Buprenorphine is an Antagonist at the κ Opioid Receptor

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Abstract: The effect of buprenorphine on bremazocine-induced diuresis was tested in the rat to determine the nature of buprenorphine's action at the κ opioid receptor. Both morphine-tolerant and naive rats were used to account for possible antidiuretic effects of buprenorphine at the μ site. Separate experiments established that the morphine pretreatment caused profound tolerance with respect to the antidiuretic action of μ agonists. Buprenorphine acted as a potent antagonist (ID_{50} = 11 μg/kg) of the diuretic action of the κ agonist bremazocine (ED_{50} = 10 μg/kg). The similar potency of buprenorphine as an antagonist of bremazocine in naive and morphine-tolerant rats further supports the hypothesis that buprenorphine exerts it's antidiuresis via an antagonistic effect at κ sites, rather than as an agonist at the μ sites. The high affinity displayed by buprenorphine at the κ opioid receptor *in vivo* is consistent with this conclusion. Hence, buprenorphine can now be classified as a partial agonist at the μ site and as an antagonist at the κ site against bremazocine induced urine flow, while its action at the δ site to which it has much lower affinity *in vivo* remains unknown.

Buprenorphine is an opioid agonist/antagonist that gives rise to a bell-shaped dose-response curve in analgesia tests with rats (1, 2). Recently, it was shown that buprenorphine saturates μ and κ sites *in vivo* at doses that also cause peak analgesia (500 μg/kg), while the downslope of the bell shaped curve occurs at doses that give rise to δ site occupancy (3). However, the relative involvement of μ and κ sites in buprenorphine analgesia remains uncertain. Martin et al. (5) characterized buprenorphine as a partial μ agonist. Alternatively, others have suggested on the basis of heat versus pressure stimulus antinociceptive assays that κ sites play a dominant role in mediating buprenorphine analgesia (5, 6).

Hence to clarify the nature of buprenorphine's action at the κ site, its influence on the diuretic response to bremazocine was tested in rats. The increased urine output in rats caused by bremazocine recently has been shown to be mediated by κ receptors in the brain. However, because strong μ agonists are known to decrease urine output (9–12), a decrease in the diuretic response to bremazocine could be caused by an antagonist or a partial agonist/antagonist effect of buprenorphine at the κ receptor, or by an agonist effect at the μ receptor. Thus, buprenorphine's action at the μ and κ sites were differentiated by comparing its effect on bremazocine stimulated urine flow in naive and morphine-tolerant rats. Buprenorphine's similar antagonistic potency against bremazocine-associated diuresis in both morphine (μ) tolerant and naive rats indicates that buprenorphine is an antagonist at the κ receptor in this pharmacological test.

Materials and Methods

Urine Collections

Urine samples were collected as previously described (7) from 28 male Sprague-Dawley albino rats, weighing 350 to 450 g. Except during urine collections, the animals were housed at 22 to 23° C in a colony room with a 12 h dark-light cycle and allowed free access to food and water. Bremazocine (± buprenorphine) was administered S.C. in 400 μl normal saline. Immediately after injection of drugs and placement of the rats in metabolism cages, urine collections were initiated and the output recorded 2 and 5 h later. Each rat was used no more than twice weekly. Upon completion of studies with normal rats, many of the same animals were then used for the morphine tolerance studies.

Induction of Morphine Tolerance

Two groups of 12 rats each were administered either 10 mg/kg morphine twice daily or 20 mg/kg morphine three times a day in 600 μl normal saline. Four rats died as a result of the initial 20 mg/kg dose of morphine. Urine flow response to bremazocine (± buprenorphine) was tested at both 3 and 5 days after beginning morphine injections and 12 to 13 h after the previous dose of morphine. Hence, there were four animal groups with somewhat different morphine treatment schedules; rats receiving either 20 or 60 mg/kg morphine daily for 3 and 5 days. Rats in all morphine treatment groups demonstrated a profound depression of activity after initial injections. In all cases, this was replaced by normal or excessive levels of activity following later morphine injections, indicating the development of tolerance.

The effectiveness of morphine administration for inducing tolerance to the inhibitory action of μ agonists on urine flow was tested in a separate experiment. Urine output response to concurrent administration of bremazocine (25 μg/kg) and etorphine (25 μg/kg), a selective μ agonist (3), was measured both in naive rats (N = 10) and in rats which had received 10 mg/kg morphine twice daily for 3 days (N = 10).

Parameter Estimation

The pharmacodynamic parameters describing the activity of bremazocine and buprenorphine on modulating the output of urine were obtained by simultaneously analyzing bremazocine's dose-response curves in the absence and presence of varying doses of buprenorphine. This was performed separately for naive and morphine tolerant rats.

Buprenorphine's ability to decrease bremazocine stimulated diuresis in morphine tolerant rats did not differ when fitting the urine output data obtained from rats receiving morphine doses (over 3 and 5 days) of either 60 mg/kg daily (buprenorphine ID_{50} = 7.1 μg/kg, S.E. = 5.9) or 20 mg/kg daily (buprenorphine ID_{50} = 11.2 μg/kg, S.E. = 5.7). Hence, the data obtained from all morphine tolerant rats were pooled. The pharmacodynamic model, derived from the law of mass action, has been described previously (7):

$$E = \frac{(EMAX - EMIN)\ A^N}{A^N + [ED\,50\ (1 + B/ID\,50)]^N} \quad (1)$$

The estimate of urine output (E) caused by a s.c. dose of bremazocine (A) in the absence or presence of a fixed dose of

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buprenorphine (B) ranges from the expected values for minimum (EMIN) and maximum (EMAX) urine output. ED_{50} represents the dose of bremazocine that will cause an output of urine equal to [0.5 (EMAX – EMIN) + EMIN]. The N parameter describes the slope of the dose-response curves as determined from the simultaneous fits. Simultaneous data fitting with a nonlinear regression program allows use of all observations from the naive and morphine-tolerant rat groups to estimate the pharmacological parameters in each group. Hence, although each bremazocine dose-response curve (±-buprenorphine) might be inadequately described when considered individually, the combined data from the 3 or 4 dose-response curves of either the naive or morphine-tolerant animals are sufficient for providing reasonably accurate parameter estimates, while permitting one to minimize sample size. Moreover, the fitting program readily converged on final parameter estimates suggesting that the competitive antagonist model and the parameter estimates are compatible with the results.

Results and Discussion

The simultaneous fits of the bremazocine (± buprenorphine) dose-response curves in naive rats (Fig. 1a, Table I) show that buprenorphine antagonizes bremazocine associated diuresis in a dose dependent manner. Similarly, buprenorphine antagonized urine output in morphine tolerant animals resulting in parameter estimates close to the values observed for naive rats (Fig. 1b, Table I).

It has been suggested that κ receptors mediate the increased urine output caused by certain opioids, i.e., bremazocine, ethylketocyclazocine and ketazocine (4, 8). This was supported more recently by comparisons of *in vivo* fractional occupancy of μ, δ and κ sites by general opioid antagonists with their ability to inhibit bremazocine-induced diuresis (7). The ID_{50} values (as defined in this paper for buprenorphine) for diprenorphine, Win 44,441-3 and MR 2266 coincided with the doses of these antagonists required to block 50 per cent of the κ receptors *in vivo*, while receptor occupancy at the ID_{50} doses was variable for the μ and δ sites. This result clearly implicates the κ site as the primary receptor for mediating bremazocine's effects on urine flow.

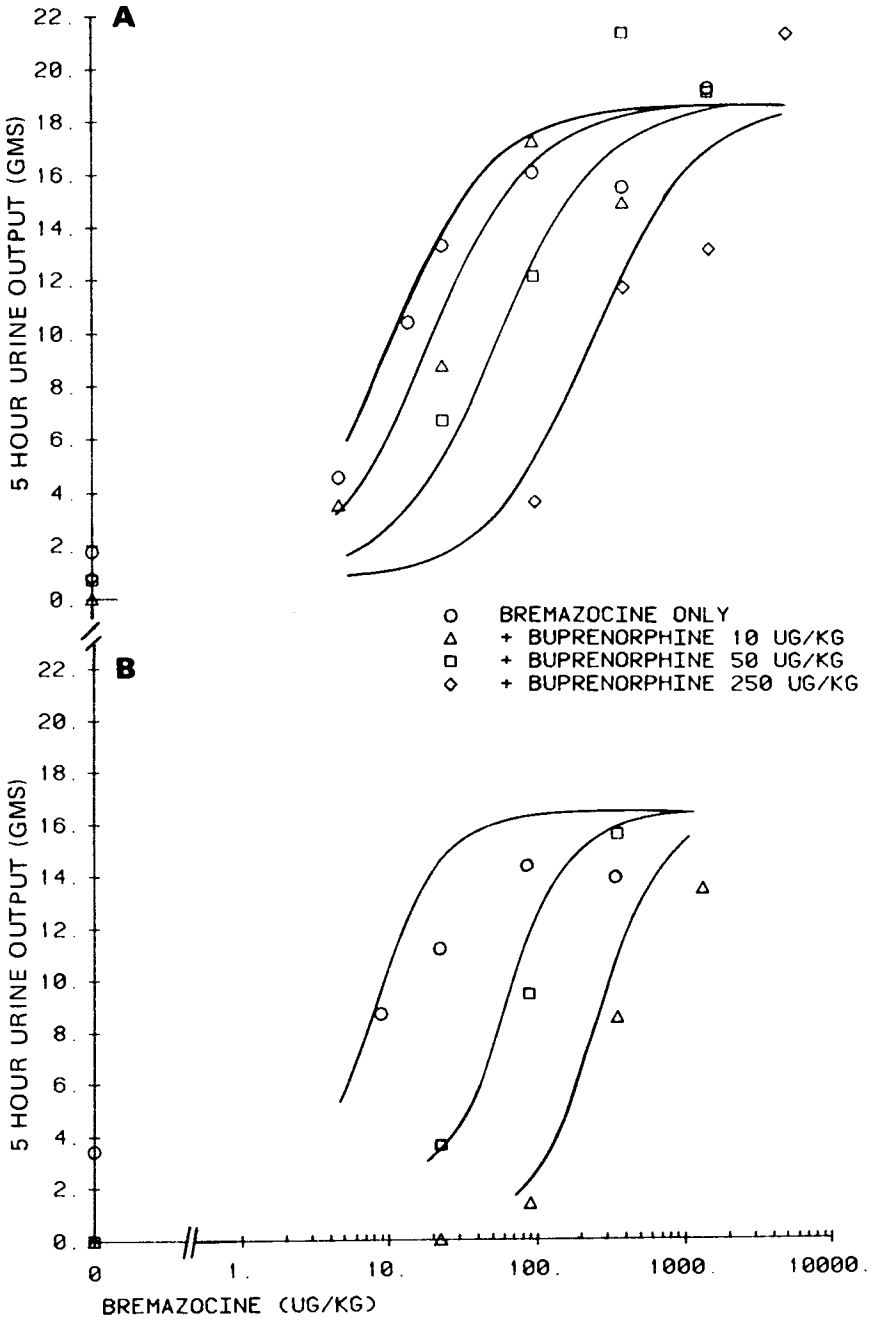

Fig. 1 Bremazocine dose-response curves in the absence and presence of buprenorphine as measured in (A) naive and (B) morphine-tolerant rats. Each data point represents the mean of two or more observations. The fitted lines are based on the parameter estimates of Table I as generated from the raw data. Note that all data points in A and in B are simultaneously fitted, which allows one to use fewer data points per dose-response curve than in cases when each dose-response curve is fitted individually.

Table I. Computer Generated Parameter Estimates Derived from Simultaneous Fitting of Urine Output Data Obtained from Naive or Morphine Tolerant-Rats (see eq. 1). Doses are Expressed as the Free Base.

	Naive	Morphine tolerant
Observations	72	38
EMAX (± S.E.)	18.7 (± 1.4) g	17 (±.6) g
EMIN	.8 g	3 g
N (± S.E.)	1.3 (±.1)	1.8 (±.2)
ED_{50} (bremazocine) (± S.E.)	10.3 (± 3.5) μg/kg	7.3 (± 2.7) μg/kg
ID_{50} (buprenorphine) (± S.E.)	11.2 (± 3.2) μg/kg	6.8 (± 3.2) μg/kg

Hence, the shift of bremazocine dose-response curves to the right by increasing doses of buprenorphine (Fig. 1 a) suggests that buprenorphine is acting as a κ antagonist. Alternatively, because strong μ agonists have been found to decrease urine output in water loaded rats (9, 10), the inhibition of bremazocine diuresis by buprenorphine could be a result of agonist activity at μ receptors. However, Leander (8) demonstrated that 20 mg/kg morphine does not alter the diuretic response to 80 μg/kg bremazocine. Because 10 mg/kg morphine and 500 μg/kg buprenorphine s.c. produce an equivalent level of analgesia in the electrically evoked vocalization test (13), it can be implied that, in the doses employed, buprenorphine's efficacy at the μ site is insufficient to antagonize the bremazocine effect on urine flow. Nonetheless, in relation to Leander's findings (8), it should be pointed out that 80 μg/kg bremazocine occupies a substantial proportion of μ sites *in vivo,* based on *in vivo* binding affinities (7), which could have prevented morphines's μ activity.

To address further the question of whether buprenorphine's inhibitory effects on bremazocine induced urine flow are mediated by its antagonistic action at the κ site or its agonistic action at the μ site, we have induced selective μ tolerance by repeated morphine administration. In the presence of tolerance to the antidiuretic action of μ agonists one would expect no diminution of buprenorphine's anti-bremazocine potency, if it primarily acts as an antagonist at the κ site. Tolerance has been reported to develop to morphine's antidiuretic effect (9); moreover, twice daily injection of 10 mg/kg morphine were shown to cause tolerance to the analgesic effects of buprenorphine at the μ site (13). Therefore, this dosage schedule as well as higher doses of morphine were chosen to induce tolerance over 3 to 5 days. To avoid saturation of the μ receptors with high doses of bremazocine (e. g. 80 μg/kg in a previous study (8)), we have chosen a rather low dose (25 μg/kg bremazocine) that elicits ~ 70 % of maximal response. In contrast to the findings of Leander (8) with 80 μg/kg bremazocine, morphine (20 mg/kg) was capable of preventing the diuresis induced by the low bremazocine dose; further, this effect was attenuated in morphine pretreated animals (data not shown). Because etorphine is considered to be a potent and rather selective μ agonist with a chemical structure similar to that of buprenorphine, it was chosen to document the degree of μ tolerance attained after 3 days of twice daily injections with 10 mg/kg morphine, i. e., the minimum pretreatment schedule for the induction of μ tolerance. At 25 μg/kg etorphine, which is at least 30-fold greater than its ED_{50} in the rat tail flick analgesia assay (14), etorphine virtually eliminated the diuretic action of bremazocine (25 μg/kg) over at least 2 h, reducing urine flow from 10.0 ± 3.2 g ($\pm$ SD) and 12.3 ± 3.1 g to 1.8 ± 1.9 g and 8.6 ± 3.2 g over 2 and 5 h, respectively. In contrast, etorphine (25 μg/kg) was ineffective in reducing bremazocine stimulated urine flow in morphine pretreated animals (8.2 ± 2.9 g over 2 h and 12.3 ± 1.9 g over 5 h). The differences between naive and morphine pretreated animals were significant to the < 0.0005 and < 0.01 levels for 2 and 5 h, respectively (paired t test). The apparent loss of etorphine's antidiuretic effect at 5 h relative to 2 h might be explained by a longer residence time of bremazocine in the brain relative to that of etorphine (15). No significant differences were observed between naive and tolerant animals treated with bremazocine alone (see also Fig. 1 and Table I) and tolerant animals treated with bremazocine plus etorphine.

These results clearly establish the development of tolerance to the antidiuretic effects of μ agonists by morphine pretreatment. Since etorphine at the selected dosage (25 μg/kg) was shown to selectively interact with the μ sites *in vivo*, with no measurable binding to δ and κ sites (3), one can conclude that the morphine induced tolerance is specific to the μ site. This finding allows one to interpret any effects of buprenorphine on bremazocine induced diuresis in morphine tolerant animals, which are shown in Fig. 1 b and Table I. The lack of any significanct change of buprenorphine's ability to antagonize bremazocine in the μ tolerant animals strongly argues against the hypothesis that its effect on urine flow is mediated via agonistic actions at the μ site. Rather, the hypothesis that buprenorphine acts as a potent antagonist at the κ site remains unchallenged.

The conclusion that buprenorphine represents a κ antagonist contrasts with that of Tyers (5) who classified buprenorphine as a κ agonist on the basis of comparing its ED_{50} values against various nociceptive stimuli. It is possible that an agonist of low efficacy could behave like an antagonist in one test systems (urine flow), while causing an agonistic response in another system (antinociception) because of differences in the required receptor activation. However, the complexity introduced by the bell shaped-analgesia curve of buprenorphine makes comparison of ED_{50}'s subject to erroneous conclusions.

The view that buprenorphine does not act as a κ agonist is supported by behavioral studies. Discriminative stimulus experiments by Shearman and Herz (16) revealed that bremazocine generalized to the effects of MR 2033, a putative κ agonist, but did not generalize to the effects of buprenorphine or etorphine, the latter being a preferential μ agonist (13).

We have recently measured the relative affinity of buprenorphine at the μ, δ and κ sites *in vivo* (3). The dose (s. c.) required to occupy 50 per cent of each receptor polulation at 60 min was approximately 20, >200 and 20 μg/kg respectively. This result appears to rule out any δ site involvement with buprenorphine's action against bremazocine. Although both μ and κ affinities are in the range that is consistent with the involvement of either of these receptor types, the combined pharmacological evidence presented here and elsewhere strongly supports the notion that buprenorphine, while acting as a partial agonist at the μ sites in analgesia tests, represents a potent κ antagonist in the bremazocine-stimulated urine flow test. However, it cannot be ruled out at present that buprenorphine displays weak agonistic κ effects in other selected pharmacological test systems. Buprenorphine's effect at the δ site remains unknown; however, because it now appears that buprenorphine's analgesic effect may predominantly be elicited through μ receptors while exerting little or no agonistic effect at the κ site, it is possible that the δ sites are involved in mitigating the action of buprenorphine at the μ site, thereby creating a bell shaped dose-analgesia curve.

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A New Method for the Determination of Partition Coefficients of Air Sensitive Copper(I) Complexes

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Abstract: Determinations of log P values of copper complexes in oil/water were performed in a new, totally closed apparatus connected with a filter-probe extractor. The results indicate that the system may be generally suitable for the determination of log P values of oxidizable and nonoxidizable metal complexes. In the case of the copper (I) complexes spectrophotometric analysis was not feasible, since 1-octanol was extracted by these complexes into the aqueous phase, resulting in a change in the extinction coefficient. To establish accurately the concentration in each phase, copper was determined by atomic absorption spectrometry.

Recently, Pijper (1) performed a Hansch analysis on the antimycoplasmal activity of compounds structurally related to 2,2'-bipyridyl. Using Σf, Taft E_S and Hammet σ an excellent correlation was found ($n = 33$, $r = 0.976$). In this relationship lipophilicity is the parameter with the strongest contribution. However, these compounds only show their activity in the presence of copper sulfate levels that by themselves are nontoxic. From studies on the mode of action, Antic et al. (2) and Smit et al. (3) assumed, that a copper(I) complex enters the cell. Therefore, it is important to test whether there exists a linear relationship between the lipophilicity of a ligand and that of the corresponding copper(I) complex. For this purpose the lipophilicity of these complexes had to be measured. As some of these are air sensitive, a procedure in which the presence of air is excluded, has to be used. Shake-flask experiments are most common, but it would be difficult to avoid oxidation by air. In a few experiments with reversed-phase TLC we observed strong tailing, so that this method was abandoned. In experiments with reversed-phase HPLC using RP-2, RP-6, RP-18 columns and oxygen free solvents, no peaks from the copper complexes could be detected.

Recently new methods for the separation of oil/water mixtures have been published. With the Segsplit (4) approach segmented flow is used, while the filter-probe extractor (5) based on the work of Mohammed and Cantwell (6) separates the two phases. We developed an apparatus using the filter-probe extractor, which allows the determination of the partition coefficient in a totally closed, oxygen free system. Using this system the results obtained with air sensitive copper(I) complexes are described.

Materials and Methods

Chemicals

2,9-Dimethyl-1,10-phenanthroline (**1**) was purchased from Aldrich Europe. 1-Amino-3-(2-pyridyl)isoquinoline (**2**), 1-amino-3-(6-methyl-2-pyridyl)isoquinoline (**3**), 1-amino-6-methyl-3-(2-pyridyl)isoquinoline (**4**), 1-amino-8-methyl-3-(2-pyridyl)isoquinoline (**5**), 3-(2-pyridyl)isoquinoline (**6**) and 1-chloro-3-(2-pyridyl)isoquinoline (**7**) were from laboratory stock (7). Bis [2,9-dimethyl-1,10-phenanthroline]copper(I) nitrate (**8**), bis[1-amino-3-(2-pyridyl)isoquinoline]copper(I) nitrate (**9**), bis[1-amino-3-(6-methyl-2-pyridyl)isoquinoline]copper(I) nitrate (**10**), bis [1-amino-6-methyl-3-(2-pyridyl)isoquinoline] copper(I) nitrate (**11**), bis[1-amino-3-(2-pyridyl)isoquinoline]copper(I) nitrate (**12**), bis [3-(2-pyridyl)isoquinoline]copper(I) nitrate (**13**) and bis[1-chloro-3-(2-pyridyl)isoquinoline]copper(I) nitrate (**14**) were prepared as described (8). 2,4-Pentanedione was obtained commercially. Bis [2,4-pentanedione]copper(II) was prepared according to Adams and Hauser (9). All other chemicals were of analytical grade (J. T. Baker) and were used without further purification.

Measurement of the Partition Coefficient of the Copper (I) Complex

Mixing chamber: The mixing chamber (Fig. 1) consisted of a 300 ml water jacket vessel with a special lid in which appropriately located holes allowed the insertion of a nitrogen inlet and outlet tube; a buret inlet connected to an autoburet (Mettler DV10) with a water jacket reservoir (250 ml) equipped with a nitrogen inlet and outlet tube; an outlet for the filter probe (with a polytef film, Mitex LC 10 mcm with 68 %

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