Cotreatment with Racemic Fenfluramine Inhibits the Development of Tolerance to Morphine Analgesia in Rats

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ABSTRACT

As a follow-up study to an earlier report that racemic fenfluramine can acutely potentiate the analgesic effects of morphine in humans, we investigated the effects of fenfluramine on the development of tolerance to morphine analgesia in rats. Antinociceptive effect, as measured by the tail-flick latency, was studied over 8 days in rats that received continuous i.v. infusion of 1) 22 mg/kg/day of morphine, 2) 20 mg/kg/day of fenfluramine, 3) both drugs concomitantly or 4) saline. Infusion with morphine alone resulted in a peak analgesia of 100% maximal possible effect, which declined with time; full tolerance was reached by day 4. Fenfluramine treatment alone had no effect. Fenfluramine confusion attenuated the development of tolerance to morphine; >70% maximal possible effect was still present on day 4. The effect of fenfluramine confusion occurred in the absence of a significant increase in plasma or brain morphine concentration, or a decrease in the accumulation of morphine’s putative antagonistic metabolite, morphine-3-glucuronide. In another set of infusion experiments, rats were challenged with a single i.p. dose of morphine to characterize the morphine dose-response curves at 10 hr following 4-day i.v. infusion of 1) 22 mg/kg/day of morphine, 2) 20 mg/kg/day fenfluramine, 3) morphine plus fenfluramine or 4) saline. An acute i.p. morphine challenge dose response experiment was also conducted in naïve control rats and in rats receiving a concomitant i.p. injection of fenfluramine (2.4 mg/kg). Coinjection of fenfluramine acutely potentiated the antinociceptive potency of morphine. However, potentiation alone does not fully account for the apparent attenuation of tolerance during morphine i.v. infusion. ED₅₀ of morphine was elevated to 7.0 mg/kg in the morphine-infused rats compared to 2.4 mg/kg in saline-infused rats. Coinfusion of fenfluramine increased ED₅₀ to only 3.7 mg/kg. These results demonstrate that fenfluramine significantly attenuates tolerance development to morphine by modulating the pharmacological process responsible for tolerance development to morphine.

Opioids remain the drugs of choice for the control of severe pain despite efforts to develop new analgesic agents. However, individual patient’s response to opioids is highly variable (Portnoy, 1994), and complete pain control often cannot be reached without causing serious side effects, such as nausea, mood disturbance and respiratory depression. Tolerance development to the analgesic effect of opioids further complicates the treatment of pain and contributes to the variation in analgesic response seen among patients (Foley, 1991). Therefore, the identification of adjuvant drugs that can attenuate or inhibit the development of tolerance to opioids and increase their therapeutic index may lead to improved management of pain.

Neurotransmission systems that couple to or modulate the opioid system offer a target for clinically useful strategies to block opioid tolerance. For example, NMDA-antagonists and nitric oxide synthase inhibitors have been shown to attenuate the tolerance development to morphine in rodents (Herman et al., 1995; Kolesnikov et al., 1992; Trujillo and Akil, 1991; Trujillo and Akil, 1994). The exact biochemical mechanism(s) underlying this effect is not fully understood. There is concern over the potential adverse effects of these new pharmacological agents that may limit their clinical applicability as adjuvants in pain management (Trujillo and Akil, 1995). Also, NMDA-antagonists and nitric oxide synthase inhibitors attenuate rather than completely block the development of morphine tolerance, which suggests that other systems also play a role in the tolerance process.

Opioid and serotonergic systems are interconnected in the circuitry underlying nociceptive neurotransmission in the CNS (Basbaum and Fields, 1984; Fields et al., 1991; Le Bars, 1988). It is well accepted that opioids establish part of their analgesic effect through stimulation of the serotonergic sys-

ABBREVIATIONS: MPE, maximal possible effect; NMDA, N-methyl-D-aspartate; CNS, central nervous system; 5-HT, 5-hydroxytryptamine; M3G, morphine-3-glucuronide; 5-HIAA, 5-hydroxyindoleacetic acid; CI, confidence interval.
tem (Sawynok, 1989). In animal models, acute morphine administration enhances serotonin turnover as evidenced by an increase in its synthesis, release and metabolism (Boadle-Biber et al., 1987; Godefroy et al., 1980; Grauer et al., 1992; Snelgar and Vogt, 1981; Tao and Auerbach, 1994; Yaksh and Tyce, 1979). Upon chronic morphine administration, a decrease in the release of serotonin from the nerve terminals is observed (Jung et al., 1994; Tao et al., 1996), along with a return of intracellular 5-HIAA, the primary metabolite of serotonin, to basal level (Desole et al., 1996; Haleem et al., 1994; Haubrich and Blake, 1973). These changes occur in parallel with the development of tolerance to the analgesic effect of morphine. Involvement of the serotonergic system in the induction of tolerance is further indicated by the ability of 5-hydroxytryptophan, the serotonin precursor or p-chlorophenylalanine, the inhibitor of tryptophan hydroxylase, to attenuate tolerance (Contreras et al., 1973; Way et al., 1968).

In addition, t-tryptophan accelerates tolerance development (Ho et al., 1975). More recently, 5-HT antagonists have been shown to inhibit the expression of tolerance (Hui et al., 1996; Zarrindast et al., 1995). These reported effects of serotonergic agents on morphine tolerance do not follow a logical pattern that could explain the precise role of the serotonergic system in the tolerance process. Therefore, at this time, the effects of a specific serotonergic agent on morphine tolerance is not predictable. Nevertheless, serotonergic agents have the potential to be used clinically to inhibit opioid tolerance.

A well investigated serotonergic agent is the appetite suppressant, fenfluramine (Rowland and Carlton, 1986). Fenfluramine is thought to exert its anorectic effect by increasing the synaptic availability of serotonin. This is accomplished both through stimulation of serotonin release as well as inhibition of synaptic serotonin reuptake (Rowland and Carlton, 1986). Previously, a human volunteer study from our laboratory showed that racemic fenfluramine can enhance the acute analgesic potency of morphine without a parallel increase in opioid side-effects (Coda et al., 1993). To further investigate the pharmacological interaction between fenfluramine and morphine, we evaluated the effects of racemic fenfluramine on the development of tolerance to morphine antinociception in rats.

Methods

Chemicals

Morphine sulfate was purchased from Mallinckrodt Chemical Co. (St. Louis, MO). Racemic fenfluramine and the internal standard, normorphine, were obtained from Sigma Chemical Co. (St. Louis, MO). All drugs for administration were dissolved in sterile physiological saline. Dodecyl sodium sulfate was purchased from Supelco Inc. (Bellefonte, PA), phosphoric acid from J.T. Baker Inc. (Phillipsburg, NJ) and acetonitrile from Fisher Scientific (Springfield, NJ). All other chemicals used for drug assays were of analytical grade.

Animals

Male Charles-River, Sprague-Dawley rats (290–310 g) were kept in individual cages in animal quarters that were maintained at a temperature of 21°C and on a 12-hr light-dark cycle (lights on 7:00 A.M. to 7:00 P.M.). The rats had free access to food and water. A PE-10 cannula was surgically implanted into the right jugular vein of each rat under ketamine/xylazine/acepromazine anesthesia. The open end of the catheter was threaded through a button/tether assembly and connected to a 22-gauge swivel (Instech Laboratories, Plymouth Meeting, PA), which in turn was connected to a microinfusion pump (Provider 5500, Abbott Laboratories, North Chicago, IL). During the 2 recovery days after surgery, the animals received a constant infusion of saline (0.2 ml/hr) and were acclimatized to the testing equipment.

Algesiometric Testing

Nociceptive response was assessed by the radiant heat tail-flick test (D’Amour and Smith, 1941). The intensity of the heat source in the tail-flick apparatus (Columbia Instruments Int. Co., Columbus, OH) was set to provide a predrug latency of 2 sec. A cut-off time of 6 sec was imposed to avoid tissue damage. Before testing, the caudal part of the tail was blackened to improve heat absorption. Each response assessment consisted of three separate measurements taken at 4.5, 3.0 and 1.5 cm from the tip of the tail. Data are expressed as the average percentage of MPE (Dewey et al., 1970) as defined by the following equation.

\[ \% \text{MPE} = \left( \frac{\text{post drug latency} - \text{baseline}}{\text{cut off latency} - \text{baseline}} \right) \times 100\% \]

Time Course of Morphine Tolerance

Drug dosing and testing protocols. Tolerance development to different rates of i.v. morphine infusion was assessed to find the optimal infusion rate for the interaction study between fenfluramine and morphine. On the morning of study day 1 (the third day post-surgery), two successive baseline measurements were taken 30 min apart. Each rat was then randomly assigned to an 8-day i.v. infusion of saline (n = 3), or one of the three morphine regimens: 22 mg/kg/day (n = 8), 44 mg/kg/day (n = 8) or 88 mg/kg/day (n = 8). During the 8-day drug or saline infusion, the tail-flick latency to heat stimulus was determined at the following times to assess the time course of tolerance development: 4 hr, 8 hr, 1 day, 2 days, 4 days, 6 days and 8 days. After the last tail-flick measurement (day 8), the animals were euthanized to collect blood and brain tissue.

Investigation of the effect of coinfusion of fenfluramine on morphine tolerance followed. Starting on day 1, after duplicate baseline measurements, rats were assigned to either 8 days of combined infusion of 22 mg/kg/day morphine and 20 mg/kg/day fenfluramine (n = 5), or 8 days of 20 mg/kg/day infusion of fenfluramine alone (n = 5). The algesiometric testing schedule was the same as that described in the previous morphine infusion rate ranging study. In a supplemental experiment to assess the ability of fenfluramine to reverse tolerance, a group of rats (n = 6) was pretreated with a 22-mg/kg/day infusion of morphine for 4 days to produce full tolerance to morphine analgesia. This was followed by 4 days of morphine infusion in combination with 20 mg/kg/day fenfluramine. Tail-flick latency was determined during morphine pretreatment (at 4 hr, 8 hr, 1 day, 2 days, 4 days) and during coinfusion of morphine and fenfluramine (at 4 days plus 4 hr, 4 days plus 8 hr, 5 days, 6 days plus 4 hr, 8 days plus 4 hr). At the end of the fenfluramine coinfusion experiments, blood and brain tissue were collected to measure the concentrations of morphine and its 3-glucuronide. An additional group of rats (n = 6) received 4 days of morphine infusion, followed by another day of coinfusion of morphine and fenfluramine before they were euthanized for blood and brain tissue to provide another time-point of drug concentration data.

Morphine pharmacokinetics during chronic infusion. A parallel study was performed to investigate the effect of fenfluramine on the pharmacokinetics of morphine and its 3-glucuronide. There were three cohorts of rats for the different drug treatments. The first cohort received a constant i.v. infusion of saline for 4 days (n = 6), after which they were euthanized for the collection of blood and brain tissue. Blood and brain samples from this group served as a check for potential chromatographic interference in the assay of morphine and its 3-glucuronide. The second cohort consisted of groups of rats that received morphine infusion (22 mg/kg/day) for one
of the following durations: 4 hr, 8 hr, 1 day, 2 days, 4 days, 6 days or 8 days (n = 4–6 per time point). At the termination of infusion, blood and brain tissue were collected. In the third cohort, groups of rats received coinfusion of 22 mg/kg/day morphine and 20 mg/kg/day fenfluramine for a duration of 4 hr, 8 hr, 1 day, 2 days, 4 days or 8 days (n = 5–6 per time point).

Morphine Dose-Response

Dose-response studies were performed to further investigate whether the apparent attenuation in morphine tolerance upon co-treatment with fenfluramine reflected acute potentiation of analgesia and/or an actual attenuation of the decrease in analgesic potency over time. Morphine dose response was determined in two groups of rats, one after acute treatment and the other after chronic drug infusions.

In the acute treatment group, each rat underwent sham cannulation of the jugular vein. Baseline tail-flick measurements were performed on the morning of the third day after surgery. Immediately after baseline testing, rats received an acute i.p. injection of morphine alone or morphine in combination with 2.4 mg/kg of fenfluramine. Literature reports of fenfluramine kinetics in rats (Caccia et al., 1982; Rowland and Carlton, 1986) suggested that an i.p. fenfluramine dose of 2.4 mg/kg would provide a mean plasma fenfluramine concentration at the time of morphine dose-response testing that would approximate the steady-state plasma level reached during i.v. infusion of 20 mg/kg/day fenfluramine. Each animal was assigned to one of the following morphine doses of 0, 0.5, 1, 2, 3 or 5 mg/kg (n = 4–6 per dose). Tail-flick latency was determined 30 min after drug injection.

The second group of rats was infused, after baseline tail-flick measurements, for 4 days with: 1) morphine (22 mg/kg/day), 2) morphine plus fenfluramine (22 and 20 mg/kg/day), 3) fenfluramine (20 mg/kg/day) or 4) saline. Antinociceptive response was measured by determining the tail-flick latency at 4 hr, 8 hr, 1 day, 2 days and 4 days. After 4 days, the rats were switched to saline infusion for 10 hr to allow washout of morphine and fenfluramine. Complete washout of fenfluramine was required to determine the degree of tolerance without the confounding potentiation of morphine analgesia. After washout, a single, i.p. dose of morphine was given in the following ranges: 1) 5, 6, 7 or 8 mg/kg in the morphine group (n = 4–7 per dose), 2) 3, 4 or 5 mg/kg in the morphine plus fenfluramine group (n = 4–7 per dose), 3) 2, 2.5, 3 mg/kg in the fenfluramine group (n = 4 per dose) and 4) 1, 2, 2.5 or 3 mg/kg in the saline group (n = 4–5 per dose). Tail-flick measurements were taken at 30 min after injection. Each infused animal was assigned to a single i.p. morphine dose.

Drug Analysis

HPLC assays. Concentration of morphine and its metabolite, M3G, in plasma were determined by high-performance liquid chromatography, using electrochemical detection for morphine and fluorescence detection for M3G. The procedure is a modification of an assay described by Milne et al. (1991). To 200 μl of plasma sample or standard, 14 ng of the internal standard normorphine were added. Plasma protein was precipitated with 4 μl of 60% perchloric, and the samples were centrifuged. The supernatant was transferred to another tube containing 2 ml of 0.5 M ammonium sulfate adjusted to pH 9.2 with sodium hydroxide. The mixture was loaded onto a Sep-Pak C18 cartridge (Waters Associates, Milford, MA). The cartridge was then washed consecutively with 5 ml of methanol, 3 ml of mobile phase, 5 ml of water and 20 ml of 5 mM ammonium sulphate (pH 9.2), under gravity flow. Excess fluids were removed from the cartridges under vacuum. Morphine, M3G and normorphine were eluted with 1 ml of mobile phase, of which 100 μl was injected onto the high-performance liquid chromatography column. The high-performance liquid chromatography consisted of a pump system and an autosampler (Hewlett Packard model 1050), a guard column (HP 5 μm ODS 2, 40 × 4 mm), an analytical column (HP 5 μm Sperisorb ODS 2, 250 × 4 mm) and an integrator (HP instrument 35900). The electrochemical detector (Environmental Science Associates, E.S.A., Coulochem model 5200A, conditioning cell model 5021) was set to +0.25 V for detector 1, +0.40 V for detector 2 and 0.80 V for the guard cell. The fluorescence detector (HP 1046A) was set to an excitation wavelength of 227 nm and an emission wavelength of 348 nm as suggested earlier (Joel et al., 1988). The mobile phase consisted of 72.5% acetonitrile and 10 mM dodecyl sodium sulfate at 0.02 M sodium dihydrogen phosphate, adjusted to pH 2.28 by phosphoric acid. The mobile phase was pumped through the column at a flow rate of 1 ml/min. The limit of quantification was 2 ng/ml for morphine and 25 ng/ml for M3G. The inter-day coefficients of variations were 8% for morphine at a level of 10 ng/ml and 7% for M3G at a level of 50 ng/ml.

Morphine concentration in brain tissue was determined by preparing a homogenate of 0.4 to 0.5 g of whole brain (without the cerebellum) in 3 ml of methanol, followed by liquid-liquid extraction using a modification of the method described previously (Borg et al., 1993). Nalorphine was used as the internal standard. A total of 50 μl of the extract was injected onto an analytical cation-exchange column. The mobile phase consisted of 5% acetonitrile, 8% methanol and 0.5 mM EDTA in 0.073 M potassium dihydrogen phosphate, adjusted to pH 3.48 by formic acid. The mobile phase flow rate was 0.8 ml/min. The average recovery of morphine and nalorphine from brain homogenate was 60%. The limit of detection was 10 ng/ml, and the inter-day coefficient of variation was <10%.

Statistical analysis. Differences in mean latency times between pairs of treatments were evaluated at individual time-points using the two-sample Student’s t test. Differences in the slopes of the overall time course of antinociceptive response were evaluated by simple factorial analysis of variance. The dose-response curves were fitted to the following sigmoidal Emax model using the nonlinear regression program PCNONLIN (version 4.2, SCI software, Lexington, KY).

\[ E = \frac{E_{\text{max}} \cdot D^N}{ED_{50}^N + D^N} \]

where E is the %MPE, D is the morphine dose, ED50 is the dose at half-maximal effect, Emax is the maximal effect and was set to 100%, N is a power constant expressing the steepness of the dose-effect relationship. The nonlinear regression program provided 95% confidence intervals (CI) for the parameters, which were used for comparison of the mean parameter estimates between treatments.

Results

The time course of antinociceptive response over an 8-day period was determined at different morphine infusion doses to identify an optimum infusion rate for the study of the interaction between fenfluramine and morphine (fig. 1). Saline-infused rats in the control groups did not exhibit any antinociceptive behavior during the experiment. In all three morphine infusion groups, near maximal antinociceptive response (80–100% MPE) was achieved within a few hours of morphine infusion. The antinociceptive response declined rapidly beyond 24 hr, and eventually returned to baseline despite continual infusion of morphine. Tail-flick latency returned to baseline values (i.e., latency of the saline treated group) in less than 4 days for the 22 mg/kg/day infusion rate, and after 6 days for the 44 and 88 mg/kg/day infusion rates. The slopes of the decrease in %MPE over time were not significantly different (P > .05) for the three infusion rates, indicating that the rate of tolerance development was independent of dose (simple factorial analysis of variance). To
avoid a ceiling effect, which could conceal an effect of fenfluramine on morphine analgesia and tolerance development, the 22 mg/kg/day morphine infusion rate which gave submaximal peak responses was used in the subsequent morphine-fenfluramine interaction experiments.

Confusion of fenfluramine at a rate of 20 mg/kg/day significantly attenuated the development of tolerance to the analgesic effect of morphine during the 8 days of morphine infusion at 22 mg/kg/day (fig. 2). At day 4, the rats infused with morphine alone did not show any measurable antinociception. In contrast, the rats that received combined infusions of morphine and fenfluramine exhibited an antinociceptive response of more than 70% MPE. After 8 days, the recorded antinociceptive response was still around 30% in rats that received combined infusions of morphine and fenfluramine. Rats infused with morphine alone frequently exhibited hyperalgesic behavior after 8 days of morphine infusion. Infusion of fenfluramine alone did not result in any detectable antinociception at the chosen infusion rate. Figure 2 also shows that the tolerance induced in rats after 4 days of morphine infusion was not immediately reversed by subsequent fenfluramine coinfusion. Nonetheless, by day 8 (4 days of morphine treatment followed by 4 days of morphine-fenfluramine coinfusion) the animals showed less inclination toward hyperalgesic behavior compared to animals infused with morphine alone (tail-flick latency significantly different at \( P = .056 \)).

We evaluated whether fenfluramine had induced an increase in plasma and/or brain morphine concentration (i.e., a pharmacokinetic interaction) that could have increased analgesia, and partly offset the expression of tolerance. Figure 3A shows that mean plasma morphine concentrations in rats infused with morphine alone or morphine plus fenfluramine, tended to increase over the 8 days with large inter-individual variation in morphine concentrations. However, the mean plasma morphine concentrations over time were not statistically significantly different between the two treatment groups (\( P > .05 \), analysis of variance). An increasing trend in brain morphine concentration over time was also observed (fig. 3B). The mean brain concentrations of morphine in rats infused with morphine plus fenfluramine were consistently higher than in rats infused with morphine alone on days 1, 2 and 8 (\( P < .05 \)). The less than 2-fold increase in brain morphine concentration in fenfluramine cotreated rats may explain part, but not the full extent, of the observed attenuation of tolerance (see “Discussion”). The mean brain/plasma concentration ratios of morphine, which varied from 0.5 to 0.8, did not differ significantly within group over time or between groups. There were no evident changes in the equilibration of morphine across the blood-brain barrier.

High doses of M3G, the major metabolite of morphine in circulation, can elicit hyperalgesia. M3G has been postulated to contribute to the development of morphine tolerance by antagonizing the antinociceptive effect of morphine (Ekblom et al., 1993; Smith and Smith, 1995; Smith et al., 1990). Therefore, we measured the M3G concentration in plasma to determine whether a decrease in mean M3G concentrations occurred in the fenfluramine coinfused rats, which could explain the attenuation in tolerance. Figure 4 shows that, in fact, the reverse was true with the M3G plasma concentrations. Mean plasma M3G concentration was significantly greater over time in rats receiving morphine and fenfluramine compared to the rats receiving morphine alone.

Potentiation of morphine’s potency by fenfluramine could have contributed to the observed attenuation of tolerance. If so, fenfluramine should acutely potentiate morphine-induced antinociception in our rat model. The dose-response data in figure 5 show that fenfluramine, which did not exhibit antinociceptive activity on its own, potentiated morphine by a modest degree; \( \text{ED}_{50} \) of morphine (mean ± S.E.) was lowered from 2.00 ± 0.10 mg/kg (95% CI, 1.71–2.29) to 1.18 ± 0.10 mg/kg (95% CI, 0.99–1.37).

We further investigated whether this immediate potentiation of morphine’s analgesic effect by fenfluramine was the only pharmacodynamic effect responsible for the attenuation in tolerance development or whether fenfluramine interfered with the intrinsic pharmacological mechanism underlying tolerance, or both. The extent of tolerance to morphine an-
The pain sensitivity with and without fenfluramine coinfusion was assessed by i.p. morphine dose-response curves (fig. 6) at 10 hr after discontinuation of infusion treatments with morphine alone, with fenfluramine alone, with the combination of morphine and fenfluramine, and with saline. The 10-hr interval was considered sufficient for the washout of the infused morphine and fenfluramine and their pharmacologically active metabolites. The dose-response curve for animals that had been subjected to a 4-day saline infusion (fig. 6) did not differ from the acute morphine dose-response curve for naïve animals; ED$_{50}$ of 2.4 ± 0.1 mg/kg vs. 2.0 ± 0.1 (compare figs. 5 and 6). This indicates that the experimental manipulations involved in drug infusion did not change the sensitivity of the rats to morphine. Fenfluramine infusion alone did not appear to alter morphine dose-response; the ED$_{50}$ in the fenfluramine-infusion group was nearly the same as in saline-infused animals (2.7 ± 0.1 mg/kg vs. 2.4 ± 0.1 mg/kg, see table 1). This suggested that residual fenfluramine, if any, did not potentiate the analgesic response of the acute morphine dose. As expected, prior infusion of morphine for 4 days caused a remarkable rightward shift of the dose-response curve relative to the curve for the saline infusion group; ED$_{50}$ increased nearly 3-fold from 2.4 ± 0.1 to 7.0 ± 0.1 mg/kg (table 1). In comparison, coinfusion of fenfluramine and morphine caused a significantly smaller rightward shift of the morphine dose-response curve. Morphine ED$_{50}$ increased to 3.7 ± 0.3 mg/kg which represents a modest 54% increase over the ED$_{50}$ of the saline-infusion group and a value 53% that of the morphine-infusion group. These data indicate that fenfluramine during the 4 days of treatment definitely interfered with the pharmacological process responsible for tolerance development to morphine.
However, unlike cholecystokinin antagonists and midazolam, potentiation of morphine analgesia cannot completely explain the apparent attenuation of morphine tolerance by fenfluramine. This conclusion is based on comparison of the morphine dose-response curves (fig. 6) that were generated in animals at 10 hr after 4 days of various drug infusion treatments. Based on pharmacokinetic data from our laboratory (unpublished data) and literature source, we estimated 10 hr to be a sufficient duration for washout of morphine, fenfluramine and their metabolites from circulation (Bhar-gava et al., 1992; Ekblom et al., 1993; Rowland and Carlton, 1986). If the observed action of fenfluramine was entirely the result of potentiation of morphine analgesia, the morphine dose-response curve generated after washout of morphine-fenfluramine coinfusion should coincide with the curve generated in animals infused with morphine alone. However, fenfluramine coinfusion lessened the development of tolerance as was evident from the leftward shift of the morphine dose-response curve in the morphine-fenfluramine coinfused rats compared to the animals infused with morphine alone (fig. 6).

It is still possible that sufficient amounts of fenfluramine or its active metabolite, norfenfluramine, remained in circulation that would have potentiated morphine analgesia 10 hr after the discontinuation of the drug infusions. This would have complicated the interpretation of the morphine dose-response curves. However, this is considered unlikely because the morphine dose-response curve of animals infused for 4 days with fenfluramine alone was not significantly different from the dose-response curve of animals infused with saline. This indicates that the attenuation of morphine tolerance seen in the morphine plus fenfluramine-infused animals was not a result of acute potentiation of morphine analgesia by residual amounts of fenfluramine or norfenfluramine.

We also demonstrated that in animals made fully tolerant by 4 days of infusion with morphine alone, subsequent coinfusion with fenfluramine did not immediately restore the sensitivity to morphine. It is expected that administering agents that offset the expression of tolerance to morphine by potentiation of morphine analgesia would immediately improve the morphine sensitivity in animals that are tolerant, as has been observed for the CCK antagonist proglumide (Watkins et al., 1984). Because we did not observe restoration of morphine sensitivity after 4 days of morphine infusion, it is possible that the acute potentiating effect of fenfluramine on morphine analgesia disappeared during chronic treatment with fenfluramine, as has been observed with LY274614 (Tiseo and Inturrisi, 1993). The time-course of disappearance of this potentiation would agree with the supplementation of the drug infusions with saline. This indicates that the attenuation of morphine tolerance seen in the morphine plus fenfluramine-infused animals was not a result of acute potentiation of morphine analgesia by residual amounts of fenfluramine or norfenfluramine.

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M3G does not appear to play a role in attenuation of morphine tolerance. During the first 4 days of morphine infusion, plasma M3G levels in the fenfluramine coinfused rats did not differ significantly from plasma M3G levels in

### Discussion

We demonstrated for the first time that racemic fenfluramine, an indirect serotonin agonist, can inhibit the development of tolerance to morphine analgesia.

The robust and rapid tolerance to morphine antinociception was attenuated by fenfluramine to a degree comparable to the attenuation of morphine tolerance seen with nitric oxide synthase inhibitors and NMDA-antagonists (Dambisya and Lee, 1996; Marek et al., 1991; Tiseo and Inturrisi, 1993; Trujillo and Akil, 1991, 1994). We showed that inhibition of morphine tolerance by fenfluramine occurs largely due to an interference with the pharmacological mechanism underlying morphine tolerance. It cannot solely be explained by an immediate and readily reversible potentiation of the analgesic effect of morphine or an alteration in the pharmacokinetics of morphine.

Previously, it has been shown that cholecystokinin antagonists (Dourish et al., 1990) and the benzodiazepine agonist midazolam (Tejwani et al., 1993) were able to alleviate morphine tolerance by acute potentiation of morphine analgesia. Therefore, we investigated whether this phenomenon should be considered as a contributing factor in attenuating tolerance development to morphine. We did find that fenfluramine potentiated morphine analgesia acutely (fig. 5). How-

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**TABLE 1**

Intraperitoneal morphine dose-response parameters in the chronic fenfluramine cotreatment groups

<table>
<thead>
<tr>
<th>Cotreatment</th>
<th>Mean ED₅₀ (mg/kg)</th>
<th>95% CI (mg/kg)</th>
<th>Ratio of Mean ED₅₀ to Naïve Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic saline</td>
<td>2.41</td>
<td>2.15–2.66</td>
<td>1.21</td>
</tr>
<tr>
<td>Chronic i.v.</td>
<td>7.03</td>
<td>6.75–7.31</td>
<td>3.52</td>
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<tr>
<td>morphine infusion alone</td>
<td>2.70</td>
<td>2.45–2.95</td>
<td>1.35</td>
</tr>
<tr>
<td>Chronic i.v.</td>
<td>3.74</td>
<td>3.19–4.28</td>
<td>1.87</td>
</tr>
<tr>
<td>fenfluramine infusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>infusion alone</td>
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<td></td>
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<tr>
<td>Chronic i.v.</td>
<td></td>
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<tr>
<td>infusion of morphine</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>+fenfluramine</td>
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</tbody>
</table>

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**Fig. 6.** Comparison of morphine dose-response curves at 10 hr after discontinuation of a 4-day infusion of saline, fenfluramine (20 mg/kg/day), morphine (22 mg/kg/day) and morphine plus fenfluramine. Each data point represents mean ± S.E. for n = 4–6. The dose-response curves were fitted to a sigmoid E₅₀ model. The continuous lines through the symbols represent model prediction.
the morphine-alone infused group (fig. 4). However, the extent of tolerance (ED$_{50}$) developed at this time point (4 days of morphine infusion) in these two groups was already quite different. Our results confirm the recent findings of Ouellet and Pollack (1997) that exposure to levels of synthetic M3G comparable to the derived M3G levels in our study did not alter morphine-induced response in rats. They conclude that systemic formation of M3G is unlikely to contribute significantly to the development of tolerance to morphine antinociception. The active metabolite of morphine, morphine-6-glucuronide, formed in humans was never an issue in this study because it is not formed in rats.

The inhibition of morphine tolerance was unlikely the result of the marginal increase in brain morphine levels that we observed in the fenfluramine coinfused animals. First, the morphine brain concentrations in these animals were comparable to the morphine concentrations in animals that received the infusion dose of 44 mg/kg/day (data not reported). In animals infused with 44 mg/kg/day of morphine, the anergic effect of morphine was completely dissipated by day 4, whereas the morphine plus fenfluramine-infused animals with comparable brain morphine concentration still exhibited 75% of the MPE. Second, there is evidence that exposure to a higher concentration of an opioid leads to a higher degree of tolerance (Stevens and Yaksh, 1989). We demonstrated that tolerance to morphine analgesia that developed after 4 days of chronic morphine administration persisted beyond 10 hr of washout. Thus, if increased levels of morphine were responsible for an apparent decrease in tolerance expression, the potency of morphine tested 10 hr postinfusion should have been lower in the morphine plus fenfluramine-treated animals compared to the animals infused with morphine alone. Instead, we showed that the potency of morphine was higher in the morphine plus fenfluramine-treated animals.

The mechanism responsible for fenfluramine's interference with the tolerance process to morphine is not known. An acute dose of fenfluramine causes a decrease of 5-HT level in the brain and a delayed decrease in the level of 5-HIAA (5-HT's primary metabolite) after the initial release of 5-HT (Mart' in and Artigas, 1992; Schwartz et al., 1989; Viana et al., 1996). This leads to an increased 5-HIAA/5-HT ratio in the brain for an extended time period (Fuller et al., 1988). A 4-day regimen of fenfluramine in a dose range comparable to ours leads to a depletion of serotonin and its metabolite dose-dependently and a continual elevation of the 5-HIAA/5-HT ratio in the brain (Fracasso et al., 1995; Zacek et al., 1990). Morphine also elevates the 5-HIAA/5-HT ratio in the brain when it is given acutely to rats. However, during chronic morphine administration, the 5-HIAA/5-HT ratio returns to normal baseline values over time as tolerance becomes evident (Desole et al., 1996; Haubrich and Blake, 1973). It is possible that fenfluramine counteracts this alteration in the serotonin balance (i.e., maintaining an elevated 5-HIAA/5-HT ratio) in morphine-tolerant rats. This may explain why fluoxetine, which dose-dependently decreases the 5-HIAA/5-HT ratio in the brain over time (Hynes and Fuller, 1982), further decreases the antinociceptive potency of morphine in morphine-tolerant rats when given acutely or chronically (Larson and Takemori, 1977). In morphine-naive rats, however, fluoxetine, like fenfluramine, acutely potentiates the antinociceptive effect of morphine which is an effect related to increased availability of synaptic serotonin due to fluoxetine's inhibition of serotonin reuptake from the synaptic cleft (Hynes and Fuller, 1982).

There is evidence that NMDA-antagonists can inhibit tolerance development to ethanol through serotonergic modulation (Khanna et al., 1994). This may suggest a possible link between NMDA-antagonists and fenfluramine in their ability to inhibit development of morphine tolerance through their shared action on the central serotonergic system, especially because fenfluramine has been shown to have an influence on ethanol intake (Yu et al., 1997).

In addition to the serotonergic system, there is the possibility that fenfluramine attenuates morphine tolerance through any of the other systems that have been shown to also affect morphine tolerance; for example, inhibition of glutamatergic transmission, GABAergic transmission or nitric oxide synthase activity.

The pharmacological mechanism of fenfluramine's inhibition of morphine tolerance requires further investigation, because it will contribute to our current understanding of opioid tolerance. Further studies on the involvement of the serotonergic system in opioid tolerance may lead to the identification of new drug targets that enable development of clinically applicable strategies to minimize tolerance to opioid analgesia.

References