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Kappa Antinociceptive Activity of Spiradoline in the Cold-Water Tail-Flick Assay in Rats

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BRIGGS, S. L., R. H. RECH AND D. C. SAWYER. *Kappa antinociceptive activity of spiradoline in the cold-water tail-flick assay in rats*. PHARMACOL BIOCHEM BEHAV **60**(2) 467–472, 1998.—Spiradoline (U62066E) a racemic mixture of the two enantiomers U63639(+) and U63640(−), appears to have kappa opioid receptor activity, but the contribution of each enantiomer toward this activity is still in question. To determine the activity of each enantiomer in comparison to the racemic mixture, the three forms were tested in the cold-water tail-flick (CWTF) assay in male Sprague–Dawley rats. Antinociception by spiradoline was completely antagonized by naloxone 0.50 mg/kg, a dose five times that required to antagonize antinociception by fentanyl in this same assay. In a second series of tests, fentanyl-induced antinociception was markedly reduced, while spiradoline-induced antinociception was essentially unchanged, in methadone-tolerant animals. Of the enantiomers, only U63640 produced antinociception, whereas U63639 failed to affect the nociceptive response. Additionally, spiradoline failed to produce antinociception in animals pretreated with norbinaltorphimine (kappa receptor specific), but antinociception was not affected in animals pretreated with beta-funaltrexamine (mu receptor specific). These results show that spiradoline is a full antinociceptive agonist in the CWTF assay and that the effects of the drug are mediated through kappa opioid receptors.

Kappa opioid Spiradoline Enantiomers Somatic pain A-delta and C fibers Antinociception nor-BNI beta-FNA

SPIRADOLINE is a racemic mixture of two enantiomers, U63639(+) and U63640(−). The racemic mixture, U62066E, appears to have kappa opioid receptor activity in rodents (18,26) and primates (11). However, the levo-enantiomer, U63639 demonstrated weak activity at mu receptors, which contributed no detectable analgesic effect in various nociceptive assays including a warm water tail flick (49.5°C) (26). Another study using monkeys (20) showed that antinociceptive dose–effect curves of the racemic mixture of spiradoline were not altered by a dose of beta-funaltrexamine (beta-FNA 8.0 mg/kg, SC) that produced marked shifts in the dose–effect curves of morphine. This evidence suggests that spiradoline acts as a selective kappa agonist in attenuating a nociceptive response in monkeys. However, the selectivity of spiradoline at the kappa receptor and its ability to produce antinociception using a cold nociceptive stimulus of −10°C has not been tested in rodents.

This study examines the hypothesis that spiradoline produces its antinociceptive effect against cold stimuli in rats via kappa opioid receptors. The nociceptive model used in this experiment was the cold-water tail-flick (CWTF) at −10°C. Cold stimuli have been used to study pain in human subjects (15) as well as animals (21). The CWTF in rats is a nociceptive assay with the advantage that many subjects can easily be tested repeatedly over short intervals with reproducible results. Furthermore, the CWTF has been shown to be sensitive to both mu (morphine) and kappa opioid receptor agonists (dynorphin A, U-50488H, and pentazocine; (21,24)]. Spiradoline has also demonstrated kappa receptor mediated antinociception in a similar CWTF assay (−3°C) (1). However, the temperature of the thermal stimuli and the maximum cutoff latency used in any CWTF assay is critical. First, the temperature of the stimulus needs to be less than −3°C to detect agonist activity at the kappa receptor. Secondly, the response latency can be modulated in a graded and quantal manner (5,7,17,28). For data in this article a 60-s cutoff latency was used because it has been shown to separate strong narcotic analgesics from mixed agonist-antagonist drugs (21). Thus,

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these experiments using a −10°C thermal stimuli with a 60-s cutoff latency were conducted for two purposes: 1) to determine whether spiradoline produces antinociception in the CWTF assay at −10°C, and 2) to explore the extent to which any antinociceptive drug effect in rats is mediated via kappa receptors. These experiments and others investigating the effects of spiradoline (2–4, 26) provide necessary information that enables further examination of the potential use of mu and kappa opioid agonist combinations to afford enhanced antinociception with minimal expression of side effects for each type of agonist (manuscripts in preparation).

METHOD

Subjects

Male Sprague–Dawley rats, weighing 300 to 500 g, were approved for use in the following experiments by the All-University Committee on Animal Use and Care of Michigan State University. All rats were trained over a 2-month period to lie quietly in a towel that was snugly secured around them. Training started for rats between the ages of 60 to 80 days. After approximately 6 weeks of training, rats accommodated to being restrained in the towels without struggling. The subjects were reinforced after training sessions by access to Cheerios® cereal "treats" and time to "play and socialize" on a large table-top among towels and plastic boxes and tunnels.

Drugs

Spiradoline racemic mixture (U62066E), U63639 (dextro-enantiomer), and U63640 (levo-enantiomer) were generously provided by P. F. VonVoigtlander from The Upjohn Company, Kalamazoo, MI. Fentanyl citrate was purchased from Elkins-Sinn, Inc., Cherry Hill, NJ. Methadone was purchased from Mallinckrodt (Mundelein, IL). All agonists were dissolved in saline. Naloxone was purchased from Mallinckrodt (Mundelein, IL) and diluted in saline. The selective antagonists beta-funaltrexamine (beta-FNA) and nor-binaltorphimine (nor-BNI) were generously provided by the National Institute on Drug Abuse. The antagonists were dissolved in sterile water.

Procedure for Log Dose–Response Analysis of Agonists in CWTF

Trained rats were restrained in towels as described earlier while their tails were dipped into tap water at 27 to 30°C (dummy stimulus) or a solution of ethylene glycol and water (1:1) maintained at −10°C (nociceptive stimulus). Nociceptive thresholds were determined by establishing the latency from the time the tail was dipped until the time the rat flicked its tail from the cold solution. Frequent tail dips using tap water (controls) were employed to extinguish any conditioned pattern of tail flicking that might develop by dipping the tail into a liquid. After four test tail dips, latencies were averaged and the mean latency was used as a baseline response. Most rats removed or "flicked" their tails from the cold solution in less than 3 s. Other studies using different temperatures (0°C) have reported longer latencies (6,25) but a 3-s latency is in agreement with studies using colder temperatures (1). After determining these thresholds, rats were released from towel restraint and injected with a coded drug (experimenter blinded). The subjects were again restrained and responses to the cold solution were recorded at 15, 30, 45, and 60 min after injection. The subjects' tails were never left in the cold solution for more than 60 s.

Procedure for Naloxone Antagonism

Nociceptive thresholds (controls) were determined as previously described. Trained rats were then injected (SC) with saline and various doses of naloxone (0.005–0.5 mg/kg) followed by either fentanyl, 0.03 mg/kg, or spiradoline, 1.0 mg/kg. Antinociceptive effects were determined at 15 min postinjection. Doses of the agonists chosen were previously determined to be the analgesic dose producing 50% attenuation of the maximum nociceptive stimulus (AD_{50}).

Procedure for Methadone Tolerance

Two groups of trained rats ($n = 15$ each) were treated every 12 h with either methadone or saline. Doses of methadone were gradually increased to 7.6 mg/kg as tolerance developed, at which period experiments were conducted. Experiments were scheduled such that residual methadone contributed no significant antinociceptive effect. On test days, both methadone-tolerant rats and saline-treated rats were randomly administered fentanyl or spiradoline and then tested 15 min later. After 3 h (time at which agonists were no longer active) those rats that received fentanyl were injected with spiradoline and rats that first received spiradoline were injected with fentanyl. Thereafter, rats were again tested at 15 min postinjection. A blinded experimenter observed and recorded latency responses.

Procedure for Enantiomers

Eight trained rats were injected randomly with a dose of coded drug or saline each week for 4 weeks. Thus, all rats received one dose each: 1.0 mg/kg SC U62066E, 1.0 mg/kg SC U63639 (dextro form), 1.0 mg/kg SC U63640 (levo form), and saline (vehicle). Nociceptive thresholds were determined as described earlier and each of the coded drugs was tested for antinociceptive effects at 15, 30, 45, and 60 min postinjection.

Procedure for Selective Antagonism

Pilot studies were conducted to verify activity of selective antagonists in the CWTF. For the first 12 h after administration, subjects treated with beta-FNA or norBNI exhibited antinociceptive responses in the CWTF. However, after 12 h the CWTF nociceptive threshold returned to predrug controls. With further testing, results showed that antinociception of fentanyl was most effectively antagonized after 24 h of the beta-FNA administration, while antinociception of spiradoline was not altered. Results of the pilot study also showed that antinociception of spiradoline was most effectively antagonized after 48 h of the nor-BNI administration, while antinociception of fentanyl remained unchanged. Results of these pilot studies were used to determine dosing times for the present study and subsequent studies to be reported later. Seven trained rats were pretreated SC with 2.5 mg/kg beta-FNA, the selective mu receptor antagonist. Twenty-four hours later, three of those rats received fentanyl (0.018 mg/kg) and four rats received spiradoline (1.0 mg/kg). Eight trained rats were pretreated SC with 10.0 mg/kg nor-BNI, a selective kappa receptor antagonist. After 48 h, four of those rats received fentanyl (0.018 mg/kg) and four received spiradoline (1.0 mg/kg). A blinded experimenter observed and recorded the latency of responses at 15 min postinjection.

Data Analysis

The AD_{50} dose of fentanyl and of spiradoline was determined by using the linear regression function of Sigma Plot® for Windows. All drug comparisons were tested using a random ANOVA, except data from the enantiomers, which were tested using a repeated measures ANOVA. Student–Neuman–Keuls method was used to determine significant group differences. Significance was set at $p < 0.05$. For graphical representation, antinociceptive data were standardized as a maximum percent effect [MPE, (12)].

$$\text{MPE} = \frac{\text{PDn} - \text{C}}{\text{Max} - \text{C}} \times 100$$

Where PDn is the stimulus level to which a subject responds at *n* minus postinjection. C is the stimulus level to which a naive subject normally responds. Max is the maximum stimulus level presented to any subject.

RESULTS

Dose–Response Patterns of the Agonists in CWTF

Results of log dose–response analyses in the CWTF demonstrated that spiradoline acted as a full agonist in producing antinociception with an AD_{50} of 0.56 mg/kg SC (Fig. 1B). This result is in good agreement with those of spiradoline tested in other nociceptive assays in the rat (26). However, the dose–response analysis of spiradoline was somewhat limited in that higher doses also produced behaviors that tended to confound observations. For reporting of accurate observations, subjects had to remain still and only "flick" their tail. Subjects administered higher doses of spiradoline often would not remain still, making observations of the subject's tail-flick response ambiguous at the highest doses tested. Only responses that were free of this interference were utilized. Results of fentanyl testing in the CWTF showed a steep log dose–response pattern and demonstrated that fentanyl acted as a full agonist with an AD_{50} of 0.004 mg/kg SC (Fig. 1A).

Naloxone Antagonism

To determine specificity of spiradoline at the kappa receptor, incremental doses of naloxone were used to antagonize antinociceptive effects of fentanyl and spiradoline. Results showed that naloxone (0.0025 mg/kg) did not affect the fentanyl dose–response pattern, while 0.005 mg/kg of naloxone reduced fentanyl's effect by 50% (Fig. 2). Naloxone, at 0.05 mg/kg, fully antagonized fentanyl, but did not significantly reduce the effect of spiradoline. However, there was a trend for spiradoline in combination with 0.05 mg/kg naloxone to exert a reduced antinociceptive effect. A naloxone dose of 0.10 mg/kg also fully antagonized fentanyl without significantly affecting spiradoline, while 0.50 mg/kg of naloxone fully antagonized both fentanyl and spiradoline.

Methadone Tolerance

The antinociceptive effect of fentanyl in methadone-tolerant rats was markedly less ($p < 0.05$) than that observed in nontolerant rats (Fig. 3). Antinociception induced by spiradoline in methadone-tolerant rats was not significantly altered from antinociception induced in nontolerant rats (Fig. 3).

Antinociceptive Activity of Enantiomers of Spiradoline

U63639(+) at 1.0 mg/kg SC was without effect as was saline over the 60-min period that testing was conducted ($p < 0.05$) (Fig. 4). However, U62066E and U63640(−) produced significant antinociception at 1.0 mg/kg SC during the 60-min period ($p < 0.05$). Also, U62066E and U63640(−) produced similar levels of antinociception at all time points with the exception of 30 min.

Selective Antagonism

From pilot studies, beta-FNA was most potent and selective as an antagonist in the CWTF at 24 h after administration, while nor-BNI was most selective and potent as an antagonist 48 h after administration. Although both beta-FNA and nor-BNI produced agonistic activity in the CWTF during the first 12 h, there was no antinociceptive activity at any time there-

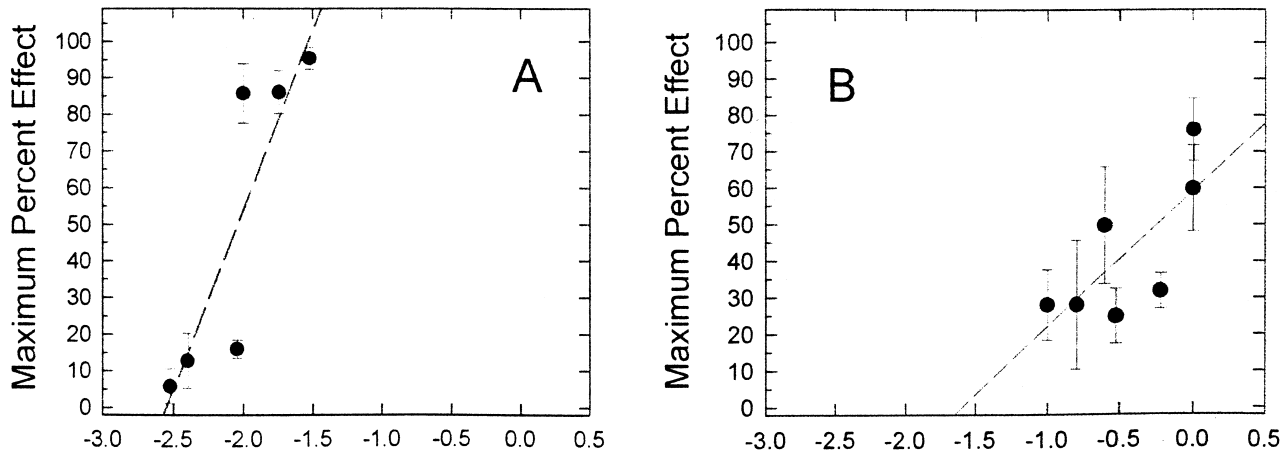

FIG. 1. Graph A: mean (±SEM) for fentanyl dose response in the CWTF assay at 15 min postinjection; AD_{50} = 0.004 mg/kg SC; n = 3 to 16 subjects per dose. Graph B: mean (±SEM) for spiradoline dose response in CWTF at 15 min postinjection; AD_{50} = 0.56 mg/kg SC; n = 3 to 12 subjects per dose.

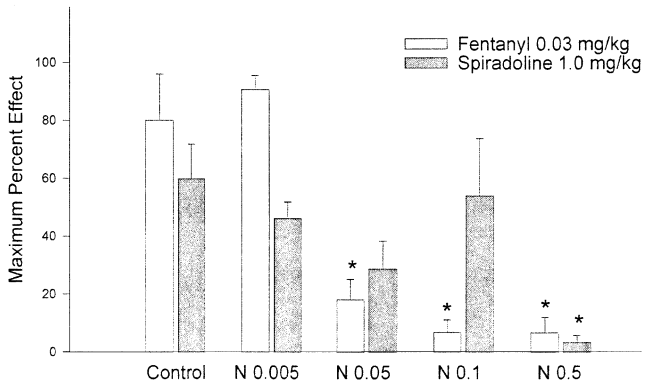

FIG. 2. Mean (±SEM) of the level of antinociception (MPE) at 15 min postinjection in subjects pretreated with saline or naloxone (N) in incremental doses (0.005, 0.05, 0.1, 0.5 mg/kg) and administered either fentanyl 0.03 mg/kg SC or spiradoline 1.0 mg/kg SC. Asterisk indicates significant differences from control ($p < 0.05$).

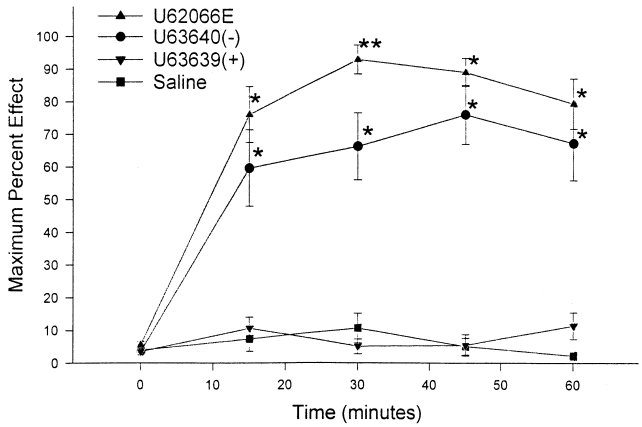

FIG. 4. Mean (±SEM) of the level of antinociception (MPE) at 15, 30, 45, and 60 min postinjection in subjects administered saline (control) and U62066E, U63640(−), or U63639(+) 1.0 mg/kg SC each. Asterisk indicates significant difference from control ($p < 0.05$). Double asterisk indicates significant difference from U63640(−) ($p < 0.05$).

after (24, 48, and 72 h). After the 24-h pretreatment, beta-FNA antagonized antinociceptive effects of fentanyl 0.018 mg/kg SC (90% analgesic dose) by 75% (Fig. 5). The antinociceptive effect of spiradoline (1.0 mg/kg SC) in these beta-FNA pretreated rats was similar to that in nonpretreated rats. After 48 h, nor-BNI did not affect fentanyl-induced antinociception, whereas spiradoline-induced antinociception was antagonized by more than 80%.

DISCUSSION

The dose–response curves of fentanyl and spiradoline described above indicate that mu and kappa agonists can be equally efficacious as antinociceptives in the CWTF at −10°C. Spiradoline appears to act as a full agonist in the CWTF at −10°C and appears to produce its antinociceptive effect by selective action at kappa receptors. It is important to note that this study used a 60-s latency cutoff, which has been shown to separate strong narcotic analgesics from mixed agonist-antagonist drugs (21). Previously, spiradoline demonstrated full agonist activity in the CWTF; however, the nociceptive stimulus used was −3°C with a 30-s latency cutoff (1). Thus, these data from the CWTF at −10°C using a 60-s latency cutoff further demonstrate that spiradoline has full agonist activity at the kappa receptor. The results with naloxone antagonism (Fig. 2) are in agreement with the literature in that spiradoline-induced

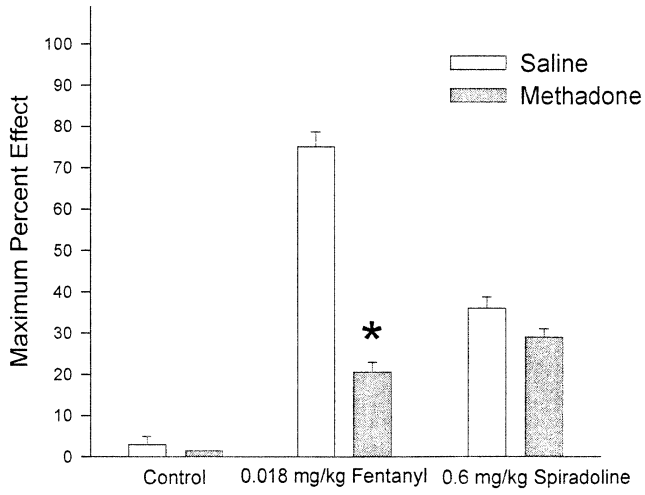

FIG 3. Mean (±SEM) of controls and of the level of antinociception (MPE) at 15 min postinjection in nontolerant (saline) vs. methadone tolerant subjects. Asterisk indicates significant difference from nontolerant (saline) treated subjects ($p < 0.05$).

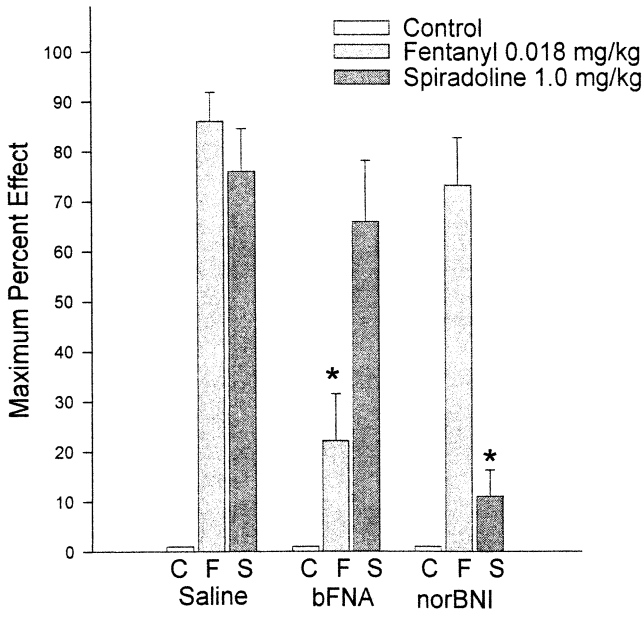

FIG. 5. Mean (±SEM) of the level of antinociception (MPE) at 15 min postinjection in subjects administered saline (control), fentanyl, or spiradoline. Subjects were pretreated with either saline, beta-FNA, or nor-BNI. Asterisk indicates significant difference from corresponding saline pretreatment ($p < 0.05$).

antinociception was antagonized by a dose approximately five times that required to antagonize fentanyl-induced antinociception (9,10,27). Results of testing the agonists in methadone-tolerant rats (Fig. 3) also demonstrated kappa selectivity for spiradoline in that spiradoline-induced antinociception remained unchanged, whereas the effect of fentanyl was significantly reduced. In addition, comparing the effects of the enantiomers and racemic mixture of spiradoline in the CWTF revealed that the proposed mu enantiomer, U63639(+), had no measurable antinociceptive effect, whereas the proposed kappa enantiomer, U63640(−), produced an antinociceptive effect very similar to the racemic mixture over a period of at least 1 h. The significant difference between U62066E and U63640 at the 30-min time point could indicate that U63639 contributed slightly to the antinociceptive effect in the racemate. Nevertheless, the antinociceptive effect of U63639(+) at 30 min postinjection was equivalent to the effect of saline. Thus, the (+) and (−) isomers may interact in some complex manner. The most convincing evidence that spiradoline is a selective kappa agonist in the CWTF came from studies using the selective antagonists, beta-funaltrexamine (beta-FNA) and nor-binaltorphimine (nor-BNI). beta-FNA antagonized fentanyl-induced antinociception without affecting the activity of spiradoline, while nor-BNI was without effect on fentanyl, but completely antagonized spiradoline-induced antinociception. The results of these experiments demonstrate that 1) a kappa agonist can be equally efficacious to a mu agonist in the CWTF nociceptive assay, and 2) spiradoline-induced antinociception in the CWTF assay in the rat is selectively mediated by activation of kappa receptors.

The conclusion that a kappa agonist can be equally efficacious to a mu agonist in the CWTF seems to be applicable at a specific temperature of −10°C of nociceptive challenge. Kappa agonists have been noted to produce poor antinociceptive effects in the CWTF (6,25) by investigators using a temperature of 0°C as a cold noxious stimulus. Others have shown that by decreasing the temperature to −10°C, a variety of kappa agonists, for example, dynorphin A, U-50488H, and pentazocine, are efficacious in producing antinociception (21,24). In an analogous manner to the difference seen between 0 and −10°C, opioid agonists in tail-flick nociceptive assays using warm (45 to 50°C) vs. hot (55°C) temperatures also show differences in efficacy depending on the type of opioid. For instance enadoline (CI-977), a kappa agonist, was shown to be 1000 times more potent than morphine as an antinociceptive agent with a challenge of 50°C, but enadoline was less efficacious than morphine when tested with water at 55°C (8). Others have shown this trend as well, i.e., that kappa agonists seem to lack antinociceptive efficacy at higher levels of thermal stimuli (13,16,25).

Single-fiber electrophysiological recordings of the saphenous nerve in anesthetized rats (14) and monkeys (23) have shown that A and C neural-related "mechanoreceptors" are excited by noxious cold, with only C nociceptors active at temperatures of 0°C and above, but both A and C nociceptors active at temperatures below 0°C. It is interesting to take note of this action because there seems to be a discriminating difference at these temperatures for kappa opioids but not mu opioids. This evidence suggests that kappa receptors may be associated with nociception mediated by A cells more than by C fibers, and that mu receptors are either associated with both or may have a closer association with C fibers. Although evidence for this hypothesis is far from conclusive, it seems attractive enough to warrant further investigation. On the basis of the above finding, we initiated similar studies of selective mu and kappa agonists in the colorectal distension (CRD) nociceptive procedure, a visceral pain model (19,22). Experiments were begun addressing the hypothesis that combinations of selective mu and kappa opioids can induce enhanced antinociception in doses that cause minimal side effects, utilizing both CWTF and CRD. The results of these additional studies will be published separately (2,3).

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