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Spinal Opioid Receptors and Adenosine Release: Neurochemical and Behavioral Characterization of Opioid Subtypes[1]

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ABSTRACT

Release of adenosine from the spinal cord contributes to spinal antinociception by morphine. Morphine induces a Ca^{++}-dependent release of adenosine from dorsal spinal cord synaptosomes, which is augmented under partially depolarizing conditions. The present study examined the opioid receptor subtypes involved in this release, and determined whether adenosine is an important mediator of antinociception induced by the spinal administration of selective opioid agonists in rats. Nanomolar and micromolar concentrations of the selective *mu* opioid agonists DAMGO {[D-Ala², N-Me-Phe⁴,Gly⁵-ol]enkephalin} and PLO17 {[N-MePhe³,D-Pro⁴]morphiceptin} induced release of adenosine in a biphasic manner in the presence of a partial depolarization (addition of 6 mM K^+ to the Krebs' medium). The *delta* opioid agonists DPDPE {[D-Pen²,D-Pen⁵] enkephalin} and DELT {[D-Ala²,Cys⁴]deltorphin} and the *kappa* opioid agonist U50488H {*trans*-(±)-3,4-dichloro-N-methyl-N-(2-(1-pyrroli-zemeacetamide} had little effect on the release of adenosine except at high micromolar concentrations. Release of adenosine by *mu* (nanomolar) and *delta* (micromolar) ligands is Ca^{++}-dependent, whereas the *kappa* (micromolar) receptor ligand releases adenosine *via* a Ca^{++}-independent mechanism. Behavioral antinociception using the hot-plate threshold test revealed that intrathecal administration of the *mu* and *delta* opioid receptor agonists produced dose-dependent antinociception with an order of potency of DAMGO, PLO17 > morphine, DELT > DPDPE. An ED_{75} dose of morphine, DAMGO or PLO17 was attenuated dose-dependently by intrathecal pretreatment with the adenosine receptor antagonist caffeine. Caffeine did not block the antinociceptive response to *delta* agonists, but in fact augmented antinociception when combined with DPDPE and DELT. This augmentation was dose-dependent. This study demonstrates that activation of the *mu* receptor subtype is responsible for the opioid-induced release of adenosine from the spinal cord, that such release contributes to the spinal antinociception by *mu* agonists and that only release evoked by low doses of opioids is behaviorally relevant.

It is well established that the spinal administration of *mu*, *delta* and *kappa* opioid receptor agonists produces spinal antinociception in human and animal studies (reviewed by Yaksh and Malmberg, 1994). Opioid receptor binding sites are present in the substantia gelatinosa of the dorsal spinal horn at sites both pre- and postsynaptic to primary afferent terminals (Besse *et al.*, 1990). One mechanism by which morphine produces antinociception is by acting presynaptically to inhibit the release of neurotransmitters/modulators such as substance P from afferent nerve terminals in the spinal cord (Jessell and Iversen, 1977; Yaksh and Noueihed, 1985). However, more recent data have indicated that opioids can exert multiple effects on such release (Mauborgne *et al.*, 1987; Pohl *et al.*, 1989; Suarez-Roca and Maixner, 1993). At nanomolar concentrations, opioids enhance the K^+-evoked

release of substance P from spinal cord slices (Mauborgne *et al.*, 1987; Pohl *et al.*, 1989) and trigeminal nucleus slices (Suarez-Roca and Maixner, 1993), in addition to exhibiting the characteristic inhibition of release at higher doses. Opioids also can produce dual effects on the release of cholecystokinin from the spinal cord (Benoliel *et al.*, 1991). Spinally mediated excitatory effects of opioids also have been demonstrated in electrophysiological paradigms in which low nanomolar doses of opioid agonists enhance C-fiber activity (Dickenson and Sullivan, 1986; Sullivan and Dickenson, 1988). In cultured dorsal root ganglion neurons, opioids produce complex effects on action potential duration with prolongation at low nanomolar concentrations and shortening at micromolar concentrations (Chen *et al.*, 1988; Shen and Crain, 1989).

Adenosine is a modulator of sensory transmission within the spinal cord after noxious stimulation (reviewed by Sawynok and Sweeney, 1989; Salter *et al.*, 1993). The i.t. admin-

ABBREVIATIONS: i.t., intrathecal; DAMGO, [d-Ala²,N-Me-Phe⁴,Gly⁵-ol]enkephalin; DPDPE, [D-Pen²,D-Pen⁵]enkephalin; DELT, [D-Ala²,Cys⁴] deltorphin; %MPE, maximum percentage of effect; PLO17, [N-mePhe³,D-Pro⁴]morphiceptin; U50488H, *trans*-(±)-3,4, dichloro-N-methyl-N-(2-(1-pyrroli-zemeacetamide); i.c.v., intracerebroventricular.

istration of adenosine receptor agonists produces antinociception in thermal threshold tests (reviewed by Sawynok, 1991), in the formalin test (Malmberg and Yaksh, 1993) and in a chemical model of allodynia (Sosnowski and Yaksh, 1989). The i.t. administration of methylxanthine adenosine receptor antagonists attenuates the antinociceptive effects of morphine (DeLander and Hopkins, 1986; Sweeney et al., 1987; Yang et al., 1994), DAMGO, DPDPE and β-endorphin (DeLander et al., 1992). The release of adenosine from the spinal cord has been proposed to contribute to opioid-induced antinociception on the basis of such behavioral observations.

This adenosine release hypothesis of opioid action is supported by neurochemical experiments, as morphine has been shown to release adenosine from the spinal cord both from synaptosomes and the intact spinal cord (Sweeney et al., 1987, 1989). This release is receptor mediated, Ca^{++}-dependent and originates from capsaicin-sensitive primary afferent neurons (Sweeney et al., 1989). Originally, release from dorsal spinal cord synaptosomes was shown to occur at 10 to 100 μM morphine (Sweeney et al., 1987) but, more recently, morphine has been shown to produce two phases of release at 10 nM and 1 to 100 μM in the presence of partial depolarization produced by the addition of 6 mM KCl to the Krebs' medium (Cahill et al., 1993b).

In the present study, we have examined the opioid receptor subtypes involved in the release of adenosine from rat dorsal spinal cord in both neurochemical and behavioral studies. This was accomplished by determining the effect of the selective mu opioid receptor agonists PLO17, DAMGO and morphine, the delta opioid receptor agonists DPDPE and DELT and the kappa opioid receptor agonist U50488H, on the release of adenosine from synaptosomes prepared from the dorsal spinal cord. In addition, the effects of caffeine, a methylxanthine adenosine antagonist, on antinociception produced by mu and delta opioid agonists in the hot-plate test after i.t. administration were determined. Caffeine was chosen as it is a broad spectrum adenosine receptor antagonist (blocking adenosine A1 and A2 receptors with comparable affinity) and is water soluble (Daly, 1993).

Recently, delta opioid receptor subtypes have been identified pharmacologically after the development of selective agonists and antagonists. DPDPE, a delta-1 agonist, and delta-2 agonists [D-Ala^2,Glu^4]deltorphin (Jiang et al., 1991; Sofuoglu et al., 1991a,b; Mattia et al., 1991, 1992) and DELT (Horan et al., 1992) induce antinociception after either i.c.v. or i.t. administration. An additional objective of this study was to examine whether different delta opioid receptor subtypes release adenosine from the spinal cord and produce a methylxanthine-sensitive antinociception. Some results presented in this paper have appeared previously in abstract form (Cahill et al., 1992, 1993c).

Methods

Animals. Male Sprague-Dawley rats (250–325 g; Charles River, Quebec, Canada) were housed in groups of two for in vitro experiments or singly for behavioral experiments. They were maintained on a 12/12 hr light/dark cycle and were allowed free access to food and water until the time of experimental testing. Experiments were carried out according to a protocol approved by the Animal Care Committee of Dalhousie University, Nova Scotia.

Neurochemical Experiments

Preparation of spinal cord synaptosomes and adenosine release. Release of adenosine from spinal cord synaptosomes was studied as described previously (Cahill et al., 1993a). Spinal cord synaptosomes were prepared from the dorsal spinal cord in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered sucrose and isolated by differential centrifugation. Synaptosomes were then preincubated in Krebs-Henseleit bicarbonate medium at 37°C before experimentation.

Aliquots of the synaptosomal (P2 fraction) suspension (350 μl) were added to microfuge tubes containing the drugs to be investigated (final volume of 365 μl) and were incubated for 10 min at 37°C. Two control tubes containing only synaptosomes and Krebs' medium were included in each experiment. One tube was centrifuged immediately before the 10-min incubation (time, 0 min) to indicate the quantity of adenosine released during the preparation of the synaptosomes. The second tube was incubated for 10 min to yield the amount of adenosine released in the absence of drugs (basal release). In all cases, release was terminated by centrifugation; the supernatant was deproteinated then derivatized with 4.5% chloroacetaldehyde to form the etheno-derivative of adenosine which was then quantitated by high-performance liquid chromatography with fluorescence detection. Adenosine release was expressed as picomoles per milligram of protein per 10 minutes. Basal adenosine values were calculated by subtracting release at 0 min from the total adenosine released in 10 min. Evoked values were calculated by subtracting the total release in the absence of drugs from total release with drugs present. When an additional 6 mM K^+ was present to partially depolarize the synaptosomes, evoked values were expressed as above the 6 mM K^+ value. In all experiments, the addition of 6 mM K^+ did not produce a significant intrinsic release.

For Ca^{++}-free experiments, synaptosomes were prepared as above except the Krebs' medium was Ca^{++}-free. Ca^{++} was added back to synaptosomes during the drug incubation stage for controls only, thereby allowing comparisons to be made between Ca^{++}-free conditions and Krebs' with normal Ca^{++} concentrations. Experiments to determine whether release of adenosine originates as adenosine per se or a nucleotide that is converted to adenosine extracellularly were performed by the addition of ecto-5'-nucleotidase inhibitors (α,β-methylene ADP and 5'GMP) at the drug incubation stage.

In all experiments, a positive control of either 24 mM K^+ or 100 μM morphine was included; both of these evoke maximal release of adenosine (Cahill et al., 1993a).

Behavioral Experiments

Acute injection. Opioid agonists were injected directly into the subarachnoid space of rats (275–300 g) by lumbar puncture according to the method of Hylden and Wilcox (1980). Injections of 20 μl were made under halothane anesthesia via a 30-gauge needle into an intravertebral space at approximately the level of the 5th or 6th lumbar vertebrae. A characteristic tail-flick response confirmed entry into the subarachnoid space. Acute i.t. injections were used to obtain dose-response relationships of each mu and delta agonist in the hot-plate test.

Intrathecal cannulation and injection. Rats were implanted with chronic i.t. cannulae under halothane anesthesia as described previously (Sweeney et al., 1987). After surgery, rats were given penicillin G (PenlongXL) i.m. and 10 ml of lactated Ringer's solution s.c. to promote recovery from surgery. Only animals exhibiting no motor deficits as the result of surgery were used for antinociception experiments. Experiments were commenced 7 to 10 days after surgery and animals were only used for one experiment. Chronic cannulae were implanted in animals used for studying the methylxanthine sensitivity of selective opioid agonists. Animals implanted with chronic cannulae were used for antagonist experiments as acute injection of caffeine did not block morphine-induced antinociception. All opioid agonists and caffeine were injected in a volume of 10 μl

(cannula volume, 8 µl) followed by 10 µl of saline flush to ensure complete delivery of the drugs. Caffeine, 10 µl i.t., was injected followed by 10 µl of saline flush, 15 min before the i.t. administration of the opioid agonist tested.

Antinociceptive testing. Antinociception was quantitated by using a constant temperature hot-plate test (50°C ± 0.5°C). The response latency to a hindpaw lick was recorded (base line, 7–12 sec; cutoff, 50 sec). In the absence of a response, the animal was removed from the hot plate at 50 sec to avoid tissue injury, and assigned this latency.

Agonist studies. The first series of experiments determined the dose-response and time course of acute i.t. *mu* and *delta* agonists on the hot-plate response. The following doses for each opioid agonist were examined on the hot-plate test after acute i.t. administration: DAMGO (0.019–5.8 nmol), PLO17 (0.19–5.6 nmol), morphine (1.5–15 nmol), DPDPE (1.35–81.2 nmol) and DELT (0.34–11.5 nmol).

Antagonist studies. For evaluation of adenosine involvement in opioid antinociception, caffeine (103–515 nmol) or a saline control was injected i.t. 15 min before the i.t. administration of the opioid agonist tested. The degree of antagonism determined subsequent doses of caffeine to obtain an IC_{50} value for the methylxanthine-sensitivity of each opioid agonist.

Statistics and calculation of data. By using the peak effect of the particular opioid agonist, dose-response curves were generated by plotting the %MPE vs.. log dose. Response latency data from the hot-plate measurements were converted to %MPE according to the formula:

$$MPE = \frac{(\text{postdrug latency} - \text{baseline latency})}{(\text{cutoff time} - \text{baseline latency})} \times 100$$

Time effect and dose-response data are presented as mean ± S.E.M. The effectiveness of an opioid agonist in producing antinociception is presented as the ED_{50} on the hot-plate test. The IC_{50} values for caffeine refer to the dose producing a 50% reduction in the effect of an ED_{75} dose of agonist. ED_{50} values were interpolated by the computer program INPLOT (Graph Pad) and confidence intervals were calculated using InSTAT (Graph Pad). Statistical comparisons were made by using analysis of variance followed by the Student's-Newman-Keuls test.

Drugs. Drugs used in this study were obtained from the following sources: DPDPE and PLO17 (Peninsula Laboratories, San Carlos, CA); α,β-methylene ADP, 5'GMP and DAMGO (Sigma Chemical Co., St. Louis, MO); morphine sulfate (British Drug Houses, Ontario, Canada); DELT (kindly supplied by Dr. Frank Porreca); and U50488H [trans-(±)-3,4-dichloro-N-methyl-N-(2-(1-pyrroli-zemeac-etamide] (Upjohn, Kalamazoo, MI). All drugs were dissolved in physiological saline (0.9% NaCl w/v).

Results

Opioid-evoked release of adenosine from dorsal spinal cord synaptosomes. After the addition of 6 mM K^+ (total K^+ concentration of 10.7 mM), which by itself did not alter the release of adenosine, a significant enhancement of the release of adenosine occurred at 10^{-8} and 10^{-6} M morphine, but not 10^{-7} M morphine (Cahill *et al.*, 1993b) (see fig. 1A). The selective *mu* opioid agonist, DAMGO, had little effect on the release of adenosine alone (fig. 1B), but the addition of 6 mM K^+ significantly augmented the release of adenosine in a biphasic manner similar to morphine. DAMGO now evoked the release of adenosine at 10^{-9} to 10^{-8} M and 10^{-6} to 10^{-4} M, but not at 10^{-7} M. PLO17, a highly selective *mu* opioid agonist, significantly increased the release of adenosine above basal levels at 10^{-7} M, and this release was enhanced in the presence of an additional 6 mM
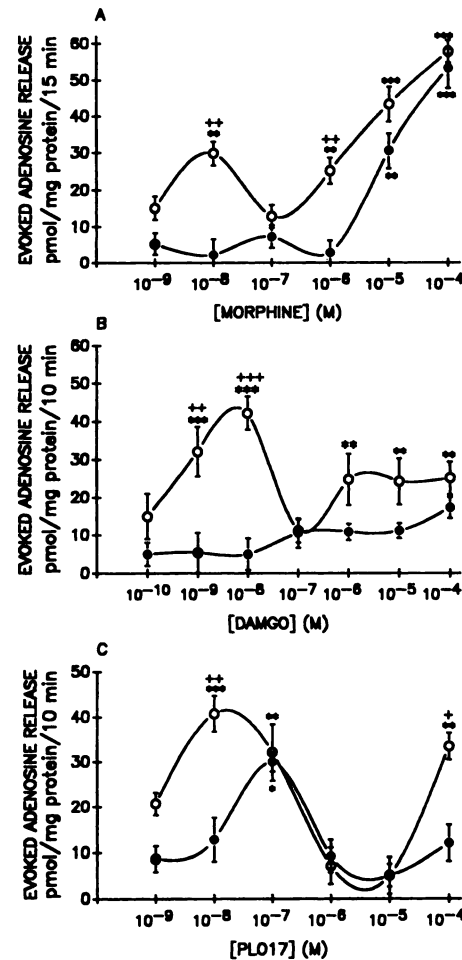

Fig. 1. Dose-related release of adenosine by the *mu* opioid agonists (A) morphine, (B) DAMGO and (C) PLO17 in the absence (●) or presence (○) of an additional 6 mM K^+ added to the Krebs-Henseleit medium (total concentration of 10.7 mM K^+). Values represent means ± S.E.M. of the adenosine released by opioids above basal release values or above values obtained after the addition of 6 mM K^+ for $n = 6$. *$P < .05$, **$P < .01$, ***$P < .001$ compared to basal release values, +$P < .05$, ++$P < .01$ and +++$P < .001$ compared to evoked adenosine release in the absence of the additional 6 mM K^+. A: basal, 172 ± 7; 6 mM K^+ 180 ± 5 pmol/mg of protein per 15 min (data from Cahill *et al.*, 1992). B: basal, 111 ± 7; 6 mM K^+ 112 ± 5 pmol/mg of protein per 10 min. C: basal, 125 ± 8; 6 mM K^+ 132 ± 10 pmol/mg of protein per 10 min. In all cases, the addition of this concentration of K^+ did not induce a significant increase in release.

K^+ at both nanomolar and micromolar doses (fig. 1C). The relative nanomolar and micromolar potencies of the *mu* opioid agonists in the presence of 6 mM K^+ in releasing adenosine from the dorsal spinal cord are shown in figure 2. At nanomolar concentrations, the more selective *mu* opioid ligands, DAMGO and PLO17, appeared to be more potent than morphine but, at micromolar concentrations, morphine was more potent than DAMGO and PLO17.

The potential role of *delta* opioid receptors in releasing adenosine from the spinal cord was examined by using the specific opioid agonists DPDPE (*delta*-1 agonist) and DELT (*delta*-2 agonist). DPDPE had no effect on adenosine release except at the highest dose (10^{-4} M) in the presence of added 6 mM K^+ (fig. 3A). DELT increased adenosine release at 10^{-4} M, but there was no additional enhancement of release in the
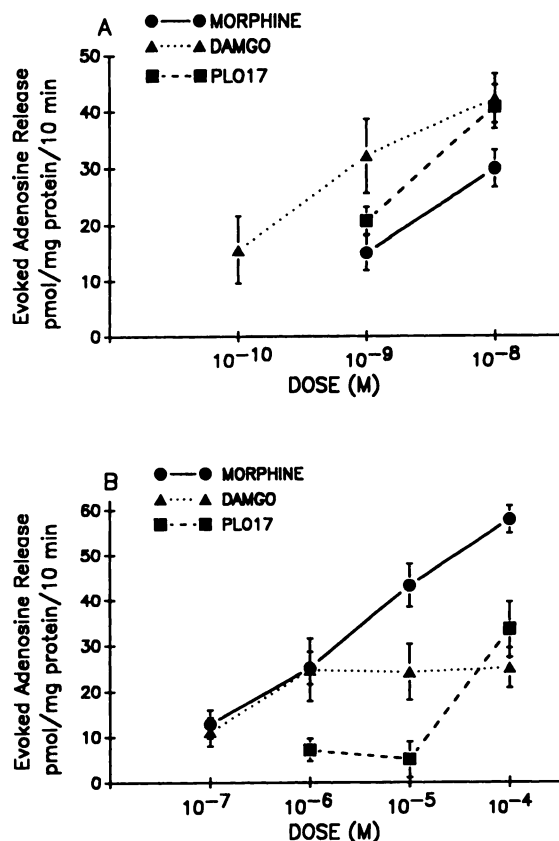
Fig. 2. Comparison of nanomolar and micromolar potencies of the *mu* opioid agonists morphine, DAMGO and PLO17 in their ability to evoke the release of adenosine from dorsal spinal cord synaptosomes. Values represent means ± S.E.M. for *n* = 6.

presence of an additional 6 mM K$^+$ (fig. 3B). Neither of the *delta* opioid agonists enhanced the release of adenosine at nanomolar concentrations, either with or without the addition of the 6 mM K$^+$.

The *kappa* opioid agonist U50488H was used to examine the potential *kappa* receptor involvement in release of adenosine from the spinal cord. U50488H had no effect on adenosine release with normal Krebs' medium, but an enhancement of release occurred at 10^{-5} to 10^{-4} M when synaptosomes were partially depolarized with 6 mM K$^+$ (fig. 3C). There was no effect at nanomolar doses either in the absence or presence of 6 mM K$^+$.

Characterization of Adenosine Release by Opioid Agonists

Calcium dependence. The release of adenosine by nanomolar doses of the *mu* opioid agonists morphine, PLO17 and DAMGO, and micromolar doses of the *delta* opioid agonists was Ca^{++}-dependent as no release was seen when synaptosomes were prepared and incubated in Ca^{++}-free medium (fig. 4). In contrast, the *kappa* opioid receptor agonist-evoked release of adenosine was Ca^{++}-independent, as release of adenosine by U50488H was still present and perhaps even augmented in the absence of Ca^{++}.

Adenosine vs. nucleotide. The release of adenosine by nanomolar concentrations of *mu* opioid receptor agonists was characterized further by determining whether the evoked
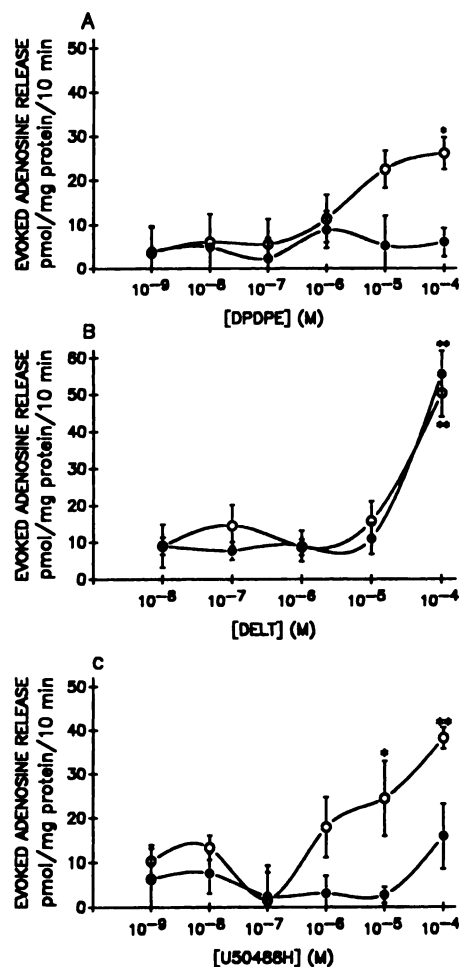

Fig. 3. Evoked release of adenosine by (A) DPDPE (*delta*-1 agonist), (B) DELT (*delta*-2 agonist) and (C) U50488H in the absence (●) and presence (○) of an additional 6 mM K$^+$. Values represent means ± S.E.M. for *n* = 6. *P < .05 and ``P < .01 compared to basal release values. Basal adenosine values for A: 124 ± 5; 6 mM K$^+$ 126 ± 8 pmol/mg of protein per 10 min. B: 130 ± 7; 6 mM K$^+$ 121 ± 5 pmol/mg of protein per 10 min, C: 120 ± 10; 6 mM K$^+$ 132 ± 10 pmol/mg of protein per 10 min.

adenosine release represents adenosine *per se* or arises from nucleotide that is converted to adenosine extracellularly. Synaptosomes were incubated with and without α,β-methylene ADP and 5'GMP, which inhibits ecto-5'-nucleotidase activity. The degree of inhibition of enzyme activity was determined by calculating the amount of conversion of 5'AMP (1 μM) to adenosine in a control tube (80%). There was no alteration in the amount of adenosine released by any *mu* agonist in the presence of the nucleotidase inhibitors, indicating that the evoked release of adenosine is the result of adenosine released *per se* rather than release of a nucleotide (fig. 5).

Spinal antinociception by selective opioid agonists. The effects of the acute i.t. injection of the *mu* opioid receptor agonists morphine, PLO17 and DAMGO in the hot-plate test are shown in figure 6. The i.t. injection of each *mu* agonist produces a dose-dependent increase in hot-plate latency; between 1.5 to 15 nmol for morphine, 0.19 to 5.6 nmol for PLO17 and 0.19 to 5.8 nmol for DAMGO. These agents differed with regard to their duration of action. The more selec-
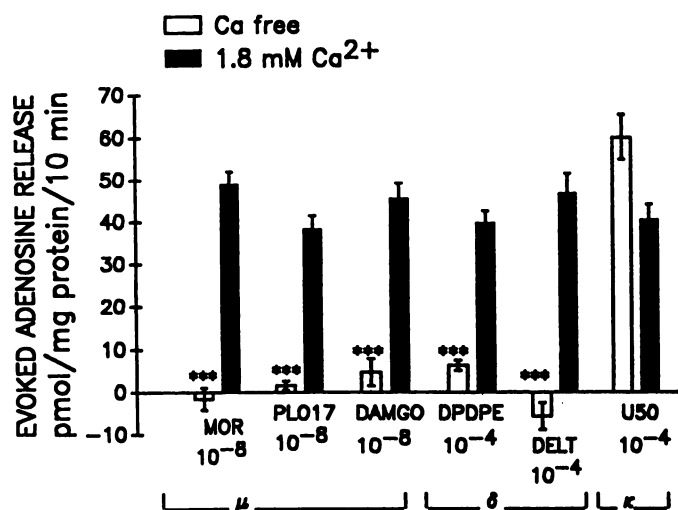
Fig. 4. Release of adenosine by the *mu* opioid agonists morphine (MOR), PLO17 and DAMGO, the *delta* opioid agonists DPDPE and DELT and the *kappa* agonist U50488H (U50) in the absence and presence of 1.8 mM Ca^{++}. Synaptosomes were prepared in Ca^{++}-free medium and the normal concentration of Ca^{++} in Krebs' medium was added back at the drug incubation stage. Values represent means ± S.E.M. for n = 4. ***P < .001. Basal values ranged between 199 to 220 pmol/mg of protein per 10 min. Release of adenosine by each opioid agonist was examined in the presence of the additional 6 mM K^+.
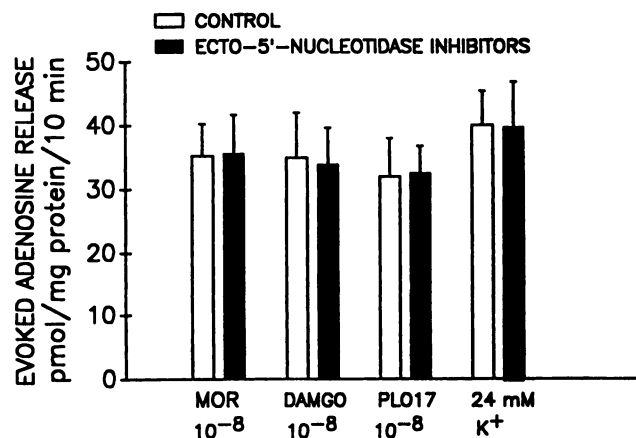

Fig. 5. Release of adenosine by nanomolar concentrations of the *mu* opioid agonists morphine (MOR), PLO17 and DAMGO in the absence and presence of ecto-5'-nucleotidase inhibitors α,β-methylene ADP and 5'GMP. Values represent means ± S.E.M. for n = 4.

tive *mu* agonists DAMGO and PLO17 produced their greatest effect at the first postdrug measurement (15 min), and this was diminished by 60 to 75 min, whereas morphine antinociception lasted throughout the test period of 90 min (fig. 6). The dose-response curves for each *mu* agonist were plotted as the peak effect for each dose examined. ED_{50} values were derived from the dose-response curves and are presented in table 1. No changes in motor function were observed except for slight hind limb rigidity at high doses (9.3 nmol) of DAMGO and PLO17, but impairment of motor function was not present at the doses presented.

The antinociceptive effects of the *delta* opioid agonists DP-DPE and DELT in the hot-plate test are presented in figure 7. Both agonists produced a dose-dependent increase in hot-plate latency between 1.35 to 81.2 nmol for DPDPE and 0.34 to 11.5 nmol for DELT. ED_{50} values were derived from the
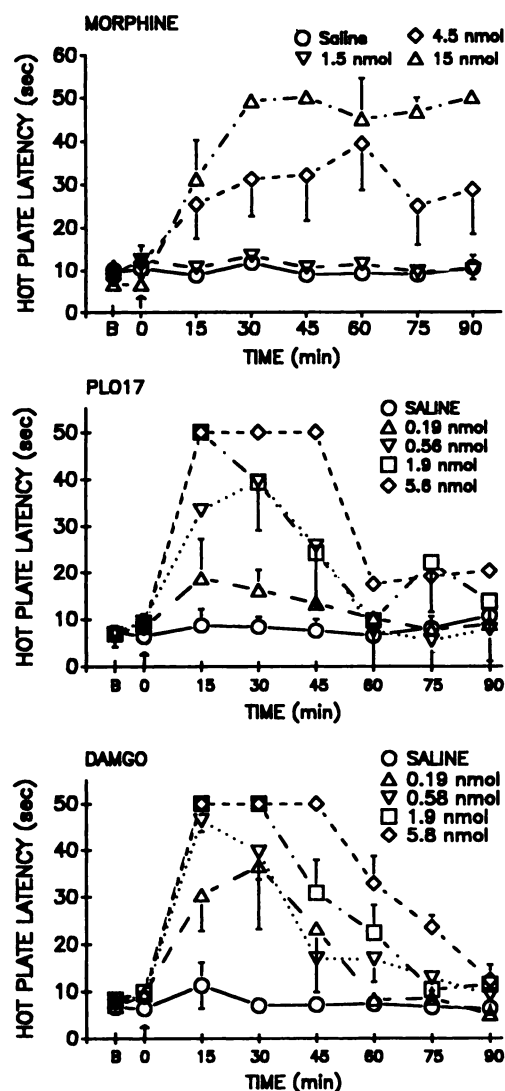

Fig. 6. Dose- and time-related antinociceptive effects of *mu* opioid receptor agonists morphine, PLO17 and DAMGO administered by acute i.t. injections. B, base-line latencies determined at 15-min intervals before the i.t. injection of drug. Each line on the graph represents the mean and S.E.M. for hind paw lick latency of n = 5 in the rat hot-plate test.

dose-response curves and are presented in table 1. Both *delta* agonists had a similar onset of action, but the analgesic effects of DELT were more prolonged compared to DPDPE. No impairment of motor function was observed at the doses presented.

The relative potencies of the *mu* and *delta* opioid receptor

TABLE 1
Effects of i.t. administered *mu* and *delta* agonists

The ED_{50} values given are expressed with the range of confidence intervals (95%) of range of upper and lower limits in parentheses. ED_{50} values were calculated from dose-response curves in figure 8.

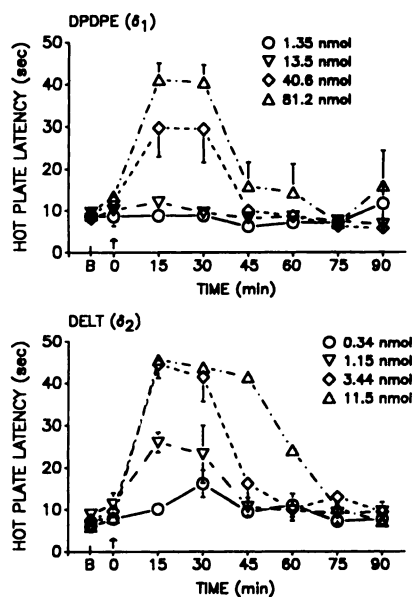|	ED_{50} Values	
	nmol	
Morphine	3.9	(1.112–6.690)
DAMGO	0.097	(0.084–0.110)
PLO17	0.22	(0.091–0.349)
DPDPE	68.7	(42.99–94.41)
DELT	1.78	(1.523–2.037)

DPDPE (δ_1)

DELT (δ_2)

Fig. 7. Dose- and time-related antinociceptive effects of *delta* opioid receptor agonists DPDPE (*delta*-1-selective) and DELT (*delta*-2-selective) administered by acute i.t. injection. B, base-line latencies determined at 15-min intervals before the i.t. injection of drug. Each line on the graph represents the mean and S.E.M. of $n = 5$.

agonists are depicted in figure 8. Peak analgesic effects of each agonist were converted to %MPE and are presented as a function of dose. The antinociceptive potency of the opioid agonists is: DAMGO, PLO17 > DELT, morphine > DPDPE. The analgesic effects of U50488H were not examined in this study as *kappa* agonists have little effect on thermal nociceptive tests.

Effects of caffeine on spinal antinociception by *mu* and *delta* agonists. Rats implanted with chronic i.t. cannulae were used to determine the role of adenosine in the antinociceptive effect elicited by an EC_{75} dose of selective opioid receptor agonists. Caffeine pretreatment dose-dependently attenuated the antinociceptive effect of each of the *mu* agonists (fig. 9). Caffeine shortened the duration of action of each ligand and attenuated the peak analgesic response. These doses of caffeine alone had no effect on hot-plate latencies and produced no overt behavioral effects at any of the doses used in this paradigm (data not shown). In the right
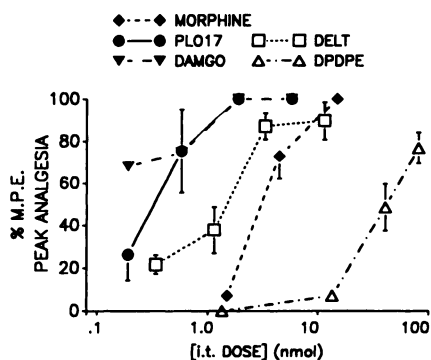

Fig. 8. Dose-response curves for acute i.t. injection of *mu* opioid receptor agonists DAMGO, PLO17 and morphine and the *delta* opioid receptor agonist DPDPE and DELT on the hot-plate test. ED_{50} values for the dose-response curves are presented in table 1. Each point on the graph represents the means ± S.E.M. for $n = 5$.

hand panels (fig. 9), the degree of antagonism is depicted by values of area under the curve of each agonist with increasing doses of caffeine. IC_{50} values were interpolated to be 82 nmol (morphine), 58 nmol (DAMGO) and 75 nmol (PLO17), indicating that caffeine had a similar ability to antagonize each *mu* agonist.

In contrast to the *mu* opioid agonists, i.t. pretreatment with caffeine did not antagonize the antinociceptive effects elicited by the *delta* opioid agonists DPDPE and DELT (fig. 10). The antinociceptive response to DELT in the antagonist studies was less effective than in the agonist studies, and this may be due to the use of two different samples of DELT. Interestingly, higher doses of caffeine (≥515 nmol) produced a significant enhancement of antinociception. These doses of caffeine also had no significant effect on hot-plate latency or produced any overt behavioral effects (data not shown).

Discussion

The aim of the present study was to characterize the primary opioid receptor subtypes (*mu* vs. *delta* vs. *kappa*) involved in the spinal release of adenosine using both behavioral and neurochemical approaches. The involvement of selective *delta* opioid receptor subtypes (*delta*-1 and *delta*-2) in adenosine release and sensitivity of their actions to caffeine was subsequently addressed.

Recent studies in this laboratory have demonstrated that morphine releases adenosine from the dorsal spinal cord in two distinct phases (nanomolar and micromolar) (Cahill *et al.*, 1993b). The nanomolar component of release is exhibited by the selective *mu* opioid agonists DAMGO and PLO17, but not by either *delta* or *kappa* agonists, indicating that this phase of release is mediated by activation of *mu* opioid receptors. PLO17, DAMGO and morphine show high selectivity for the *mu* opioid receptor subtype in binding studies compared to *delta* receptors (Chang *et al.*, 1983; James and Goldstein, 1984). The micromolar component of release may involve activation of multiple opioid receptors as all of the opioid agonists for *mu*, *delta* and *kappa* receptors release adenosine at 100 μM. Alternatively, at these doses, the *delta* and *kappa* opioid agonists may have lost their selectivity and act at *mu* opioid receptors (Chang *et al.*, 1983; Goldstein, 1987; Erspamer *et al.*, 1989).

Although opioids are generally considered to produce inhibitory actions on cellular function, a growing number of studies have reported excitatory effects at nanomolar doses of opioids. Stimulating effects of low concentrations of *mu* opioid receptor agonists in particular have been noted (Mauborgne *et al.*, 1987; Pohl *et al.*, 1989; Suarez-Roca and Maixner, 1991, 1992). Within trigeminal nucleus caudalis slices, morphine produces multiphasic effects on the K^+-evoked release of substance P from the rat (Suarez-Roca *et al.*, 1992), with the different phases being attributed to activation of different opioid receptors (Suarez-Roca and Maixner, 1992). Thus, activation of *mu*-1 and *delta* opioid receptors inhibit K^+-evoked release of substance P, whereby activation of *mu* and *kappa* opioid receptors facilitates K^+-evoked substance P release (Suarez-Roca and Maixner, 1992). Electrophysiological studies represent another paradigm in which opioids produce excitatory actions. Thus, i.t. administration of morphine (Wiesenfeld-Hallin *et al.*, 1990; Strimbu-Gozariu *et al.*, 1993) or other *mu* agonists (Dicken-
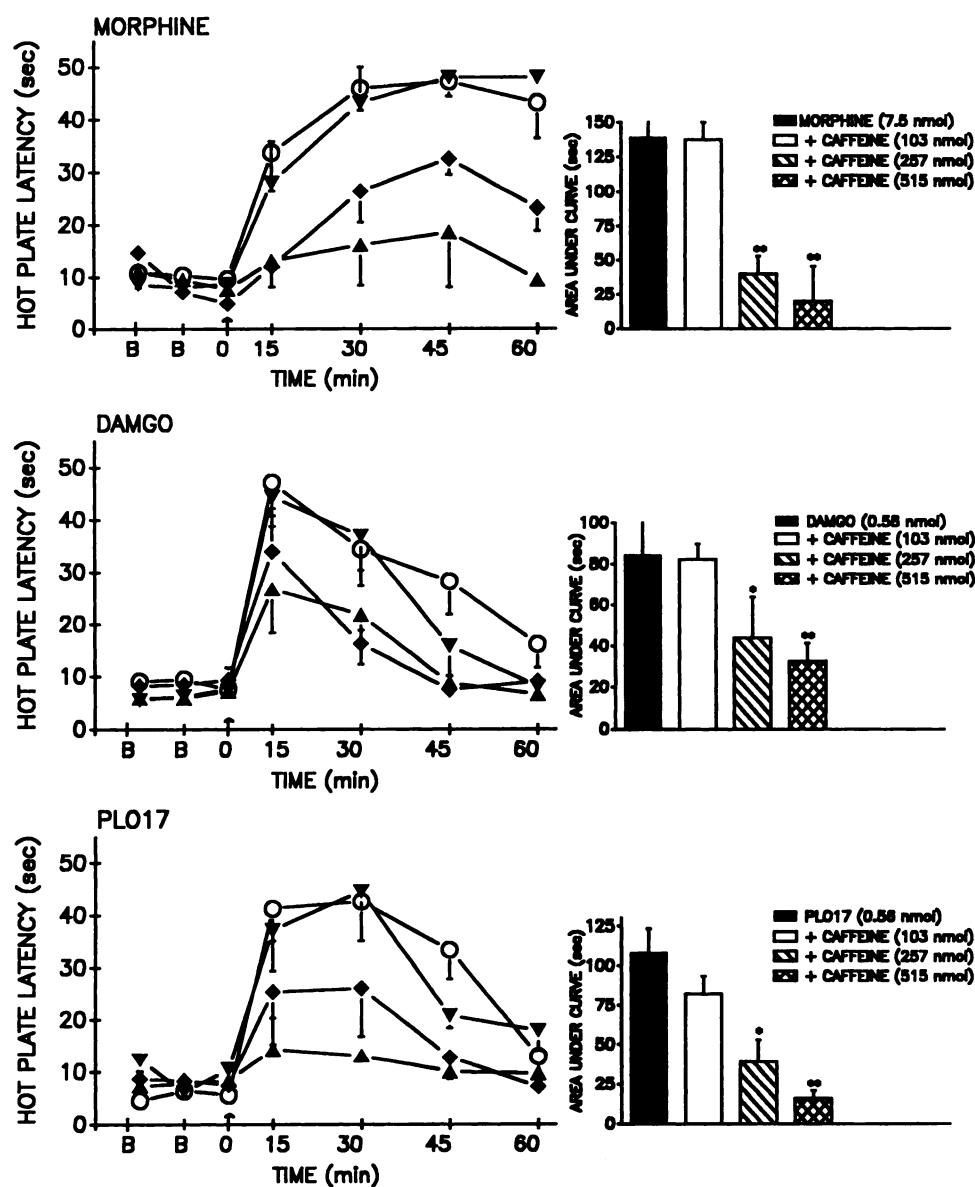
Fig. 9. Effects of graded doses of i.t. caffeine administered 15 min before opioid agonist on the antinociceptive response to a constant dose of either morphine (7.5 nmol), DAMGO (0.58 nmol) or PLO17 (0.56 nmol). O, Agonist control; ▼, +caffeine, 103 nmol; ◆, +caffeine, 257 nmol; ▲, +caffeine, 515 nmol. B, base-line latencies determined at 15-min intervals before the i.t. injection of drug. The data are expressed as the means ± S.E.M. for the latency in the hot-plate test of $n = 5$; *$P < .05$ and **$P < .01$.

son and Sullivan, 1986; Dickenson et al., 1987; Sullivan and Dickenson, 1988) at low concentrations produce a facilitory effect to enhance C-fiber-evoked activity. Finally, morphine has dual effects on the release of cholecystokinin from the spinal cord (Benoliel et al., 1991), although in this case stimulation is mediated by delta receptor activation, whereas inhibition is mediated by mu receptor activation.

Dual excitatory and inhibitory effects of opioids have been observed in other studies. Opioids produce complex effects on cultured dorsal root ganglion neurons, as action potential duration is prolonged by low nanomolar concentrations of opioid receptor agonists, whereas it is shortened by micromolar concentrations (Chen et al., 1988; Shen and Crain, 1989). Opioids produce a similar biphasic effect on electrically evoked release of [met]-enkephalin from myenteric plexus of the guinea pig ileum (Xu et al., 1989). Whereas these studies demonstrate dual excitatory and inhibitory effects of opioids, the effects are not discriminated by selective agonists, as mu, delta and kappa ligands produce each effect.

The relative potency of mu opioid receptor-selective ago-

nists to induce adenosine release in partially depolarized synaptosomes at micromolar concentrations (morphine > PLO17 and DAMGO) is opposite to that seen with nanomolar concentrations (DAMGO and PLO17 > morphine). Thus, morphine is more active at micromolar concentrations than the more selective mu ligands DAMGO and PLO17. This may be the result of morphine acting at both delta and mu opioid receptors in this concentration range, thus producing synergistic effects to elicit the release of adenosine. There are many recent reports providing evidence for synergistic spinal antinociceptive interactions among mu and delta opioid receptor ligands (e.g., Malmberg and Yaksh, 1992; reviewed by Solomon and Gebhart, 1994). Indeed, low nanomolar doses of morphine, PLO17 or DAMGO when combined with inactive doses of either DELT or DPDPE act synergistically to enhance the release of adenosine from spinal cord synaptosomes (Cahill et al., 1993c), indicating that mu/delta synergy is expressed at the synaptosomal level as well as in more integrated systems utilized in behavioral studies.

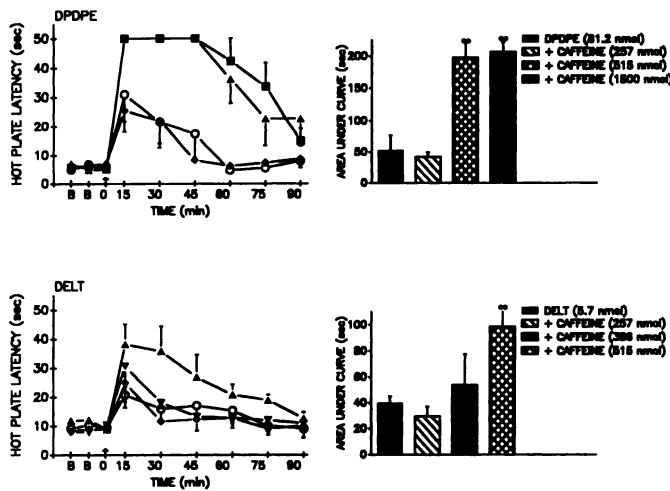The present study shows that the release of adenosine by

Fig. 10. Effects of graded doses of i.t. caffeine administered 15 min before opioid agonist on the antinociceptive response to a constant dose of either DPDPE (81.2 nmol) or DELT (5.7 nmol). ○, Agonist control; ◆, +caffeine, 257 nmol; ▼, +caffeine, 386 nmol; ▲, +caffeine, 515 nmol; ■, +caffeine, 1500 nmol. B, base-line latencies determined at 15-min intervals before the i.t. injection of drug. The data are expressed as the means ± S.E.M. for the latency in the hot-plate test of $n = 5$; **P < .01.

both *mu* opioid (nanomolar) and *delta* opioid agonists (micromolar) release adenosine *via* a Ca^{++}-dependent mechanism (release by the *kappa* opioid agonist is Ca^{++}-independent), and that nanomolar doses of *mu* agonists release adenosine *per se* rather than a nucleotide. This is consistent with previous observations whereby morphine-evoked release of adenosine at both nanomolar and micromolar concentrations occurs as adenosine *per se* and *via* a Ca^{++}-dependent mechanism (Sweeney *et al.*, 1989; Cahill *et al.*, 1993b). Opioid-induced release of adenosine involves activation of ω-conotoxin-sensitive voltage-dependent Ca^{++} channels (Cahill *et al.*, 1992, 1993b). Ca^{++} entry through voltage-sensitive Ca^{++} channels has been implicated in the mechanism by which opioids produce spinal analgesia (Porzig, 1990). Whereas most earlier studies examining the effects of opioids on Ca^{++} currents have demonstrated inhibition of Ca^{++} entry into neurons (Moises *et al.*, 1994), a number of recent studies have described mechanisms by which opioids may enhance Ca^{++} entry into cells or intracellular levels of Ca^{++}. Thus, studies utilizing Ca^{++} imaging techniques have shown that opioids can increase intracellular Ca^{++} levels in cultured neurons (Jin *et al.*, 1992; Tang *et al.*, 1994). In some cells, this effect is mediated by *delta* opioid receptor activation (Jin *et al.*, 1992; Tang *et al.*, 1994) but, in other cells, it is mediated by *mu* opioid receptors (Smart *et al.*, 1994).

The second messenger system involved in the opioid-enhanced intracellular Ca^{++} levels may be the phospholipase *C*-phosphoinositol system (Lambert *et al.*, 1990; Jin *et al.*, 1994; Smart *et al.*, 1994). Thus, opioids produce a Ca^{++}-dependent and pertussis toxin-sensitive G protein-dependent increase in phosphoinositol levels (Smart *et al.*, 1994). Morphine evoked release of adenosine is both Ca^{++}-dependent (Sweeney *et al.*, 1989) and is sensitive to pertussis toxin pretreatment (Sawynok *et al.*, 1990). As activation of protein kinase *C* increases the release of neuropeptides from sensory neurons (Barber and Vasko, 1994), it is quite possible that the ability of morphine to increase adenosine release is me-

diated by the protein kinase *C* second messenger system. Whereas stimulatory effects of opioids in cultured dorsal root ganglion cells have been attributed to stimulation of cyclic AMP (Crain and Shen, 1990), this effect does not appear to be involved in adenosine release from synaptosomes (Nicholson *et al.*, 1991).

Behavioral testing of the selective agonists for *mu* and *delta* opioid receptors demonstrated a dose-dependent antinociceptive effect for each ligand in the hot-plate test. The duration of effect produced by each agonist was variable, with morphine having the longest antinociceptive effect of each of the ligands examined and DPDPE having the shortest. The relative potency for these agonists was DAMGO, PLO17 > DELT and morphine > DPDPE. This is similar to the order of potency of the *mu* and *delta* agonists in our synaptosomal release study as well as that already reported for the hot-plate test (DAMGO > PLO17 > morphine > DPDPE) (Malmberg and Yaksh, 1992). Caffeine blocks antinociception induced by *mu* but not *delta* agonists, and this suggests that only the release of adenosine which occurs at nanomolar concentrations of opioids is relevant to the expression of behavioral actions.

Activation of *delta* opioid receptors produces antinociception which was not antagonized but augmented by caffeine. DeLander and colleagues (1992) examined the possible involvement of adenosine in opioid modulation of nociceptive processing in mice. They demonstrated that theophylline inhibited the antinociceptive actions produced by i.t. morphine and DAMGO as well as the action of DPDPE, although in the latter case dose-response curves were shifted in a nonparallel manner. Whether the difference in methylxanthine-sensitivity of *delta* agonists is due to species (rats *vs.* mice), or to a difference in the nociceptive test (hot-plate *vs.* tail-flick) is not clear. In a subsequent study, DeLander and Keil (1994) showed that i.t. adenosine agonists produced antinociceptive synergy when combined with *delta* opioid agonists but only additivity with *mu* opioid agonists. It was argued that this observation supported a *mu* opioid receptor but not a *delta* opioid receptor-mediated release of adenosine.

The present study revealed the interesting observation that antinociception produced by *delta* opioid receptor activation was augmented by pretreatment with the adenosine receptor antagonist caffeine. Some other studies also have reported augmentation of opioid induced antinociception with methylxanthines. Thus, after i.c.v. administration of morphine to rats, antinociception in the hot-plate and tail-flick tests was attenuated by a low dose of i.t. theophylline, but potentiated by a high dose (Sweeney *et al.*, 1991). High doses of methylxanthines have pharmacological actions other than adenosine receptor blockade, including inhibition of cyclic AMP phosphodiesterase (reviewed by Daly, 1993). Non-xanthine phosphodiesterase inhibitors potentiate antinociception produced by i.t. morphine (Nicholson *et al.*, 1991), such that this action may contribute to caffeine effects at high doses. However, the doses of caffeine that augmented antinociception by *delta* opioid agonists were the same as those which antagonized morphine and *mu* opioid-induced antinociception in this study. Whether this reveals some kind of paradoxical facilitatory effect of adenosine which is not normally expressed remains to be resolved.

Delta opioid receptor subtypes (*delta*-1 and *delta*-2) regulate nociceptive transmission at the spinal level. The exis-

tence of distinct and functionally significant *delta* receptor subtypes at the spinal level in rats is based on the antinociceptive effect of selective *delta* antagonists in behavioral tests (Sofuoglu *et al.*, 1991b; Malmberg and Yaksh, 1992; Mattia *et al.*, 1992; Sofuoglu *et al.*, 1993; Stewart and Hammond, 1993a), and by the lack of antinociceptive cross-tolerance after i.t. administration of *delta* subtype selective agonists in mice (Sofuoglu *et al.*, 1991a). The present study provides additional evidence that the *delta* opioid receptor is important in modulating nociceptive input, as the *delta*-2 agonist, DELT, is as potent as morphine in producing analgesia. Dose-response data in mice have shown that [D-Ala^2,Glu^4]deltorphin is 6- to 10-fold more potent after i.c.v. administration (Jiang *et al.*, 1991), but equipotent with DPDPE after i.t. injections (Mattia *et al.*, 1991, 1992). A more recent study examining the antinociceptive effects of i.t.-selective *delta* opioid receptor subtype agonists in the carrageenin-induced model of thermal hyperalgesia in rats demonstrated that both *delta*-1 and *delta*-2 receptors produce analgesia with no difference in potency (Stewart and Hammond, 1993b). Deltorphin II did not increase hot-plate response latencies in the rat, except at doses that produced adverse motor effects (Stewart and Hammond, 1993a). In the present study, no motor impairment was observed by the *delta*-2 agonist, DELT, at doses 0.34 to 11.5 nmol. The present study demonstrates that, in rats, DELT is ~10 times more potent than DPDPE after the i.t. route of administration, so the Cys^4− derivative appears to be a more potent analog than the Glu^4 deltorphin derivative, with no motor impairment at analgesic doses.

In summary, the opioid receptor involved in adenosine release from the rat spinal cord appears to be a *mu* receptor subtype with little evidence for a selective involvement of *delta* or *kappa* receptor subtypes. In behavioral experiments, this conclusion is substantiated by the observation that only the *mu* opioid agonists are attenuated by adenosine receptor antagonists. This study provides further evidence of the importance of adenosine in opioid-induced antinociception at the spinal level, and demonstrates that only the opioid-induced release of adenosine that occurs at low (nanomolar) concentrations contributes to behavioral effects.

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