Loperamide (ADL 2-1294), an Opioid Antihyperalgesic Agent with Peripheral Selectivity


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Accepted for publication November 19, 1998

This paper is available online at http://www.jpet.org

ABSTRACT

The antihyperalgesic properties of the opiate antidiarrheal agent loperamide (ADL 2-1294) were investigated in a variety of inflammatory pain models in rodents. Loperamide exhibited potent affinity and selectivity for the cloned μ (Kᵢ = 3 nM) compared with δ (Kᵢ = 48 nM) and κ (Kᵢ = 1156 nM) human opioid receptors. Loperamide potently stimulated [35S]guanosine-5’-O-(3-thio)triphosphate binding (EC5₀ = 56 nM), and inhibited forskolin-stimulated cAMP accumulation (IC5₀ = 25 nM) in Chinese hamster ovary cells transfected with the human μ opioid receptor. The injection of 0.3 mg of loperamide into the intra-articular space of the inflamed rat knee joint resulted in potent antinociception to knee compression that was antagonized by naloxone, whereas injection into the contralateral knee joint or via the i.m. route failed to inhibit compression-induced changes in blood pressure.

Recent studies have challenged the notion that opiates act to produce antinociception exclusively through central mechanisms. The peripheral antinociceptive effects of opiates in response to painful chemical, thermal, or mechanical insult have been demonstrated in a variety of models and species in which the injection of opiates directly into inflamed tissue results in antinociception at doses that are systemically inactive (for reviews, see Hargreaves and Joris, 1993; Stein, 1995). The local actions of opiates are mediated via the μ, δ, and κ receptor subtypes (Stein et al., 1989; Hargreaves and Joris, 1993; Nagasaka et al., 1996), are stereoselective (Stein et al., 1989; Hargreaves and Joris, 1993), and are reversible with antagonist treatment (Stein et al., 1989; Nagasaka et al., 1996), and exhibit greater potency and efficacy compared with local (Cortes Burgos and DeHaven-Hudkins, 1996) or systemic (Wheeler-Aceto, 1995) administration of nonsteroidal anti-inflammatory agents.

Antinociception is commonly not observed when opiates are administered locally into uninflamed tissues (Stein et al., 1989), suggesting that the inflammatory component is necessary for the full expression of peripheral antinociception. The greater efficacy of opiate agonists after inflammation is believed to be due to the opening of the perineurial sheath, allowing access to opioid receptors present on the peripheral nerve terminal (Antonijevic et al., 1995). After the administration of mannitol, a compound that causes no inflammation but increases perineurial permeability, the antinociceptive effect of locally administered [d-Ala2,N-MePhe4,Gly-ol5]-enkephalin (DAMGO), fentanyl, [d-Pen2,d-Pen5]-enkephalin, or U-50,488H into a mannitol-treated paw was similar to that observed in the inflamed paw of rats when Freund's adjuvant (FCA) was used as the inflammatory stimulus (Antonijevic et al., 1995). Increased numbers of binding sites and increased axonal transport of opioid receptors (shown by labeling with [125I]β-endorphin) occurred in the inflamed paw, and the receptors were associated with cutaneous nerves and infiltration of immune cells (Hassan et al., 1993). In addition, approximately one third of cutaneous sensory afferents are not protected by the perineurial sheath but are

ABBREVIATIONS: A50, concentration of drug yielding 50% antagonism of the formalin-induced flinching response; CHO, Chinese hamster ovary; CNS, central nervous system; DAMGO, [d-Ala²,N-MePhe⁴,Gly-ol⁵]-enkephalin; FCA, Freund's complete adjuvant; β-FNA, β-funaltrexamine; GTPγS, guanosine-5’-O-(3-thio)triphosphate; PPT, paw pressure threshold.

Received for publication August 4, 1998.

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responsive to direct application of the \( \mu \) agonist DAMGO (Coggeshall et al., 1997), suggesting an additional receptor population that is not protected by the perineural sheath and that may play a role in peripherally mediated antinociception.

The local actions of endogenous opioid peptides are important compensatory mechanisms in response to noxious inflammatory stimuli. Increased expression of mRNA for the opioid precursor peptides (Przewlocki et al., 1992) and increased levels of immunoreactive \( \beta \)-endorphin and met-enkephalin have been observed in the paws of rats where inflammation was induced by FCA, with localization of immunoreactivity to immune cells (Stein et al., 1990; Przewlocki et al., 1992; Cabot et al., 1997). Furthermore, the local injection of corticotropin-releasing factor, interleukin-1\( \beta \), interleukin-6, or tumor necrosis factor-\( \alpha \) into inflamed paws produces antinociception that is a result of local release of opiate peptides and can be antagonized by opiate antagonists, antibodies to the endogenous opiate peptides or immunosuppressants (Czlonkowski et al., 1993). Lymphocytes, which can produce \( \beta \)-endorphin, migrate to inflamed tissue, where the peptide is secreted at the site of inflammation (Cabot et al., 1997). Collectively, the data support the notions that opiates play a prominent role in mediating antinociception locally at the level of the primary afferent nerve terminal and that these local effects can be dissociated from the centrally mediated analgesia produced by certain opiate agonists.

In humans, the local administration of morphine to inflamed tissues produces antinociception at doses well below those that result in systemic effects. Local injection of 1 to 5 mg of morphine into the intra-articular space of the knee joint after surgery resulted in significant antinociception that was of extended duration and antagonized by administration of naloxone (Stein et al., 1991; Reuben and Connelly, 1996; Whitford et al., 1997). The efficacy and prolonged duration of action of intra-articular morphine have been confirmed in a study of patients with osteoarthritis (Likar et al., 1997). In contrast, the i.v. administration of 1 mg of morphine was far less efficacious (Stein et al., 1991). Hyperalgesia to both heat and pressure stimuli produced by an experimental second-degree burn injury to the leg was ameliorated by the local injection of 2 mg of morphine at the site of the burn (Moiniche et al., 1993). The direct application of a solution of 0.5% morphine to the abraded corneas of patients after intraocular surgery resulted in significant antinociception as measured by sensitivity to corneal pressure, whereas topical application to the unabraded eye failed to alleviate pain in the injured eye (Peyman et al., 1994). The clinical data suggest that local administration of opiate agonists results in potent antinociception under a variety of inflammatory conditions without the occurrence of concomitant side effects. Furthermore, in each of these clinical studies, injury and inflammation had occurred before drug treatment, corroborating the data from studies in animals that suggest that inflammation is necessary for the complete exposure of opioid receptors in the periphery.

The potential therapeutic use of an opiate agonist that antagonizes the hyperalgesia resulting from inflammation is obvious. Such a compound is best described as an antihyperalgesic agent because it reduces pain responses to baseline levels without producing analgesia via central mechanisms and without compromising normal function. This work describes the antihyperalgesia produced by loperamide hydrochloride (ADL 2-1294), an opiate agonist with selectivity for the \( \mu \) subtype of the opioid receptor. This compound was originally developed as an antidiarrheal agent (for a review see Niemegeers et al., 1981) and is the active ingredient in Imodium AD, an over-the-counter agent for the treatment of diarrhea. The compound does not penetrate into the brain in appreciable amounts (Heykants et al., 1974; Wuster and Herz, 1978; Schinkel et al., 1996), and it is the only marketed opiate agonist that is available over-the-counter in the United States. The safety profile of this agent has been established over many years of use (Ericsson and Johnson, 1990), and clinical studies have shown that it does not possess abuse potential (Jaffe et al., 1980) or dependence liability (Korey et al., 1980). In this report, we describe the in vitro profile of loperamide at cloned human opioid receptors and the potent antihyperalgesia produced by the administration of loperamide in various in vivo models of inflammatory pain.

### Experimental Procedures

**Materials.** \( [3\text{H}] \)Guanosine-5\'O-3-thio)triphosphate (\( [3\text{H}] \)GTP\( \gamma \)S) and \( [3\text{H}] \)diprenorphine were obtained from Amersham Life Science Inc. (Arlington Heights, IL), and flash plates were obtained from NEN Life Science Products (Boston, MA). Peptidase inhibitors were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemical reagents were purchased from Sigma Chemical Co. or BASF (Walbrzych, NJ).

**Animals.** Experiments were performed in male Sprague-Dawley rats (Ace Animals, Boyertown, PA, or Harlan Industries, Indianapolis, IN) or male ICR mice (Ace Animals) that were housed in groups in polypropylene cages lined with Bed-o-cobs. Standard laboratory rodent chow and water were available on an ad libitum basis. Room temperature and relative humidity were maintained at 22 ± 0.5°C and 60%, respectively. A 12:12 h light/dark cycle (6:00 a.m./6:00 p.m.) was used. All testing was performed during the light phase. All protocols using animals were approved by the Adolor Institutional Animal Care and Use Committee in accordance with the guidelines of the “Guide for the Care and Use of Laboratory Animals” (Institute of Lab Animal Resources, NRC, 1996).

**Drugs.** Loperamide HCl, \( \beta \)-funaltrexamine (\( \beta \)-FNA), and naloxone HCl were obtained from Research Biochemicals, Inc. (Natick, MA). Morphine sulfate was from Merck, Sharp and Dohme (West Point, PA) or Research Biochemicals, Inc. (Natick, MA). Loperamide was diluted in dimethyl sulfoxide in 0.9% saline containing 5% methycellulose or 20% Cremophor EL in 0.9% saline. All other drugs were solubilized in saline. The routes and volumes of administration for drugs were 200 \( \mu l \) for intra-articular, 200 \( \mu l \) for i.m., 50 \( \mu l \) for intrapaw, 50 \( \mu l \) for intraplantar, and 1 ml/kg for s.c.

**Binding to Cloned Human \( \mu \), \( \delta \), and \( \kappa \) Opioid Receptors.** Stable cell lines expressing the individual full-length human \( \mu \), \( \delta \), and \( \kappa \) opioid receptor cDNAs were generated by transfecting 70% confluent Chinese hamster ovary (CHO)-K1 cells in 35-mm dishes with the appropriate cDNA construct as described by Bare et al. (1994). The cells were transfected with DNA and Lipofectamine for 5 h under serum-free conditions in Ham’s F-12 medium. After 5 h, the medium was made 10% with respect to FCS, and after 24 h, the medium was replaced. At 48 h after transfection, the cells were split 1:50 in medium containing 1 mg/ml geneticin (GIBCO BRL, Gaithersburg, MD) and cultured for 14 days; subsequently, healthy cell foci were selected and cultured to confluence in 24-well microtiter dishes. Receptor expression in these selected cells was confirmed using \( [3\text{H}] \)diprenorphine as a ligand in whole-cell binding assays as described by Law et al. (1983). The binding was performed in 300 \( \mu l \) of serum-free F-12 medium containing the labeled ligand at 10 \( \times K_{D} \) for each area.
concentrations. Cell cultures showing the highest amount of binding were selected and characterized in greater detail.

Receptor binding studies were conducted in membranes essentially as described in Raynor et al. (1994). Cells harvested 72 h after transfection were centrifuged at 1000 × g for 10 min; resuspended in 50 mM Tris-HCl, pH 7.8, containing 1 mM EGTA, 5 mM MgCl₂, 10 mg/ml leupeptin, 10 mg/ml pepstatin A, 200 mg/ml bacitracin, and 0.5 mg/ml aprotinin; and centrifuged as before. The pellet was resuspended in the same buffer, and the cells were homogenized using a Polytron homogenizer (Brinkmann, Westbury, NY). The homogenate was centrifuged at 48,000 × g for 10 min at 4°C. The membrane pellet was resuspended at 1 mg protein/ml in the same buffer as before, and the aliquots were stored at −80°C until use. Routine experiments were conducted by incubating final concentration of 25 to 100 μg of protein and 1 nM \(^{(35)S}\)GTPγS binding was determined according to the method of Selley et al. (1997). Membranes prepared from CHO cells expressing human \(\mu\) opioid receptor (100 μg of protein) were added to assay mixtures containing agonist (lormetrexol or morphine) at concentrations ranging from 3.2 × 10⁻⁴ M to 1 × 10⁻¹ M with or without naloxone, approximately 100,000 dpm \(^{(35)S}\)GTPγS, 30 μM GDP, 100 mM NaCl, 3.0 mM MgCl₂, 200 μM EGTA, 100 μM dithiothreitol, 10 mg/ml leupeptin, 10 mg/ml pepstatin A, 200 mg/ml bacitracin, and 0.5 mg/ml aprotinin in 50 mM Tris-HCl buffer, pH 7.4. After incubation at 30°C for 1 h, the reaction was terminated by filtration through Whatman GF/B filters prepared from CHO cells expressing human opioid receptors, and the filters were rinsed three times with 1.0 ml each of 50 mM Tris-HCl, pH 7.4. After incubation at 30°C for 1 h, the reaction was terminated by filtration through Whatman GF/B filters, and the filters were rinsed three times with 1 ml of cold 50 mM Tris-HCl, pH 7.8. Then, 30 μl of MicroScint 20 (Packard, Downers Grove, IL) was added to each filter, and radioactivity on the filters was determined by scintillation spectrometry in a Packard Top-Count.

**Stimulation of \(^{(35)S}\)GTPγS Binding.** \(^{(35)S}\)GTPγS binding was determined according to the method of Blake et al. (1997). Membranes prepared from CHO cells expressing human \(\mu\) opioid receptor (100 μg of protein) were added to assay mixtures containing agonist (lormetrexol or morphine) at concentrations ranging from 3.2 × 10⁻⁴ M to 1 × 10⁻¹ M with or without naloxone, approximately 100,000 dpm \(^{(35)S}\)GTPγS, 30 μM GDP, 100 mM NaCl, 3.0 mM MgCl₂, 200 μM EGTA, 100 μM dithiothreitol, 10 mg/ml leupeptin, 10 mg/ml pepstatin A, 200 mg/ml bacitracin, and 0.5 mg/ml aprotinin in 50 mM Tris-HCl buffer, pH 7.4. After incubation at 30°C for 1 h, the reaction was terminated by filtration through Whatman GF/B filters, and the filters were rinsed three times with 1 ml each of 50 mM Tris-HCl, pH 7.8. Then, 3 ml of Ready Safe was added to each well, and radioactivity on the filters was determined by scintillation spectrometry in a Beckman LSC counter.

**Inhibition of Forskolin-Stimulated cAMP Accumulation.** The procedure was a modification of the methods of Blake et al. (1997). CHO cells stably transfected with the human \(\mu\) opioid receptor were seeded onto 24-well plates (5 × 10⁴ cells/well) and cultured for 72 h. The growth medium was then removed and replaced with medium containing 500 μM 3-isobutyl-1-methylxanthine. After a 15-min incubation, the medium was replaced with medium containing 3-isobutyl-1-methylxanthine and 25 μM forskolin with vehicle and the agonist or with the agonist and the antagonist β-FNA at appropriate concentrations and incubated for an additional 15 min. The reactions were stopped by adding lysis buffer (Promega, Madison, WI) in 0.1 M HCl. Aliquots were removed, and the cAMP content was determined using a commercially available radioimmunoassay kit (NEN Life Science Products, Boston, MA).

**Kaolin-Carrageenan-Induced Hyperalgesia.** The model has been previously described in detail (Nagasaki et al., 1996). Briefly, rats weighing 300 to 340 g were anesthetized with 2% halothane, and 0.2 ml of a 4% kaolin-carrageenan mixture was injected into the right knee joint cavity through the patellar ligament with a 21-gauge needle. The rat was allowed to recover from anesthesia, and 3.5 h later, anesthesia was again induced and the tail artery was cannulated for monitoring of blood pressure. Blood pressure was recorded continuously, and body temperature was monitored and maintained at 37°C. A pediatric blood pressure cuff was used to produce compression of the inflamed knee joint. For stimulation, the cuff was rapidly elevated to 200 mm Hg with a syringe pump, and each inflation was sustained for 2 min. Loperamide or morphine was injected by the intra-articular route at 3 h after the induction of inflammation. Blood pressure was expressed as the percent change of the baseline before drug administration. Data were expressed as the percent of the preinjection change induced by compression, where percent change in blood pressure = (change in blood pressure postdrug/change in blood pressure predrug) × 100. In separate groups of unanesthetized rats, loperamide (0.3 mg) or morphine (3 mg) was injected i.m., and the amount of time that each rat stood before dismounting from a 4-cm-high bar was recorded as a measure of catalepsy.

**Formalin-Induced Nociception.** The methods of Wheeler-Aceto and Cowan (1991) were used. Inflammation was induced by s.c. injection of 50 μl of a 5% formalin solution into the dorsal surface of the right hind paw (intrapaw) of rats weighing 70 to 130 g. Drugs were administered by the intrapaw route at 10 min before formalin injection except where noted, and injections were counterbalanced across treatment groups with respect to time of treatment. Flenching behavior was quantified by counting the number of flinching responses that occurred during either the acute (early) or the tonic (late) phase of pain, measured from 0 to 10 min or from 20 to 35 min, respectively, after formalin injection. Results were expressed as the mean percent antagonism (%A) of formalin-induced flinching calculated for individual drug-treated, formalin-injected rats using the formula \[(\text{mean formalin response} – \text{mean saline response}) / \text{mean formalin response} \times 100\] (Wheeler-Aceto and Cowan, 1991). The mean formalin response is the mean behavioral score of vehicle-treated and formalin-injected rats. The mean saline response is the pooled behavioral score from rats injected with vehicle followed by 50 μl of saline into the paw. The A₅₀ values were calculated by linear regression analysis of the mean %A values from each treatment group.

**FCA-Induced Hyperalgesia.** A modification of the methods of Stein et al. (1988a, 1989) was used, where hyperalgesia in response to inflammation was measured by determining the paw pressure threshold (PPT) of inflamed and uninflamed paws of rats weighing 200 to 250 g at the time of treatment. Drug was injected under light ether anesthesia by the intraplantar route into the inflamed paw approximately 24 h after intraplantar injection of 150 μl of modified FCA (Calbiochem, La Jolla, CA). PPT values were measured in conscious rats before and after injection of drug. Rats were restrained in a guaze wrap, and pressure was applied to the dorsal surface of the inflamed or the uninflamed paw with a conical piston using a pressure analgesia apparatus (Stoelting Instruments, Wood Dale, IL). The pressure that resulted in paw withdrawal, or the PPT, was measured by applying a cutoff PPT value of 250 g. Testing was alternated between inflamed and uninflamed paws for each rat. Time course data were analyzed with respect to the change in PPT after drug administration compared with the baseline value obtained before drug treatment in either the inflamed or the uninflamed paw. Dose-response data were expressed as the percent of baseline, calculated as \[(\text{postdrug PPT} / \text{baseline PPT}) \times 100\].

**Tape Stripping-Induced Hyperalgesia.** Rats weighing 200 to 300 g were used. After anesthesia, the hair on the dorsal surface of the right hind paw was removed by depilation with commercial hair remover. Tape stripping of the area was performed by repeated application and removal of fresh pieces of Scotch Brand 810 tape to the hairless area for a total of 20 times to remove the stratum corneum and to produce hyperalgesia. At approximately 24 h after tape stripping, hyperalgesia was quantified by measurement of PPT values in both paws before and after injection of drug into the inflamed paw of rats under light ether anesthesia. Time course data were analyzed with respect to the change in PPT after drug administration compared with the baseline value obtained before drug treatment in either the inflamed or the uninflamed paw. Dose-response data were expressed as the percentage of maximal effect, calculated as \[(\text{postdrug PPT} - \text{baseline PPT}) / \text{baseline PPT} \times 100\].
the program Prism (GraphPad Software, San Diego, CA). ED50 values and 95% confidence intervals were calculated by the procedures of Tallarida and Murray (1987). Group comparisons and time course data analyses were performed using ANOVA and appropriate post hoc tests with GraphPad Prism.

Results

In Vitro Profile of Loperamide at Cloned Human μ, δ, and κ Opioid Receptors. Loperamide potently inhibited binding to the cloned human μ opioid receptor, with a $K_i$ value of 3.3 nM (Table 1). Loperamide was 15-fold selective for the μ versus the δ subtype and 350-fold more potent for the μ versus the κ subtype of the opioid receptor as determined by competition with the binding of [3H]diprenorphine.

The agonism produced by loperamide was demonstrated by its potent stimulatory effects on the binding of [35S]GTPγS mediated by the cloned human μ opioid receptor (Fig. 1). The mean EC50 value of loperamide was 19 nM compared with a mean EC50 value of 115 nM for morphine and 234 nM for DAMGO. The approximate maximum stimulation by loperamide and DAMGO was 100%, whereas that produced by morphine was 58%. Naloxone appeared to be a competitive inhibitor of the stimulation of [35S]GTPγS binding that was produced by loperamide (Fig. 2). Increasing concentrations of naloxone caused a parallel shift to the right of the dose-response curve for loperamide, without decreasing the maximal stimulation. The mean EC50 values were 1.2 μM in the presence of 100 nM naloxone and 16 μM in the presence of 1 μM naloxone.

Loperamide inhibited cAMP accumulation in a concentration-dependent manner, with a mean IC50 value of 27 nM and maximal inhibition of 54 ± 6% (Table 1). The dose-response curve for loperamide was shifted to the right in the presence of 1 μM concentration of the μ-opioid-specific antagonist β-FNA, with a mean IC50 value for inhibition of cAMP synthesis of 4.8 ± 1.2 μM and no change in the percent maximal inhibition (data not shown).

Kaolin-Carrageenan-Induced Hyperalgesia. Both loperamide (0.3 mg) and morphine (3 mg) inhibited the compression-evoked changes in blood pressure after intra-articular injection (one-way ANOVA, $F = 125, p < .0001$), and the effect of loperamide was antagonized by naloxone (Fig. 3). Intramuscular injection of morphine at a dose of 3 mg, but not loperamide (0.3 mg), blocked the cuffed-evoked increases in blood pressure and resulted in catalepsy. After i.m. administration, the ratio of the percentage of change in the compression-evoked blood pressure postdrg to the percentage of change in the compression-evoked blood pressure predrg was 67 ± 4 for morphine and 98 ± 5 for loperamide ($n = 4$/group, paired t test, $p = .012$), and the time to dismount from an elevated bar was $37 ± 11$ s for morphine and $2 ± 1$ s for loperamide ($n = 4$/group, paired t test, $p = .0075$). Injection of morphine, but not loperamide, into the contralateral knee joint also blocked the compression-evoked increases in blood pressure in the inflamed joint (data not shown).

Formalin-Induced Nociception. The administration of loperamide by the intrapaw route at a dose of 100 μg produced long-lasting antinociception during the late phase of flinching (Fig. 4). In this study, loperamide or vehicle was injected at various times before, simultaneously with, or 10 min after the intrapaw injection of formalin. Two-way ANOVA revealed significant effects due to treatment ($F = 56.65, p < .0001$), time ($F = 6.4, p = .0001$), and treatment × time interaction ($F = 10.75, p < .0001$). Post hoc analyses indicated that the mean flinching response for rats treated with loperamide significantly differed from that for vehicle-

TABLE 1

The in vitro profile of loperamide, morphine, and DAMGO at cloned human opioid receptors

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_i$ μM</th>
<th>$K_i$ δM</th>
<th>$K_i$ κM</th>
<th>Receptor Selectivity μ/δ</th>
<th>μ/κ</th>
<th>EC50 GTPγS nM</th>
<th>IC50 cAMP nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loperamide</td>
<td>3.3 ± 1.6 (3)</td>
<td>48 ± 6 (4)</td>
<td>1156 ± 124 (3)</td>
<td>15</td>
<td>350</td>
<td>19 ± 1.1 (7)</td>
<td>27 ± 6 (3)</td>
</tr>
<tr>
<td>Morphine</td>
<td>19.3 ± 4.3 (4)</td>
<td>171 ± 55 (4)</td>
<td>273 ± 56 (3)</td>
<td>10</td>
<td>14</td>
<td>115 ± 1.4 (7)</td>
<td>N.D.</td>
</tr>
<tr>
<td>DAMGO</td>
<td>17.9 ± 2.1 (25)</td>
<td>&gt;10,000 (2)</td>
<td>&gt;10,000 (3)</td>
<td>&gt;559</td>
<td>&gt;559</td>
<td>234 ± 1.3 (7)</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Data are mean ± S.E.M., with the number of determinations given in parentheses.

N.D., not determined.
Fig. 3. Effect of intra-articular loperamide or morphine on blood pressure (BP) changes evoked by compression of the inflamed knee joint. Loperamide (0.3 mg) or morphine (3 mg) was injected by the intra-articular route at 3 h after the induction of inflammation (n = 4/group). Naloxone was injected i.p. at a dose of 1 mg/kg (n = 4). **Significantly different from vehicle, Dunnett’s test, p < .01.

Fig. 4. Time course of the antagonism by loperamide of late-phase formalin-induced flinching. Loperamide at a dose of 100 μg or vehicle (n = 8–11/group) was injected by the intrapaw route at various times before or after the injection of 50 μl of 5% formalin. The number of flinches occurring during the 20- to 35-min interval after the injection of formalin was counted. Each point is the mean ± S.E.M. of the average number of flinches per 5-min observation interval. *p < .05, **p < .01, significantly different from vehicle, Bonferroni’s multiple comparison test.

The effects of loperamide at doses of 0.3, 3, 30, or 300 μg by the intrapaw route on early-phase flinching were assessed to evaluate possible local anesthetic effects and to confirm the peripheral nature of the antihyperalgesia. One-way ANOVA indicated a significant effect due to treatment (F = 6.91, p < .001), which was primarily due to the lack of flinching in the vehicle-vehicle group. No significant dose-related effects of loperamide on early-phase flinching were observed at any of the doses tested (Fig. 6), with the exception of a slight suppression of flinching at the 30-μg dose, which was due to the low responding of one rat. In contrast, morphine inhibited flinching during the early phase in a dose-dependent manner, with an A50 of 180 μg and full efficacy at 1000 μg (n = 9/dose; data not shown).

Additional evidence for the peripheral selectivity of the antihyperalgesia produced by loperamide in the formalin assay was obtained by examining late-phase flinching behavior after injection of drug into the contralateral paw. If loperamide possessed central or systemic analgesic activity as a result of intrapaw injection, antinociception would be observed when loperamide was injected into the paw opposite to the paw that was injected with formalin. The results of this experiment are presented in Table 2, where loperamide at a dose of 100 μg produced antinociception when injected into the paw contralateral to formalin but failed to produce antinociception when injected into the paw contralateral to formalin (one-way ANOVA, F = 10.02, p < .001).

FCA-Induced Hyperalgesia. Loperamide at a dose of 100 μg produced a significant attenuation of the hyperalgesia induced by FCA in the inflamed paw, lasting from 5 min to 6 h after a single injection (one-way ANOVA, F = 9.948, p < .001; Fig. 7). Significant effects of drug were not observed in the unflamed paw at any time point compared with baseline values in the unflamed paw, confirming the peripheral selectivity of the compound. In contrast, morphine at a dose of 300 μg by the intraplantar route produced antinociception in the inflamed paw at 15 min, 30 min, and 1 h postinjection (one-way ANOVA, F = 18.94, p < .001) and in the unflamed paw at 30 min postinjection (one-way ANOVA, F = 5.774, p < .001) compared with the respective baseline values.

The ED50 value, which is defined as the dose that produced a 50% increase over baseline, was 21 μg for loperamide when evaluated at 2 h after injection, the peak time of antihyperalgesia (Fig. 5). The ED50 value for loperamide was comparable to that of morphine (14 μg) when evaluated at 15 min after injection, its peak time of antihyperalgesia. Trend analyses of the dose-response data were F = 7.84, p < .0001, for loperamide, and F = 7.55, p = .0002, for morphine. Linear regression analyses of the dose-response data yielded similar results: loperamide, F = 11.41, p = .0431; morphine F = 22.5, p = .0045. Note in Figs. 5 and 7 that treatment with loperamide elevated the PPT in the inflamed paw to a level comparable to that of an untreated and uninnflamed paw, without producing further elevations that may be indicative of systemic or central nervous system (CNS) effects. In contrast, morphine, which can produce antinociception by systemic and CNS mechanisms, elevated PPT values in both inflamed and uninnflamed paws. Indeed, the mean percentages of base-
The antihyperalgesia produced by intraplantar injection of loperamide into the inflamed paw was completely antagonized by pretreatment with naloxone at a dose of 10 mg/kg s.c. given 15 min before loperamide at a dose of 300 mg/kg by the intraplantar route (one-way ANOVA, $F = 7.952$, $p = .0031$). The mean PPT of 133 ± 40 g ($n = 5$) after treatment with loperamide was significantly different from the PPT of 54 ± 5 g ($n = 6$) with naloxone before treatment and the mean baseline PPT of 44 ± 3 g ($n = 11$) (Tukey’s test; $p < .01$).

Loperamide ($n = 13–15$) or morphine ($n = 15–18$) was then injected by the intraplantar route into the inflamed paw ($t = 0$). The PPT (in g) was read in each paw before drug injection and at 2 h after treatment with loperamide or 15 min after treatment with morphine. Each point is the mean ± S.E.M. percent increase of the baseline value. To determine tape stripping-induced hyperalgesia, tape stripping was performed, and hyperalgesia was allowed to develop for 24 h. Loperamide ($n = 8$) or morphine ($n = 12–19$) was injected by the intrapaw route into the tape-stripped paw, and the PPT (in g) was read in each paw before drug injection and 15 min after treatment. Each point is the mean ± S.E.M. percent maximal effect.

**TABLE 2**

Effects of loperamide injected into the paw on late-phase formalin-induced flinching

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Number of Flinches per Observation Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle, ipsilateral paw</td>
<td>29 ± 5</td>
</tr>
<tr>
<td>Loperamide, ipsilateral paw</td>
<td>6 ± 4*</td>
</tr>
<tr>
<td>Vehicle, contralateral paw</td>
<td>27 ± 4</td>
</tr>
<tr>
<td>Loperamide, contralateral paw</td>
<td>35 ± 4</td>
</tr>
</tbody>
</table>

Loperamide at a dose of 100 μg or vehicle ($n = 11$/group) was injected intrapaw 10 min before the injection of 50 μl of 5% formalin. Ipsilateral injection was performed into the same paw as that receiving formalin; contralateral injection was performed into the paw opposite that receiving formalin. The number of flinches occurring during the 20- to 35-min period after the injection of formalin was counted. Each point is the mean ± S.E.M. of the average number of flinches per 5-min observation interval.

*Significantly different from all other treatment groups, Tukey’s test; $p < .01$. 

The antihyperalgesia produced by intraplantar injection of loperamide into the inflamed paw was completely antagonized by pretreatment with naloxone at a dose of 10 mg/kg s.c. given 15 min before loperamide at a dose of 300 μg by the intraplantar route (one-way ANOVA, $F = 7.952$, $p = .0031$). The mean PPT of 133 ± 40 g ($n = 5$) after treatment with loperamide was significantly different from the PPT of 54 ± 5 g ($n = 6$) with naloxone before treatment and the mean baseline PPT of 44 ± 3 g ($n = 11$) (Tukey’s test; $p < .01$).
**Fig. 7.** Time course of the antagonism by loperamide or morphine of FCA-induced hyperalgesia. FCA was injected by the intraplantar route, and hyperalgesia was allowed to develop for 24 h. Loperamide at a dose of 100 μg (n = 11–13) or morphine at a dose of 300 μg (n = 8–13) was then injected by the intraplantar route into the inflamed paw (t = 0). The PPT (in g) was read in each paw before drug injection and at various times up to 24 h after treatment. Each point is the mean ± S.E.M. PPT at the respective time points. The PPT values in the inflamed paw were significantly different from baseline at 15 min, 30 min, and 1 h after treatment with loperamide (p < .01, Dunnett’s test). Morphine also produced significant antinoceception in the uninflamed paw at 30 min (p < .01, Dunnett’s test).

**Tape Stripping-Induced Hyperalgesia.** After tape stripping, loperamide at a dose of 100 μg was injected by the intrapaw route and PPT values were measured at various times after injection. Antihyperalgesia in the inflamed paw was observed at 15 min, 30 min, and 1 h after injection (one-way ANOVA, F = 16.86, p < .0001; Fig. 8). Antihyperalgesia due to injection of loperamide into the inflamed paw was not observed in the uninflamed paw.

Loperamide produced dose-dependent antihyperalgesia in this model, with an ED50 value of 71 μg (35–168 μg) (Fig. 5) when measured at 15 min after intrapaw injection, the time of maximal antihyperalgesia. Morphine was 4-fold less potent when tested at 15 min after injection, with an ED50 value of 293 μg (136–531 μg). When 300 μg of loperamide was injected into the inflamed paw, antinoceception was not observed in the uninflamed paw, where the mean PPT in the uninflamed paw was 126 ± 11 g, compared with 225 ± 15 g in the inflamed paw. However, the values for morphine were similar in the inflamed and uninflamed paws (inflamed, 136 ± 16 g; uninflamed, 167 ± 24 g).

The antihyperalgesia produced by loperamide against tape stripping-induced hyperalgesia at 15 min after intrapaw injection was blocked by pretreatment with naloxone at 10 mg/kg s.c. administered 15 min before loperamide. The mean PPT of 225 ± 15 g (n = 8) after treatment with loperamide was significantly different from that observed with naloxone pretreatment (45 ± 7, n = 6) or the mean baseline PPT of 49 ± 3 g (n = 6) (Tukey’s test; p < .01).

**Discussion**

Loperamide is a potent and fully efficacious antihyperalgesic agent when administered locally under conditions of inflammatory pain. The mechanism of action of loperamide is consistent with agonist activity at the μ opioid receptor in that it inhibits binding to the receptor with nanomolar potency, and is a full agonist in the [35S]GTPγS and cAMP assays and its effects in vitro and in vivo are naloxone reversible. The antihyperalgesic effects resulting from the administration of loperamide are due to local actions at the nociceptor, and loperamide lacks the side effects commonly associated with administration of centrally acting opiate agonists.

Compression of the inflamed knee joint is a model in which robust hyperalgesia can be measured by the change in blood pressure that results from the sympathetic response to the painful stimulus of knee compression (Nagasaka et al., 1996). After intra-articular injection, opiate agonists with μ or κ, but not δ, selectivity produce antinoceception via a peripheral mechanism and at doses much lower than those that are efficacious by systemic routes (Nagasaka et al., 1996). When injected directly into the inflamed joint, loperamide reduced...
the pain response evoked by knee joint compression to a level comparable to that induced by morphine and at a 10-fold lower dose than that of morphine. The effect of loperamide was naloxone reversible and, in contrast to morphine, did not result in catalepsy. The lack of effect of loperamide after i.m. or contralateral joint injection supports the notion that treatment with loperamide directly at the site of inflammation resulted in potent antihyperalgesia that was not due to systemic or central effects of the compound.

After local administration, loperamide inhibited late-phase formalin-induced flinching, with an $A_{50}$ value of 6 $\mu$g, and produced a prolonged antihyperalgesia. The lack of effect of loperamide on early-phase flinching or after injection into the contralateral paw supports the notion that loperamide is a peripherally selective compound that does not act as a local anesthetic and does not produce centrally mediated analgesia after systemic absorption. The lack of effect of the peripherally selective compound loperamide on early-phase flinching is consistent with the data on other opiate agonists that do not cross the blood-brain barrier. Methylmorphine, a quaternary morphine analog that does not penetrate the CNS, was efficacious against late-phase formalin-induced flinching only when administered i.p. to the rat (Oloyemi et al., 1992). In contrast, morphine produces comparable antinoiception in both the early and late phases when given by either intrapaw injection or the s.c. route of administration (Wheeler-Aceto and Cowan, 1991; Wheeler-Aceto, 1995).

Loperamide was also efficacious against inflammatory pain induced by an inflammation in deep tissue with injection of FCA or by a superficial abrasion-like injury caused by tape stripping. In both models, the lack of effect on paw pressure thresholds in the uninflamed paw indicated that the effect of loperamide was local and peripheral. The antihyperalgesia produced by loperamide was blocked by pretreatment with naloxone, demonstrating an opiate mechanism of action. In studies using the FCA model, Stein et al. (1988b, 1989) demonstrated that peripheral antinociceptive effects are produced by local injection of opioids into inflamed tissue. Although antinociception resulted from activation of all three opioid receptor subtypes, the $\mu$ agonists fentanyl (Stein et al., 1988b) and DAMGO were more potent and efficacious than the $\delta$ agonist [D-Pen$^2$,D-Pen$^5$]-enkephalin or the $\kappa$ agonist U-50,488H when injected locally (Stein et al., 1989). The effects of agonists were dose dependent, stereoselective, and naloxone reversible and did not occur at equivalent systemic doses (Stein et al., 1988b, 1989).

Tape stripping-induced hyperalgesia is a model of inflammatory pain developed in our laboratory (Cortes Burgos and DeHaven-Hudkins, 1996) that uses tape stripping to produce an abrasion injury by removal of the stratum corneum layer of the epidermis. The potency of loperamide was 4-fold greater than the $\mu$ agonists DAMGO or morphine in the tape-stripping model (Cortes Burgos and DeHaven-Hudkins, 1996). As shown with the FCA-induced hyperalgesia model, the antinoiception produced by loperamide was not observed in the uninflamed paw. The hyperalgesia that results from the tape-stripping procedure was antagonized by the local injection of $\mu$ agonists or by $\kappa$ agonists with nanomolar affinity for the $\mu$ receptor but not by agonists selective for the $\delta$ or $\kappa$ subtypes (Cortes Burgos and DeHaven-Hudkins, 1996). These data suggest that the tape-stripping model of hyperalgesia is selective for $\mu$ agonists administered locally and that loperamide exhibits peripheral antihyperalgesia in this model as well.

In recent work, it was shown that the topical application of loperamide in a cream-base formulation reduced the thermal hyperalgesia induced by a mild thermal injury to the plantar surface of the rat paw. This effect was dose dependent and readily reversed by pretreatment with systemic naloxone and showed a partial cross-tolerance to systemic morphine. Importantly, as in other models, loperamide itself had little effect on nonhyperalgesic thermal escape latencies, and the local action was emphasized by the lack of effect on hyperalgesic escape latencies when applied to the uninjured paw (Nozaki-Taguchi and Yaksh, 1999).

The peripheral nature of the antihyperalgesia produced by loperamide was substantiated by pharmacokinetic data that indicated that loperamide does not cross the blood-brain barrier (Heykants et al., 1974; Schinkel et al., 1996). After the oral dosing of 1.25 mg/kg to rats, the majority of drug was localized to the stomach and intestines, with extremely low levels (<0.022 $\mu$g/g wet weight) in the brain (Heykants et al., 1974). When 5 mg/kg $[^{3}H]$loperamide was injected i.v. in mice, the majority of radioactivity was found in the lung, liver, and kidney, with levels in brain of <1 $\mu$g/g (Wuster and Herz, 1978). Recently, Schinkel et al. (1996) reported that loperamide, as well as other peripherally acting drugs, are substrates for the drug-transporting P-glycoproteins (MDR) in brain, meaning that loperamide and other peripherally selective compounds may be effectively prevented from crossing the blood-brain barrier by the activity of the MDR transporter. Mice lacking the mdr transporter gene exhibited opiate-like behaviors after the oral administration of loperamide, and compared with wild-type mice, the mdr$^{-/-}$ mice exhibited a 14-fold increase in the levels of loperamide in brain after an oral dose of 1 mg/kg (Schinkel et al., 1996).

The unique pharmacokinetic properties that make loperamide an effective antidiarrheal agent are the same properties that make it an ideal antihyperalgesic agent for local administration. The elements of the antihyperalgesia produced by loperamide that differentiate it from centrally acting opiate analgesics are 1) potency with local administration, 2) lack of efficacy at distal sites of injection, and 3) lack of efficacy in central measures of antinociception. After local administration, loperamide produces antihyperalgesia through its accessibility to the peripheral opioid receptor. It is not distributed systemically, as demonstrated by the lack of effect when it is injected into the contralateral knee in the compression model or into the contralateral paw in the formalin assay and by its lack of effect on uninflamed paw pressure thresholds in the FCA- and tape stripping-induced hyperalgesia assays. The potency and efficacy of loperamide after local administration are comparable to or better than those of morphine administered locally. In contrast, when loperamide was administered i.v. to both mice and rats, antinociception was not observed until doses were achieved that approach the LD$_{50}$ dose (Wuster and Herz, 1978; Hurwitz et al., 1994). After oral administration in rats, loperamide failed to exhibit antinociception in the tail withdrawal test at doses up to 160 mg/kg (Niemegeers et al., 1974a) and in the tail pinch test at doses up to 80 mg/kg and was inactive in the Randall-Selitto test at doses up to 16 mg/kg (Bianchi and Gai, 1977). Similarly, loperamide was a weak analgesic in the hot-plate test with an ED$_{50}$ value of 42 mg/kg p.o. in the
mouse (Bianchi and Goi, 1977) and exhibited efficacy only at very high doses in the tail withdrawal test in the mouse after s.c. or p.o. administration (Hurwitz et al., 1994). In mice, morphine-like side effects were not observed with loperamide at doses of 80 mg/kg s.c. or 20 mg/kg i.p., whereas the LD50 was 75 mg/kg s.c. and 28 mg/kg i.p. (Niemegeers et al., 1974b).

In summary, loperamide has potential therapeutic usefulness as a peripherally selective topical or local opiate antihyperalgesic agent that lacks many of the side effects associated with opiate administration. Loperamide demonstrated antihyperalgesia in four models of inflammatory pain. In the studies in which dose-response relationships were determined, loperamide exhibited potency and efficacy equal to or better than those of morphine after local administration. When evaluated for efficacy against formalin-induced flinching, loperamide did not produce antihyperalgesia in the early phase or after injection into the contralateral paw. This was unlike the effects observed with morphine, which inhibited early-phase flinching and produced antinociception when injected into the contralateral paw (Wheeler-Aceto, 1995). Similar results were obtained for the antihyperalgesia produced by intra-articular injection of loperamide into an inflamed knee joint. Loperamide produced antihyperalgesia by local effects in the FCA- and tape stripping-induced models of hyperalgesia, whereas the effects of morphine were mediated by local, systemic, and CNS actions. Because loperamide does not cross the blood-brain barrier or produce its effects systemically, it is a superior drug of choice for the treatment of inflammatory pain where the administration of drug directly at the site of injury is possible.

Acknowledgments

We thank Dr. Alan Cowan and Dr. Alan Maycock for their critical reviews of the manuscript.

References


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