κ-opioid receptor agonists (κ-ORAs) have been shown to modulate visceral nociception through an interaction with a peripheral, possibly novel, κ-opioid-like receptor. We used in the present experiments an antisense strategy to further explore the hypothesis that κ-opioid effects in the colon are produced at a site different from the cloned κ-opioid receptor (KOR). An antisense oligodeoxynucleotide (ODN) to the cloned rat KOR was administered intrathecally (12.5 μg, twice daily for 4 d) to specifically knock-down the cloned KOR. Efficacy of the KOR antisense ODN treatment was behaviorally evaluated by assessing the antinociceptive effects of peripherally administered κ- (EMD 61,753 and U 69,593), μ- (DAMGO) and δ- (deltorphin) ORAs in the formalin test. Intrathecal antisense, but not mismatch ODN blocked the actions of EMD 61,753 and U 69,593 without affecting the actions of DAMGO or deltorphin; a complete recovery of antinociceptive actions of the κ-ORA EMD 61,753 was observed 10 d after the termination of antisense ODN treatment. In contrast, the ability of EMD 61,753 to dose-dependently attenuate responses of pelvic nerve afferent fibers to noxious colonic distension was unaffected in the same rats in which the antisense ODN effectively knocked-down the KOR as assessed in the formalin test. Additionally, Western blot analysis demonstrated a significant downregulation of KOR protein in the L4-S1 dorsal root ganglia of antisense, but not mismatch ODN-treated rats. The present results support the existence of a non-κ-opioid receptor site of action localized in the colon.

Key words: peripheral opioids; nociception; colorectal distension; formalin test; antisense; visceral pain

Effects of opioid receptor agonists (ORAs) are mediated by one of the three opioid receptors: μ, δ, or κ. Because of undesirable effects like respiratory depression, tolerance, constipation, and abuse potential associated with μ-ORAs, there is considerable interest in developing therapeutically useful agonists at other opioid receptors. κ-ORAs exert potent visceral antinociceptive effects by acting at peripheral sites. κ-ORAs, but not μ- or δ-ORAs, dose-dependently attenuate responses of decentralized pelvic nerve afferent fibers to noxious colonic as well as urinary bladder distension, providing evidence for a peripheral site of action for these agonists (Sengupta et al., 1996; Su et al., 1997a,b). In addition, κ-ORAs are effective when injected directly into a peripheral site (e.g., tail; Kolesnikov et al., 1996) or intracolonically (Su et al., 2000).

Although only one κ-opioid receptor (KOR) has been cloned and characterized from rat CNS (Minami et al., 1993), binding studies suggest the existence of at least four different KOR subtypes (Pasternak, 1993). The existence of multiple subtypes of KOR is further supported by data obtained in assays of cutaneous nociception (Zukin et al., 1988; Clark et al., 1989). We have previously reported that κ-ORAs inhibit the response of pelvic nerve afferent fibers to noxious colonic or bladder distension (Sengupta et al., 1996; Su et al., 1997a,b) as well as the pressor and visceromotor responses to colonic distension in awake, unrestrained rats (Burton and Gebhart, 1998). In all these experiments, naloxone partially antagonized the actions of the κ-ORAs tested, but two κ-receptor selective antagonists, nor-binaltorphimine (nor-BNI) and 2-(3,4-dichlorophenyl)-N-methyl-N-[13-(1-3-isothiocyanato phenyl) 2-(1-pyrrolidinyl)-ethyl]acetamide (DIPPA), were ineffective. Furthermore, the mean effective doses for the receptor-selective κ-ORAs examined in the electrophysiological studies were virtually the same, ranging between 2 and 10 mg/kg, which is in contrast to 100-fold differences reported in the literature for these same κ-ORAs in other models (Chang et al., 1984; Devlin and Shoemaker, 1990; Nock et al., 1990; Paul et al., 1990). These findings led us to speculate that the κ-ORAs tested modulate visceral nociception through an interaction with a peripheral, novel, site of action that is different from the cloned KOR.

Antisense oligodeoxynucleotides (ODNs) have proven to be a valuable tool to study the pharmacology of opioid receptors (Pasternak and Standifer, 1995). There is evidence that intrathecal administration of antisense ODNs can also cause a “knock-down” of peripheral opioid receptors, presumably by inhibiting the synthesis of receptors in the dorsal root ganglion (Bilsky et al., 1996; Khasar et al., 1996). In the present study, we have used antisense strategy to both assess the peripheral visceral antinociceptive role, if any, of the cloned KOR and further explore the hypothesis that the κ-ORA effects in the colon are produced at a site different from the cloned KOR.

Parts of this paper have been published previously in abstract form (Joshi et al., 1999).

MATERIALS AND METHODS

Animals. Male Sprague Dawley rats (Harlan, Indianapolis, IN) were housed one or two per cage with ad libitum access to food and water and were maintained on a 12 hr light/dark cycle (lights on 6:00 A.M. to 6:00 P.M.) in the Association For Assessment and Accreditation of Laboratory Animal Care-approved animal care facility. All experimental procedures were approved by the Institutional Animal Care and Use Committee, The University of Iowa.

Experimental objectives. The goal of the present experiments was to evaluate the role of the cloned KOR in peripheral somatic (hindpaw) and visceral (colon) tissues using antisense ODN-mediated receptor knock-down. Rats received antisense or mismatch ODNs targeting the cloned KOR or saline intrathecally for 4 d. The antinociceptive effects of κ-, μ-, and δ-ORAs, administered peripherally, were tested in the formalin test. After testing the peripheral actions of κ-ORAs in antisense and mismatch ODN-treated rats in the formalin test, electrophysiological evaluation of a κ-ORA in visceral nociception was tested in the same rats. To verify whether the ODNs caused a decrease in KOR expression in the dorsal root ganglia, Western blot analysis was performed. In a separate group of animals, the recovery of antinociceptive actions of κ-ORAs 6 and 10 d after the termination of antisense ODN treatment was also assessed in the formalin test. The experimental strategy is diagrammed in Figure 1.

Synthesis and administration of ODNs. Antisense and mismatch ODNs...
Joshi et al. • Non-Opioid, Peripheral Site of \( \kappa \)-Opioid Action

**RESULTS**

**Effects of selective ORAs on formalin-produced flinching in KOR antisense ODN-treated rats**

Injection of formalin into the dorsal surface of the hindpaw produced characteristic biphasic flinching behavior with clear first (0–15 min) and second (15–50 min) phases (Figs. 2A, 3A, 4A, 5A). On average, rats that received pretreatment with saline (10 min before formalin) flinched a total of 345 ± 20 times between 15 and 50 min after formalin. Pretreatment with 100 nmoles of selective \( \kappa \)- (EMD 61,753 and U 69,593), \( \mu \)- (DAMGO), or \( \delta \)- (deltorphin) opioid agonists or saline alone did not significantly alter the number of second phase flinches after their administration, 10 min before formalin at the same site (Figs. 2B, 3B, 4B). The first phase of the response to formalin was not significantly altered by pretreatment with the ORAs tested.

The second phase antinoceptive actions of the two \( \kappa \)-ORAs tested were significantly blocked by intrathecal treatment with KOR antisense, but not mismatch ODN treatment (Figs. 2B, 3B). In contrast, neither KOR antisense nor mismatch ODN treatment had any effect on the antinoceptive actions of either DAMGO or deltorphin (Fig. 4). Additionally, administration of KOR antisense ODN alone also had no effect on formalin-induced flinching behavior (Figs. 2B, 3B, 4B). The antinoceptive action of EMD 61,753 was also assessed in a separate group of animals 6 and 10 d after the onset of distension. Dose–response relationships in antisense and mismatch ODN-treated rats were obtained by giving cumulative doses of EMD 61,753 (0.5, 1, 2, 4, 8, 16, and 32 mg/kg; doses administered at 4.5 min intervals).

**Figure 1.** Diagram illustrating the experimental strategy to study the effects of KOR knock-down using antisense ODNs. Antisense ODN targeting the cloned rat KOR was administered intrathecally into the lumbar-sacral area twice daily (dosing interval 10–12 hr) for 4 consecutive days, each dose containing 12.5 \( \mu \)g of ODN in a 5 \( \mu \)l volume followed by a 10 \( \mu \)l saline flush. The efficacy of the antisense ODN treatment to cause a knock-down of the peripheral KOR was evaluated using the formalin test. Rats showing a block of the peripheral antinoceptive actions of the \( \kappa \)-ORAs in the formalin test were subsequently examined electrophysiologically to test \( \kappa \)-ORIA modulation of visceral nociception.

- **Behavioral study.** The formalin test was performed on antisense and mismatch ODN-treated and saline-treated rats on the morning of the fifth day. -12 hr after the last intrathecal injection of ODN or saline. Different groups of rats (n = 5–10) received 100 nmol in 5 \( \mu \)l of saline, EMD 61,753 and U 69,593, \( \mu \)- (DAMGO), or \( \delta \)- (deltorphin) opioid agonists or saline, injected subcutaneously into the dorsum of the right hindpaw, 10 min before formalin injection (0–15 min) and between 15 and 50 min, respectively. In some experiments, the selective \( \kappa \)-opioid receptor antagonist nor-BNI (50 nmol in 5 \( \mu \)l) was injected into the hindpaw simultaneously with EMD 61,753. Recovery of antinoceptive actions of EMD 61,753 was analyzed in a separate group of animals 6 and 10 d after the termination of antisense ODN treatment.

**Electrophysiological study.** The experimental procedures for recording responses of pelvic nerve afferent fibers to noxious colorectal distension have been described in detail (Sengupta et al., 1996; Su et al., 1997b) and are only briefly summarized here. Rats treated with either antisense or mismatch ODN and previously tested with formalin were anesthetized with sodium pentobarbital (Nembutal) and mechanically ventilated with room air. A lumbar laminectomy was performed, and the lumbar theca were catherized for measurement of arterial pressure and administration of pentobarbital, respectively. The left carotid artery was catherized for subsequent drug administration. Core body temperature was maintained at 37°C. The lower abdomen was exposed by a 3- to 4-cm incision laterally at the left flank. A flexible latex balloon 6–7-cm-long and 2.5–3 cm in diameter was inserted via-intrally into the descending colon and rectum. The balloon catheter was connected to a distension control device via a low-volume pressure transducer, and the left pelvic nerve was isolated from the surrounding fatty tissues, and a pair of Teflon-coated stainless steel wires that were stripped at the tips were wrapped around the pelvic nerve and sealed with a nonreactive silicon gel (Wacker Silicone Corporation, Adrian, MI). The hypogastric, pudendal, and femoral nerves were isolated and transected. The sciatic nerve was approached through the ischiatric notch and transected.

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**Localization of \( \kappa \)-OPIA in L4, L5, L6, and S1 dorsal root ganglia.** The lumbosacral spinal cord was exposed by laminectomy (T13–S1), and the dorsal root ganglia (DRG) were collected from the ischiatic notch and transected. The pelvic nerve and sealed with a nonreactive silicon gel (Wacker Silicone Corporation, Adrian, MI). The hypogastric, pudendal, and femoral nerves were isolated from the surrounding fatty tissues, and a pair of Teflon-coated stainless steel wires that were stripped at the tips were wrapped around the pelvic nerve and sealed with a nonreactive silicon gel (Wacker Silicone Corporation, Adrian, MI). The hypogastric, pudendal, and femoral nerves were isolated and transected. The sciatic nerve was approached through the ischiatric notch and transected.

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To verify that the blockade of antinociceptive actions of k-ORAs is not attributable to a generalized neurotoxicity brought about by antisense ODN administration, effects of EMD 61,753 were assessed in the formalin test 6 and 10 d after the termination of antisense ODN treatment. A partial recovery of the antinociceptive actions of EMD 61,753 was seen 6 d after the last antisense ODN injection, and a complete recovery was observed after 10 d (Fig. 5).

Electrophysiological evaluation of k-ORA modulation of pelvic nerve afferent fiber responses to colonic distension was performed in mismatch and antisense ODN-treated rats after the formalin test. Recordings were made from a total of 14 pelvic nerve sensory fibers in the decentralized S1 dorsal root. In contrast to the observations in the formalin test, in which the antinociceptive actions of the k-ORA EMD 61,753 were blocked by KOR antisense ODN treatment, EMD 61,753 dose-dependently attenuated the responses of pelvic nerve afferent fibers to noxious colonic distension (80 mmHg) in both KOR antisense and mismatch ODN-treated rats (Fig. 6). The estimated ED50 values (dose-attenuating response magnitude to 50% of control) and 95% confidence intervals for EMD 61,753 in antisense (7 mg/kg; 1.7–12.4 mg/kg) and mismatch (6 mg/kg; 0.6–11.37 mg/kg) ODN-treated rats did not differ from each other or from the ED50 of EMD 61,753 determined in other experiments (7.9 mg/kg; Sengupta et al., 1999).

**DISCUSSION**

The present experiments support the hypothesis that there exists in the colon a novel site at which k-ORAs significantly attenuate visceral nociception. Intrathecal administration of antisense, but not mismatch ODNs to the cloned KOR blocked the antinociceptive effects of k-ORAs injected into the paw without affecting the actions of DAMGO (µ-ORA) or deltorphin (δ-ORA). Significantly, the ability of the k-ORA EMD 61,753 to dose-dependently attenuate responses of pelvic nerve afferent fibers to colonic distension was unaffected by the same ODN treatment. We used
Western blot analysis to verify that intrathecal ODN administration causes a KOR protein knock-down in the L4-S1 DRG.

**Antisense strategies in opioid pharmacology**

Antisense ODNs have been extensively used to correlate the pharmacology and molecular biology of opioid receptors (Pasternak and Standifer, 1995; Hutcheson et al., 1999). The turnover rate of opioid receptors is 4 d in vivo (Pasternak, 1993), which necessitated administration of the KOR antisense ODN for 4 d so that not only new protein synthesis was blocked, but also the pre-existing protein was cycled out.

Antisense ODNs targeting the KOR have been shown to selectively inhibit κ-ORA antinociception in rats (Adams et al., 1994) and, more recently, to cause hypertension after injection into the hippocampus (Wright et al., 1999). Intrathecal administration of antisense ODNs has also been demonstrated to cause a knock-down of peripheral proteins. For example, intrathecal administration of antisense ODN targeting the μ-opioid receptor significantly decreased peripheral DAMGO-produced inhibition of prostaglandin E2 hyperalgesia and also DAMGO-induced inhibition of voltage-gated Ca2+ currents in cultured rat DRG neurons (Khasar et al., 1996). Intrathecal administration of δ-opioid receptor (DOR) ODN selectively blocks the antinociceptive effects of peripherally administered δ-ORAs (Bilsky et al., 1996). Radioligand binding experiments in the same study indicated an ~50% decrease in δ-opioid receptors in the lumbar spinal cord. A large proportion of δ-opioid receptors in rat spinal cord are located on primary afferent nerve terminals (Dado et al., 1993), suggesting that one of the sites of action of intrathecally administered DOR antisense ODN is the DRG, where the opioid receptors are synthesized and subsequently transported to central and peripheral terminals. Likewise, we verified using Western blot analysis that lumbarocaudal intrathecal administration of antisense ODNs directed toward the cloned KOR causes a KOR knock-down in the L4-S1 DRG. Although the KOR downregulation we observed was almost complete, it is possible that the antibody could not detect some KOR protein that persisted in DRG after the antisense ODN treatment. We have also behaviorally demonstrated the efficacy of the antisense ODN, which selectively blocked the antinociceptive actions of EMD (p < 0.05). A partial recovery of antinociceptive actions of EMD was seen 6 d after the termination of κ-ODN treatment, and a complete recovery was observed after 10 d.

**Role of κ-ORAs in modulating visceral nociception**

Whereas κ-ORAs are well documented to be antinociceptive in cutaneous models of pain (Leighton et al., 1988; Herraro and Headley, 1993), several recent studies point toward a significant role in modulating visceral pain. The role of the KOR gene product in the perception of visceral chemosensitivity has been demonstrated in KOR-deficient mice, which have a decreased threshold to a noxious visceral chemical stimulus when compared to their wild-type littermates (Simonin et al., 1998). A peripheral site of action of κ-ORAs in the viscera has been recently documented in reports
colonic distension by acting at a receptor that is distinct from the KOR cloned in the CNS. The present experiments neither document nor localize such a receptor. An immunohistochemical study using antibodies raised to the cloned KOR demonstrated that \( \kappa \)-opioid receptors, although not present in the smooth muscle cells in the rat colon, were present on myenteric and submucosal plexus neurons as well as on interstitial cells of Cajal (Bagnol et al., 1997). At present, there is no evidence for the existence of any opioid receptor associated with the peripheral endings of pelvic nerve sensory fibers. Accordingly, it is possible that \( \kappa \)-ORAs effects on pelvic nerve sensory fibers arise indirectly through action at the \( \kappa \)-opioid receptor associated with neurons of the intrinsic nervous system of the gut. Intrinsic and extrinsic primary afferent nerve terminals, however, are not anatomically organized in a manner that would make such an interaction likely. That is, intrinsic primary afferent nerve terminals are not “presynaptic” to terminals of extrinsic neurons. Intrinsic primary afferent neurons have been shown to interact with each other and with second order neurons (interneurons and motor neurons) of the enteric nervous system, but not with extrinsic primary afferent neurons such as studied here (Furness et al., 1998).

The existence of KOR 1 splice variants has been suggested in a recent report (Pasternak et al., 1999). In this study, the analgesic actions of \( \alpha \)-neoeendorphin and dynorphin B in mice were antagonized by nor-BNI but not blocked by antisense ODN targeting exon 1 of the KOR. Although we find nor-BNI to be without effect in our electrophysiology studies, we cannot rule out the possibility that \( \kappa \)-ORA effects in the viscera are mediated by a splice variant encoded by the KOR 1 gene. Such a splice variant would neither be affected by the present antisense ODN treatment nor be recognized by the antibody used in the Western blot analysis. Further studies using antisense ODN sequences targeting other regions of the cloned KOR mRNA would therefore be informative, although an antibody that can be used to assess the differential knock-down of various KOR 1 splice variants is not currently available. Alternately, the receptor in the viscera may be heterodimeric. A recent report (Jordan and Devl, 1999) demonstrated the ability of \( \kappa \)- and \( \delta \)-opioid receptors to form a heterodimeric receptor with altered ligand binding and functional properties. However, the inability of \( \delta \)-ORAs to modulate the responses of mechanosensitive pelvic nerve afferent fibers innervating the colon (Sengupta et al., 1996) and the comparable efficacy of subtype-selective \( \kappa \)-ORAs (Su et al., 1997b) makes us believe that the presently investigated visceral receptor does not represent such a \( \kappa \)-\( \delta \) heterodimer. We have previously eliminated the possibility that this novel receptor may be an orphan receptor like ORL 1 at which the endogenous orphanin FQ/nociceptin peptide acts (Meunier et al., 1995; Reinscheid et al., 1995) because nociceptin had no effect on pelvic nerve afferent fibers to colorectal distension (V. Julia and G. F. Gehlhart, unpublished observations). It remains to be tested whether this novel receptor maybe an opioid-somatostatin-like receptor.

Two novel, related genes, named GPR7 and GPR8, have been described which encode receptors with structural features in common with both opioid and somatostatin receptors and also bind several opioid drugs (O’Dowd et al., 1995). Finally, several \( \kappa \)-ORAs, in addition to their opioid effects, have been demonstrated to possess Na\(^+\) channel blocking properties (Wong et al., 1990; Pugsley et al., 1993). Although \( \kappa \)-ORAs have no effect on conduction velocity or amplitude of action potentials of pelvic nerve afferent fibers (Sengupta et al., 1996; Su et al., 1997a,b), and \( \kappa \)-ORA effects are partially reversed by naloxone, we cannot completely rule out the possibility that \( \kappa \)-ORA actions are mediated by Na\(^+\) channel blockade.

In summary, the present experiments provide further evidence for the existence of a novel, peripheral \( \kappa \)-opioid-like receptor localized in the colon. Agonists directed toward this receptor are attractive targets as analgesics, potentially free of the undesirable side effects associated with activation of central \( \kappa \)-opioid receptors and could provide relief for visceral pain states like inflammatory pain.

**Visceral \( \kappa \)-opioid agonist site of action**

The present results, combined with our previous electrophysiological data, suggest that \( \kappa \)-ORAs attenuate responses to noxious visceral pain in the colon. In this study, we have demonstrated that ODN-mediated knock-down of the cloned KOR is sufficient to block the effects of \( \kappa \)-ORAs on mechanosensitive afferent fibers. This is consistent with our previous report (Jordan and Devl, 1999) that the effects of \( \kappa \)-ORAs on colorectal distension are blocked by the \( \kappa \)-opioid receptor antagonist nor-BNI. In contrast, the effects of \( \kappa \)-ORAs on noxious colonic distension are not blocked by naloxone, suggesting that these effects are mediated by a receptor distinct from the \( \kappa \)-opioid receptor. This is consistent with our previous finding that \( \kappa \)-ORAs act through a heterodimeric receptor that is not blocked by naloxone.

**Figure 6.** Effect of KOR antisense and mismatch ODN treatment on EMD 61,753 modulation of visceral nociception. ODN-treated rats, previously tested with formalin, were deeply anesthetized and were examined electrophysiologically. EMD 61,753 (10 μg/kg, s.c.) was injected into the pelvic nerve afferent fibers to noxious colonic distension (80 mmHg, 30 sec). There were no differences in effects of the drug in antisense or mismatch ODN-treated rats.

**Figure 7.** Immunoblot showing the effects of ODN treatment on KOR protein levels in rat DRG. Tissue extracts were prepared from L4, L5, L6, and S1 DRG of saline (C), antisense ODN-treated (AS), and mismatch ODN-treated (MM) rats. Samples were normalized for protein content using BCA quantification. Each lane was loaded with ~100 μg of protein, and immunoreactive bands were revealed at 43 and 70 kDa with an antibody directed against an internal region of the cloned rat KOR. Antisense ODN, but not mismatch ODN treatment, caused a clear downregulation of KOR protein at all the DRGs examined. Similar results were obtained from duplicate experiments.
bowel disease, for which satisfactory treatment is not currently available.

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Joshi et al. • Non-Opioid, Peripheral Site of k-Opioid Action J. Neurosci., August 1, 2000, 20(15):5874–5829 5879