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Differential Antagonism of U69,593- and Bremazocine-Induced Antinociception by (−)-UPHIT: Evidence of *Kappa* Opioid Receptor Multiplicity in Mice[1]

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ABSTRACT

The effect of pretreatment with the *kappa* receptor nonequilibrium antagonist, (−)-UPHIT {1*S*,2*S*-*trans*-2-isothiocyanato-4,5-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide}, on U69,593 {(5α,7α,8β)-(−)-N-methyl-N-(7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl)benzeneacetamide}- and bremazocine-induced antinociception was examined in mice. Both U69,593 and bremazocine produced antinociception in the warm water tail-flick test after i.c.v. administration. Pretreatment with the *kappa* antagonist, nor-binaltorphimine, at doses shown not to affect [D-Ala^2, NMePhe^4, Gly-ol]enkephalin- (*mu*-agonist) or [D-Pen^2, D-Pen^5]enkephalin (*delta*-agonist)-induced antinociception, significanlly attenuated the effects of U69,593 and bremazocine, suggesting actions of these agonists at *kappa* receptors. Furthermore, β-funaltrxamine (*mu* antagonist) and ICI 174,864 {N,N,-diallyl-Tyr-(α-aminoisobutyric acid)$_2$-Phe-Leu-OH} (*delta* antagonist), had no effect on U69,593 or bremazocine in this test providing further evidence of *kappa* receptor-mediated activity. Pretreatment with (−)-UPHIT produced no effect alone and a long-lasting (up to 48 hr) antagonism of U69,593, but not bremazocine, antinociception. The antagonist actions of (−)-UPHIT did not alter the antinociceptive effects of [D-Ala^2, NMePhe^4, Gly-ol]enkephalin or [D-Pen^2, D-Pen^5]enkephalin. These data suggest that (−)-UPHIT is a selective, long-lasting *kappa* antagonist which can differentially antagonize the antinocicieption produced by these two *kappa* agonists. These data provide evidence *in vivo* supportive of *kappa* receptor subtypes in the mouse, and suggest that (−)-UPHIT may be a useful probe for the exploration of *kappa* receptor heterogeneity.

The existence of multiple opioid receptor types termed *mu*, *delta* and *kappa* has been recognized for some time (Martin *et al.*, 1976; Lord *et al.*, 1977). Since these initial discoveries, however, both biochemical and pharmacological data have accumulated which cannot be easily explained based on this simple classification of opioid receptors. Radioligand binding and autoradiographic studies, for example, have provided evidence for possible differences in the characteristics of opioid *delta* receptors (Bowen *et al.*, 1981; Rothman *et al.*, 1984, 1985). Additionally, recent data have suggested differences between opioid *delta* receptors found in rat brain and mouse vas deferens (Shimohigashi *et al.*, 1987; Vaughn *et al.*, 1990), and pharmacological studies suggest that opioid *delta* receptor subtypes exist in mouse brain (Jiang *et al.*, 1991; Mattia *et al.*, 1991). Similarly, both evidence *in vitro* (Rothman *et al.*, 1983, 1987; Wolozin and Pastenak, 1981) and *in vivo* (Heyman *et al.*, 1988; Ling *et al.*, 1985; Takemori and Portoghese, 1989) exists which would support the notion of *mu*-opioid receptor multiplicity.

As with both opioid *mu* and *delta* receptors, data from both biochemical and pharmacological studies (Attali *et al.*, 1982; Iyenger *et al.*, 1986; Rothman *et al.*, 1990; Tiberi and Magnan, 1990) suggest the presence of at least two opioid *kappa* receptors. These receptor subpopulations have been classified as *kappa*$_1$, or those which preferentially bind the *kappa*-selective benzeneacetamides such as U69,593 {(5α,7α,8β)-(−)-N-methyl-N-(7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl)benzeneacetamide} (Lahti *et al.*, 1985) and *kappa*$_2$, or those that preferentially bind *kappa*-selective benzomorphans such as bremazocine (Romer *et al.*, 1980). Definitive pharmacological evidence for *kappa* receptor multiplicity has been lacking, however, mainly due to the absence of a *kappa* receptor subtype selective antagonist. Recently, de Costa *et al.* (1989) have described the synthesis and preliminary characterization *in vitro* of (−)-UPHIT {1*S*,2*S*-*trans*-2-isothiocyanato-4,5-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide}, an isothiocyanate benzeneacetamide, which appears to selectively bind

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ABBREVIATIONS: (−)-UPHIT, 1*S*,2*S*-*trans*-2-isothiocyanato-4,5-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide; β-FNA, β-funaltrexamine; DAMGO, Tyr-D-Ala-Gly-NMe-Gly-ol; DPDPE, [D-Pen^2, D-Pen^5]enkephalin; nor-BNI, nor-binaltorphamine; DMSO, dimethylsulfoxide.

in an apparent nonequilibrium fashion to the $kappa_1$ opioid receptor subtype in guinea pig brain. Such a profile might allow (−)-UPHIT to be utilized in the differentiation of *kappa* receptor subtypes in tests of antinociception *in vivo*.

In order to explore this hypothesis, the present study has evaluated the effects of (−)-UPHIT pretreatment *in vivo* on both U69,593- and bremazocine-induced antinociception in the mouse. The data provide pharmacological support for the concept of two *kappa*-opioid receptor subtypes in the mouse central nervous system.

Materials and Methods

Animals. Male, ICR mice (20–30 g, Harlan Industries, Cleveland, OH) were used in these studies. Mice were caged in groups of five, maintained on a 12/12-hr light/dark cycle and allowed free access to food and water until the time of nociceptive testing.

Intracerebroventricular injections. All compounds used in this study were administered into the right lateral ventricle using the method of Haley and McCormick (1957), as modified by Porreca *et al.* (1984). Under light ether anesthesia, a small midsagittal incision was made to the scalp, and injections were made 2 mm lateral and 2 mm caudal to bregma, 3 mm ventral from the skull surface using a 10-μl Hamilton syringe (Hamilton Corporation, Reno, NV). All compounds were administered in a volume of 5 μl.

Tail-flick test. The tail-flick test used in this study was the standard tail-flick test that has been described previously (Heyman *et al.*, 1987) which utilizes warm water (55°C) as the noxious stimulus, and the latency to a rapid flick of the tail as the endpoint. In order to minimize tissue damage, a 15-sec cutoff time was used. Mice which had predrug tail-flick latencies, greater than 5 sec were discarded from the study. Animals were tested only once before drug treatment. Data were expressed as percentage of maximal antinociception and were normalized to the control reaction time before dosing, percentage of antinociception = 100 × (test latency − control latency)/(15 − control latency). For time course studies, tail-flick latencies were determined at 10, 20, 30, 45, 60 and 90 min after U69,593 or bremazocine administration.

Test of sedation. Ten minutes after dosing with U69,593 or bremazocine, animals were placed on a plastic platform (10 × 8.3 × 2.5 cm), and the time spent on the platform determined, with latency (in seconds) to step off the platform taken as the endpoint. The test was terminated when either the animal stepped of the platform with all four paws or a time of 60 sec was spent on the surface of the platform. Any animal which took longer than 15 sec to step off the test platform before dosing was discarded from the study and a time of 60 sec spent on the testing device was taken as the point of maximal sedation. Data were expressed as percentage of sedation which was calculated as 100 × (test latency − control latency)/(60 − control latency).

Antagonism studies. In order to determine the *kappa* receptor-selectivity of the antinociceptive responses to U69,593 and bremazocine, animals were administered the *mu*-selective antagonist, β-FNA (18 nmol i.c.v. at −24 hr) and/or the *delta*-selective antagonist, ICI 174,864 (4.4 nmol i.c.v., −10 min) before antinociceptive testing. These dosing regimens of β-FNA and ICI 174,864 have been shown previously to selectively antagonize DAMGO- and DPDPE-induced antinociception, respectively (Heyman *et al.*, 1987). To explore the selectivity of nor-BNI, a proposed *kappa*-selective antagonist (Takemori *et al.*, 1988), this compound was given i.c.v. (0.3, 1, 3 or 10 nmol/mouse) 20 min before dosing with equieffective doses of DAMGO (0.1 nmol i.c.v.) or DPDPE (30 nmol i.c.v.). In dose-response studies with (−)-UPHIT, animals were pretreated with vehicle (50% DMSO, 5 μl i.c.v.) or (−)-UPHIT (2.2, 6.5, 22 or 65 nmol i.c.v.) 24 hr before the administration of equieffective doses of U69,593 (70 nmol) or bremazocine (25 nmol). In time course studies which examined the duration of the antagonistic action of (−)-UPHIT, animals received (−)-UPHIT (65 nmol i.c.v.) at 0, 1, 2, 4, 8, 12, 24, 48 or 96 hr before i.c.v. dosing with U69,593 (70 nmol) or bremazocine (25 nmol).

Chemicals. Bremazocine hydrochloride (racemic) (a generous gift from Dr. Dietmar Roemer, Sandoz Inc., Basel, Switzerland) and U69,593 {(5α,7α,8β)-(−)-N-methyl-N-(7-(1-pyrrolidinyl)-1-oxaspiro-(4,5)dec-8-yl)benzeneacetamide} (Sigma Chemical, St. Louis, MO) were dissolved in distilled water acidified with a small quantity (10 μl/ml) of glacial acetic acid in order to facilitate dissolution. Nor-BNI and β-FNA (Research Biochemicals Inc., Wayland MA), ICI 174,864 {N,N,-dialiyl-Tyr-(α-aminoisobutyric acid)$_2$-Phe-Leu-OH} (Cambridge Research Biochemicals, Atlantic Beach, NJ), DAMGO and DPDPE (Bachem, Torrance, CA) were dissolved in distilled water before use. (−)-UPHIT {1*S*,2*S*-*trans*-2-isothiocyanato-4,5-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide} (synthesized as described previously (de Costa *et al.*, 1989)) was dissolved in 50% DMSO before drug administration. Control studies with the 50% DMSO vehicle were also done.

Statistics. Regression lines and A_{50} values with 95% confidence intervals were determined using the computer program as described by

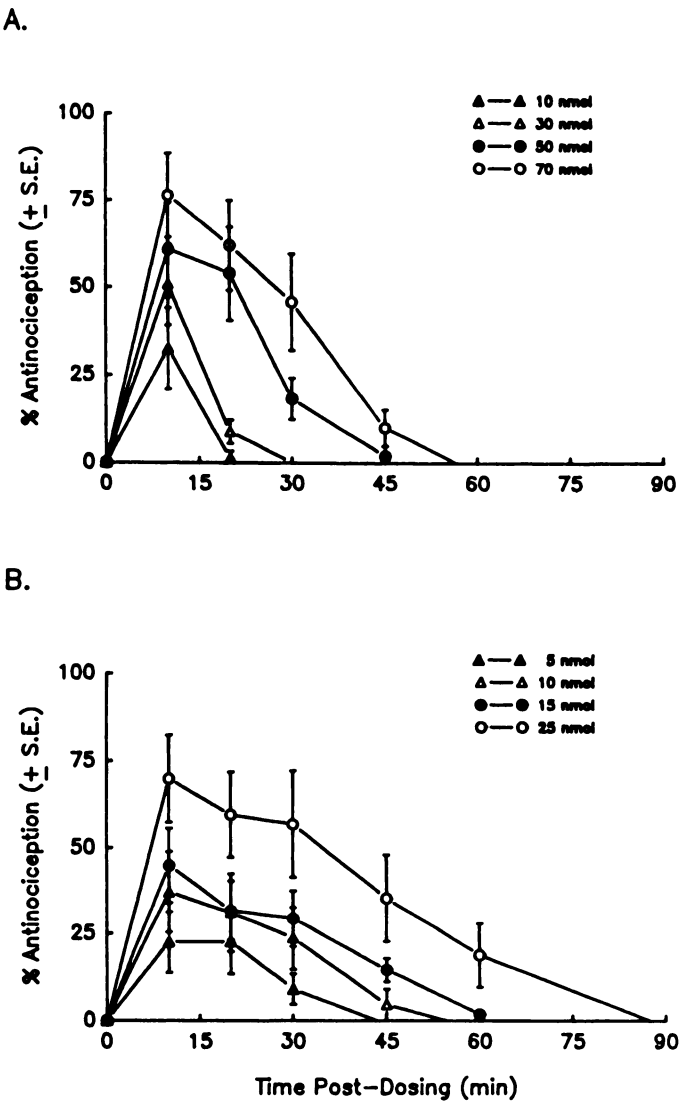

Fig. 1. A, time course of U69593-induced antinociception in the tail-flick test. B, time course of bremazocine-induced antinociception in the tail-flick test. Animals were dosed with U69593 (10, 30, 50 or 70 nmol i.c.v.) or bremazocine (5, 10, 15 or 25 nmol i.c.v.) and the percentage of antinociception in each dosing group determined at 10, 20, 30, 45, 60 and 90 min postdosing. At least 10 animals were used per dose of compound.

Tallarida and Murray (1986) (procedure 8). All data points shown are expressed as the mean ± S.E., and were obtained from 8 to 20 mice per data point, with at least four data points per dose-response curve. Comparisons of the antinociceptive responses of control and treatment groups were made using Dunnett's test (Tallarida and Murray, 1986) (procedure 44).

Results

Both U69,593 and bremazocine produced antinociception in the mouse warm water tail-flick test after i.c.v. administration (fig. 1). The antinociception produced by these compounds persisted for approximately 45 min for U69,593 and 90 min for bremazocine at the highest doses tested. The A_{50} value (95% confidence interval) to elicit this response calculated at the time of peak effect (10 min for both compounds) was 31.9 (25.9–39.2) and 13.7 (11.8–15.8) nmol/mouse for U69,593 and bremazocine, respectively. The sedative effects of each of these compounds were also evaluated (fig. 2). Both U69,593 and bremazocine produced sedation, but at doses significantly higher than those needed to induce antinociception; the A_{50} value (95% confidence interval) for sedation was 72.9 (50.2–106.0) and 108.5 (80.0 and 166.0) for U69,593 and bremazocine, respectively.

In order to explore the *kappa* receptor-selectivity of the antinociceptive responses to each of these compounds, animals were pretreated with the *mu*-selective alkylating agent, β-FNA (Ward *et al.*, 1985) at a dose and time (18 nmol, −24 hr) shown previously to be selective for antagonism of *mu* agonists (Heyman *et al.*, 1987) or the *delta*-selective antagonist, ICI 174,864 (Cotton *et al.*, 1984), at a dose and time (4.4 nmol at −10 min) shown previously to be selective for antagonism of *delta* agonists (Heyman *et al.*, 1987). Neither of these compounds were found to produce any significant effect on either U69,593- or bremazocine-induced antinociception (figs. 3 and 4). Moreover, ICI 174,864 had no effect on bremazocine-induced antinociception in β-FNA-pretreated animals as its A_{50} value was not different from that in vehicle-treated animals, 13.9 (7.6–25.5), providing further evidence of bremazocine's *kappa*-mediated actions in this test of antinociception.

Pretreatment with (−)-UPHIT at a dose of 65 nmol/mouse i.c.v., significantly attenuated U69,593-induced antinociception (fig. 5). The antagonist actions of (−)-UPHIT were significant at 8 hr postdosing, reached a maximum at 24 hr and persisted for up to 48 hr (fig. 6). In contrast, the same pretreatment with (−)-UPHIT did not alter the effects of an equiantinociceptive dose of bremazocine (fig. 5) over the same time period (fig. 6). The antagonism of U69,593, but not bremazocine, by (−)-UPHIT also appeared selective for the *kappa* opioid receptor, as pretreatment with this antagonist had no effect on the antinociceptive properties of DAMGO or DPDPE (fig. 7). Pretreatment with higher doses of (−)-UPHIT (220 nmol i.c.v.) was not possible as toxic effects were observed with the antagonist alone.

In order to more completely explore the differential antagonism by (−)-UPHIT of U69,593, but not bremazocine, full dose-response curves of the antinociceptive actions of both agonists were generated in both (−)-UPHIT- and non-(−)-UPHIT-treated animals. In addition to evaluating further both the extent and *kappa* selectivity of the (−)-UPHIT antagonistic action, the effect of the *kappa*-selective antagonist, nor-BNI on the dose-response curves of U69,593-and bremazocine-mediated antinociception were determined in both (−)-UPHIT- and non-(−)-UPHIT-treated mice. A dose of nor-BNI was selected (1 nmol i.c.v.) which had no significant effect on the antinociceptive effects of either DAMGO or DPDPE (fig. 8). Pretreatment with this dose of nor-BNI resulted in approximately 3- and 7-fold rightward shifts of the dose-response curves for U69,593 and bremazocine antinociception in non-(−)-UPHIT-pretreated mice (table 1). Pretreatment with (−)-UPHIT again had no effect on the antinociceptive responses to bremazocine, whereas producing approximately a 3-fold rightward shift in the dose-response curve of U69,593. In (−)-UPHIT- pretreated animals, nor-BNI produced no further antagonism of the U69,593 dose-effect curve (table 1). In contrast, nor-BNI was still effective in antagonizing bremazocine antinociception in (−)-UPHIT-pretreated mice (table 1).

The effect of (−)-UPHIT pretreatment on U69,593 and bremazocine-induced sedation was also evaluated (table 2). The sedation produced by U69,593 or bremazocine was not statis-

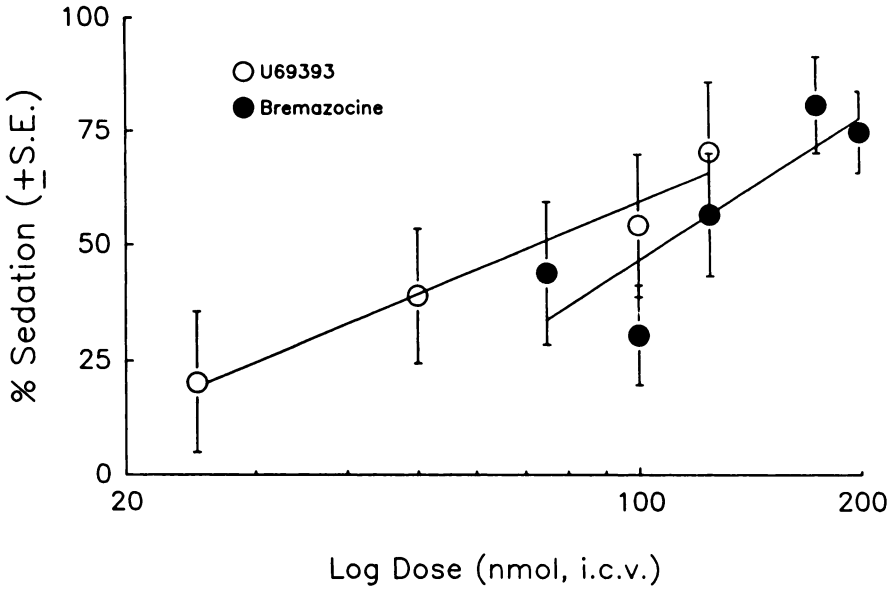

Fig. 2. Dose-response curve for U69,593- and bremazocine-induced sedation. Animals were dosed with U69,593 (25, 50, 100 or 120 nmol i.c.v.) or bremazocine (75, 100, 125, 175 or 200 nmol i.c.v.). Animals were then tested for sedation at 10 min after the administration of U69593 or bremazocine. At least 10 animals were used per dose of U69,593 or bremazocine.

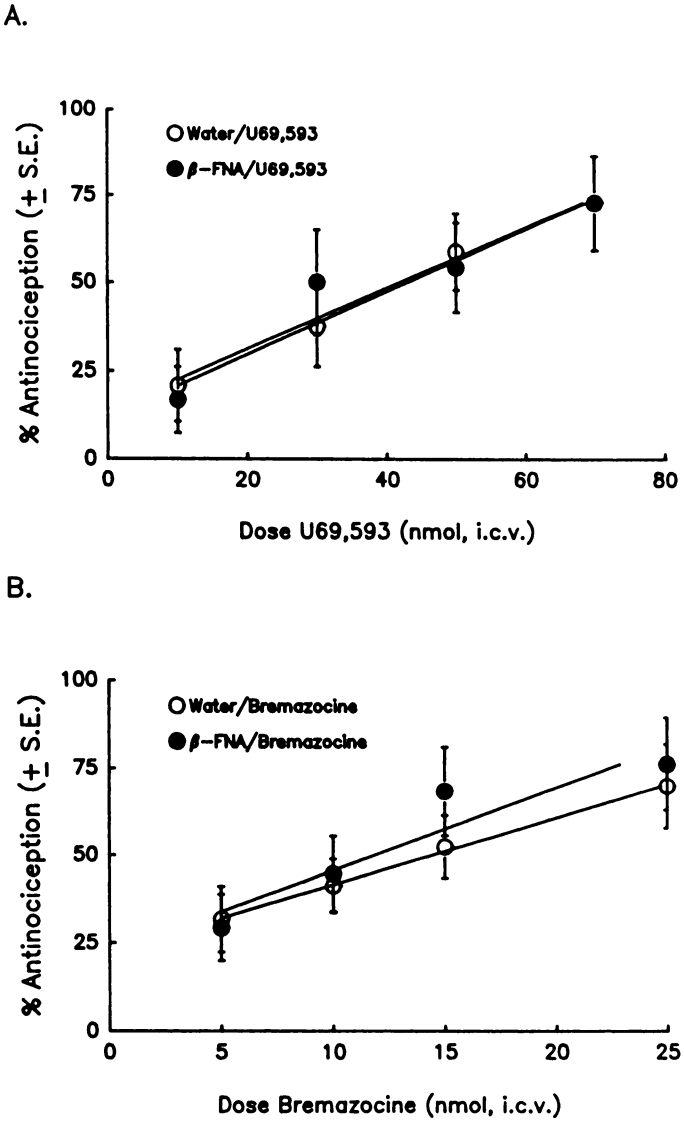

Fig. 3. A, effect of β-FNA on U69,593-mediated antinociception in the tail-flick test. B, effect of β-FNA on bremazocine-mediated antinociception in the tail-flick test. Animals were administered β-FNA (18 nmol i.c.v.) or distilled water (5 μl i.c.v.) 24 hr before dosing with U69,593 (15, 30, 45 or 70 nmol i.c.v.) or bremazocine (5, 10, 15 or 25 nmol i.c.v.). The percentage of antinociception in each treatment group was then evaluated 10 min after either U69,593 or bremazocine. At least 10 animals were used per dose of compound.

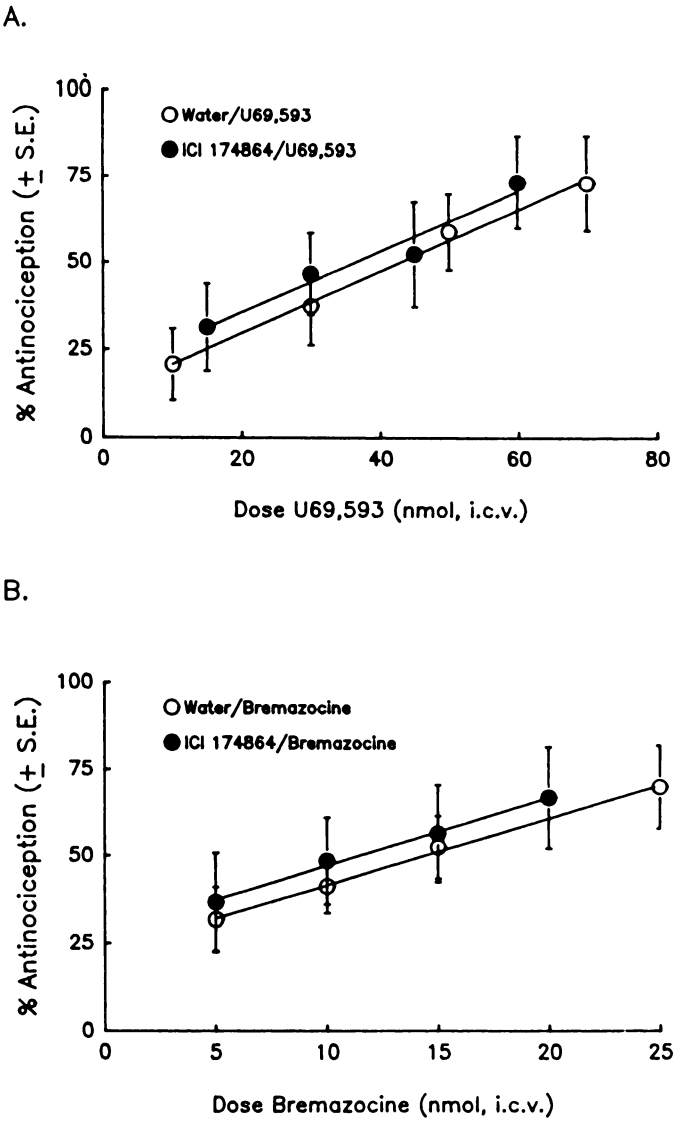

Fig. 4. A, effect of ICI 174,864 on U69,593-induced antinociception in the tail-flick test. B, effect of ICI 174,864 on bremazocine-induced antinociception in the tail-flick test. Mice were coadministered ICI 174,864 (4.4 nmol i.c.v.) or distilled water (5 μl i.c.v.) and U69,593 (10, 30, 50 or 70 nmol i.c.v., vehicle-treated) or 15, 30, 45 or 60 nmol i.c.v., ICI 174,864-treated) or bremazocine (5, 10, 15 or 25 nmol i.c.v., vehicle-treated) or 5, 10, 15 or 20 nmol, i.c.v., ICI 174,864-treated). Animals were then tested for antinociception 10 min after dosing with U69,593 or bremazocine. At least 10 animals were used per dose of U69,593 or bremazocine.

tically antagonized by pretreatment with (−)-UPHIT, nor-BNI or by nor-BNI in (−)-UPHIT-pretreated animals.

Discussion

In this study (−)-UPHIT selectively antagonized the antinociception induced by the *kappa*-selective benzeneacetamide, U69,593 (Lahti *et al.*, 1985), whereas having no effect on the antinociceptive properties of the 6,7-benzomorphan, bremazocine, which has been shown to have significant activity at the opioid *kappa* receptor (Roemer *et al.*, 1980; Hayes and Kelly, 1985). Data *in vitro* demonstrate that bremazocine is relatively nonselective, binding to *mu*, *delta* and *kappa* opioid receptors (Gillian and Kosterlitz, 1982). The action of bremazocine as an agonist or antagonist, however, at each of these receptor types remains somewhat unclear. The results of the present study suggest that bremazocine has little, if any, agonist actions at opioid *mu* or *delta* receptors in this endpoint as the observed antinociceptive effect was not blocked by either pretreatment with the irreversible *mu*-selective antagonist, β-FNA, by the coadministration of the *delta* antagonist, ICI 174,864 or by both β-FNA and ICI 174,864.

Bremazocine has also been shown to be an antagonist at the opioid *mu* receptor in the guinea pig ileum (Corbett and Kosterlitz, 1986) and at the opioid *delta* receptor in the hamster vas deferens (McKnight *et al.*, 1985). A recent study (Picker *et al.*, 1990) examining the discriminative stimulus properties of bremazocine in rats trained to discriminate morphine from water suggests that the pharmacological profile of this compound may also include some degree of agonist activity at the opioid *mu* receptor. As emphasized above, however, agonist

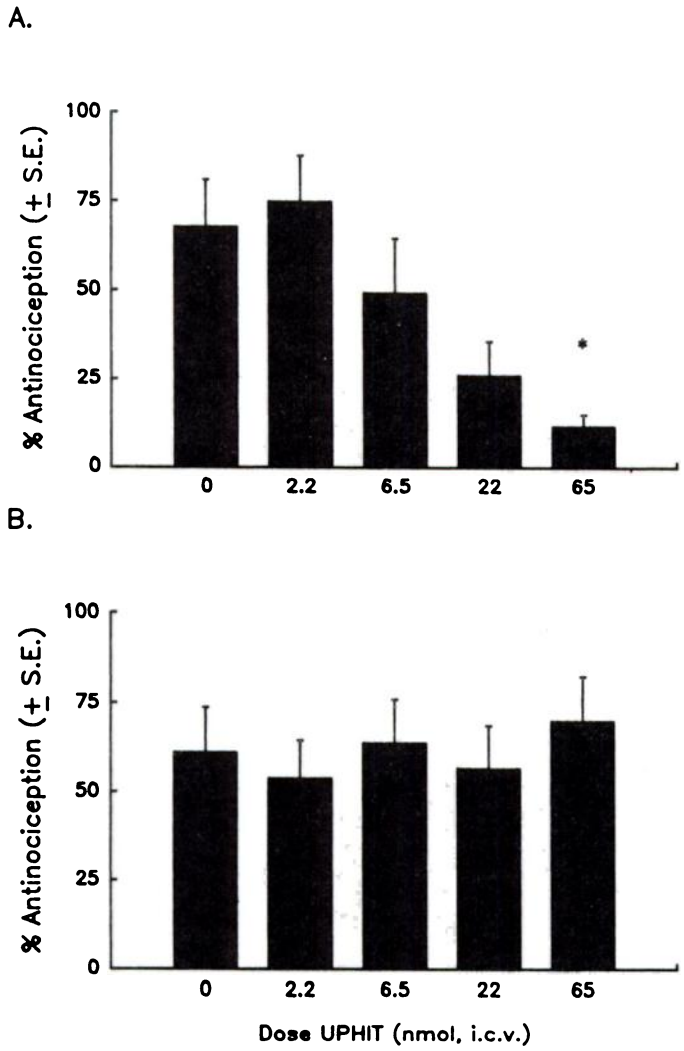

Fig. 5. A, dose-response of (−)-UPHIT-induced antagonism of the antinociceptive effect of U69,593 in the tail-flick test. B, dose-response of (−)-UPHIT-induced antagonism of the antinociceptive effects of bremazocine in the tail-flick test. Animals received vehicle (50% DMSO, 5 μl i.c.v.) or (−)-UPHIT (2.2, 6.5, 22 or 65 nmol i.c.v.) 24 hr before the administration of equieffective doses of U69,593 (70 nmol) or bremazocine (25 nmol). The percentage of antinociception was then determined 10 min after either U69,593 or bremazocine administration. Ten animals were used per dose of (−)-UPHIT. *P < .05 *vs.* vehicle-treated.

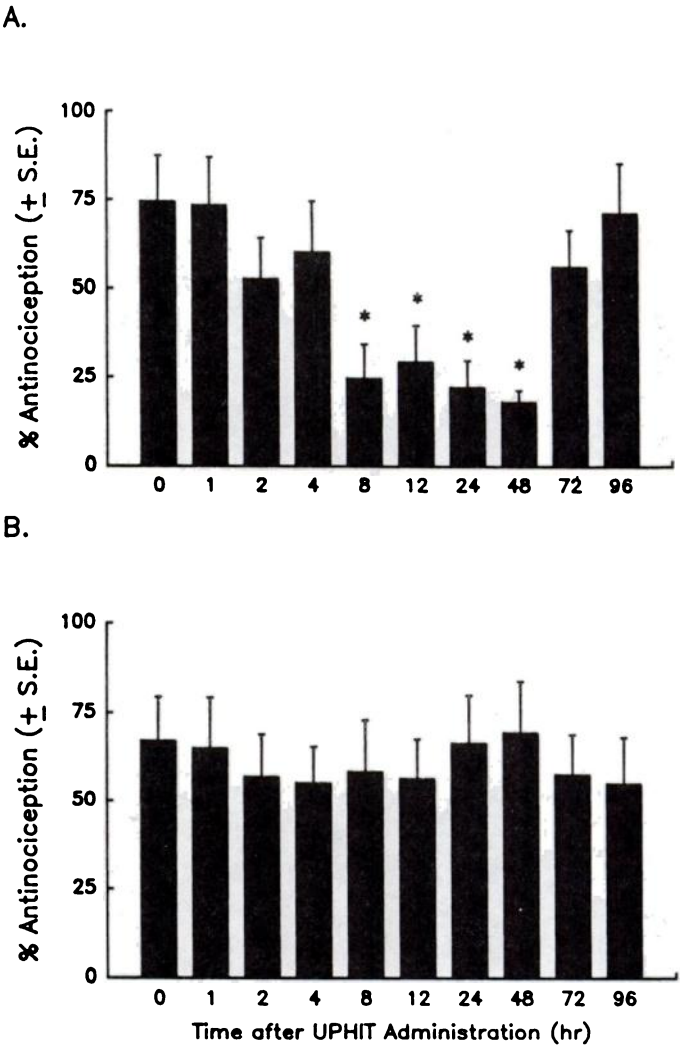

Fig. 6. A, effect of (−)-UPHIT on U69,593-mediated antinociception in the tail-flick test. B, effect of (−)-UPHIT on bremazocine-induced antinociception in the tail-flick test. Animals received (−)-UPHIT (65 nmol) at 0, 1, 2, 4, 8, 12, 24, 48, 72 or 96 hr before treatment with equiantinociceptive doses of U69,593 (70 nmol i.c.v.) or bremazocine (25 nmol i.c.v.). Antinociceptive testing was commenced 10 min postdosing with U69593 or bremazocine. Ten aniamls were used per time point per dose of U69,593 or bremazocine. *P < .05 *vs.* control.

actions of bremazocine at opioid receptors other than the *kappa* receptor were not detected in the present study.

As the present experiment relied on assessment of antinociceptive activity using a motor endpoint, these compounds were also tested for their ability to produce sedation. Our data indicate that the sedative doses for these compounds were significantly higher than those needed to produce antinociception. Additionally, it should be noted that although (−)-UPHIT and/or nor-BNI pretreatment blocked U69,593 antinociception, these compounds did not block the sedative properties of U69,593 or of bremazocine, suggesting that the antinociceptive effects of both U69,593 and bremazocine were not related to sedation, at least *via* a *kappa*-mediated mechanism.

Earlier studies (Wood *et al.*, 1981; Porreca *et al.*, 1984; Schmauss and Yaksh, 1984) have suggested that *kappa* agonists were relatively ineffective antinociceptive agents when given i.c.v., especially when animals were challenged with thermal nociceptive stimuli. However, others have shown an apparent dissociation between sedation and antinociception in mice and rats (Belknap *et al.*, 1987; Fennessy and Sawynok, 1973; Gray *et al.*, 1970; Millan, 1989). More recent studies have shown that *kappa* agonists do produce antinociception, with their activity dependent on the intensity of the nociceptive stimulus (Millan, 1989; Parsons and Headley, 1989; Piercey and Einspahr, 1989). These considerations, coupled with the finding of differential antagonism of U69,593 antinociception and sedation by (−)-UPHIT and/or nor-BNI, as well as the antinociceptive actions of bremazocine at doses 8-fold lower than those required to produce sedation, argue for a significant role of the *kappa* opioid receptor in supraspinal antinociception.

The blockade of U69,593-induced antinociception by (−)-UPHIT was demonstrated to be both dose- and time-dependent, persisting for up to 48 hr. This blockade also appeared to be selective for the *kappa* opioid receptor, as (−)-UPHIT was unable to affect either DAMGO- or DPDPE-induced antino-

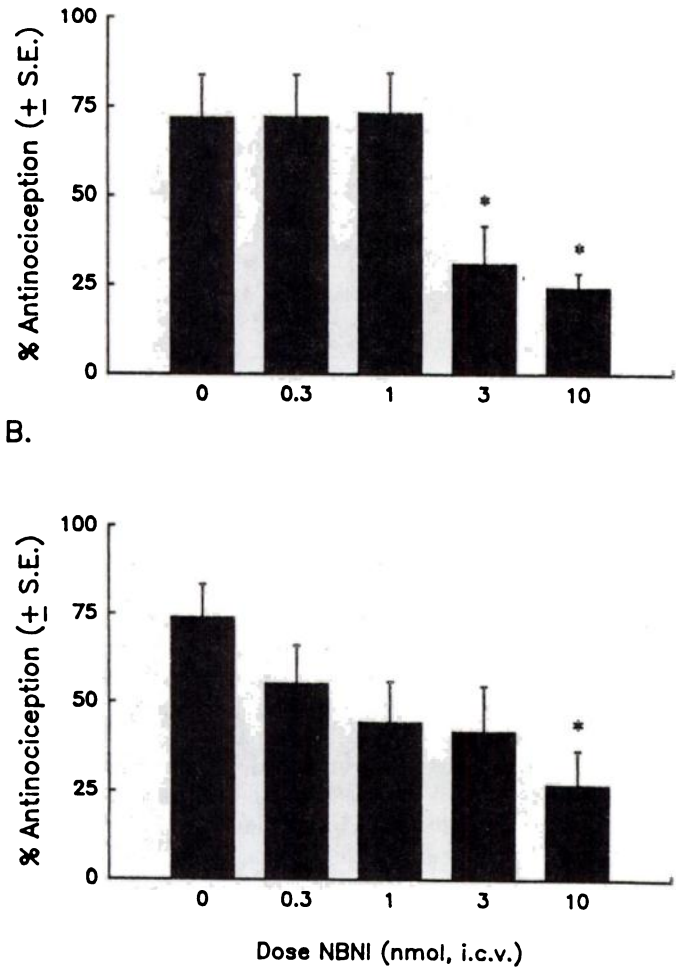

Fig. 7. A, effect of nor-BNI against DAMGO-induced antinociception in the tail-flick test. B, effect of nor-BNI against DPDPE-induced antinociception in the tail-flick test. Mice were pretreated with distilled water (5 μl i.c.v.) or nor-BNI (0.3, 1, 3 or 10 nmol i.c.v.). Twenty minutes later, the percentage of antinociceptive response to equieffective doses of DAMGO (0.1 nmol i.c.v.) or DPDPE (30 nmol i.c.v.) was determined. Ten animals were used per dose of DAMGO or DPDPE. *P < .05 *vs.* vehicle-treated.

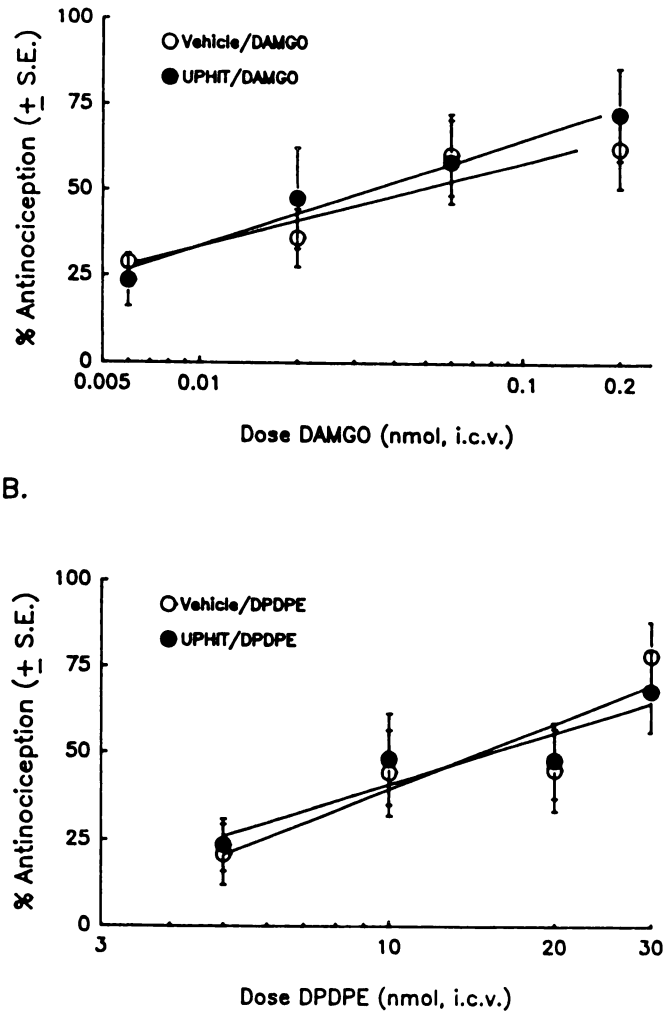

Fig. 8. A, effect of (−)-UPHIT on DAMGO-mediated antinociception in the tail-flick test. B, effect of (−)-UPHIT on DPDPE-mediated antinociception in the tail-flick test. Mice were pretreated with vehicle (50% DMSO, 5 μl i.c.v.) or (−)-UPHIT (65 nmol i.c.v.). Twenty four hours later, antinociceptive dose-effect curves were determined in animals dosed with DAMGO (0.006, 0.02, 0.06 or 0.2 nmol i.c.v.) or DPDPE (5, 10, 20 or 30 nmol i.c.v.) 10 min after their administration. At least 10 animals were used per dosing group.

ception at the dose and pretreatment time selected in these studies. In order to evaluate more fully the differential blockade by (−)-UPHIT of the antinociceptive actions of U69,593 and bremazocine, nor-BNI, a *kappa*-selective antagonist, was studied in (−)-UPHIT- and non-(−)-UPHIT-pretreated animals. Nor-BNI produced approximately a 3- and 7-fold rightward displacement in the U69,593- and bremazocine dose-response lines in non-(−)-UPHIT-pretreated mice. Although nor-BNI was not effective in antagonizing U69,593 in (−)-UPHIT-pretreated mice, this compound remained effective against bremazocine. At the doses required to produce antinociception under these conditions, it was noted that the animals were markedly sedated, which may be expected at the doses of U69,593 and bremazocine utilized after the administration of (−)-UPHIT and/or nor-BNI. However, (−)-UPHIT and/or nor-BNI were unable to block the sedation associated with bremazocine and U69,593 suggesting the possibility of a non-opioid sedative effect of these compounds. This possibility requires further evaluation using more general opioid antagonists such as naloxone.

Whereas the differential antagonism of the antinociceptive actions of U69,593 and bremazocine by (−)-UPHIT pretreatment would support the concept of separate types of *kappa* receptors, an alternative explanation involving differences in efficacy must be considered. If one hypothesized that the efficacy of bremazocine at the *kappa* receptor was much greater than that of U69,593, then partial inactivation of this receptor by (−)-UPHIT might result in an attenuation of U69,593, but not bremazocine, antinociception. In such a situation, the conclusion of heterogeneity of *kappa* opioid receptors would be incorrect. Whereas evaluation of efficacy *in vivo* is not readily achievable, it should be noted that even if bremazocine was a compound of high efficacy and was acting at the same receptor as U69,593, treatment with (−)-UPHIT would nevertheless

TABLE 1

A_{50} values with 95% confidence intervals for U69,593 and bremazocine-induced antinociception after (−)-UPHIT and/or nor-BNI pretreatment

Animals were pretreated with 50% DMSO (5 μl i.c.v.) or (−)-UPHIT (65 nmol i.c.v.) 24 hr before dosing with U69,593 or bremazocine. In nor-BNI- and/or (−)-UPHIT-treated animals, animals received (−)-UPHIT (65 nmol i.c.v.) 24 hr before antinociceptive testing and/or nor-BNI (1 nmol i.c.v.) 20 min before the administration of U69,593 or bremazocine. Ten animals were used per dose of U69,593 or bremazocine.

Agonist	Pretreatment			
	Vehicle	(−)-UPHIT	Nor-BNI	(−)-UPHIT/nor-BNI
U69,593	36.2 (25.5–51.3)	153.7 (83.5–283.1)	121.4 (57.8–255.0)	159.9 (88.7–288.4)
Bremazocine	14.0 (8.4–23.4)	14.9 (11.9–18.6)	96.8 (51.5–181.9)	97.9 (56.4–170.1)

TABLE 2

A_{50} values with 95% confidence intervals for U69,593- and bremazocine-induced sedation

Animals were pretreated with 50% DMSO (5 μl i.c.v.) or (−)-UPHIT (65 nmol i.c.v.) 24 hr before dosing with U69,593 or bremazocine. In nor-BNI- and/or (−)-UPHIT-treated animals, animals received (−)-UPHIT (65 nmol i.c.v.) 24 hr before sedative testing and/or nor-BNI (1 nmol i.c.v.) 20 min before the administration of U69,593 or bremazocine. At least 10 animals were used per dose of U69,593 or bremazocine.

Agonist	Pretreatment			
	Vehicle	(−)-UPHIT	Nor-BNI	(−)-UPHIT/nor-BNI
U69,593	62.9 (41.9–94.4)	62.3 (42.9–90.4)	104.2 (52.5–202.9)	80.8 (47.9–136.3)
Bremazocine	76.7 (45.8–128.5)	94.3 (79.1–112.4)	86.6 (59.3–125.5)	66.9 (48.0–94.0)

block some fraction of these receptor sites, as demonstrated by the observed antagonism of U69,593. In this instance, in spite of the ability of bremazocine to achieve a maximal response in the presence of some fraction of (−)-UPHIT occupied receptors, one would also expect to see a rightward displacement of the dose-response line resulting from the fewer available receptor sites. As no rightward displacement of the bremazocine dose-response line was observed after pretreatment with (−)-UPHIT, the possibility of differential antagonism resulting from differences in efficacy appears to be unlikely. Furthermore, the possibility of differential efficacy has been explored in our laboratory by a comparison of the relative efficacy of U69,593 and bremazocine in an isolated tissue preparation, the guinea pig longitudinal muscle-myenteric plexus. Our results (K. D. Wild, P. J. Horan and F. Porreca, unpublished observations), indicate that the relative efficacy of U69,593 and bremazocine, as measured by the relative fraction of receptors occupied to produce an equivalent effect, do not differ in this isolated tissue preparation. Thus, although the absolute efficacy of these compounds in producing antinociception will, of course, be different from the efficacy needed to inhibit electrically induced contractions in a bioassay tissue, the relative efficacy between U69,593 and bremazocine should remain constant, given the unknown validity of the assumption that the *kappa* receptor in the two situations is the same. Additionally, Hunter *et al.* (1990) have compared recently the effects of U69,593 and bremazocine in the rabbit vas deferens and found that, unlike U69,593, bremazocine does not elicit a full effect in this tissue. This finding would suggest that bremazocine has, if anything, lower efficacy than U69,593. In this regard, it should be noted, however, that Verlinde and de Ranter (1988) have also evaluated bremazocine in the rabbit vas deferens and show a full response for this compound. Given these findings using techniques *in vitro* as well as the failure of (−)-UPHIT to produce a rightward displacement of the bremazocine dose-response line, interpretation of the present data as reflective of *kappa* subtypes appears to be appropriate.

The present data also suggest that (−)-UPHIT selectively acts at the receptor site mediating U69,593 ($kappa_1$,) but not bremazocine ($kappa_2$) antinociception, whereas nor-BNI acts at both receptor subtypes. However, radioligand binding data in guinea pig brain after (−)-UPHIT pretreatment suggest that this compound produced a partial blockade of the $kappa_2$ binding site, or that bremazocine interacts with both $kappa_1$ and $kappa_2$ sites. The latter possibility would appear more likely based on binding studies in guinea pig brain (Rothman *et al.*, 1990, Tiberi and Magan, 1990) and rat brain (Zukin *et al.*, 1988). Additionally, binding studies in guinea pig brain (Rothman *et al.*, 1990, Tiberi and Magan, 1990) also suggest that nor-BNI is a nonselective antagonist of these receptor subtypes. Further studies *in vitro* are needed in the mouse to determine more fully the selectivity of the interaction of both (−)-UPHIT and nor-BNI with these receptor subtypes.

Substantial data *in vivo* (Iyengar *et al.*, 1986), as well as more recent autoradiographic and radioligand binding data (Nock *et al.*, 1988, 1989; Tiberi and Magan, 1990b; Zukin *et al.*, 1988) support the existence of at least two *kappa* receptor subtypes, and perhaps others (Rothman *et al.*, 1989, 1990). Collectively, these data, together with the present observations, provide evidence *in vivo* for two subtypes of the *kappa* opioid receptor. Differential antagonism has historically provided the strongest pharmacological evidence for the existence of receptor subtypes. It is tempting to speculate on the function of each of these receptor subtypes, although this will require further study with compounds such as (−)-UPHIT which, from these data obtained in the present study, selectively and perhaps *via* a nonequilibrium mechanism, blocks the $kappa_1$-opioid receptor subtype. (−)-UPHIT appears to be a useful tool for investigations into subtypes of the *kappa* opioid receptor, and may provide inroads for the development of a clinically useful *kappa* opioid analgesic, as well as promote a further understanding of *kappa*-opioid receptor multiplicity.

References

ATTALI, B., GOUARDERES, C., MAZARGUIL, H., AUDIGER, Y. AND CROS, J.: Evidence for multiple "kappa" binding sites by use of opioid peptides in the guinea-pig lumbo-sacral spinal cord. Neuropeptides. **3:** 53–64, 1982.

BELKNAP, J. K., DANIELSON, P. W., LAURSEN, S. E. AND NOORDEWIER, B.: Selective breeding for levorphanol-induced antinociception on the hot-plate assay: Commonalities in mechanism of action with morphine, pentazocine, ethylketocyclazocine, U-50488H and clonidine in mice. J. Pharmacol. Exp. Ther. **241:** 477–481, 1987.

BOWEN, W. D., GENTLEMAN, S., HERKENHAM, M. AND PERT, C. B.: Interconverting mu and delta forms of the opiate receptors in rat striatal patches. Proc. Natl. Acad. Sci. U.S.A. **78:** 4818–4822, 1981.

CORBETT, A. D. AND KOSTERLITZ, H. W.: Bremazocine is an agonist at κ-opioid

receptors and an antagonist at μ-opioid receptors in the guinea-pig myenteric plexus. Br. J. Pharmacol. **89:** 245–249, 1986.

COTTON, R., GILES, M. G., MILLER, L., SHAW, J. S. AND TIMMS, D.: ICI 174864: A highly selective antagonist for opioid δ receptor. Eur. J. Pharmacol. **97:** 331–332, 1984.

DE COSTA, B. R., BAND, L., ROTHMAN, R. B., JACOBSON, A. E., BYKOV, V., PERT, A. AND RICE, K. C.: Synthesis of an affinity ligand ('UPHIT') for *in vivo* acylation of the κ-opioid receptor. FEBS Lett. **249:** 178–182, 1989.

FENNESSY, M. R. AND SAWYNOR, J.: The effect of benzodiazepines on the analgesic effect of morphine and sodium salicylate. Arch. Int. Pharmacodyn. Ther. **204:** 77–85, 1973.

GILLAN, M. C. G. AND KOSTERLITZ, H. W.: Spectrum of the μ-, δ-, and κ-binding sites in homogenates of rat brain. Br. J. Pharmacol. **77:** 461–469, 1982.

GRAY, W. D., OSTERBERG, A. C. AND SCUTO, T. J.: Measurement of the analgesic efficacy and potency of pentazocine by the D'Amour and Smith method. J. Pharmacol. Exp. Ther. **172:** 154–162, 1970.

HALEY, T. I. AND MCCORMICK, W. G.: Pharmacological effects produced by intracerebral injections of drugs in the conscious mouse. Br. J. Pharmacol. Chemother. **12:** 12–15, 1957.

HAYES, A. AND KELLY, A.: Profile of activity of κ receptor agonists in the rabbit vas deferens. Eur. J. Pharmacol. **110:** 317–322, 1985.

HEYMAN, J. S., MULVANEY, S. A., MOSBERG, H. I. AND PORRECA, F.: Opioid δ receptor involvement in supraspinal and spinal antinociception. Brain Res. **420:** 100–108, 1987.

HEYMAN, J. S., WILLIAMS, C. L., BURKS, T. F., MOSBERG, H. I. AND PORRECA, F.: Dissociation of opioid antinociception and central gastrointestinal propulsion in the mouse: Studies with naloxazine. J. Pharmacol. Exp. Ther. **245:** 238–243, 1988.

HUNTER, J. C., LEIGHTON, G. E., MEECHAM, K. G., BOYLE, S. J., HORWELL, D. C., REES, D. C. AND HUGHES, J.: CI-977, a novel and selective agonist for the κ-opioid receptor. Br. J. Pharmacol. **101:** 103–109, 1990.

IYENGER, S., KIM, H. S. AND WOOD, P. L.: Effects of kappa opiate agonists on neurochemical and neuroendocrine indices: Evidence for kappa receptor subtypes. Life Sci. **39:** 637–644, 1986.

JIANG, Q., TAKEMORI, A. E., SULTANA, M., PORTOGHESE, P. S., BOWEN, W. D., MOSBERG, H. I. AND PORRECA, F.: Differential antagonism of opioid delta antinociception by [D-Ala^2, Leu^5, Cys^6]enkephalin (DALCE) and naltrindole 5′-isothiocyanate (NTII): Evidence for *delta* receptor subtypes. J. Pharmacol. Exp. Ther. **257:** 1069–1075, 1991.

LAHTI, R. A., MICKELSON, M. M. AND VON VOIGTLANDER, P. F.: [^{3}H]U-69593: A highly selective ligand for the opioid κ receptor. Eur. J. Pharmacol. **109:** 281–284, 1985.

LING, G. S., SPEIGEL, K., LOCKHART, S. H. AND PASTERNAK, G. W.: Separation of opioid analgesia from respiratory depression: Evidence for different receptor mechanisms. J. Pharmacol. Exp. Ther. **232:** 149–155, 1985.

LORD, J. A. H., WATERFIELD, A. A., HUGHES, J. AND KOSTERLITZ, H. W.: Endogenous opioid peptides, multiple agonists and receptors. Nature (Lond.) **267:** 495–497, 1977.

MARTIN, W. R., EADES, C. G., THOMPSON, J. A., HUPPLER, R. E. AND GILBERT, P. E.: The effects of morphine- and nalorphine-like drugs in the nondependent and morphine-dependent chronic spinal dog. J. Pharmacol. Exp. Ther. **197:** 517–532, 1976.

MATTIA, A., VANDERAH, T., MOSBERG, H. I. AND PORRECA, F.: Lack of antinociceptive cross-tolerance between [D-Pen^2, D-Pen^5]enkephalin and [D-ALA^2]-deltorphin II in mice: Evidence for *delta* opioid receptor subtypes. J. Pharmacol. Exp. Ther., in press, 1991.

MCKNIGHT, A. T., CORBETT, A. D., MARCOLI, M. AND KOSTERLITZ, H. W.: The opioid receptors in the hamster vas deferens are of the δ-type. Neuropharmacol. **24:** 1011–1017, 1985.

MILLAN, M. J.: *Kappa*-opioid receptor-mediated antinociception in the rat. I. Comparative actions of *mu*- and *kappa*-opioids against noxious thermal, pressure and electrical stimuli. J. Pharmacol. Exp. Ther. **251:** 334–341, 1989.

NOCK, B., GIORDANO, A. L. AND CICERO, T. J.: ICI 197067 is a selective ligand for the U-69593-sensitive κ opiate binding site. Eur. J. Pharmacol. **162:** 385–386, 1989.

NOCK, B., RAJPARA, A., O'CONNOR, J. H. AND CICERO, T. J.: Autoradiography of [^{3}H]U-69593 binding sites in rat brain: Evidence for κ opioid receptor subtypes. Eur. J. Pharmacol. **154:** 27–34, 1988.

PARSONS, C. G. AND HEADLEY, P. M.: Spinal antinociceptive actions of μ-and κ-opioids: The importance of stimulus intensity in determining 'selectivity' between reflexes to different modalities of noxious stimulus. Br. J. Pharmacol. **98:** 523–532, 1989.

PICKER, M. I., DOTY, P., NEGUS, S. S., MATTOX, S. R. AND DYKSTRA, L. A.: Discriminative stimulus properties of U50,488 and morphine: Effects of training dose on stimulus substitution patterns produced by *mu* and *kappa* opioid agonists. J. Pharmacol. Exp. Ther. **254:** 13–22, 1990.

PIERCEY, M. F. AND EINSPAHR, F. I.: Spinal analgesic actions of *kappa* receptor agonists, U-50488H and spiradoline (U-62066). J. Pharmacol. Exp. Ther. **251:** 267–271, 1989.

PORRECA, F., MOSBERG, H. I., HURST, R., HRUBY, V. J. AND BURKS, T. F.: Roles of *mu*, *delta* and *kappa* opioid receptors in spinal and supraspinal mediation of gastrointestinal transit effects and hot-plate analgesia in the mouse. J. Pharmacol. Exp. Ther. **230:** 341–348, 1984.

ROMER, D., BUSCHER, H., HILL, R. C., MAURER, R., PETCHNER, T. I., WELLE, H. B. A., BAKEL, H. C. C. K. AND AKKERMAN, A. M.: Bremazocine, a potent, long-acting opiate kappa-agonist. Life. Sci. **27:** 971–978, 1980.

ROTHMAN, R. B., BOWEN, W. D., BYKOV, V., SCHUMACHER, U. K., PERT, C. B., JACOBSON, A. E., BURKE, T. R., JR. AND RICE, K. C.: Preparation of rat brain membranes greatly enriched with either type-I-delta or type-II-delta opiate binding sites using site directed alkylating agents: Evidence for a two-site allosteric model. Neuropeptides **4:** 201–215, 1984.

ROTHMAN, R. B., BOWEN, W. D., HERKENHAM, M., JACOBSON, A. E., RICE, K. C. AND PERT, C.: A quantitative study of [^{3}H]D-Ala^2-D-Leu^5-enkephalin binding to rat brain membranes: Evidence that oxymorphone is a noncompetitive inhibitor of the lower affinity δ binding site. Mol. Pharmacol. **27:** 399–408, 1985.

ROTHMAN, R. B., BOWEN, W. D., SCHUMACHER, U. K. AND PERT, C. B.: Effect of beta-FNA on opiate receptor binding: Preliminary evidence for two types of mu receptors. Eur. J. Pharmacol. **95:** 147–148, 1983.

ROTHMAN, R. B., BYKOV, V., de COSTA, B. R., JACOBSON, A. E., RICE, K. C. AND BRADY, L. S.: Interaction of endogenous opioid peptides and other drugs with four kappa opioid binding sites in guinea pig brain. Peptides **11:** 311–331, 1990.

ROTHMAN, R. B., FRANCE, C. P., BYKOV, V., de COSTA, B. R., JACOBSON, A. E., WOODS, J. H. AND RICE, K. C.: Pharmacological activities of optically pure enantiomers of the *kappa* opioid agonist, U50,488, and its cis diastereomer: Evidence for 3 κ receptor subtypes. Eur. J. Pharmacol. **167:** 345–353, 1989.

ROTHMAN, R. B., JACOBSON, A. E., RICE, K. C. AND HERKENHAM, M.: Autoradiographic evidence for two classes of mu opioid binding sites in rat brain using [^{125}I]FK33824. Peptides **8:** 1015–1021, 1987.

SCHMAUSS, C. AND YAKSH, T. L.: *In vivo* studies on spinal opiate receptor systems mediating antinociception. II. Pharmacological profiles suggesting a differential association of *mu*, *delta* and *kappa* receptors with visceral chemical and cutaneous thermal stimuli in the rat. J. Pharmacol. Exp. Ther. **228:** 1–12, 1984.

SHIMOHIGASHI, Y., COSTA, T., PFEIFFER, A., HERZ, A., KIMURA, H. AND STAMMER, C. H.: D-Phe^4-enkephalin analogs: Delta receptors in rat brain are different from those in mouse vas deferens. FEBS Lett. **222:** 71–74, 1987.

TAKEMORI, A. E., HO, B. Y., NAESETH, J. S. AND PORTOGHESE, P. S.: Norbinaltorphamine, a highly selective *kappa*-opioid antagonist in analgesic and receptor binding assays. J. Pharmacol. Exp. Ther. **246:** 255–258, 1988.

TAKEMORI, A. E. AND PORTOGHESE, P. S.: The use of μ opioid receptors in the mouse vas deferens for the study of opiate tolerance. Eur. J. Pharmacol. **174:** 267–271, 1989.

TALLARIDA, R. J. AND MURRAY, R. B.:: Manual of Pharmacologic Calculations, Springer-Verlag, New York, 1986.

TIBERI, M. AND MAGNAN, J.: Demonstration of the heterogeneity of the κ-opioid receptors in guinea-pig cerebellum using selective and nonselective drugs. Eur. J. Pharmacol. **188:** 379–389, 1990.

VAUGHN, L. K., WIRE, W. S., DAVIS, P., SHIMOHIGASHI, Y., TOTH, G., KNAPP, R. J., HRUBY, V. J., BURKS, T. F. AND YAMAMURA, H. I.: Differentiation between rat brain and mouse vas deferens δ opioid receptors. Eur. J. Pharmacol. **177:** 99–101, 1990.

VERLINDE, C. AND DE RANTER, C.: Assessment of the κ-opioid activity of a series of 6,7-benzomorphans in the rabbit vas deferens. Eur. J. Pharmacol. **153:** 83–87, 1988.

WARD, S. J., FRIES, D. S., LARSON, D. L., PORTOGHESE, P. S. AND TAKEMORI, A. E.: Opioid receptor binding characteristics of the non-equilibrium antagonist, β-funaltrexamine (β-FNA). Eur. J. Pharmacol. **107:** 232–330, 1985.

WOLOZIN, B. L. AND PASTENAK, G. W.: Classification of multiple morphine and enkephalin binding sites in the central nervous system. J. Pharmacol. Exp. Ther. **238:** 625–631, 1981.

WOOD, P. L., RACKHAM, A. AND RICHARD, J.: Spinal analgesia: Comparison of the mu agonist morphine and the kappa agonist, ethylketocyclazocine. Life Sci. **28:** 2119–2225, 1981.

ZUKIN, R. S., EGHBALI, M., OLIVE, D., UNTERWALD, E. M. AND TEMPEL, A.: Characterization and visualization of rat and guinea pig brain κ opioid receptors: Evidence for κ_1 and κ_2 opioid receptors. Proc. Natl. Acad. Sci. U.S.A. **85:** 4061–4065, 1988.

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