Morphine Tolerance in the Mouse Ileum and Colon


Department of Pharmacology and Toxicology (G.R.R., B.H.G., W.L.D., H.I.A.), Virginia Commonwealth University Program in Enteric Neuromuscular Sciences (G.R.R., H.I.A.), Virginia Commonwealth University, Richmond, Virginia

Received July 10, 2008; accepted August 4, 2008

ABSTRACT

Repeated administration of morphine is associated with tolerance to its antinociceptive properties. However, constipation remains the major side effect of chronic exposure to morphine. In contrast, previous studies suggest that tolerance to opioids develops in the ileum of several species. In this study, we provide evidence that constipation may arise due to a lack of tolerance development to morphine in the colon. Mice received implants with either placebo or 75 mg of morphine pellets, and they were examined for morphine tolerance to antinociception, defecation, and intestinal and colonic transit after 72 h. Tissues were obtained from the ileum and distal colon, and contractile responses were measured from longitudinal and circular muscle preparations. In morphine-pelleted mice, a 5.5-fold tolerance developed to antinociception after 72 h, and a 53.2-fold tolerance developed in mice that received an additional daily morphine injection. In both models, intestinal transit but not defecation or colonic transit developed tolerance. In isolated longitudinal muscles, electrical field stimulation-induced cholinergic contractions were dose-dependently inhibited by morphine in both the ileum and colon of placebo pelleted with a pD2 of 7.1 ± 0.4 and 7.8 ± 0.4, respectively. However, the dose response to morphine inhibition was shifted to the right for the ileum from morphine-pelleted mice (pD2 = 5.1 ± 0.4) but not the colon (pD2 = 6.9 ± 0.4). In circular muscle preparations, morphine induced atropine-insensitive contractions in both tissue segments. Tolerance to morphine developed in the ileum but not the colon upon repeated administration of morphine. These findings indicate that a lack of tolerance development in the colon is the basis for opioid bowel dysfunction.

Morphine is one of the most frequently prescribed drugs for the treatment of moderate to severe pain. Upon repeated administration, tolerance develops to many of its effects including analgesia, nausea, vomiting, and respiratory depression (Thompson and Ray, 2003). However, clinical evidence suggests that tolerance does not develop to morphine-induced constipation, which limits the chronic use of this excellent pain reliever in man (Ling et al., 1989; Yuan et al., 1998; Thompson and Ray, 2003; Gutstein and Akil, 2006). Indeed, many terminally ill patients choose to discontinue opioid treatment to alleviate the discomfort of chronic opioid-induced bowel dysfunction. Opiate analogs such as morphine act centrally as well as on peripheral sites within the enteric nervous system. Although gastrointestinal effects may partly be the result of activation of opioid receptors at spinal and supraspinal sites to slow intestinal transit and inhibit secretion, a large body of evidence indicates a direct peripheral activation (Wood and Galligan, 2004). The mechanism of morphine-induced constipation involves inhibition of gastrointestinal peristalsis, which occurs as a result of presynaptic inhibition of excitatory cholinergic neurons within the myenteric plexus (Paton, 1957) and may also involve increased tone and nonmigrating contractions (Matsumoto et al., 1986; Frantzides et al., 1992). Since the early work of Paton (1957), the guinea pig longitudinal muscle-myenteric plexus (LMMP) preparation has been the tissue preparation of choice to study the in vitro effects of morphine and related opioids in the gastrointestinal tract. Both tolerance and dependence have been demonstrated in this preparation (Rezvani et al., 1983; Leedham et al., 1989, 1992; David et al., 1993) and in other species (Weisbrodt et al., 1977). This contrasts with the clinical findings of the lack of tolerance to constipation and raises the question of whether similar tolerance occurs within the colon, an important site for the induction of constipation. It is remarkable that there are only a few studies addressing this issue, although the first indication of the lack of morphine-induced tolerance toward colonic motility was noted in dogs in 1926 (Plant and Miller, 1926). In the rat colon, Burks and colleagues (Williams et al., 1997) demonstrated that continuous infusion of mor-
phrine resulted in tolerance in the proximal colon in vivo. In contrast, others have found the lack of tolerance to the anti-transit effects of morphine in mice pretreated twice daily for 10 days (Tan-No et al., 2003) or over 8 h of continuous infusion (Ling et al., 1989), although in these studies the effects of morphine were examined toward inhibition of small intestinal transit. Whereas clinical observations strongly suggest that morphine-induced constipation remains resistant to the development of tolerance (Kreek, 1973), studies defining tolerance in the colon are lacking. The objective of this study was to compare the development of morphine tolerance in the mouse ileum and colon using both in vivo and in vitro methods.

Materials and Methods

Animals. Male Swiss-Webster mice (Harlan, Indianapolis, IN) that weighed 25 to 30 g were housed six per cage in animal care quarters and maintained at 22 ± 2°C on a 12-h light/dark cycle. Food and water were available ad libitum. The mice were brought to a test room (22 ± 2°C, 12-h light/dark cycle), marked for identification, and allowed 18 h to recover from transport and handling. Protocols and procedures were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University Medical Center and comply with the recommendations of the International Association for the Study of Pain.

Surgical Implantation of Pellets. Mice were anesthetized with 2.5% isoflurane before shaving the hair on the back of the neck. Adequate anesthesia was noted by the absence of the righting reflex and lack of response to a toe pinch, according to Institutional Animal Care and Use Committee guidelines. The skin was cleansed with 10% povidone iodine (General Medical Corp., Prichard, WV) and rinsed with water. Maintenance of a stringent aseptic technique was observed. All instruments and solutions were prepared using aseptic technique. Before giving the mice injections, a baseline (control) latency was determined. Only mice with a control reaction time of 2 to 4 s were used. The average baseline latency for these experiments was 3.0 ± 0.1 s. The test latency after drug treatment was assessed at the appropriate time, and a 10-s maximal cutoff time was imposed to prevent tissue damage. Antinociception was quantified according to the method of Harris and Pierson (1964) as the percentage of maximum possible effect (%MPE), which was calculated as follows: 

\[
\%MPE = \left(\frac{[\text{test latency} - \text{control latency}] - 10}{\text{control latency}}\right) \times 100.
\]

Percentage MPE was calculated for each mouse, using at least six mice per group.

Charcoal Meal Test for Gastrointestinal Transit Analysis. Forty-eight hours before testing, mice were placed in cages with raised mesh wire to suspend them above their bedding and prevent ingestion of feces or bedding. The animals were habituated for 24 h in the presence of food and water and then fasted for 24 h with free access to water (Roy et al., 1998; Raenal et al., 2005). This time frame was chosen to deplete the intestine and colon of any feces. To maintain caloric intake and to avoid hypoglycemia, mice had access to a sugar water solution consisting of a final concentration of 5% dextrose for the first 8 h of the fasting period. Mice were treated with either saline (10 μl/g s.c.) or morphine (10 mg/kg s.c.), and 20 min later they were given an oral gavage consisting of 5% aqueous suspension of charcoal in a 10% gum Arabic solution. At 30 min after the administration of the charcoal meal, the mice were euthanized by cervical dislocation, and the small intestine from the jejunum to the cecum was dissected and placed in cold saline to stop peristalsis. The distance traveled by the leading edge of the charcoal meal was measured relative to the total length of the small intestine, and the percentage of intestinal transit for each animal was calculated as 

\[
\text{percentage transit} = \left(\frac{\text{charcoal distance}}{\text{small intestinal length}}\right) \times 100.
\]

This is referred to as intestinal transit in the text.

Bead Expulsion Test for Colonic Transit Analysis. Mice were habituated and fasted as described above for the small intestine transit analysis. Mice were given an injection of either saline (10 μl/g b.wt.) or morphine (10 mg/kg s.c.). At 20 min postinjection, animals were anesthetized with isoflurane (1–2 min) to insert a single 2-mm glass bead into the distal colon at a distance of 3 cm from the anus. Bead insertion was accomplished using a glass rod with a fire-polished end and marked at 3 cm to avoid tissue damage (Raffa et al., 1987; Martinez et al., 2004). After bead insertion, mice were placed in individual cages and the time to bead expulsion was monitored. Animals were monitored for a maximum of 5 h unless the bead expulsion occurred sooner. The quantity of fecal matter expelled was measured and counted as number of fecal boli and the total weight of fecal boli every 24 h over a 72-h period.

Evaluation of Defecation. Mice were placed in individual cages with raised wire mesh to suspend them above their bedding and prevent ingestion of feces. The amount of food consumed and fecal boli were weighed at 24, 48, and 72 h after pellet implantation. The ratio of the weight of fecal boli to the amount of food consumed was calculated for each group.

Isometric Tension Recording. One-centimeter segments of ileum and distal colon (approximately 1 cm proximal to the anus) were dissected, flushed of their contents, and trimmed of mesentery. Preparations were suspended in the axis of the circular muscle with a metal triangle or longitudinal muscle tied to a glass hook under 1 of g passive tension in 15 ml of siliconized organ baths containing Krebs solution (118 mM NaCl, 4.6 mM KCl, 1.3 mM NaH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, 11 mM glucose, and 2.5 mM CaCl₂) maintained at 37°C and bubbled with 95% O₂ and 5% CO₂, and tissues were allowed to equilibrate for 60 min before start of experiments, with Krebs solution changed every 15 min. Isometric contractions were recorded by a force transducer (GR-FT03; Radnoti, Monrovia, CA) connected to a personal computer using Acqknowledge 382 software (BIOPAC Systems, Inc., Santa Barbara, CA). Acetylcholine (3 μM) contractions were measured at the beginning of each experiment as the reference control.

Neurogenic Contractions by Transmural Field Stimulation. Electrical field stimulation (EFS; 50 V, 7.5 Hz, unless stated otherwise) was applied through concentric electrodes over longitudinal muscles or L-type stimulating electrodes over circular muscle strips to produce neurogenic contractions/relaxations. Single or cumulative doses of morphine or other specific opioid agonists were added over the EFS contractions/relaxations to determine their inhibitory effects on the neurogenic responses.

Drugs and Chemicals. The placebo and 75 mg of morphine pellets were obtained from the National Institute on Drug Abuse (Bethesda, MD). Morphine sulfate (Mallinckrodt, Hazelwood, MO) was dissolved in pyrogen-free isotonic saline (Baxter Healthcare, Deerfield, IL). Activated charcoal, gum Arabic, atropine sulfate, and acetylcholine were purchased from Sigma-Aldrich (St. Louis, MO). Morphine sulfate (Mallinckrodt, Hazelwood, MO) was dissolved in pyrogen-free isotonic saline (Baxter Healthcare, Deerfield, IL). Activated charcoal, gum Arabic, atropine sulfate, and acetylcholine were purchased from Sigma-Aldrich (St. Louis, MO).

Data Analysis. The in vivo data are expressed as mean ± S.E. Analysis of variance (ANOVA) followed by the post hoc Student Newman-Keuls test were performed to assess significance using the InStat 3.0 software (GraphPad Software Inc., San Diego, CA). P < 0.05 was considered significant.

Contractile responses after repeated administration of morphine were analyzed by repeated measures ANOVA followed by the Bon-
Results

Tolerance to Morphine Antinociception

Tolerance developed to morphine antinociception in morphine-pelleted mice, as measured in the tail-immersion test. The baseline latency for tail immersion in tolerant mice was similar to that of placebo-pelleted mice at 72 h after pellet implantation (Fig. 1, A and B). Morphine administered subcutaneously elicited dose-dependent antinociception in the tail-immersion test in both placebo- and morphine-pellet mice at 72 h after pellet implantation. Morphine-pelleted mice (with no ramping morphine injections) showed a 5.5-fold tolerance to morphine antinociception compared with placebo-pelleted mice. The calculated ED50 value for morphine was 38.2 mg/kg (95% confidence limit (C.L.), 34.4–42.4) in morphine-pelleted mice compared with 6.9 mg/kg (95% C.L., 6.3–7.6) in placebo-pelleted mice. In a similar but more pronounced fashion, morphine-pelleted mice that received ramping morphine injections were 53.2-fold tolerant (ED50 = 228.9 mg/kg (95% C.L., 196.8–266.2)) to the antinociceptive effect of acute morphine compared with the placebo-pelleted mice (ED50 = 4.3 mg/kg (95% C.L., 3.9–4.7)).

Effect of Morphine on Defecation

To establish whether tolerance develops to constipating effects of morphine as it develops to antinociception, defecation was measured as the weight of fecal boli over the 72-h duration of pellet implantation in the 5.5-fold antinociceptive tolerance model. Figure 2A shows that defecation was markedly reduced in morphine-pelleted mice compared with the placebo-pelleted mice. The average amount of food consumed by placebo-pelleted mice was 672.3 ± 112.1, 818.2 ± 115.2, and 705.1 ± 72.6 mg at 24, 48, and 72 h after pellet implantation, respectively, compared with 33.5 ± 5.3, 6.1 ± 1.8, and 8.3 ± 2.1 mg in morphine-pelleted mice. Because morphine induced a decrease in food consumption, the ratio of the weight of fecal boli to the amount of food consumed was calculated for each group. Even though morphine-pelleted mice showed a decrease in their food intake, we observed a significant decrease in the ratio of fecal output per gram of food intake in this same group (Fig. 2B). The percentage of weight of fecal boli per gram of food consumed was 10 ± 0.8, 4.1 ± 0.5, and 3.4 ± 0.6% at 24, 48, and 72 h after pellet implantation, respectively, compared with 34.7 ± 0.6, 22.5 ± 0.5, and 17.6 ± 0.7% in the placebo-pelleted mice. This indicates that constipation is maintained throughout the 72-h period while tolerance developed to morphine antinociceptive effects.

Gastrointestinal Transit

The distance traveled by the leading edge of the charcoal meal was reduced by more than 50% upon acute subcutaneous administration of morphine (10 mg/kg) in placebo-pelleted mice (Fig. 3, A and B). In the 5.5-fold antinociceptive tolerance model, there was approximately 30% reduction in the baseline distance traveled after saline administration. However, acute subcutaneous administration of this same dose of morphine in this antinociceptive tolerant model did not produce any further reduction in the gastrointestinal transit (Fig. 3A). Likewise, in the

---

![Fig. 1. Antinociceptive effects of morphine in placebo-pelleted (PP) and morphine-pelleted (MP) mice. Experiments were conducted 72 h after the implantation of the placebo or the 75-mg morphine pellet. Baseline latencies were obtained in the warm water tail-immersion test, mice were challenged with various doses of morphine subcutaneously, and their tail-immersion latencies were reassessed for construction of dose-response curves. A, mice implanted with morphine pellets and administered saline (5.3-fold tolerance); B, mice implanted with the morphine pellets and administered ramping morphine injections (53.2-fold tolerance). Data are expressed as mean %MPE ± S.E.M. Each data point represents 6 to 10 mice.](image-url)
52.3-fold antinociception-tolerant group, whereas basal gastrointestinal transit was reduced in mice that received saline injections, acute administration of morphine (10 mg/kg s.c.) did not further alter the intestinal transit, suggesting that tolerance developed to the effect of morphine on intestinal transit (Fig. 3B). An important criterion for tolerance development is that higher concentrations are required to produce a similar effect. Consistent with this fact, morphine at 30 and 50 mg/kg significantly reduced intestinal transit in the 53-fold antinociceptive tolerant mice. At these high doses of morphine, the inhibition of intestinal transit was equal to that achieved with acute atropine (5 mg/kg) over a 30-min period, suggesting that tolerance develops to the opioid but not the cholinergic receptor (Fig. 3B).

Fig. 2. Analysis of defecation in morphine antinociceptive tolerant mice. Mice were implanted with a placebo pellet (PP) or a 75-mg morphine pellet (MP) for 72 h. Equal amounts of water and chow were available for each group, and mice were placed on grid cages. A, total weight of fecal boli and percentage of weight of fecal boli per gram of food consumed (B) were calculated at 24, 48, and 72 h. Data are expressed as mean ± S.E.M. (n = 6–8). ***, significantly different from the placebo-pelleted group at P < 0.001.

Fig. 3. A, effect of morphine on gastrointestinal transit in moderate and high (B) morphine antinociception tolerance models. Experiments were conducted 72 h after the implantation of the placebo pellet (PP) or the 75-mg morphine pellet (MP). The low (5.5-fold) tolerant mice were pelleted with a 75-mg morphine pellet, whereas the high (53.2-fold) tolerant mice received ramping morphine injections in addition to the pellet. Mice were fasted for 14 h before testing. A charcoal meal was administered by oral gavage, 20 min after the injection of saline or morphine (10 mg/kg s.c.). Mice were sacrificed 30 min later, and the distance traveled by the leading edge of the charcoal was calculated as percentage of the total length from the pylorus to the ileocecal junction. Data are expressed as mean ± S.E.M. (n = 6–12). ***, significantly different from PP-saline group at P < 0.001; ++, significantly different from PP-morphine group at P < 0.01; $, significantly different from PP-morphine group at P < 0.05; and ##, significantly different from MP-saline group at P < 0.05.
Colonic Transit

We constructed a dose-response curve for the inhibitory effects of morphine on colonic transit in naive mice using the bead expulsion test. As shown in Fig. 4A, morphine induced a dose-dependent increase in bead expulsion time, with a maximal effect being achieved at a dose of 10 mg/kg. This dose was equivalent to the maximal analgesia previously identified in the tail-immersion assay (data not shown). We next identified whether morphine tolerance develops to colonic transit. In both the 5.5- and 53.2-fold antinociception tolerance models, acute subcutaneous administration of morphine (10 mg/kg) significantly retarded the expulsion time in morphine-pelleted mice in a similar manner to that in placebo-pelleted mice (Fig. 4, B and C). In the low antinociception

Fig. 4. Effect of morphine on colonic transit. A, dose response to the inhibitory effects of morphine on colonic transit in drug-naive mice. Mice (n = 6) received injections with either saline or morphine (0.5–10 mg/kg s.c.). At 20 min postinjection, a single 2-mm glass bead was inserted into the distal colon, and the time for bead expulsion was determined. B, effect of morphine on colonic transit in low and high (C) morphine antinociception tolerance models. Experiments were conducted 72 h after the implantation of the placebo pellet (PP) or the 75-mg morphine pellet (MP). The low (5.5-fold) tolerant mice were pelleted with a 75-mg morphine pellet, whereas the high (53.2-fold) tolerant mice received ramping morphine injections in addition to the pellet. Mice were fasted for 14 h before testing. Saline or morphine (10 mg/kg) was administered subcutaneously, and 30 min later a 2-mm glass bead was inserted into the distal colon at a distance of 3 cm from the anus. The time for expulsion of the bead was determined as an indication of colonic transit. Data are expressed as mean ± S.E.M. (n = 6–12). ***, significantly different from PP-saline group at P < 0.001; ***, significantly different from PP-morphine group at P < 0.001; ***, significantly different from PP-morphine group at P < 0.05; and $$$, significantly different from MP-saline group at P < 0.001.
tolerance model (5.5-fold), the average bead expulsion time in morphine-pelleted mice was 100.8 ± 11.2 min compared with 127.8 ± 6.5 min in placebo-pelleted mice after morphine administration. In the high antinociception tolerance model (52.3-fold), the morphine-pelleted mice expelled the bead within an average of 76.8 ± 10.4 min compared with 109.8 ± 6.5 min for the placebo-pelleted mice group. This finding suggested that tolerance does not occur to the effects of acute morphine in the colon of morphine-pelleted mice, which contrasts with the development of tolerance to morphine antinociception and morphine-induced reduction in gastrointestinal transit.

These in vivo data show that whereas 72-h exposure to morphine induced tolerance to antinociception and intestinal transit, colonic transit remained responsive to morphine inhibition, thus resulting in prolonged constipation.

**Morphine Tolerance in Vitro.** Constipation can generally be ascribed to both a decrease in the propulsive activity and increased nonpropulsive contractions. Whereas propulsive activity is largely neurally mediated, nonpropulsive contractions are likely to include a myogenic component. To determine the effects of morphine on each of these components in both ileum and colon, we used an in vitro whole tissue approach. Longitudinal muscle preparations were used to study the effect of morphine on neurally mediated cholinergic contractions, whereas circular muscle preparations were used to test the effect of morphine on muscle contraction.

**LMMP Preparations.** In the first set of experiments, we examined the effect of morphine on field-stimulated (FS) cholinergic contractions in the longitudinal muscle preparations from the ileum and colon. The FS contractions were abolished by tetrodotoxin (TTX; 100 nM) and atropine (100 nM) (data not shown). Morphine dose-dependently inhibited FS contractions in both ileum and colon (Fig. 5A), consistent with its widely known effects on inhibition of acetylcholine release from enteric neurons. The pD₂ values for morphine-induced inhibition were 7.8 ± 0.4 in the colon and 7.1 ± 0.4 in the ileum. The inhibition of FS contractions in the colon from the 5.5-fold antinociceptive tolerant mice was similar between the placebo and morphine-pelleted mice (pD₂ in placebo 7.8 ± 0.4 and 6.9 ± 0.4 morphine-pelleted). In contrast, there was a significant rightward shift in morphine sensitivity in the ileum from the morphine-tolerant mice, with the pD₂ value being 7.1 ± 0.4 in placebo and 5.1 ± 0.4 in morphine-pelleted (Fig. 5B). This indicates that tolerance to morphine develops in the ileum but not the colon to the inhibition of cholinergic-mediated contractions and is consistent with the above in vivo data on intestinal and colonic transit. The pD₂ values for morphine were similar in the ileum (7.1 ± 0.4) and colon (7.8 ± 0.4) from the placebo-pelleted mice, indicating that there is no difference in the morphine receptor sensitivity toward inhibition of cholinergic neurons in these two tissue segments.

There was no difference in the sensitivity to exogenous acetylcholine between the placebo and morphine-tolerant mice [ED₅₀ of 11.4 nM (placebo) and 10.9 nM (morphine-pelleted)] (Fig. 6A) or to neurally mediated, frequency-dependent contractions (Fig. 6B). Furthermore, acetylcholine-induced contractions in the presence of morphine (1 μM) were also not affected (Fig. 6C). Morphine-dependent inhibition of FS contractions was reversed by naloxone (Fig. 6D).

To further verify that morphine-pellet implantation for 3 days did not alter smooth muscle contractility, we measured longitudinal muscle contractions to calcium in depolarizing K⁺ solution. Figure 7 shows that in either colon or ileum,
calcium-dependent contractions are similar in placebo- and morphine-pelleted mice.

To test whether acute tolerance occurred upon repeated administration, FS-stimulated contractions were measured in drug-naive mice, i.e., tissues isolated from nonpelleted mice. As shown in Fig. 8, repeated administration of morphine continues to inhibit FS contractions in the colon, whereas in the ileum FS contractions become resistant to morphine by the third application.

**Circular Muscle Preparations.** Unlike the longitudinal muscle, circular smooth muscle cells are known to express opiate receptors (Bitar and Makhlouf, 1982, 1985). To test the effect of morphine on the circular muscle, tissues were set up as ring preparations in the organ bath. After 1 h of equilibration, morphine (applied in a cumulative fashion) induced contractions in both the distal colon and ileum from placebo-pelleted mice. As shown in Fig. 9, the response to morphine markedly differed between the colon and ileum. Whereas morphine induced a sustained increase in the phasic and tonic contractions in the colon, those in the ileum consisted of an initial increase in tone and phasic contraction amplitudes, which gradually diminished toward baseline val-

---

Fig. 6. Contractility of the muscle strips were unaffected by acute or chronic morphine (pellets) treatment. A, dose-response curve of acetylcholine-induced contractions and frequency (electrical field)-dependent contractions were different between placebo- and morphine-pelleted mice. B, C, dose-response to acetylcholine in the absence and presence of 1 μM morphine for 10 min. Acute administration of morphine did not affect the acetylcholine-induced contractions, and the reversal of morphine-induced inhibition by naloxone of field-stimulated contractions in distal colon longitudinal muscle preparations is illustrated (D). Data points are mean responses ± S.E.M.; n = values in parentheses.

Fig. 7. Calcium-induced contractions of muscle strip preparations from colon (A) and ileum (B) in a depolarizing (80 mM K⁺ physiological saline) solution in both placebo- and morphine-pelleted mice. There was no difference in the contractile ability of either muscle strip between placebo- and morphine-pelleted groups. Data points are mean ± S.E.M.; n = values in parentheses.
ues within 30 min. The pD$_2$ values for morphine-induced phasic contractions were 6.9 ± 0.3 and 7.2 ± 0.1 ($n = 9$) in the colon and ileum, respectively. We have found that exposure of mouse colon to morphine causes an increase in spontaneous contractions for at least 2 h, whereas in the ileum the increase in contractions lasts for only 10 to 15 min. These findings suggested that changes in receptor desensitization may be more prominent in the ileum than the colon.

To determine whether colonic contractions were resistant to morphine-induced tolerance and compare it with tolerance in the ileum, repeated administrations of morphine (a submaximal dose of 3 μM) were applied every 20 min, with in-between washes in drug-naive mice. Morphine induced contractions of equal or greater magnitude to all repeated administrations (3 μM) in the colon but showed desensitization in the ileum (Fig. 10). By the fourth administration, the total contractions in the colon seemed to be enhanced, whereas those in the ileum were reduced by almost 40%.

To test whether tolerance was evident in the gastrointestinal tract in morphine-pelleted mice, colon and ileum preparations were subjected to repeated administration of morphine (300 nM) as described above. The amplitude of peak contraction, measured as the percentage of acetylcholine-induced contraction, was enhanced by the third application in the colon in both the placebo- and morphine-pelleted mice. In the ileum, the contractions in the placebo declined by the third application, but they remained constant in the tolerant mice (Fig. 11).

**Discussion**

In this study, we have systematically compared the development of tolerance to morphine in the mouse small intestine and colon. Whereas both the ileum and colon from several species have been studied with respect to the acute effects of morphine, studies on the development of tolerance have largely been limited to the upper gastrointestinal tract, particularly the small intestine (Rezvani et al., 1983; Leedham et al., 1989; Ling et al., 1989). There are only a few studies that have looked at the chronic effect of morphine in the colon.
(Williams et al., 1997). The lack of tolerance to morphine toward its constipating effects in man is widely accepted (Gutstein and Akil, 2006). To the best of our knowledge, the effects of chronic opioids on the ileum and colon have not been comprehensively evaluated when combining an in vivo and in vitro approach in any species.

It is well known that acute and chronic morphine results in constipation. We now show that constipation also exists in morphine antinociceptive tolerant mice. In addition, we found that tolerance exists to the effects of chronic morphine to intestinal transit but not to bead expulsion in the colon. In this study, we measured intestinal transit using charcoal meal as a marker. Acute morphine-induced inhibition of small intestinal transit has also been observed with radioactive chromium (Weisbrodt et al., 1980) and by fluorescence markers (Schmidt et al., 2008). We found that whereas basal intestinal transit was significantly reduced in the morphine-pelleted compared with the placebo-pelleted mice, the administration of morphine did not further decrease intestinal transit. This indicated that tolerance to an additional bolus of morphine occurs toward intestinal transit. In contrast to the effects in the ileum, tolerance did not occur in the colon. Colonic transit time was significantly retarded by morphine in placebo- as well as in morphine-pelleted mice. However, it is noteworthy that basal transit time was not significantly retarded in the morphine-pelleted mice. The reason for this is not obvious because further administration of morphine to these mice resulted in inhibition of colonic transit time, negating the possibility of tolerance. It is possible that the colon may have become partially tolerant to low concentrations of morphine, as may occur at 72 h after morphine-pellet implantation, and that a 10 mg/kg dose is sufficient to further retard colonic transit. Nevertheless, this development differs from that observed in the ileum in vivo, and the difference was further confirmed in isolated tissue segments.

Two preparations were used to study the effects of chronic morphine in vitro. In the longitudinal muscle preparation, morphine inhibits field-stimulated release of acetylcholine from excitatory enteric nerves (Paton, 1957), whereas in the circular muscle preparations morphine induces contractions (Grider and Makhlof, 1987; Iwata et al., 2007). In both types of preparations, there was no difference in the sensitivity to the effects of morphine between the ileum and colon in the naive mice. However, repeated administration of morphine to drug-naive mice, or those with placebo-pellet implantations, resulted in tolerance development in the ileum. This was
observed as a rightward shift of the concentration-dependent inhibition of field-stimulated contractions by morphine in morphine-pelleted mice and in decreased ability to inhibit the neurogenic contractions upon repeated administration in the ileum from drug-naive mice. Likewise, in circular muscle, contractions to morphine were reduced by repeated administration in drug-naive mice, and they remained suppressed in the ileum but not the colon from morphine-pelleted mice. Our findings in the mouse ileum using chronic morphine treatment and repeated administration in the organ bath confirm other studies that have demonstrated tolerance to morphine to neurogenic contractions in the longitudinal muscle preparations, especially in the guinea pig ileum (Collier et al., 1981; Rezvani et al., 1983). These studies show for the first time that tolerance also occurs to circular muscle contractions in the ileum but not the colon.

The decrease in neuronal excitability primarily results in the suppression of peristaltic propulsion. Morphine also increases phasic contractions and resting contractile tone of circular muscle in both the ileum and colon. In the rat colon, Grider and Makhlouf (1987) demonstrated that phasic contractions of circular muscle strips were induced by tetrodotoxin and vasoactive intestinal polypeptide (VIP) antiserum, suggesting that inhibition of an inhibitory VIPergic component by morphine leads to the unmasking of a myogenic phasic activity of the circular muscle. However, a TTX-insensitive component was also iden-

Fig. 10. A, representative isometric tension-recording traces from colon (top trace) and ileum (bottom trace) circular muscle tissue ring preparations showing effects of repeated administration of morphine; paired experiments in placebo-pelleted mice. Bar graphs depict peak contractions (mean ± S.E.M.) of circular muscle strips from colon (B) and ileum (C) induced by repeated administration of same concentration of morphine (3 μM) at an interval of 20 to 30 min, keeping the initial response as 100%. The repeated administration of morphine was less effective (to cause contractions) compared with the first administration of morphine in the ileum, whereas the colon exhibited similar responsiveness to all of the repeated administrations of morphine, demonstrating a lack of tolerance development to morphine in the colon. *, P < 0.05; **, P < 0.01 is considered significant compared with the corresponding initial response; n = 3 (repeated measures ANOVA).

Fig. 11. Peak amplitude of morphine-induced contractions in distal colon and ileum upon repeated administration of same concentration of morphine (3 μM) in placebo and morphine-pelleted mice. A, contractions were slightly augmented (no tolerance development) in the colon after each morphine administration in both placebo and morphine-pelleted mice. B, in contrast, the morphine-induced contractions of ileum preparations in the placebo group declined substantially, but they remained constant in morphine-pelleted mice. Data are expressed as respective mean contraction ± S.E.M.; n = values in parentheses. **, P < 0.01 versus initial contraction (unpaired t test).
ified by these authors, consistent with a possible direct smooth muscle stimulation by morphine. Early work by Bitar and Maklhouf (1982, 1985) showed opiate-mediated contractions of isolated smooth muscle cells from the guinea pig and human stomach, intestine, and gall-bladder, and evidence for specific opiate receptors on circular smooth muscle was further identified by receptor protection assays (Grider and Maklhouf, 1991).

Thus, morphine-induced contractile responses in the circular muscle may include both a direct smooth muscle component as well as inhibition of an inhibitory tone. Nonpropulsive segmental contractions in addition to inhibition of peristalsis contribute toward constipation (Parsiach, 2006). In addition to the effect of morphine on inhibition of acetylcholine release, enhanced contractions are likely to exacerbate constipation. In several species, including humans, morphine increases nonpropulsive activity (Plant and Miller, 1926; Frantzides et al., 1990, 1992).

The present studies were done in mice, and further studies are necessary to establish that there is differential ileal versus colonic morphine tolerance across species. In humans, colonic propulsion and peristalsis are reduced, and tone increases that may lead to spasms. This may occur as a result of disinhibition of inhibitory neurons (Burleigh and Trout, 1986), an effect that may be reflected as an increase in contractions of isolated circular muscle. Furthermore, differences in the absorptive/secretory functions between the ileum and colon as well the intrinsic and extrinsic neuronal input may account for the differential tolerance. Future studies addressing these issues will be required to delineate the differential basis for tolerance development.

The rewarding and analgesic effects of morphine occur through the activation of μ-opioid receptors. Inhibition of gastrointestinal transit by morphine is also a μ-opioid receptor function. In μ-receptor knockout mice, inhibition of intestinal transit by morphine is absent (Roy et al., 1998). In this study, subcutaneous administration of morphine significantly inhibited gastrointestinal transit in wild-type and heterozygous mice but not in homozygous mice. Furthermore, δ and κ agonists given either intracerebroventricularly or subcutaneously did not produce significant gastrointestinal transit effect in μ-receptor knockout mice. Although gastrointestinal effects may be the result of activation of opioid receptors at spinal and supraspinal sites to slow intestinal transit and inhibit secretion, a large body of evidence indicates a direct peripheral activation (Wood and Galligan, 2004). Our findings that in vivo tolerance correlates with those from isolated tissue segments further confirms a peripheral mechanism for morphine tolerance.

The mechanisms by which tolerance to antinociception or any other effect occurs are complex and still not well understood (Ueda et al., 2003). In the intact animal, morphine tolerance may include adaptive changes of the opioid receptor and/or changes in gene expression (Williams et al., 2001). There is still controversy concerning the role of receptor desensitization in tolerance to morphine in vivo (Finn and Whistler, 2001). This may be further complicated because morphine tolerance can develop to different effects at different rates depending upon the dose and frequency of dose administration (Smith et al., 1999, 2003). The finding that the colon does not develop tolerance is consistent with the clinical impression of the lack of tolerance toward constipation (Gutstein and Akil, 2006). The present study highlights the need for further defining the cellular basis for the differential expression of morphine tolerance in the ileum and colon.

Acknowledgments

We thank Dr. Krsita L. Scoggins, David L. Stevens, Joshua A. Seager, and Sanaa M. Akbarali for their input in the in vivo studies.

References


Pharmacological Basis of Therapeutics


Address correspondence to: Dr. Hamid I. Akbarali, Department of Pharmacology and Toxicology, Virginia Commonwealth University, 1112 E. Clay St., McGuire Hall 317, Richmond, VA 23298. E-mail: hiakbarali@vcu.edu