balloon was removed and 1.5–2 ml of 5% mustard oil (MO) in peanut oil was instilled via the anus into the descending colon. The MO was retained in the colon for 15 min, and the colon was then washed with warm saline. The balloon was reinserted and responses of the previously characterized fiber to graded CRD were determined 30 and 60 min after instillation of MO. At the end of an experiment, both untreated and treated colons were removed and fixed in 10% formaldehyde. After 7 days, the tissues were transferred to a cold (4°C) 30% sucrose in 0.1 M phosphate buffer solution for cryoprotection. After 24–48 h, sections (20 μm) of the tissues were cut on a cryostat and stained with hematoxylin and eosin for histological examination.

The effects of putative κ₁-ORA (U50,488, U69,593, U62,066), κ₂-ORA (bremazocine), and κ₁-ORA (naloxone benzoylhydrazide, nalBzoH) were tested on responses of mechanosensitiveafferents to 80 mmHg CRD. All drugs were administered intravenously using a cumulative dose paradigm. To reduce possible contributions of drug metabolism and/or excretion on effects of subsequent, cumulative doses of drug, each dose of drug was given 90 s before the onset of distension. In previous work, a predistension interval of 120 s was used effectively (Sengupta et al. 1996a). Cumulative dose-response relationships for U50,488, U69,593, U62,066, and nalBzoH were obtained by giving 0.5, 0.5, 1, 2, 4, 8, and (except for U50,488) 16 mg/kg (maximum cumulative dose, 32 mg/kg): bremazocine was given in cumulative doses of 0.2, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 mg/kg. Drugs were tested on responses of fibers recorded from untreated and irritant-treated colons.

To determine whether drug effects were produced at opioid receptors, the effects of U62,066 (κ₁), bremazocine (κ₂), and nalBzoH (κ₁) were tested before and 10–25 min after intraarterial administration of a nonreceptor-selective dose of naloxone (2 mg/kg). In addition, cumulative, dose-dependent effects of U50,488 also were studied in the presence of 2-(3,4-dichlorobenzyl)-N-methyl-N-[1(1 s)-1-(3-isothiocyanatophenyl)-2-(1-pyrrolidinyl)ethyl]acetamide (DIPPA), a selective κ-opioid receptor antagonist. In three experiments, DIPPA (0.25 mg/kg s.c.) was injected

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four hours before the experiment and in another three experiments, two doses (10 mg/kg sc) of DIPPA were injected 48 and 24 h before the experiment. Pretreatment with another selective κ-opioid receptor antagonist, nor-binaltorphimine dihydrochloride (nor-BNI, 2 mg/kg, 4 h, tested with U62,066; 20 mg/kg, 24 h and 48 h, tested with U50,488) also was studied.

**In vitro study of colonic smooth muscle**

To examine potential effects of κ-ORAs on colonic longitudinal and circular smooth muscle, three rats were anesthetized with sodium pentobarbital (40–45 mg/kg ip). The lower abdomen was opened by a midline incision and the descending colon was removed and placed in a dish containing oxygenated (95% O₂:5% CO₂) Kreb’s solution [which contained (in mM) 118.07 NaCl, 4.69 KCl, 2.52 CaCl₂·H₂O, 0.57 MgSO₄·H₂O, 1.01 NaH₂PO₄·H₂O, 25 NaHCO₃, and 1.11 glucose]. The colon was opened longitudinally along the mesenteric border and pinned flat. An unstretched muscle strip 1.5 mm wide and 1 cm long was cut in either a longitudinal direction to obtain a longitudinal muscle strip or perpendicular to the longitudinal direction to obtain a circular muscle strip. One end of each strip was tied to a stainless steel tissue holder and suspended in a 100-ml double-jacketed tissue bath containing Kreb’s solution that was oxygenated continuously. The outer jacket of the tissue bath was circulated with warm (37°C) water to maintain the temperature of the Kreb’s solution. The other end of the tissue strip was connected to an isometric force displacement transducer with a silk thread. The transducer was mounted on a two-directional manipulator. The tissues were given a preload tension of 1.5 g and allowed to equilibrate for 1 h before testing. Contractions of the muscles were recorded on a rectilinear polygraph.

**Data analysis**

The resting activity of an afferent fiber was counted for 60 s before colonic distention, and the response to distension was determined as the increase in discharge during distension above its resting activity. Stimulus-response functions (SRF) to graded CRD were plotted for each individual fiber and a least-squares regression line was obtained from the linear part of the SRF. The regression line then was extrapolated to the ordinate (representing distension pressure) to estimate response threshold.

For the in vitro smooth muscle study, the maximum contractile response of the tissue was obtained by adding acetylcholine (10⁻⁵ M) to the bath. The effects of opioid receptor agonists were expressed as a percentage of the maximum response of the tissue to acetylcholine.

All data are expressed as means ± SE. Results were analyzed using Student’s t-test or an analysis of variance (ANOVA) for repeated measures. The inhibitory dose 50 (ID₅₀; dose to produce 50% inhibition of the response to distension) and 95% confidence intervals were calculated from the 20–80% component of the cumulative dose-response curve (Tallarida and Murray 1991). A value of P < 0.05 was considered statistically significant.

**Drugs**

Acetylcholine bromide (MW: 226, Sigma Chemical, St. Louis, MO), bremazocine [MW: 351.9, Research Biochemicals, (RBI)], Natick, MA], morphine sulphate (MW: 668.7, Merck Chemical Division, Merck, Rahway, NJ), and U50,488 (MW: 465.5, RBI) were dissolved in distilled water. U69,593 (MW: 521.5, RBI) was dissolvd in 0.1 N HCl. SNC-80 (MW: 449.6, Tocris Cookson, St. Louis, MO) and U62,066 (MW: 365.5, RBI) were dissolved in 10% methanol. NaBzOH (MW: 445.52, RBI) was dissolved in 2.5% acetic acid. DIPPA (MW: 449.6, Tocris Cookson) was dissolved in 10 mM dimethyl sulfoxide. A 5% solution of mustard oil (MW: 99.6, Fluka, Rokonkoma, NY) was made in peanut oil.

**RESULTS**

**Fiber sample**

A total of 572 afferent fibers in the S1 dorsal root were identified by electrical stimulation of the pelvic nerve. Of the 572 afferent fibers, 348 (60%) were myelinated Aδ-fibers (mean CV: 10.1 ± 0.4 m/s) and 224 (40%) were unmyelinated C-fibers (mean CV: 1.88 ± 0.02 m/s). Two hundred-fifty-two (44%) fibers responded to noxious CRD (80 mmHg); 122 were C fibers (mean CV: 2 ± 0.03 m/s), and 130 were myelinated Aδ-fibers (mean CV: 6.3 ± 0.3 m/s). One hundred sixty-six (29%) fibers responded to noxious urinary bladder distention (UBD; 80 mmHg); 76 were C-fibers (mean CV: 1.9 ± 0.05 m/s) and 90 were Aδ-fibers (mean CV: 9.5 ± 0.7 m/s). Fourteen (2%) fibers responded to mechanical probing of the anal mucosa, all of which were Aδ-fibers (CV: 15.5 ± 2.7 m/s). One hundred-forty (25%) fibers did not respond to UBD, CRD, or mechanical probing of the anal mucosa; 26 were C fibers (mean CV: 1.8 ± 0.06 m/s); and 114 were Aδ-fibers (mean CV: 14.2 ± 0.8 m/s). The characteristics of this sample of S1 pelvic nerve afferent fibers are similar to what we found in earlier studies (Sengupta and Gebhart 1994; Sengupta et al. 1996a).

**Responses to CRD**

Of the 252 fibers that responded to CRD, 100 fibers were characterized for responses to graded intensities of CRD (5–100 mmHg).

**Resting activity**

In the presence of the balloon in the descending colon, 95 fibers exhibited an ongoing discharge (mean: 0.8 ± 0.1 impulses/s; range: 0.01–6.0 impulses/s); 5 fibers had no resting activity. There was no significant difference (P > 0.05) between the resting activities of C fibers (mean: 1.0 ± 0.2 impulses/s; n = 40) and Aδ-fibers (mean: 0.8 ± 0.2 impulses/s; n = 44).

**Stimulus-response functions**

All fibers gave monotonic increases in response to increasing pressures of CRD. Extrapolation of the linear portion of SRFs of these fibers revealed two distinct populations of fibers: a large group of fibers had low thresholds (LT) for response (mean: 3.1 ± 0.5 mmHg; n = 88) and a smaller group of fibers had high thresholds (HT) for response (mean: 28.4 ± 1.4 mmHg; n = 12). Figure 1 illustrates typical responses of LT and HT afferent fibers to graded, phasic CRD; the LT fiber had no ongoing activity but clearly responded to the least intensity of CRD (5 mmHg), whereas the HT fiber was spontaneously active, but first responded to 30 mmHg CRD. SRFs of individual fibers are plotted in Fig. 2; the insets illustrate the mean SRFs of each group of fibers.

**Effects of MO**

The mechanosensitive properties of 17 fibers were tested after instillation of 5% MO into the colon. The resting activity of 16/17 fibers increased significantly from 0.5 ± 0.2 to 5.3 ± 1.4 and 4.1 ± 1.1 impulses/s 30 and 60 min after...
MO instillation, respectively (Fig. 3). Thirteen of the 17 fibers (10/14 LT and 3/3 HT) also exhibited sensitization of responses to graded CRD 30 min after MO instillation. Responses of the 13 sensitized fibers to graded CRD were tested subsequently 60 min after MO instillation; none of these fibers exhibited further sensitization or desensitization of responses at 60 min. Figure 3 illustrates the mean SRFs of these sensitized fibers before and 60 min after intracolonic MO instillation.

Microscopic examination of MO-treated colons removed at the end of experiments revealed clear, acute damage relative to colons taken from untreated rats. Damage consisted of loss of surface epithelium; neutrophils and protein rich fibers (10/14 LT and 3/3 HT) also exhibited sensitization of responses to graded CRD 30 min after MO instillation. Fluid were present in the superfi
cial lamina propria. Neutrophils also were present in the lumen in MO-treated rats and there was evidence of submucosal edema in some sections.

**Effects of κ-ORAs on responses to noxious CRD**

All κ-ORAs tested (U50,488H, U69,593, U62,066, bremazocine, and nalBzoH) dose-dependently inhibited responses of mechanosensitive pelvic nerve afferent fibers to colonic distension (CRD). Responses of fibers are illustrated topmost in each pair as peristimulus time histograms (PSTH; 1-s binwidth); phasic distending pressure is presented below. A: responses of a low-threshold fiber. Extrapolated threshold for response of this fiber was 5 mmHg. B: responses of a high-threshold fiber. Extrapolated threshold for response of this fiber was 27 mmHg.

**FIG. 1.** Responses of 2 pelvic nerve afferent fibers to graded intensities (5–100 mmHg; 30 s) of colorectal distension (CRD). Responses of fibers are illustrated topmost in each pair as peristimulus time histograms (PSTH; 1-s binwidth); phasic distending pressure is presented below. A: responses of a low-threshold fiber. Extrapolated threshold for response of this fiber was 5 mmHg. B: responses of a high-threshold fiber. Extrapolated threshold for response of this fiber was 27 mmHg.

**FIG. 2.** Stimulus-response functions (SRFs) of 100 pelvic nerve afferent fibers to graded intensities of CRD (5–100 mmHg). A: individual SRFs of 88 low-threshold afferent fibers. Inset: mean SRF; mean extrapolated response threshold was 3.1 ± 0.5 mmHg. B: individual SRFs of 12 afferent fibers that responded at a high-threshold to CRD. Inset: mean SRF; mean extrapolated response threshold was 28.4 ± 1.4 mmHg.
FIG. 3. A: resting nerve activity before and after mustard oil (MO) treatment. Of 17 MO-treated fibers tested, 16 (94%) exhibited a significant increase ($P < 0.05$) in resting activity at 30 min that was sustained at 60 min. B: responses of a high-threshold pelvic nerve afferent fiber to graded CRD (5–100 mmHg) before and after 60 min after instillation of 5% MO into colon. Top row of PSTHs (1-s binwidth) represents responses of fiber before treatment, and bottom row represents responses of same fiber 60 min after MO treatment; distending pressures are shown below. Note that both resting activity and responses at greater distending pressures increased after MO treatment.

C: summary of SRFs of low-threshold (LT; ◀ and ◤) and high-threshold (HT; △ and ◥) pelvic nerve afferent fibers before (pre, ◀ and ◤) and after (post, ●) instillation of MO into colon. Of 14 LT fibers tested, 10 fibers (◀) exhibited sensitization after MO treatment (●). All 3 MO-treated HT fibers (△) exhibited an increase in response magnitude (≀), and their thresholds for response decreased significantly (27.1 ± 0.9 mmHg to 5.5 ± 2.4 mmHg; $P < 0.05$) 60 min after MO treatment.

noxious CRD. Figure 4 illustrates examples of dose-dependent inhibition of responses of three fibers by each major subtype of $\kappa$-ORA tested. The slopes of the dose-regression functions and the doses producing inhibition to 50% of the control response to 80 mmHg CRD did not differ among the five agonists (Fig. 5, Table 1). There were also no differences in the dose-response relationships of these drugs on LT or HT fibers (data not shown) or on fibers from untreated and MO-treated colons.

We have documented previously that the nonselective opioid receptor antagonist naloxone antagonizes the effects of $\kappa$-ORAs (Segupta et al. 1996a). In the present study, naloxone (2 mg/kg, given intravenously 10–15 min before an agonist) attenuated the effects of the $\kappa_1$-ORA U62,066, the $\kappa_2$-ORA bremazocine, and the $\kappa_3$-ORA nalBzoH (Fig. 6A). The effects of DIPPA, an irreversible $\kappa$-opioid receptor-selective antagonist (Chang et al. 1994), on the inhibitory effects of U50,488 were tested in several experiments. In three experiments, DIPPA (0.25 mg/kg sc) was injected 4 h before the experiment. In another three experiments, DIPPA (10 mg/kg sc) was injected 48 and again 24 h before the experiment. Neither pretreatment regimen antagonized the inhibitory effects of U50,488 on responses to CRD (Fig. 6B). The effect of pretreatment with another $\kappa$-opioid receptor-selective antagonist, nor-BNI (Takemori et al. 1988; 2 mg/kg, 4 h; 20 mg/kg, 48 h and 24 h), was tested against U50,488 and U62,066. Nor-BNI also failed to antagonize the inhibitory effect of these $\kappa$-ORAs (data not shown).

Effects of $\kappa$-ORAs on CV

To examine whether the inhibitory effects of these drugs on responses to CRD could be explained by an action on axonal conduction, CVs of fibers were measured before and after $\kappa$-opioid receptor agonists (ORAs) on responses of pelvic nerve afferent fibers to CRD (80 mmHg, 30 s every 4 min shown at bottom). Drugs were injected intraarterially in a cumulative dose as indicated (Fig. 4A). A: responses of a LT fiber to CRD after a cumulative 16 mg/kg dose of $\kappa_1$-opioid receptor-selective agonist U50,488. B: responses of a LT fiber to CRD after a cumulative 12.8 mg/kg dose of $\kappa_2$-opioid receptor-prefering agonist bremazocine. C: responses of a LT fiber to CRD after a cumulative 32 mg/kg dose of $\kappa_3$-opioid receptor-prefering agonist nalBzoH.
produced by acetylcholine (ACh), respectively. In circular muscle, the same concentrations of morphine and U50,488 produced 8.3 ± 1.2 and 12.5 ± 2.9% of the maximum response to ACh, respectively. SNC-80 did not change the tone of longitudinal or circular muscle.

**DISCUSSION**

We previously reported that U50,488 (κ₁) and fedotozine (a mixed κ/μ-ORA), but neither μ-ORA (morphine and fentanyl) nor δ-ORA (DPDPE and SNC-80) dose-dependently attenuated responses of mechanosensitive pelvic nerve afferent fibers to noxious CRD (Sengupta et al. 1996a). Similarly, only κ-ORAs and not either μ- or δ-ORAs dose-dependently attenuated responses of mechanosensitive pelvic nerve fibers to noxious urinary bladder distension (Su et al. 1997). In both studies, naloxone was shown to antagonize the effect of the κ-ORAs tested, providing evidence that the effects observed were produced at opioid receptors. We have not, however, in either of the two previous studies or in the present study, been able to antagonize the effects of κ-ORAs with antagonists (nor-BNI, DIPPA) selective for κ-opioid receptors. We addressed this in two ways in the present study. The range of agonists tested were extended to include other κ₁ receptor subtypes as well as those reported to be relatively selective for what are termed κ₂ (bremazocine) and κ₃ (nalBzoH) receptor subtypes. Second, several doses and pretreatment regimens of the selective κ-opioid receptor antagonists were studied. DIPPA (0.25 mg/kg, 4 h; 10 mg/kg, 48 and 24 h before the test) and nor-BNI (2 mg/kg, 4 h; 20 mg/kg, 48 and 24 h before the test) both failed to antagonize the effects of the κ₁ receptor agonists U50,488 and U62,066. It is appreciated widely that the latency to effect of nor-BNI can be long and the duration of effect days to weeks (e.g., Broadbear et al. 1994; Horan 2002).

**Effects of opioids on colonic muscle**

We also determined whether the selective μ-, δ-, or κ-ORAs morphine, SNC-80 and U50,488, respectively, affected the tone or contractility of longitudinal and circular smooth muscles of the colon. In longitudinal muscle, morphine (10⁻³ M) and U50,488 (10⁻⁴ M) produced 9.6 ± 2.4 and 10.8 ± 1.8% of the control (maximum) contraction after cumulative administration of the greatest dose tested. None of the κ-ORAs tested significantly affected CV. The mean CVs of the fibers before and after κ-ORAs were: 2.5 ± 0.3 to 2.4 ± 0.3 (n = 8, 16 mg/kg U50,488), 6.6 ± 2.1 to 6.2 ± 2.0 (n = 8, 32 mg/kg U69,593), 5.2 ± 1.5 to 4.8 ± 1.3 (n = 7, 32 mg/kg U62,066), 5.9 ± 1.5 to 5.6 ± 1.5 (n = 13, 12.8 mg/kg bremazocine), and 4.1 ± 0.6 to 4.1 ± 0.61 (n = 5, 32 mg/kg nalBzoH).

**TABLE 1. Responses to 80 mmHg CRD produced by κ-ORAs**

<table>
<thead>
<tr>
<th>Agonist and Treatments</th>
<th>n</th>
<th>ID₅₀*</th>
<th>I₅₀±SE</th>
<th>C 0.3 1 3 10 30 100 % control</th>
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</thead>
<tbody>
<tr>
<td>U50,488</td>
<td>8</td>
<td>9.8 (8.4–11.3)</td>
<td>39.9 ± 4.2</td>
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<tr>
<td>MO-treated colon</td>
<td>3</td>
<td>6.7 (3.7–12)</td>
<td>30.9 ± 5.5</td>
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<tr>
<td>DIPPA pretreated</td>
<td>3</td>
<td>11.3 (9.2–13.5)</td>
<td>44.6 ± 6.0</td>
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</tr>
<tr>
<td>MO-treated colon</td>
<td>3</td>
<td>12.3 (11.4–13.2)</td>
<td>41.9 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>U62,066</td>
<td>7</td>
<td>6.7 (5.8–7.6)</td>
<td>6.4 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>MO-treated colon</td>
<td>9</td>
<td>6.5 (4.6–9.2)</td>
<td>10.5 ± 1.4</td>
<td></td>
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<tr>
<td>U69,593</td>
<td>8</td>
<td>9.8 (8–12)</td>
<td>23.6 ± 3.4</td>
<td></td>
</tr>
<tr>
<td>MO-treated colon</td>
<td>8</td>
<td>13.2 (7.8–22.2)</td>
<td>30.6 ± 5</td>
<td></td>
</tr>
<tr>
<td>Bremazocine</td>
<td>3</td>
<td>7.0 (3.7–7.9)</td>
<td>28.1 ± 3.0</td>
<td></td>
</tr>
<tr>
<td>MO-treated colon</td>
<td>3</td>
<td>10.2 (1–20.4)</td>
<td>44.6 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>U62,066</td>
<td>7</td>
<td>14.5 (9.7–21.7)</td>
<td>28.1 ± 6.2</td>
<td></td>
</tr>
<tr>
<td>MO-treated colon</td>
<td>3</td>
<td>12.2 (10.3–14.3)</td>
<td>30.3 ± 13.4</td>
<td></td>
</tr>
</tbody>
</table>

**MO, mustard oil; DIPPA, 2-(3,4-dichlorophenyl)-N-methyl-N-[(1s)-1-(3-isothiocyanato-phenyl)-2-(1-pyrroldinyl)ethylyl] acetamide; CRD, colorectal distension. * Inhibitory dose 50s in milligrams per kilogram with 95% confidence intervals in parentheses. † Mean percentage inhibition expressed as means ± SE.**
which their effects in the periphery are produced is unlike the κ-opioid receptor characterized in the CNS.

Because Kosterlitz and associates (1981) presented evidence for the existence of a κ-type opioid receptor in guinea pig brain, multiple κ receptors have been described. For example, κ₁ receptor (U69593-sensitive) and κ₂ receptor (U69593-insensitive) binding sites were reported (Nock et al. 1990; Zukin et al. 1988). In the guinea-pig brain, U50,488 and U69,593 binding at κ₁a and κ₁b receptors, respectively, and bremazocine, binding with greater affinity at κ₂a and κ₂b receptors have been reported (Rothman et al. 1990; see Rothman 1994 for review). Binding studies also have identified a κ₃ binding site that is nalBzoH-sensitive (Clark et al. 1989; Gistrik et al. 1989; Paul et al. 1990; Price et al. 1989). In the present study, all κ-ORAs tested had similar dose-response functions and similar effective, inhibitory dose ranges. Given the broad range of effective antinociceptive doses reported in the literature for κ-ORAs (e.g., Leighton et al. 1988; Millan 1989; Piercey and Einspahr 1989; Schmauss 1987), including visceromotor responses to CRD (Burton and Gebhart 1995b), the present results suggest that none of the putative κ₁, κ₂, or κ₃ opioid receptors characterized in binding studies are sites at which the present results all produced. Further, the selective κ-opioid receptor antagonist nor-BNI possesses high affinity for κ₁ receptors relative to other κ-receptor subtypes, yet was ineffective against the three κ₁ receptor agonists tested here. Butelman et al. (1993) examined the effects of nor-BNI in a warm-water tail-withdrawal procedure in rhesus monkeys. Nor-BNI was an effective antagonist of U50,488 and U69,593, producing a half-log or greater shift in their dose-effect curves, but this same dose had no effect on the dose-effect curve for the putative κ₂ receptor agonist bremazocine. Thus whereas differential antagonism by nor-BNI of different κ-ORAs suggests actions of agonists at different κ-opioid receptor subtypes, no evidence for an effect, much less a selective effect, was found here.

The inhibitory effects of the κ-ORAs documented here could be due to: inhibition of transduction processes at the neuron sensory ending (receptor), axon, or cell body by an action at κ-like opioid receptors; blocking conduction of nerve action potentials; or a change in compliance of the agonist bremazocine. Therefore, differential antagonism by nor-BNI of different κ-ORAs suggests actions of agonists at different κ-opioid receptor subtypes, no evidence for an effect, much less a selective effect, was found here.

Nor-BNI has been reported to produce peak antagonist effects against U50,488, U69,593, and bremazocine within the 24- to 48-h pretreatment time tested here (Broadbear et al. 1994; Horan et al. 1992). DIPPA is a newer κ-opioid receptor antagonist similarly characterized by a relatively long latency to effect and a long duration of action (Chang et al. 1994). Accordingly, the two antagonists were tested after relatively short and long pretreatment intervals. Because nonreceptor-selective doses of naloxone reliably antagonized the effects of κ-ORAs on mechanosensitive pelvic nerve afferent fiber responses to distension (Sengupta et al. 1996a; Su et al. 1997; present study), the most parsimonious explanation is that the κ-like receptor at
an allosteric coupling between central κ-opioid receptors and L-type Ca²⁺ channels (Gandhi and Ross 1987, 1988). Electrophysiological studies also suggest an interaction between κ-opioid receptors and voltage-dependent Ca²⁺ channels (see North 1993 for overview). Activation of κ-opioid receptors reduces a voltage-dependent Ca²⁺ conductance in dorsal root ganglion and myenteric neurons (Cherubini and North 1985; MacDonald and Werz 1986). Attali et al. (1989) concluded, on the basis of effects of κ-ORAs on stimulated Ca²⁺ influx in spinal cord-dorsal root ganglion cocultures, that κ-opioid receptors are coupled negatively to the dihydropyridine class of voltage-dependent Ca²⁺ channels and that their effects are receptor mediated. In behavioral studies, Barro et al. (1995) similarly conclude that κ-opioid receptors are linked functionally to dihydropyridine-sensitive Ca²⁺ channels (i.e., L-type). Kappa-ORAs also have been reported to inhibit P-type (Kanemasa et al. 1995) and N-type (Tallent et al. 1994) Ca²⁺ channels. Attenuation of responses of pelvic nerve afferent fibers to noxious colorectal stimulation by κ-ORAs, which, moreover, have virtually indistinguishable effective dose ranges could arise if similar mechanisms are operative in the periphery.

Acute inflammation of the colon was produced in the present experiments by exposing the colon to 5% MO. Hübner et al. (1990) reported that a large number of unmyelinated, mechanically insensitive afferent fibers in the cat S2 dorsal root could be activated by instillation of MO or turpentine oil into the urinary bladder. In a subsequent study, these investigators demonstrated excitation and sensitization of myelinated mechanosensitive afferent fibers by the same irritants (Häbler et al. 1993a). Excitation of afferent fibers by irritant chemicals is a direct effect and not secondary to altered mechanical transduction properties or bladder hyperreflexia (Häbler et al. 1993b; McMahon and Abel 1987). In the present study, MO increased the resting activity of 16/17 fibers tested and increased the magnitude of response to noxious CRD of 13/17 fibers (10 LT and 3 HT). The enhanced response magnitude of mechanosensitive afferent fibers to CRD was observed when tested 30 min after MO instillation into the colon and was unchanged after 60 min. MO can produce, depending on the duration of exposure and concentration, desquamation (loss of surface epithelium), hemorrhagic patches, polymorphonuclear (PMN) leukocyte infiltration, edema, and plasma extravasation in colonic tissue, consistent with the present histological findings.

Accumulating evidence reveals that immune-competent cells in inflamed peripheral tissue can express endogenous opioid peptides. In rats with unilateral, localized inflammation of a hindpaw, mRNAs encoding proopiomelanocortin and proenkephalin and their respective peptide products, β-endorphin and met-enkephalin, are found; the opioid-containing cells are T and B lymphocytes, monocytes, and macrophages (Przewlocki et al. 1992; Stein et al. 1990). Suppression of the immune system abolishes these effects (Schafer et al. 1994; Sharp and Linner 1993; Stein et al. 1990). The effects of opioids, principally μ-opioid receptor agonists, on peripheral sensory nerves have been reported to be enhanced during inflammation (e.g., Ji et al. 1995; Stein et al. 1990; see Stein 1995 for overview). In the present study, immune cell infiltration stimulated by colonic irritation could lead to the synthesis of opioid peptides at the site of colonic tissue injury, contributing a local antinociceptive effect. Normally, tight intercellular contacts in the innermost layer of the perineurium act as a barrier to the diffusion of peptides or larger molecules. Inflammation disrupts this barrier, enabling trans-perineural passage of larger molecules, conceivably allowing endogenous opioid peptides derived from immune cells to act on opioid receptors located on sensory nerves (Stein 1995). Within the ~2-h period of study after colonic irritation in the present experiments, the potency of κ-ORAs was not increased after colonic irritation. Similarly, although pseudoafffective responses to CRD are increased significantly after acute colonic inflammation (Burton and Gebhart 1995a,b; Coutinho et al. 1996), the antinociceptive effects of κ-ORAs on visceromotor and presynaptic responses to CRD were not enhanced in rats 6 h after acetic acid-induced irritation of the colon (Burton and Gebhart 1995b). Because drug effects were tested within 2 h of irritant instillation into colon, it may be that the failure of the dose-response functions for κ-ORAs to shift leftward reflects an inadequate amount of time for de novo receptor synthesis or up-regulation or for infiltration of sufficient immune-competent cells to produce sufficient endogenous opioid peptides to contribute to the effects reported here. We recently studied the effects of κ-ORAs on pelvic nerve afferent fiber responses to noxious CRD four days after chronic inflammation of the colon and found a leftward shift in the dose-response function (Sengupta et al. 1996b); morphine was still unable to attenuate responses to CRD.

The present and previous results lead us to speculate that the peripheral receptor at which κ-ORAs produce their effects is an orphan opiate receptor. The receptor is an opiate receptor because naloxone can attenuate significantly the effects of κ-ORAs. The receptor is not a known κ-opioid receptor for two reasons: the effects of κ-ORAs are unaffected by κ-receptor-selective antagonists (nor-BNI and DIPPA), and the effective dose range of the κ-ORAs tested is unexpectedly tight (ranging between 4 and 15 mg/kg instead of 100-fold differences reported for these same κ-ORAs in other models).

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