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Differential tolerance to antinociceptive effects of μ opioids during repeated treatment with etonitazene, morphine, or buprenorphine in rats

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Abstract *Rationale:* Repeated treatment experiments with high and low efficacy agonists provide critical insight into possible mechanisms underlying development of opioid tolerance. *Objective:* Experiments in a tail-withdrawal assay tested the hypothesis that magnitude of tolerance to antinociceptive effects is inversely related to agonist relative efficacy in rats intermittently treated with etonitazene, morphine, or buprenorphine. *Methods:* The antinociceptive effects of five μ opioid agonists were tested in male, Sprague-Dawley rats in a warm-water tail-withdrawal assay. To induce tolerance, escalating doses of the higher efficacy agonist etonitazene, the high efficacy agonist morphine, or the lower efficacy agonist buprenorphine were administered twice daily for 2–8 weeks. *Results:* Etonitazene, etorphine, morphine, buprenorphine, and GPA 1657 [(1)- β -2'-hydroxy-2,9-dimethyl-5-phenyl-6,7-benzomorphan] produced dose-dependent increases in tail-withdrawal latency until 100% maximum possible effect (%MPE) was obtained. Treatment with escalating doses of etonitazene, morphine, or buprenorphine produced greater tolerance to the lower efficacy agonists buprenorphine and GPA 1657 than to the higher efficacy agonists etonitazene, etorphine, and morphine. Treatment with buprenorphine, a lower efficacy agonist, produced greater tolerance than did treatment with equivalent doses of the higher efficacy agonists morphine or etonitazene. *Conclusions:* Taken together, these data suggest that magnitude of antinociceptive tolerance is inversely related to relative efficacy of μ agonists, with lower efficacy agonists being more susceptible to tolerance than are higher efficacy agonists under these intermittent dosing conditions.

Keywords Efficacy · Tolerance · Etonitazene · Morphine · Buprenorphine · Etorphine · Rat

Introduction

The phenomenon of tolerance is defined as the attenuation of the effects of an agonist after repeated administration of the same or a related agonist, yielding a rightward shift and/or flattening of the agonist dose-response curve (Fernandes et al. 1977; Jaffe and Martin 1985). Opioid tolerance appears to be receptor-selective, in that subjects tolerant to morphine are cross-tolerant to opioid agonists that act through μ opioid receptors but not to agonists that act through non- μ receptors (Craft and Dykstra 1990). Opioid tolerance does not develop equally to all agonists that act through μ opioid receptors, however. Within a given assay and treatment condition, opioid agonists that produce effects through common μ opioid receptor mechanisms may show asymmetrical cross-tolerance. Such asymmetrical cross-tolerance may result from differences in agonist relative efficacy, i.e., a drug's ability to produce a biological response (Bläsigg et al. 1979; Paronis and Holtzman 1992).

At the opioid receptor level, agonists have different binding domains and may yield different receptor conformations after binding to the receptor (Law and Loh 1999). Additionally, μ opioid agonists possess varying abilities to activate G proteins in studies of receptor-stimulated guanosine-5'-O-(3-[³⁵S]-thio)triphosphate ([³⁵S]-GTP γ S) binding, and this ability appears to be related to agonist relative efficacy (Traynor and Nahorski 1995; Emmerson et al. 1996; Selley et al. 1997). In vitro tolerance experiments indicate that repeated treatment of SH-SY5Y neuroblastoma cells with morphine causes the low efficacy agonist pentazocine to lose its ability to stimulate [³⁵S]-GTP γ S binding but does not alter the ability of the higher efficacy agonists DAMGO and morphine to stimulate binding (Elliott et al. 1997). These in vitro studies indicate a role for relative efficacy in asymmetrical patterns of tolerance and cross-tolerance.

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Within *in vivo* behavioral assays as well, higher efficacy μ agonists appear more resistant to tolerance than do lower efficacy agonists. For example, repeated morphine treatment in monkeys, pigeons, and rats produces less cross-tolerance to the higher efficacy agonists etorphine (Tiano et al. 1998), methadone (Craft and Dykstra 1990; Young et al. 1991), or sufentanil (Sosnowski and Yaksh 1990) than to morphine. Additionally, repeated treatment with higher efficacy agonists can produce less tolerance than does treatment with equivalent doses of lower efficacy agonists (Stevens and Yaksh 1989). For example, continuous infusion of the higher efficacy agonist fentanyl produces less tolerance than does infusion of equiactive doses of the lower efficacy agonists morphine or meperidine (Paronis and Holtzman 1992). Thus, *in vivo* studies also indicate a role for relative efficacy in asymmetrical tolerance and cross-tolerance.

In the present experiments, the antinociceptive effects of five agonists that vary widely in relative efficacy [etonitazene=etorphine >morphine >GPA 1657=buprenorphine] (Walker et al. 1998) were examined after treatment with escalating doses of etonitazene, morphine, or buprenorphine. Previous experiments using the rat tail-withdrawal procedure demonstrated that naltrexone is equipotent as an antagonist of these five agonists, suggesting that all five produce antinociceptive effects through common, presumably μ opioid, receptors (Walker et al. 1994, 1998). Patterns of tolerance and cross-tolerance were compared after treatment with etonitazene, a selective higher efficacy μ agonist (Moolten et al. 1993; Emmerson et al. 1994; Walker et al. 1998), morphine, a high efficacy agonist (Walker et al. 1998), and buprenorphine, a low efficacy agonist (Walker et al. 1995, 1998). Etonitazene and buprenorphine were chosen as the higher and lower efficacy treatment agonists for a number of reasons. High efficacy agonists such as fentanyl, sufentanil, or etorphine are often used as treatment agonists but fentanyl and sufentanil have a shorter duration of action than etonitazene (Foley 1993; Walker et al. 1993) and etorphine is less selective than etonitazene (Richards and Sadee 1985; Su 1985; Emmerson et al. 1994). In previous tolerance studies, a single agonist is examined after treatment with buprenorphine (Cowan et al. 1977; Dykstra 1985; Berthold and Moerschbaeher 1988) or etonitazene (Tang 1982; Elmer et al. 1993; Meisch 1995). In the present experiments, multiple treatment doses of etonitazene, morphine, and buprenorphine are examined with five opioid agonists in the same assay under similar conditions and routes of administration.

Materials and methods

Subjects

Male Sprague-Dawley rats were housed individually in a humidity and temperature-controlled room with lights on from 0700–2000 hours. Rats were fed a daily ration of 20–25 g to maintain body weights of approximately 340–360 g and were given contin-

uous access to water. Cumulative-dose tests occurred approximately once a week for an individual rat. The rats used in this study were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee of Wayne State University and the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, National Academic Press 1996; NIH publication number 85–23, revised 1996).

Apparatus

Eight rodent restraint tubes were used. A Precision Model 181 water bath maintained the temperature of the water. The water from the water bath was mixed with tap water in a Thermos brand wide-mouth thermos (diameter=8 cm) to obtain the desired water temperature. Water temperature was measured by a Sensortek Model BAT-12 with Bailey/Sensortek Type T thermocouple, and tail-withdrawal latency was measured with a hand-operated digital stopwatch with time resolution of 1/100 s.

Procedure

All antinociceptive testing occurred in the morning within a 4-h period. Rats were placed into the restraining tubes with their tails hanging freely. The last 5–10 cm of the tail was immersed into a Thermos containing either 40 or 55°C water, and the latency for tail withdrawal was measured. A cut-off time of 15 s was imposed so that if the rat failed to remove its tail within 15 s, the experimenter removed the stimulus.

To control for a rat that removed its tail from the water independent of water temperature, the first three stimulus presentations during a test were 40°C. If the tail was not removed within 15 s on two out of three presentations of 40°C, the rat remained in the experiment. A 2-min interval occurred between each stimulus presentation. Next, one control latency value for the 55°C water temperature was obtained, followed by the first injection of test compound *s.c.* in the dorsal flank. After a 15-min pretreatment period, latency to withdraw the tail from both 40 and 55°C water was re-determined. On this and all succeeding trials, each temperature was presented once, with 2 min between presentations, and the order of presentation of 40 and 55°C water was varied randomly. At the conclusion of the 10-min testing period, another injection of test compound was administered so that the total dose was increased by 0.5 log unit. After another 15-min pretreatment period, latency values for 40 and 55°C were taken again during a 10-min testing period. As with similar studies using tail withdrawal and cumulative dosing (Adams and Holtzman 1990; Paronis and Holtzman 1992), dosing was stopped when a subject reached maximum effect (15 s) at 55°C water, the solubility limits of a compound were reached, or another behavior interfered with the measurements (i.e., convulsions).

Tolerance and cross-tolerance studies

All etonitazene, morphine, and buprenorphine tolerance and cross-tolerance studies followed the same basic treatment regimen (Table 1), with the exceptions noted below. Generally, three control dose-response curves were determined in each of five groups designated for etonitazene, etorphine, morphine, buprenorphine, and GPA 1657 tests. A treatment dose was selected and administered twice daily at 12-h intervals (± 2 h) for 7 days. On day 8, 12 h after the last injection, agonists were tested in their respective groups. Beginning 12 h after this test, the treatment dose was increased two-, four-, or eightfold for the next 7 days. Agonists were tested again on day 15, 12 h after the last injection. Twelve hours after this test, the treatment dose was further increased for 7 days. This procedure was repeated for 2–6 weeks, depending on experimental outcomes. Twelve hours after the last test, saline was administered twice daily at 12-h intervals for 7 or 14 days. Agonists were tested weekly to assess recovery of sensitivity.

Table 1 Schedule of etonitazene, morphine, and buprenorphine treatment regimens and tests. Daily treatment doses were divided in half and administered as two *s.c.* injections at 12-h intervals

	Tests once/week	Treatment week					
		Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Test agonist		Daily dose of etonitazene (mg/kg per day)					
Etonitazene <i>n</i> =8	Control tests	0.002	0.008	0.032	0.128	Saline	Saline
Etorphine <i>n</i> =8	Control tests	0.002	0.008	0.032	0.128	Saline	Saline
Morphine <i>n</i> =8	Control tests	0.001	0.008	0.032	0.128	Saline	Saline
Buprenorphine <i>n</i> =8	Control tests	0.001	0.002	0.032	Saline	Saline	
GPA 1657 <i>n</i> =8	Control tests	0.001	0.002	0.032	Saline	Saline	
Test agonist		Daily dose of morphine (mg/kg per day)					
Etonitazene <i>n</i> =6	Control tests	40	80	160			
Etorphine <i>n</i> =5	Control tests	20	20	40	Saline		
Morphine <i>n</i> =5	Control tests	20	20	40	Saline		
Buprenorphine <i>n</i> =5	Control tests	20	20	40	Saline		
GPA 1657 <i>n</i> =4	Control tests	20	20	40			
Test agonist		Daily dose of buprenorphine (mg/kg per day)					
Etonitazene <i>n</i> =6	Control tests	0.2	0.4	0.8	Saline	Saline	
Etorphine <i>n</i> =5	Control tests	0.2	0.4	0.8	Saline	Saline	
Morphine <i>n</i> =6	Control tests	0.2	0.4	0.8	Saline	Saline	
Buprenorphine <i>n</i> =8	Control tests	0.2	Saline				
GPA 1657 <i>n</i> =5	Control tests	0.2	Saline	Saline			
Buprenorphine <i>n</i> =4	Control tests	0.05	0.1	0.2	Saline	Saline	
GPA 1657 <i>n</i> =5	Control tests	0.05	0.1	0.2			

Etonitazene tolerance and cross-tolerance studies

Forty rats were assigned to five groups as shown in Table 1.

Morphine tolerance and cross-tolerance studies

Twenty-five rats were assigned to five groups as shown in Table 1. All rats were injected for 7 days with 20 mg/kg morphine and tested on day 8. The treatment dose of morphine was then doubled for rats tested with etonitazene. In groups tested with the other agonists, 20 mg/kg per day morphine was administered for an additional 7 days in order to determine if further tolerance would develop with a longer treatment period. Agonists were retested on day 15. The morphine treatment dose was then doubled to 40 mg/kg per day for the next 7 days. Severe withdrawal signs from 160 mg/kg per day morphine precluded recovery tests in the etonitazene group.

Buprenorphine tolerance and cross-tolerance studies

Initially, 30 rats were assigned to the first five groups shown in Table 1. After treatment with 0.2 mg/kg per day buprenorphine for 7 days, no dose of GPA 1657 or buprenorphine produced antinociceptive effects. Therefore, during the next 7 or 14 days, respectively, rats in these groups received saline injections and were tested weekly. To further characterize the patterns of tolerance and cross-tolerance to buprenorphine and to test for recovery of original antinociceptive sensitivity, two new groups of rats were designated for buprenorphine or GPA 1657 tests during treatment with lower doses of buprenorphine. Because 100 mg/kg GPA 1657 was lethal in the GPA 1657 group during the final treatment test, only the buprenorphine group was studied for recovery of initial agonist sensitivity after saline administration for 7 days. To determine if the final cumulative dose of 10 mg/kg buprenorphine was producing long-lasting antagonism, saline treatment was continued for an additional 21 days. On days 36 and 43, morphine was tested.

Data analysis

Latencies for tail withdrawal after administration of drug were converted into percent maximum possible effect (%MPE) by the formula:

$$\%MPE = \frac{\text{test latency} - \text{control latency}}{(15 \text{ sec} - \text{control latency})} \times 100 \quad (1)$$

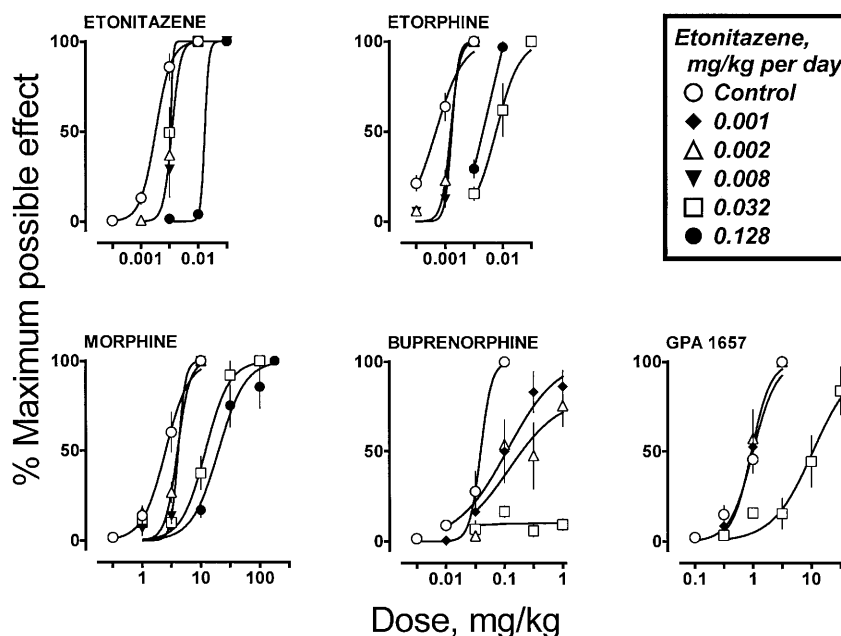
using the control latency measured at the beginning of each cumulative dose test. Each rat served as its own control. A value of zero was assigned if the rat withdrew its tail faster than the control latency. For each agonist studied, the last two dose-response curves before the beginning of treatment were used to construct the control dose-response curves used in analyses.

All dose-response curves for individual rats were fitted using the following semilogarithmic form of the logistic dose-response equation:

$$E = \frac{(E_{\max} - E_{\min}) \cdot 10^{(\log[X] \cdot h)}}{10^{(\log(ED_{50}) \cdot h)} + 10^{(\log[X] \cdot h)}} + E_{\min} \quad (2)$$

where E is the %MPE, and E_{min} and E_{max} are the minimum and maximum of the sigmoid dose-response curve. E_{min} and E_{max} were generally set at 0 and 100, respectively, unless a compound failed to reach 100% MPE. In these cases, E_{max} was not constrained to 100%. X is the dose of agonist (in mg/kg) at a particular effect level, ED₅₀ is the agonist dose causing a 50% maximal effect, and h is a slope factor. For each test, individual ED₅₀ were averaged for each group and a 95% C.L. calculated with the *t* statistic. To determine the significance of ED₅₀ differences *within* treatment regimens, individual ED₅₀ were analyzed by a one-way repeated measures analysis of variance and Tukey's multiple comparison test for post hoc analyses. To compare the magnitude of tolerance *across* agonists and treatment regimens, a potency ratio was calculated for each rat by dividing the ED₅₀ after treatment by the ED₅₀ before treatment. If a drug did not produce antinociceptive effects during treatment, the ratio of the highest dose tested to the ED₅₀ before treatment was used as a conservative estimate of the potency ratio. Individual log potency ratios were analyzed by one- or two-way analysis of variance (ANOVA), as appropriate.

Fig. 1 Tolerance and cross-tolerance to the antinociceptive effects of etonitazene, etorphine, morphine, buprenorphine, and GPA 1657 in rats ($n=8$ per group) repeatedly treated with etonitazene. Ordinate Latency measures converted into the percent maximum effect, abscissa dose of agonist in mg/kg. Control points (○) are the average of two determinations. Vertical bars represent SEM



Tukey's multiple comparison test was used for post hoc analyses. Significance was set at $P<0.05$.

Drugs

The following compounds were used: buprenorphine hydrochloride, etorphine hydrochloride, etonitazene hydrochloride, morphine sulfate (National Institute on Drug Abuse, Rockville, Md., USA; RTI), and GPA 1657 (a gift from James H. Woods, University of Michigan, Ann Arbor, Mich., USA).

Morphine, etonitazene, and etorphine were dissolved in physiological saline. GPA 1657 and buprenorphine were dissolved in sterile water. All solutions were prepared to administer each injection in a volume of 0.1–2.0 ml per 100 g body weight. Doses are expressed as the forms listed above, and injections were administered s.c. along the dorsal flank. Saline was injected in a volume of 1 ml/kg body weight.

Results

Repeated etonitazene treatment

Etonitazene, etorphine, morphine, buprenorphine, and GPA 1657 each produced dose-dependent increases in tail-withdrawal latencies until the maximum possible effect was achieved in all rats tested (Figs. 1, 2, 3). Treatment with 0.002 or 0.008 mg/kg per day etonitazene increased the ED_{50} for etonitazene and etorphine, but not for morphine (Fig. 1; Table 2). Increasing the treatment dose of etonitazene fourfold, to 0.032 mg/kg per day, increased the magnitude of tolerance to etorphine and morphine, but not etonitazene. An additional fourfold increase in treatment dose, to 0.128 mg/kg per day, further increased the magnitude of tolerance to etonitazene and etorphine, but not morphine. Throughout repeated treatment with etonitazene, these three agonists maintained the capacity to produce a 100% maximum effect. The

higher treatment doses of etonitazene, 0.032 and 0.128 mg/kg per day, produced severe stove-pipe rigidity, shallow breathing, and self-directed chewing in all rats for the first 3–4 days of treatment.

Treatment with a low dose of etonitazene, 0.001 mg/kg per day, failed to alter the ED_{50} for buprenorphine or GPA 1657 (Fig. 1; Table 2). Doubling the treatment dose to 0.002 mg/kg per day increased the ED_{50} for buprenorphine by 11-fold but failed to alter the ED_{50} for GPA 1657. A 16-fold increase in treatment dose, to 0.032 mg/kg per day, increased the ED_{50} for both buprenorphine and GPA 1657 by at least 30-fold and abolished the ability of buprenorphine to produce a 100% maximum effect.

Rats did not fully recover initial sensitivity to etonitazene, etorphine, or morphine within 2 weeks after termination of treatment with 0.128 mg/kg per day etonitazene (Table 2). Rats did recover initial sensitivity to buprenorphine and GPA 1657 within 2 weeks after termination of treatment with the lower dose of 0.032 mg/kg etonitazene per day.

Because multiple agonists were tested before and after treatment with 0.002 and 0.032 mg/kg per day etonitazene, it was possible to compare the magnitude of tolerance as a function of test agonist and treatment dose. Analysis of the log potency ratios revealed both significant main effects of the treatment dose of etonitazene [$F(1,70)=25.05$, $P<0.0001$] and the agonist tested for antinociception [$F(4,70)=8.78$, $P<0.0001$] and a significant interaction of test agonist and treatment dose [$F(4,70)=3.17$, $P=0.0187$]. Thus, treatment with 0.032 mg/kg per day etonitazene produced greater tolerance than did treatment with 0.002 mg/kg per day, agonists differed in sensitivity to tolerance, and the impact of increasing the etonitazene treatment dose varied among agonists. One-way ANOVA and *post hoc* tests showed that treatment

Table 2 ED₅₀ (mg/kg) for antinociceptive effects of μ opioids before, during, and after treatment with etonitazene. Total daily doses of etonitazene are listed below

	ED ₅₀	95% C.L.	Ratio ^a	95% C.L.
Etonitazene				
Control	0.0017	(0.0012–0.0023)		
0.002 mg/kg	0.0030 ^b	(0.0020–0.0044)	1.8	(1.3–2.5)
0.008 mg/kg	0.0039 ^b	(0.0034–0.0044)	2.3	(1.7–3.2)
0.032 mg/kg	0.0032 ^b	(0.0028–0.0037)	1.9	(1.3–2.7)
0.128 mg/kg	0.015 ^{b,d-f}	(0.013–0.018)	9.1	(5.9–14)
Saline; week 5	0.0063 ^{b,d-g}	(0.0039–0.010)	3.8	(1.9–7.5)
Saline; week 6	0.0049 ^{b,d,g}	(0.0042–0.0057)	2.9	(2.1–4.0)
Etorphine				
Control	0.00065	(0.00048–0.00090)		
0.002 mg/kg	0.0012 ^b	(0.0011–0.0013)	1.8	(1.3–2.6)
0.008 mg/kg	0.0012 ^b	(0.0011–0.0013)	1.9	(1.3–2.6)
0.032 mg/kg	0.0065 ^{b,d,e}	(0.0036–0.012)	9.9	(4.8–20)
0.128 mg/kg	0.0031 ^{b,d-f}	(0.0027–0.0036)	4.8	(3.6–6.5)
Saline; week 5	0.0030 ^{b,d-f}	(0.0020–0.0043)	4.5	(3.3–6.3)
Saline; week 6	0.0013 ^{b,f-h}	(0.0011–0.0017)	2.0	(1.3–3.2)
Morphine				
Control	2.3	(1.5–3.5)		
0.002 mg/kg	3.6	(2.7–4.7)	1.5	(1.1–2.1)
0.008 mg/kg	4.2	(3.7–4.8)	1.8	(1.1–3.0)
0.032 mg/kg	11 ^{b,d,e}	(6.9–19)	4.9	(3.0–7.9)
0.128 mg/kg	21 ^{b,d,e}	(11–40)	8.9	(3.4–23)
Saline; week 5	11 ^{b,d,e}	(8.4–14)	4.6	(2.3–9.4)
Saline; week 6	4.6 ^{b,f-h}	(3.2–6.6)	2.0	(1.1–3.7)
Buprenorphine				
Control	0.035	(0.024–0.050)		
0.001 mg/kg	0.14	(0.037–0.51)	4.0	(1.1–14)
0.002 mg/kg	0.39 ^b	(0.062–2.5)	11	(1.4–93)
0.032 mg/kg	>1.0	–	>29	–
Saline; week 4	0.063	(0.036–0.11)	1.8	(0.95–3.4)
Saline; week 5	0.069	(0.038–0.12)	2.0	(0.88–4.4)
GPA 1657				
Control	0.90	(0.74–1.1)		
0.001 mg/kg	0.75	(0.44–1.3)	0.84	(0.54–1.3)
0.002 mg/kg	1.7	(0.89–3.3)	1.9	(0.96–3.8)
0.032 mg/kg	36 ^{b-d}	(4.5–292)	40	(5.0–329)
Saline; week 4	4.2 ^f	(1.9–9.4)	4.7	(1.7–13.)
Saline; week 5	1.2 ^f	(0.52–2.8)	1.3	(0.49–3.7)

^a Potency ratio (ED₅₀ after treatment/ED₅₀ control)

^b Different from control

^c Different from treatment with 0.001 mg/kg etonitazene per day

^d Different from treatment with 0.002 mg/kg etonitazene per day

^e Different from treatment with 0.008 mg/kg etonitazene per day

^f Different from treatment with 0.032 mg/kg etonitazene per day

^g Different from treatment with 0.128 mg/kg etonitazene per day

^h Different from treatment with saline (week 1)

with 0.002 mg/kg per day etonitazene produced greater tolerance to buprenorphine than to any other agonist, but did not differentiate among the other agonists. Treatment with 0.032 mg/kg per day etonitazene produced greater tolerance to buprenorphine and GPA 1657 than to etonitazene, and greater tolerance to GPA 1657 than to morphine. The lack of difference among etonitazene, etorphine, and morphine with respect to the magnitude of tolerance produced by etonitazene was confirmed by a final comparison of their log potency ratios across the entire 64-fold range of treatment doses. There was a significant main effect of the treatment dose of etonitazene [$F(3,84)=38.63$, $P<0.0001$] and a significant interaction of test agonist and treatment dose [$F(6,84)=7.26$, $P<0.0001$], but no effect of the agonist tested for antinociception [$F(2,84)=1.19$, n.s.]. Thus, the magnitude of tolerance to all agonists increased as treatment dose increased, and the impact of increasing the etonitazene

treatment dose varied among agonists, but these three agonists did not in sensitivity to tolerance.

Repeated morphine treatment

Treatment with 20 or 40 mg/kg per day morphine increased the ED₅₀ for morphine, buprenorphine, and GPA 1657, but not etorphine (Fig. 2; Table 3). The impact of treatment with 20 mg/kg per day morphine did not change as the duration of treatment increased from 1 to 2 weeks, and all agonists maintained the capacity to produce a 100% maximum effect. During the 3rd week, the treatment dose of morphine increased from 20 to 40 mg/kg per day. This increase in treatment dose increased the magnitude of tolerance to morphine and GPA 1657 and diminished the maximal effects of buprenorphine and GPA 1657. Buprenorphine lost the capacity

Fig. 2 Tolerance and cross-tolerance to the antinociceptive effects of etonitazene, etorphine, morphine ($n=6$), buprenorphine ($n=5$), and GPA 1657 ($n=4$) in rats repeatedly treated with morphine. Control points (○) are the average of two determinations. Other details as in Fig. 1

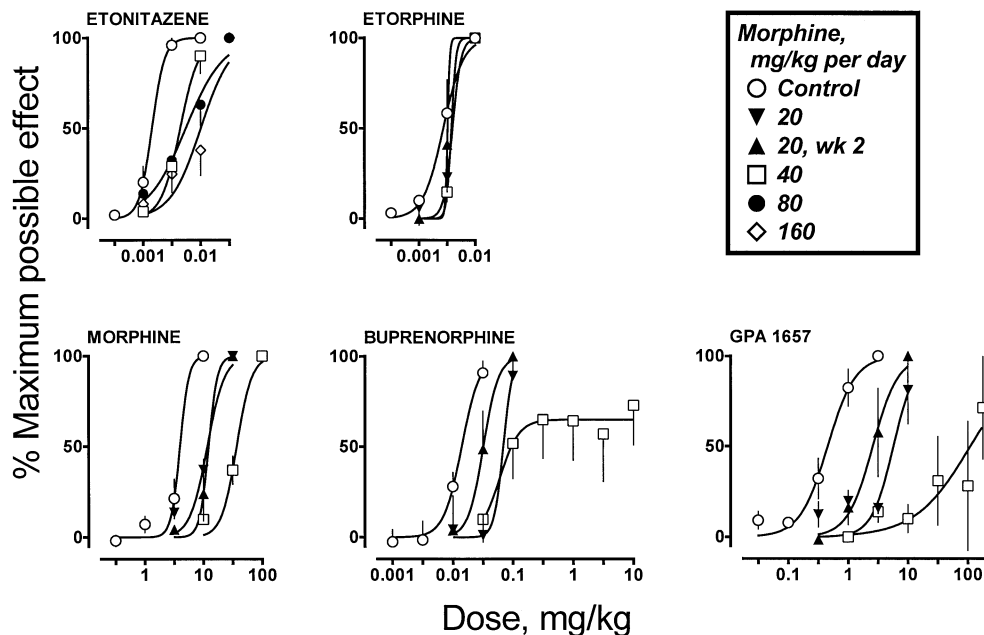


Table 3 ED₅₀ (mg/kg) for antinociceptive effects of μ opioids before, during, and after treatment with morphine. Total daily doses of morphine are listed below

	ED ₅₀	95% C.L.	Ratio ^a	95% C.L.
Etonitazene				
Control	0.0013	(0.00088–0.0019)		
40 mg/kg	0.0038 ^b	(0.0013–0.011)	2.9	(1.1–8.2)
80 mg/kg	0.0051 ^b	(0.0015–0.017)	3.9	(1.3–12)
160 mg/kg	0.0092 ^b	(0.0051–0.016)	7.0	(4.0–12)
Saline; week 4 ^f				
Etorphine				
Control	0.0023	(0.0011–0.0048)		
20 mg/kg; week 1	0.0037	(0.0031–0.0044)	1.6	(0.90–2.8)
20 mg/kg; week 2	0.0033	(0.0026–0.0043)	1.4	(0.87–2.3)
40 mg/kg; week 3	0.0038	(0.0034–0.0042)	1.6	(0.86–3.0)
Saline; week 4	0.0022	(0.00099–0.0048)	0.93	(0.40–2.2)
Morphine				
Control	3.6	(2.6–5.0)		
20 mg/kg; week 1	11 ^b	(8.5–14)	3.0	(2.1–4.2)
20 mg/kg; week 2	11 ^b	(6.8–16)	2.9	(1.5–5.5)
40 mg/kg; week 3	35 ^{b-d}	(30–41)	9.7	(6.8–14)
Saline; week 4	3.8 ^{c-e}	(1.6–9.2)	1.0	(0.40–2.8)
Buprenorphine				
Control	0.011	(0.0063–0.018)		
20 mg/kg; week 1	0.039 ^b	(0.029–0.052)	3.6	(2.1–6.3)
20 mg/kg; week 2	0.028	(0.014–0.058)	2.6	(1.0–6.8)
40 mg/kg; week 3	0.87 ^b	(0.027–0.29)	8.1	(2.8–24.)
Saline; week 4	0.013 ^e	(0.0038–0.44)	1.2	(0.27–5.3)
GPA 1657				
Control	0.46	(0.18–1.2)		
20 mg/kg; week 1	4.0 ^b	(2.5–6.4)	8.8	(2.9–27)
20 mg/kg; week 2	2.4 ^b	(0.60–9.2)	5.1	(0.92–29)
40 mg/kg; week 3	21 ^{b,c}	(8.4–51)	45	(7.8–260)
Saline; week 4 ^f				

^a Potency ratio (ED₅₀ after treatment/ED₅₀ control)

^b Different from control

^c Different from 20 mg/kg morphine per day (week 1)

^d Different from 20 mg/kg morphine per day (week 2)

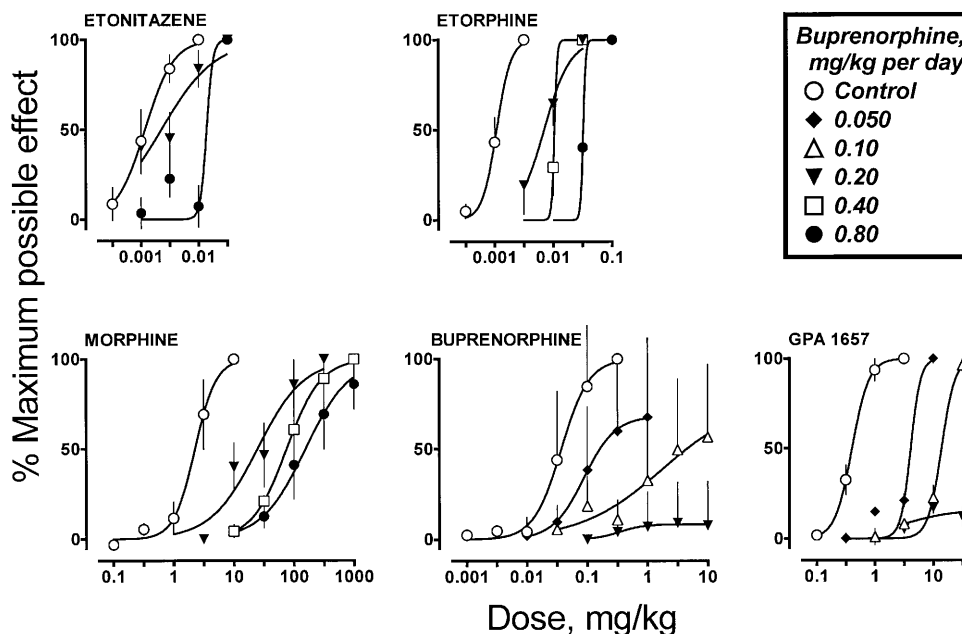
^e Different from 40 mg/kg morphine per day (week 2)

^f Not tested. Severe withdrawal signs were observed in the etonitazene group, and high doses of GPA 1657 were lethal in the GPA 1657 group

to produce full antinociception in two of five rats, and GPA 1657 lost such capacity in two of four rats. Rats recovered their initial sensitivity to etorphine, morphine, and buprenorphine after a week of saline injections

(Table 3). The GPA 1657 group could not be tested for recovery after treatment with 40 mg/kg morphine because a dose of 180 mg/kg GPA 1657 was lethal within 2–12 h after testing.

Fig. 3 Tolerance and cross-tolerance to the antinociceptive effects of etonitazene, etorphine, morphine, buprenorphine ($n=4-8$), and GPA 1657 ($n=5$) in rats repeatedly treated with buprenorphine. Control points (○) are the average of two determinations. The 0.2 mg/kg per day buprenorphine data are from buprenorphine group 1 ($n=8$) and the 0.05 and 0.1 mg/kg per day buprenorphine data are from buprenorphine group 2 ($n=4$). Other details as in Fig. 1



In the etonitazene group, the initial treatment dose of morphine, 40 mg/kg per day, increased the ED₅₀ for etonitazene (Fig. 2; Table 3). No further increase in ED₅₀ was observed as the treatment dose increased to 80 or 160 mg/kg per day. Withdrawal from 160 mg/kg per day morphine produced severe diarrhea and weight loss, which responded to treatment with subcutaneous fluids and low doses of morphine.

Because multiple agonists were tested before and after treatment with 20 and 40 mg/kg per day morphine, it was possible to compare the magnitude of tolerance as a function of test agonist and treatment dose. Because the effects of treatment with 20 mg/kg per day morphine were similar across treatment weeks, potency ratios for individual rats were averaged. Analysis of the log₁₀ potency ratios revealed both significant main effects of the treatment dose of morphine [$F(1,30)=25.34$, $P<0.0001$] and the agonist tested for antinociception [$F(3,30)=23.08$, $P<0.0001$], and a significant interaction of test agonist and treatment dose [$F(3,30)=3.38$, $P=0.031$]. Thus, treatment with 40 mg/kg per day morphine produced greater tolerance than did treatment with 20 mg/kg per day, agonists differed in sensitivity to tolerance, and the impact of increasing the treatment dose varied among agonists. One-way ANOVA and post hoc tests showed that treatment with 20 mg/kg per day morphine produced greater tolerance to GPA 1657 than to etorphine, but did not differentiate among other agonists. Treatment with 40 mg/kg per day morphine produced greater tolerance to GPA 1657 than to etonitazene, etorphine, or buprenorphine, and greater tolerance to morphine or buprenorphine than to etorphine.

Repeated buprenorphine treatment

Treatment with 0.2, 0.4, or 0.8 mg/kg per day buprenorphine produced dose-dependent increases in ED₅₀ for etonitazene, etorphine, and morphine (Fig. 3; Table 4). During the initial experiment, treatment with 0.2 mg/kg per day buprenorphine abolished the capacity of buprenorphine or GPA 1657 to produce antinociception in all rats (GPA 1657 data not shown). Therefore, these two groups of rats did not receive the higher treatment doses of buprenorphine, but rather received saline for the next 2 weeks (Table 4). In two new groups of rats, treatment with 0.05 or 0.10 mg/kg per day buprenorphine produced dose-dependent increases in the ED₅₀ for buprenorphine and GPA 1657 and decreased the maximal effects of both agonists. The final buprenorphine treatment dose of 0.2 mg/kg per day abolished the capacity of buprenorphine or GPA 1657 to produce antinociception in all rats, providing systematic replication of the findings in the first groups of rats.

Rats tested with etorphine, morphine, and GPA 1657 recovered their initial agonist sensitivity 1 week after buprenorphine treatment ended (Table 4). Sensitivity to etonitazene did not recover fully within 2 weeks. For buprenorphine, the antinociceptive response also did not recover within 1 (Fig. 4 left panel) or 2 weeks (Table 4). This loss of agonist response may have resulted from a long-lasting antagonism produced by the cumulative dose of 10 mg/kg buprenorphine tested on the last day of buprenorphine treatment. To test this possibility, rats in the second buprenorphine group were tested with morphine instead of buprenorphine after 7 days of saline injections. In this second group of rats, 320 mg/kg morphine failed to produce greater than 25% MPE (Fig. 4 right panel). However, after a 2nd week of saline treatment, the morphine dose-response curve did not differ

Table 4 ED₅₀ (mg/kg) for antinociceptive effects of μ opioids before, during, and after treatment with buprenorphine. Total daily doses of buprenorphine are listed below. Recovery tests performed in two different groups of rats (for group 2 see Fig. 4)

	ED ₅₀	95% C.L.	Ratio ^a	95% C.L.
Etonitazene				
Control	0.00099	(0.00038–0.0026)		
0.2 mg/kg	0.0034 ^b	(0.0013–0.0092)	3.4	(0.85–14)
0.4 mg/kg ^h				
0.8 mg/kg	0.015 ^{b,e}	(0.0098–0.022)	15	(5.9–37)
Saline; week 4	0.0046 ^{b,g}	(0.0035–0.0059)	4.6	(1.6–13)
Saline; week 5	0.0057 ^b	(0.0044–0.0074)	5.8	(1.7–20)
Etorphine				
Control	0.00092	(0.00060–0.0014)		
0.2 mg/kg; week 1	0.0082 ^b	(0.0054–0.012)	9.0	(6.4–13)
0.4 mg/kg; week 2	0.012 ^b	(0.0087–0.017)	13	(10–17)
0.8 mg/kg; week 3	0.036 ^{b,e,f}	(0.023–0.056)	39	(25–61)
Saline; week 4	0.0027 ^{b,e-g}	(0.0011–0.0070)	3.0	(1.1–8.4)
Saline; week 5	0.0029 ^{b,e-g}	(0.0011–0.0082)	3.2	(1.3–8.2)
Morphine				
Control	2.1	(0.96–4.6)		
0.2 mg/kg; week 1	25 ^b	(8.9–68)	12	(4.6–30)
0.4 mg/kg; week 2	86 ^b	(43–172)	41	(13–125)
0.8 mg/kg; week 3	168 ^{b,e}	(46–615)	80	(12–525)
Saline; week 4	6.8 ^{f,g}	(2.9–16)	3.2	(1.0–9.9)
Saline; week 5	3.7 ^{f,g}	(2.5–5.2)	1.1	(0.49–2.6)
Buprenorphine				
Control	0.042	(0.018–0.099)		
0.05 mg/kg; week 1	0.20 ^b	(0.046–0.91)	4.9	(1.4–17)
0.1 mg/kg; week 2	1.5 ^{b,c}	(0.15–15)	36	(7.1–184)
0.2 mg/kg; week 3	>10 ^d	–	>238	–
Saline; week 2 (group 1) ⁱ	>10	–	>238	–
Saline; week 3 (group 1) ⁱ	>10	–	>238	–
GPA 1657				
Control	0.40	(0.26–0.63)		
0.05 mg/kg; week 1	4.1 ^b	(3.9–4.3)	10	(6.4–16)
0.1 mg/kg; week 2	13 ^{b,c}	(9.9–17)		(32–57)
0.2 mg/kg; week 3	>32 ^d	–	>80	–
Saline; week 2 (group 1)	0.40	(0.27–0.61)	1.0	

^a Potency ratio (ED₅₀ after treatment/ED₅₀ control)

^b Different from control

^c Different from treatment with 0.05 mg/kg buprenorphine per day

^d Different from treatment with 0.1 mg/kg buprenorphine per day

^e Different from treatment with 0.2 mg/kg buprenorphine per day

^f Different from treatment with 0.4 mg/kg buprenorphine per day

^g Different from treatment with 0.8 mg/kg buprenorphine per day

^h First test dose during test produced 100% maximum effect in all rats

ⁱ Recovery tests performed in two different groups of rats (Fig. 4)

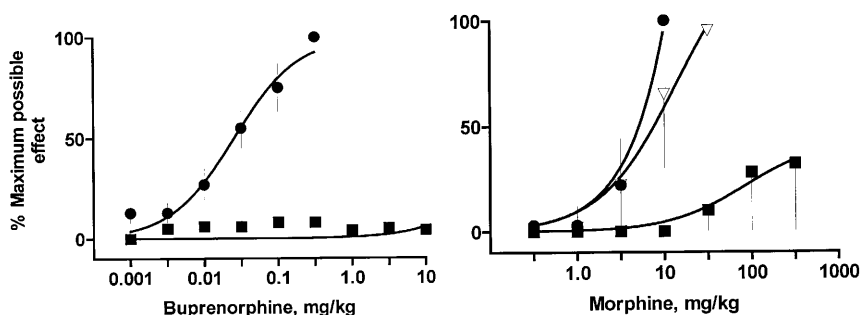


Fig. 4 Buprenorphine ($n=8$) and morphine ($n=4$) recovery experiments in rats treated with buprenorphine and tested with cumulative doses of 10 mg/kg buprenorphine or 320 mg/kg morphine. Closed circles are the average of two control buprenorphine (left panel) or morphine (right panel) determinations. Closed squares show effects of buprenorphine or morphine in buprenorphine group 1 or 2, respectively, after termination of 0.2 mg/kg per day buprenorphine treatment and 14 days of saline treatment. Intervening tests conducted on days 0 and 8 of saline treatment administered cumulative doses of 10 mg/kg buprenorphine. A final test of morphine (open triangles) was conducted on day 22, following 7 additional days of saline administration

from control morphine dose-response curves determined in this study (see control data in Figs. 3, 4; Table 4). These data suggest that a cumulative dose of 10 mg/kg buprenorphine can produce a long-lasting antagonism of μ agonists that dissipates within 2 weeks. Therefore, the tests of buprenorphine during treatment with 0.2 mg/kg per day buprenorphine in the second group of rats treated with buprenorphine were not included in the data analysis because 10 mg/kg buprenorphine was administered during the week immediately preceding the test. The figures and tables present tests of buprenorphine conducted

in the first group of animals treated with 0.2 mg/kg per day buprenorphine. Note, however, that a long-lasting antagonism was not observed after lower doses of buprenorphine, inasmuch as sensitivity to etorphine and morphine recovered 1 week after treatment with 0.80 mg/kg per day buprenorphine ended. In other tests, acute pretreatment of 1.0 mg/kg buprenorphine 7 days prior to testing failed to alter morphine's antinociceptive potency or maximum effect in other rats not treated with buprenorphine (data not shown).

Because multiple agonists were tested before and after treatment with 0.20 and 0.80 mg/kg per day buprenorphine, it was possible to compare the magnitude of tolerance as a function of test agonist and treatment dose. For the three agonists tested during treatment with both doses of buprenorphine (i.e., etonitazene, etorphine, and morphine), there was a significant effect of treatment dose of buprenorphine [$F(1,30)=20.63$, $P<0.0001$] and agonist tested [$F(2,30)=5.84$, $P=0.0072$], but no interaction of treatment dose and test agonist [$F(2,30)=0.18$]. Thus, treatment with 0.80 mg/kg per day buprenorphine produced greater tolerance than did treatment with 0.20 mg/kg per day, and the agonists differed in sensitivity to buprenorphine treatment. Subsequent one-way ANOVA and post hoc tests, incorporating tests of buprenorphine and GPA 1657, showed that treatment with 0.20 mg/kg per day buprenorphine produced greater tolerance to buprenorphine or GPA 1657 than to etonitazene, etorphine, and morphine, but did not differentiate among the former two agonists or the latter three. Treatment with 0.20 mg/kg per day buprenorphine also suppressed the maximal antinociceptive effects produced by buprenorphine and GPA 1657, but not the maximal effects of the other agonists. In contrast, treatment with 0.80 mg/kg per day buprenorphine did not differentiate among etonitazene, etorphine, and morphine with respect to magnitude of tolerance or lack of effect on their maximal antinociceptive effects.

Discussion

In the present experiments, three different opioid agonists (etonitazene, morphine, and buprenorphine) were repeatedly administered and the potencies of five agonists were examined at weekly intervals. Regardless of the opioid agonist repeatedly administered, the potencies of the lower efficacy agonists buprenorphine and GPA 1657 generally decreased to a greater degree than did the potencies of the higher efficacy agonists morphine, etorphine, and etonitazene. For example, treatment with 0.032 mg/kg per day etonitazene increased the ED_{50} for buprenorphine and GPA 1657 by 29- to 40-fold and those for morphine and etorphine by 4.9- to 9.9-fold, yet failed to alter the ED_{50} for etonitazene (Table 2). Similarly, treatment with 40 mg/kg per day morphine increased the ED_{50} for buprenorphine and GPA 1657 by 8.1- to 45-fold, the ED_{50} for morphine by 9.7-fold, and those for etonitazene and etorphine by approximately 2-

fold (Table 3). Finally, treatment with 0.2 mg/kg per day buprenorphine completely flattened the dose response curves for GPA 1657 and buprenorphine and shifted the ED_{50} for morphine by 12-fold and those for etonitazene and etorphine by 3.4- and 9-fold, respectively (Table 4). The rank order of sensitivity to repeated agonist treatment was preserved across treatment conditions so that, in general, all treatments produced the greatest tolerance to buprenorphine and GPA 1657 and the least tolerance to morphine, etorphine, and etonitazene. However, the magnitude of sensitivity to repeated agonist treatment depended on the agonist administered. For example, ANOVA analyses revealed that repeated morphine treatment, but not repeated etonitazene or buprenorphine treatments, differentiated morphine from etorphine and etonitazene under some dosing conditions.

Other studies with different treatment and test agonists have also reported greater tolerance to lower efficacy opioid agonists than to higher efficacy agonists in antinociception assays (Duttaroy and Yoburn 1995). For example, Paronis and Holtzman (1992) found greater tolerance to the low efficacy agonist meperidine than to the higher efficacy agonists morphine and etorphine after continuous infusion of fentanyl, morphine, or meperidine. Tiano et al. (1998) found greater tolerance to the low efficacy agonists buprenorphine and dezocine than to etorphine and *l*-methadone after repeated, intermittent injections of etorphine. Although the magnitude of tolerance to low and high efficacy agonists can depend on the dosing protocol (Duttaroy and Yoburn 1995), most results support the hypothesis that the magnitude of tolerance is inversely related to agonist relative efficacy.

The present results also supported the hypothesis that lower efficacy agonists produce greater tolerance than do higher efficacy agonists. There appeared to be an inverse relationship between the relative efficacy of the opioid treatment agonist and the magnitude of tolerance to the antinociceptive effects of any given test agonist. This is illustrated by comparisons of the effects of treatment doses approximately ten times the respective antinociceptive ED_{50} s for etonitazene, morphine, or buprenorphine. These treatments can be ranked in an approximate order with respect to the alterations that they produced in the potency of a majority of the test agonists: 0.032 mg/kg per day etonitazene < 40 mg/kg per day morphine < 0.2 mg/kg per day buprenorphine. For example, 0.032 mg/kg per day etonitazene, 40 mg/kg morphine, and 0.2 mg/kg per day buprenorphine produced 4.9-, 9.7-, and 12-fold reductions, respectively, in the antinociceptive potency of morphine (Tables 2, 3, 4). In other investigations, treatment with etorphine, a higher efficacy agonist, produces less tolerance to antinociceptive effects of etorphine and morphine than does treatment with morphine (Tiano et al. 1998). In a comparison of equiactive doses of meperidine, morphine, and fentanyl, continuous infusion of the lower efficacy agonist meperidine produced the greatest alterations in agonist potency, whereas the higher efficacy agonist fentanyl produced the smallest alterations (Paronis and Holtzman

1992). Similarly, treatment with lower efficacy agonist buprenorphine abolished the maximum antinociceptive effects of buprenorphine, but not those of the higher efficacy morphine (Dum et al. 1981). Taken together, these studies further substantiate the hypothesis that the degree of tolerance to the antinociceptive effects of μ opioids is an inverse function of the relative efficacy of both the agonist used for treatment and the agonist tested for activity.

However, there were exceptions to this hypothesis in the present study. For example, treatment with 0.032 mg/kg per day etonitazene and 0.2 mg/kg per day buprenorphine produced larger reductions in the potency of etorphine (9.9- and 9-fold) and buprenorphine (>29- and >238-fold) than did treatment with an equieffective dose of 40 mg/kg per day morphine (1.6- and 8.1-fold, respectively). This observation suggests that morphine treatment may interact with etorphine and buprenorphine differently than with the other agonists. Etorphine and buprenorphine are both thebaine-orphavines with high affinity for μ , κ , and δ opioid receptors (Richards and Sadee 1985; Su 1985). In mouse tail-flick assays, μ , κ , and δ antagonists antagonized the effects of etorphine (Xu et al. 1992) and the κ antagonist nor-BNI antagonized those of buprenorphine (Pick et al. 1997). When μ receptors are disabled either by β -funaltrexamine or repeated morphine administration, buprenorphine can act as a κ antagonist (Gmerek et al. 1987; Leander 1988; Negus et al. 1989). Additionally, δ antagonist properties for buprenorphine have also been demonstrated (Kajiwara et al. 1986). These observations suggest that other opioid receptors may have contributed to the effects of etorphine and buprenorphine under the treatment conditions studied here. Furthermore, the results from these experiments underscore the importance of examining more than one agonist in tolerance and cross-tolerance studies.

In the present study, treatment with buprenorphine produced greater tolerance to all five agonists than did treatment with morphine or etonitazene. Although these data support the hypothesis that treatment with lower efficacy agonists produces greater tolerance than does treatment with higher efficacy agonists, another interpretation might be that buprenorphine is also acting to block κ and δ opioid receptors. Although both etorphine (Law et al. 1983; Tao et al. 1987) and GPA 1657 (Walker et al. 1993) have κ and δ agonist activities in some reports, other behavioral studies find that both etorphine (Young et al. 1984, 1992) and GPA 1657 (Jasinski et al. 1971; Young et al. 1984) produce behavioral effects through μ receptor mechanisms. Additionally, in the rat warm-water tail-withdrawal procedure used here, naltrexone is equipotent as an antagonist of etonitazene, etorphine, morphine, buprenorphine, and GPA 1657, suggesting that the antinociceptive effects of these agonists are mediated predominantly through μ opioid receptors (Walker et al. 1994, 1998). Moreover, the increases in ED_{50} for etonitazene, morphine, and buprenorphine, which show little agonist activity at κ or δ receptors in rats, were also

larger after buprenorphine treatment. These data suggest that the larger alterations in μ agonist dose-response curves after buprenorphine treatment result primarily from the lower relative efficacy of buprenorphine.

Control experiments indicated that a high dose of 10 mg/kg buprenorphine could block the antinociceptive effects of buprenorphine or morphine 7 days after administration (Fig. 4). This blockade was only observed after a high dose of buprenorphine, however, because a high dose of 320 mg/kg morphine, failed to alter the antinociceptive effects of morphine 7 days after administration. Buprenorphine displays unusual long-term activity in pigeons (France et al. 1984; Versage et al. 1990), rats (Tallarida and Cowan 1982), monkeys (Dykstra 1983), and humans (Walsh et al. 1995; Schuh et al. 1999). Other behavioral experiments demonstrate unusual kinetics for buprenorphine, such as a bell-shaped dose-response curve for antinociception in rats (Cowan et al. 1977), rhesus monkeys (Walker et al. 1995), and humans (Schmidt et al. 1985). In the present study, only one group of rats was exposed to 10 mg/kg buprenorphine during tests. Twelve- or 24-h pretreatment with lower doses of buprenorphine, including the doses used for treatment in the present study, did not produce either short- or long-term antagonism (Walker and Young, unpublished observations; present study). For example, in the other groups treated with 0.2, 0.4, and 0.8 mg/kg per day buprenorphine, rats recovered their initial sensitivity to etorphine, morphine, and GPA 1657 after 1 week saline treatment. Based on the unusually long antagonism of morphine and buprenorphine by a dose of 10 mg/kg buprenorphine, buprenorphine dose-response curves were analyzed in the present study only if the maximum dose tested 7 days earlier was 1.0 mg/kg buprenorphine or less. However, despite buprenorphine's unusual kinetics (Boas and Villiger 1985), the effects of buprenorphine treatment still paralleled those of etonitazene and morphine treatment. For all agonists, an inverse relationship was obtained between magnitude of tolerance and relative efficacy. Further experiments with low efficacy agonists need to be performed to further substantiate this relationship.

The present study incorporated tolerance and cross-tolerance experiments with etonitazene, a high efficacy agonist (Walker et al. 1997) with excellent selectivity for the μ receptor (Moolten et al. 1993; Emmerson et al. 1994). Etonitazene was selected for these experiments because its duration of action resembles that of morphine and buprenorphine (Foley 1993; Walker et al. 1993). Although etonitazene has been used in studies of oral self-administration in rats (Suzuki et al. 1992) and monkeys (Tang 1982) and a conditioned tolerance study in mice (Elmer et al. 1993), the present study appears to be the first to describe tolerance and cross-tolerance to etonitazene using multiple agonists and dose treatments. The data in the present experiment indicate that etonitazene treatment, similar to etorphine treatment (Roerig et al. 1985; Tiano et al. 1998), produces less tolerance than does treatment with lower efficacy drugs such as mor-

phine and buprenorphine. In general, less tolerance was observed to all five agonists despite etonitazene treatment doses approximately 13 times the antinociceptive dose of etonitazene. Furthermore, etonitazene treatment doses were increased fourfold and only small changes were observed in the magnitude of tolerance. These data indicate etonitazene is an excellent agonist for tolerance and cross-tolerance studies.

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