THE ROLE OF ADENOSINE IN SPINAL OPIOID ANTINOCICEPTION

by

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Industrial 0624
Personality 0625
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Psychobiology 0349
Psychometrics 0632
Social 0451

TABLE OF CONTENTS

PAGE NUMBER

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Release of adenosine from the spinal cord contributes to spinal opioid-induced antinociception. The present study examined: 1) whether morphine-evoked release of adenosine is enhanced by agents which depolarize primary afferent nerve terminals, 2) the opioid receptor subtypes and possible interactions between μ and subclasses of δ opioid receptors involved in the release of adenosine from dorsal spinal cord synaptosomes, 3) the methylxanthine-sensitivity of selective opioid receptor agonists to determine which adenosine receptor subtype induces spinal antinociception in rats. Substance P evoked the release of adenosine in a biphasic manner; this was Ca^{2+}-dependent and originated from capsaicin-sensitive nerve terminals. Substance P augmented morphine-evoked release of adenosine from dorsal spinal cord synatosomes similar to partial depolarization with K^+. Nanomolar and micromolar concentrations of the selective μ opioid agonists DAMGO and PLO17 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 δ opioid agonists DPDPE and DELT and the κ opioid agonist U50488H had little effect on the release of adenosine except at high micromolar concentrations. DPDPE and DELT, at doses which exhibited no intrinsic effects, shifted the dose response curve for μ opioid receptor-evoked adenosine release to the left in a dose-dependent manner so that release was now expressed at subnanomolar concentrations of the μ opioid receptor agonists. Simultaneous activation of μ and δ opioid receptors thus generates a synergistic release of adenosine from spinal cord synaptosomes. Release of adenosine by μ (nanomolar) and δ (micromolar) ligands is Ca^{2+}-dependent, whereas the κ receptor ligand (micromolar) releases adenosine via a Ca^{2+}-independent mechanism. Evoked release of adenosine by nanomolar concentrations of μ opioid receptor agonists originated as adenosine *per se* from capsaicin-sensitive primary afferent neurons. Behavioural antinociception using the hot plate threshold test revealed that intrathecal (i.t.) administration of the μ and δ 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 dose-dependently attenuated by i.t. pretreatment with the adenosine receptor antagonist caffeine. Caffeine did not block the antinociceptive response to δ agonists, but in fact augmented antinociception when combined with DPDPE and DELT. This augmentation was dose-dependent. Adenosine-induced antinociception appears to be mediated by activation of A_1 adenosine receptors. This study demonstrates that activation of the μ receptor subtype is responsible for the opioid-induced release of adenosine from the spinal cord, that such release contributes to the spinal antinociception by μ agonists, and that only release evoked by low doses of opioids is behaviourally relevant.

ABBREVIATIONS

β-FNA	β-funaltrexazine
δ	delta
κ	kappa
μ	mu
2-CADO	2-chloroadenosine
ADP	Adenosine diphosphate
APNEA	N^6-2-(4-Aminophenyl)ethyladenosine
ATP	Adenosine triphosphate
cyclic AMP	cyclic Adenosine monophosphate
CGS 21680	2-[p-(2-carboxyethyl)phenethylamino]-5'-N-ethylcarboxyaminoadenosine
CGS 15943	9-Chloro-2-(2-furyl)[1,2,4]triazolo[1,5-c]quinazolin-5-amine
CHA	N^6-cyclohexyladenosine
CGRP	Calcitonin gene-related peptide
CPA	N^6-cyclopentyladenosine
CPT	8-cyclopentyltheophylline
CPX	1,3-dipropyl-8-cyclopenylxanthine
CTOP	Cys^2-Tyr^3-Orn^5-Pen^7 amide

CV1808	2-(arylamino)adenosine
CV1674	2-[4-(methyloxy)phenyl]aminoadenosine
DALCE	[D-Ala^2,Leu^5,Cys^6]enkephalin
DAMGO	[D-Ala^2,N-Me-Phe^4,Gly^5-ol]enkephalin
DELT	[D-Ala^2,Cys^4]deltorphin
Deltorphin II	[D-Ala^2,Glu^4]deltorphin
DMPX	3,7-Dimethyl-1-propargylxanthine
DPDPE	[D-Pen^2,D-Pen^5]enkephalin
GABA	γ-Aminobutyric acid
GTP	guanosine-5'-triphosphate
HPLC	High performance liquid chromatography
IP_3	inositol trisphosphate
i.t.	intrathecal
ICI 174,864	N,N-diallyl-Tyr-Aib-Aib-Phe-Leu
MPE	maximum possible effect
NECA	5'-N-ethylcarboxamideadenosine
nor-BNI	nor-binaltorphimine
PLO17	[N-MePhe^3,D-Pro^4]morphiceptin
RO 20 1724	4-[(3-Butoxy-4-methoxyphenyl)methyl]-2-imidazolidinone

R-PIA	R-N^6-phenylisopropyladenosine
U50488H	trans-(+/-)-3,4-dichloro-N-methyl-N-(2-(1-pyrrolidinyl)-cyclohexyl) benzemeacetamide methanesulfonate hydrate
U69593	(+)-5α,7α,8β)-3,4-Dichloro-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide mesylate

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PUBLICATIONS

Portions of this thesis have previously been published.

Papers

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2. J. Sawynok, **C.M. Cahill** and T.D. White. Interactions between opioid and adenosine systems. Analgesia 1: 695-698 (1995).

3. **Cahill** C.M., T.D. White and J. Sawynok. Mu/delta opioid receptor interactions mediate adenosine release from spinal cord synaptosomes. Eur. J. Pharmacol. 298: 45-49 (1996).

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1. **Cahill C.M.**, T.D. White and J. Sawynok (1992) Mu and mu/delta interactions mediate opioid-induced release of adenosine via N-type Ca^{2+} channels from dorsal spinal cord synaptosomes. Soc. Neurosci. Abstr. 18: 1019.

2. **Cahill C.M.**, T.D. White and J. Sawynok (1993) Mu/delta opioid receptor interactions mediate adenosine release from spinal cord synaptosomes. Proceedings 7th World Congress on Pain, Paris, p.215.

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INTRODUCTION

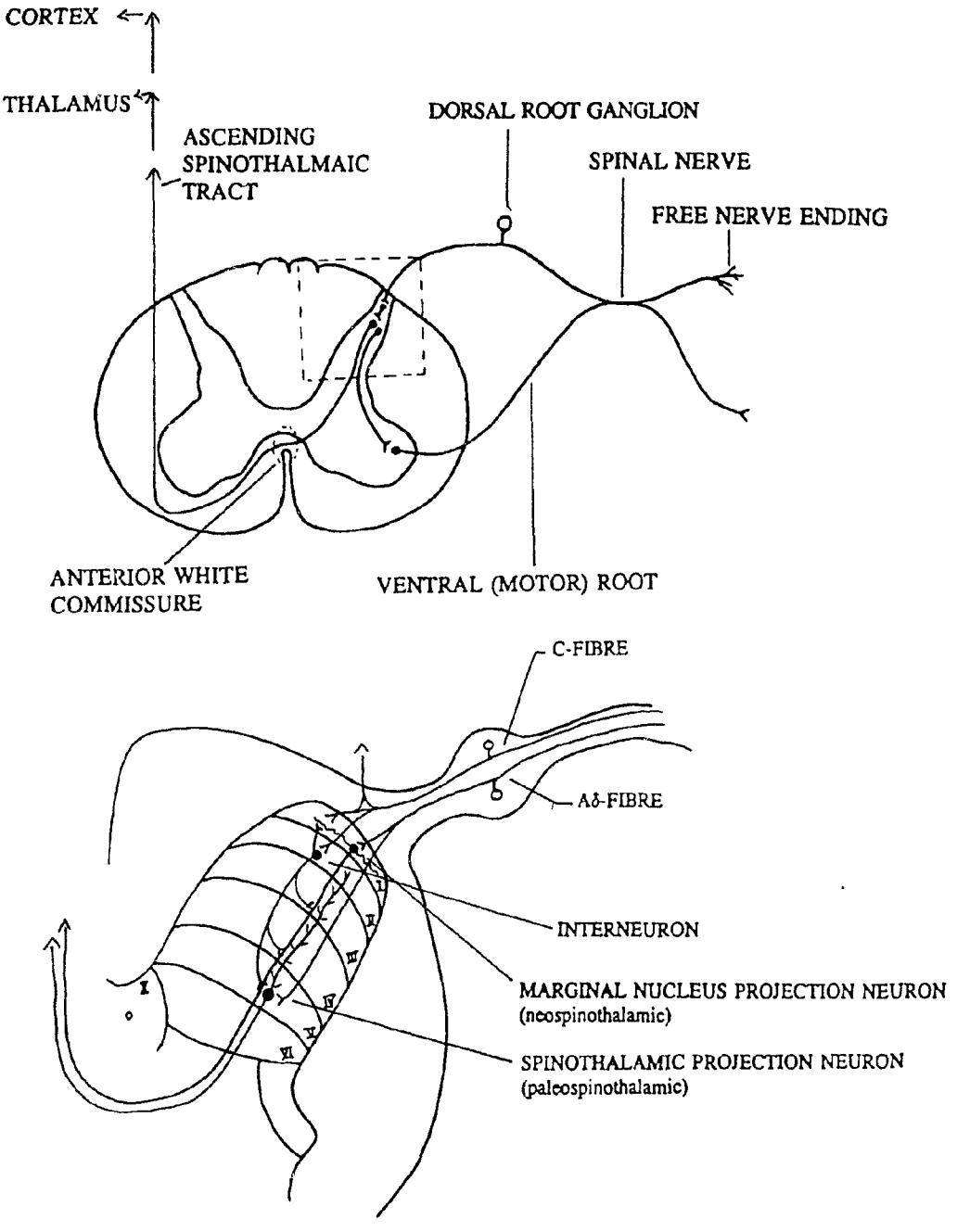
1 PAIN TRANSMISSION IN THE SPINAL CORD

The International Association for the Study of Pain (1993) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage". To understand the mechanisms that mediate behaviour interpreted as painful, comprehension of the basic mechanisms of sensory transmission is essential. Afferent pathways involved in pain transmission have been thoroughly reviewed (Besson and Chaouch, 1987). Pain is perceived following mechanical, thermal or chemical activation of nociceptors; free nerve endings of sensory afferent neurons whose cell bodies are located in the dorsal root ganglia. Subsequent transmission of noxious information to the dorsal horn of the spinal cord and then brain stem and supraspinal sites for neural processing results in the sensation of pain (Figure 1). Activation of these nociceptors transmits sensory information via small diameter primary afferents (C-fibre and/cr Aδ-fibres) to the dorsal horn of the spinal cord. Thermal or mechanical Aδ fibres are fast conducting and thinly myelinated. Activation of these nociceptors conduct influences perceived as sharp localized pain. Polymodal C fibre nociceptors are activated by a wide variety of high-intensity mechanical, chemical and hot or cold stimuli. C-fibre afferents are slow conducting, unmyelinated and are involved in diffuse pain. An extensive review of the organization of inputs to the dorsal horn has been presented (Woolf, 1994).

The Aδ and C nociceptive fibres bifurcate upon entering the dorsal spinal cord

FIGURE 1

Schematic diagram of afferent sensory transmission in the spinal cord.



as the lateral bundle of the dorsal root. These branches ascend and descend a few segments in the tract of Lissauer situated in the dorsolateral area of the white matter of the dorsal horn, while axon collaterals synapse with neurons in the dorsal horn. Nociceptive stimulation in the periphery generates low and high threshold currents within sensory afferents which terminate in various laminae of the spinal cord (Woolf, 1994 and citations therein). In general, C-fibres terminate predominantly in lamina II while Aδ fibres synapse with neurons in lamina I, II and V (Noback et al., 1991; Jessel and Kelly, 1991). The majority of nociceptive transmitters and receptors are concentrated in lamina II (substantia gelatinosa); a crucial site of termination of small diameter primary afferent neurons that respond to noxious stimuli and process information related to the transmission and modulation of sensory signals including pain (reviewed Dickenson, 1995; Randic et al., 1995 and citations therein).

Nociceptive afferents make synaptic connections with three major classes of neurons within the dorsal horn: (1) projection neurons that relay incoming sensory information to higher centers in the brain; (2) inhibitory interneurons that regulate the transmission of nociceptive information to higher centers; (3) excitatory interneurons that relay sensory input to projection neurons. Lamina I of the dorsal horn contains a high density of projection neurons that process nociceptive information (Figure 1). One class is solely excited by nociceptors (Aδ- and C-fibres) and is termed nociceptive-specific. Other projection neurons in lamina I receive input from low threshold mechanoreceptors in addition to those from nociceptors. These cells are termed wide dynamic range neurons. A second major population of wide dynamic range projection neurons is

located in lamina V (Figure 1).

Nociceptive signals in the dorsal horn are transmitted by chemicals (neurotransmitters). Neurotransmitters released by nociceptive afferents associated with pain transmission in the spinal cord include a variety of neuropeptides such as substance P and excitatory amino acids (Table 1). Both Aδ- and C-fibres release glutamate that evokes fast synaptic potentials in dorsal horn neurons and neuropeptides that elicit slow excitatory postsynaptic potentials.

Sensory afferent fibre tracts (projection neurons) within the spinal cord are components of the anterolateral pathway. They comprise the spinothalamic, spinoreticular, spinomesencephalic and spinocervical tracts as well as postsynaptic dorsal column pathways (Woolf, 1994). The most prominent ascending pathway relaying nociceptive information related to pain is the spinothalamic tract which is composed of nociceptive-specific and wide dynamic range neurons that terminate in the thalamus and reticular formation. The location of these projection neurons is primarily in laminae I, IV and V of the dorsal cord. Collateral fibres from the spinothalamic tracts also project to specific areas in the brain stem that activate descending analgesic systems. Evidence that this pathway mediates pain has arisen from studies demonstrating that stimulation of this tract frequently elicits pain sensations, while lesioning this tract can result in a marked loss of pain and temperature sensation. Spinothalamic tract fibres that terminate in the lateral thalamus are usually involved in sensory discriminative aspects of pain, whereas the fibres that terminate in the medial thalamus involving the spinoreticular pathways are implicated in the motivational and affective aspects of pain (Woolf, 1994).

TABLE 1

Neuropeptides found in small diameter neurons.

Peptide	Cellular Effect
Substance P	A neurokinin which excites dorsal horn neurons and evokes pain behaviour
Somatostatin	An inhibitor that hyperpolarizes dorsal horn neurons and blocks acute pain behaviour
CGRP	Excites dorsal horn neurons and involved in hyperalgesia
Bombesin	Can evoke pain behaviour
Vasoactive intestinal peptide	Causes excitation of nociceptive and non-nociceptive dorsal horn neurons
Galanin	inhibitory peptide having antinociceptive effects
Dynorphin	Inhibition or excitation of dorsal horn neurons
Cholecystokinin	Causes excitation of postsynaptic neurons

Summarized from Rang et al., 1994; Willis and Coggeshall, 1991; Dickenson, 1995.

Other ascending pathways contain nociceptive neurons, but it is not clear to what extent these pathways contribute to pain sensation.

Control of nociceptive transmission at the spinal level can occur through segmental control within the spinal cord as well as modulation from higher brain centres via descending tracts. Melzack and Wall (1965) proposed the 'gate control' theory which hypothesized that stimulation of peripheral non-pain transmitting fibres activate interneurons that depress pain transmission. Segmental control is characteristically inhibition produced by large-diameter fibres on the response of spinal neurons to noxious stimulation. This modulation of pain transmission occurs in the substantia gelatinosa and is mediated in part by presynaptic inhibition of the primary afferent fibres. Neurotransmitters associated with the inhibitory mechanisms of presynaptic control include endogenous opioids, γ-aminobutyric acid and ATP (Salter et al., 1993; Besson and Chaouch, 1987). Neurotransmitters, neuropeptides and neuromodulators associated with the relay of nociceptive transmission are summarized in Table 2.

Pain can be controlled by central mechanisms. Stimulation of various cortical and forebrain areas is able to modulate the responses of dorsal horn neurons to various types of peripheral stimulation (Fields et al., 1991). Direct electrical stimulation of the periaqueductal gray suppresses activity in nociceptive pathways. Brainstem stimulation inhibits nociceptive neurons in the dorsal horn of the spinal cord via the descending inhibitory dorsolateral funiculus pathway. The descending influences from higher centres modulating pain are presumed to be organized in the following way. Output from the frontal cortex and hypothalamus activates centres in the periaqueductal gray and adjacent

TABLE 2

Chemical Transduction of Pain

Primary Afferent Neurons	**Interneurons**	**Descending Pathways**
Peptides (See Table 1)	Substance P	Noradrenaline
Excitatory amino acids	Neurotensin	Enkephalin
Nitric oxide	Enkephalins	Somatostatin
ATP, adenosine	GABA	Serotonin
		Cholecystokinin
		Substance P
		Thyrotropin releasing hormone

areas of the midbrain, which have connections with tegmental nuclei of the rostromedial medulla. Another area involved with pain modulation is located in the dorsal and dorsolateral pons. Fibres from these pontine and medullary tegmental nuclei project to the spinal trigeminal nucleus, and via the pain-modulating dorsolateral tract to laminae I and II of the spinal cord. Many studies of the descending systems have implicated serotonin, noradrenaline and endogenous opioids in descending controls (Besson and Chaouch, 1987).

2 SPINAL OPIOID PHARMACOLOGY

Opioid drugs and opioid peptides produce their behavioural effects, including antinociception, by interactions with opioid receptors in the central nervous system. Aside from effects on nociception, opioids are also associated with spinal mechanisms related to other aspects of sensory, autonomic and motor function (reviewed Sabbe and Yaksh, 1990). This section will review the pharmacology of opioids and their receptors. Comprehensive recent reviews on spinal cord opioid pharmacology include Yaksh and Malmberg (1994) and Dickenson (1995), while a concise history of opioids and their use can be found in Brownstein (1993).

One of the more important current issues in pain research has been to identify the spinal opioid receptors acted upon by opioid peptides to alter nociceptive responses. The development of selective agonists and antagonists for opioid receptors has established their differential distribution in the central nervous system and allowed their pharmacology to be studied.

2.1 Classification of Opioid Receptors

The existence of specific opioid binding sites was originally suggested by behavioural and clinical studies and confirmation ensued with biochemical identification (Pert and Snyder, 1973). Several types of opioid receptors were postulated; the first definitive pharmacological evidence supporting subclasses of opioid receptors was reported by Martin and colleagues (1976), describing three classes of receptors (μ, κ and σ). *In vitro* methodologies confirmed the existence of multiple opioid binding sites and also led to the identification of the δ opioid receptor (Lord et al., 1977). A fifth receptor, the ε receptor, was proposed on the basis of a unique high potency of β-endorphin (Schulz et al., 1981). Since these early studies, multiple populations of opioid receptors have been described by a number of bioassays and binding systems (reviewed Atcheson and Lambert, 1994).

Only μ, δ and κ receptors are currently recognized as opioid receptors and are found in both the central nervous system and periphery. The effects associated with the σ receptor are not blocked by naloxone, a broadly selective opioid antagonist, therefore, the receptor is no longer considered part of the opioid family (Quirion et al., 1987). The molecular cloning of μ, δ and κ opioid receptors has confirmed the heterogeneity of opioid receptors derived from traditional and pharmacological approaches (reviewed by Knapp et al., 1995).

There is evidence for receptor subtypes of the μ (Pasternak and Wood, 1986), δ (Traynor and Elliott, 1993) and κ (Wollemann et al., 1993) opioid receptors. The classification of opioid receptor subtypes and their analgesic actions has been established

by the aid of selective agonists and antagonists, molecular cloning and antisense mapping (Pasternak, 1993; Pasternak et al., 1995; Knapp et al., 1995). In summary, two μ opioid receptor subtypes exist (μ_1, supraspinal and μ_2, spinal), three κ opioid receptor subtypes have been proposed, κ_1 (spinal), κ_2 (unknown) and κ_3 (supraspinal), and finally δ opioid receptor subtypes have been classified as δ_1 (supraspinal) and δ_2 (spinal and supraspinal).

2.2 Location of Opioid Binding Sites in the Spinal Cord

Three opioid receptor subtypes, μ, δ and κ, are present in the superficial layers of the rat dorsal spinal cord as determined by binding studies ($\mu > \delta > \kappa$) (Besse et al., 1991; Stevens et al., 1991). The cloning of the opioid receptors has profoundly affected the understanding of opioid receptor expression, regulation and function (Mansour et al., 1995; reviewed Knapp et al., 1995). The identification of μ (Chen et al., 1993), δ (Kieffer et al., 1992; Evans et al., 1992) and κ (Yasuda et al., 1993) cDNAs from rodent brain has confirmed that opioid receptors in the central nervous system belong to the G protein-coupled receptor family. The pharmacology of the three opioid receptors correlates well with the previous μ, δ and κ classification (Raynor et al., 1994) and their expression pattern parallels binding site distribution in the central nervous system (Mansour et al., 1995).

The anatomical distributions of opioid receptors throughout the central nervous system has been demonstrated by autoradiographic techniques and opioid receptor mRNA expression (George et al., 1994; Mansour et al., 1994). There is now evidence that three distinct opioid binding sites for each receptor subtype are present in the dorsal horn of

the spinal cord. Biochemical studies have demonstrated specific binding sites for μ, δ and κ opioids in homogenates of spinal cord (Stevens and Seybold, 1995 and citations therein). Receptor autoradiographic studies have revealed the distribution of opioid binding sites, with the highest levels commonly being observed in the dorsal gray matter of the spinal cord (Besse et al., 1990; 1991; Stevens et al., 1991). 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 (Atweh and Kuhar, 1977; Fields et al., 1980; Gouarderes et al., 1985; Morris and Herz, 1987). In general, μ, δ and κ opioid binding sites are concentrated in laminae I-II of the rat spinal cord, with lesser binding detected in the deeper laminae (Stevens and Seybold, 1995). The proportions of the opioid binding sites in laminae I-II were found to be homogenous at each segmental level of the rostrocaudal axis of the rat spinal cord: 70-74%, 18-20% and 7-10% for μ, δ and κ sites, respectively (Besse et al., 1991).

Several studies other than autoradiographic binding studies have demonstrated the presence of opioid receptors on the terminals of primary afferents. Biochemical studies of spinal cord tissue after dorsal rhizotomy has provided evidence that specific opioid receptors exist on pre- and postsynaptic elements within the spinal cord. When the effects of unilateral rhizotomy of animals were assessed, the side ipsilateral to the rhizotomy showed decreases in the binding of all three opioid receptors (Stevens and Seybold, 1995). Similarly, treatment of animals with the neurotoxin capsaicin produced a reduction in dorsal horn opioid binding to levels comparable to rhizotomy (Gamse et al., 1979).

Expression of μ opioid receptor mRNA was found to be intense in the laminae I and II of the lumbar spinal cord (Maekawa et al., 1994). In the same study, there were moderate to intense signals of μ opioid receptor mRNA in laminae VII and VIII of the lumbar ventral horn. In contrast, others reported that cells expressing μ opioid receptor mRNA are localized predominantly in laminae IV, V, VII, VIII and X with fewer cells observed in laminae II and III (Mansour et al., 1994). They demonstrated that a similar pattern was seen for δ opioid mRNA, however comparatively few cells expressed κ receptor mRNA with no cells detected in laminae I-III. This is consistent with another study demonstrating that δ opioid receptor mRNA was low to moderate throughout laminae I-VI, but intense in laminae IX (Satoh and Minami, 1995). It is unclear why these studies demonstrated inconsistency in the expression of μ opioid receptor mRNA; however, mRNA expression for both μ and δ opioid receptors has been identified in deeper laminae than that shown by autoradiographic binding studies. Cells in laminae I and II intensely expressed κ opioid receptor mRNA and moderate to intense mRNA was also found in laminae III-IV (Maekawa et al., 1994).

μ, δ and κ Receptor mRNA also was localized in cell bodies of the dorsal root ganglia. The opioid-receptor mRNAs are localized in different populations of dorsal root ganglion neurons, with the expression of μ receptors in the medium and large diameter cells, κ receptors in the small and medium neurons, and δ receptors predominantly in large diameter neurons, suggesting that the different opioid receptors might process different kinds of nociceptive information (reviewed by Mansour et al., 1995).

2.3 Spinal Opioid-Induced Antinociception

The first demonstration that opioids could elicit direct spinal analgesia in animals was made by Yaksh and Rudy (1976) where antinociception was induced following intrathecal opioid administration. The use of spinally administered opioids has subsequently ensued in man for both acute and chronic pain (Cousins and Mather, 1984). Spinal administration of opioids has provided greater pain relief than conventional routes and fewer side effects (Twycross, 1994). The assessment of opioid-induced antinociceptive effects and mechanistic insight have been accomplished by both clinical and experimental pain studies where analgesic effects of i.t. opioids has been assessed in thermal, electrical, pressure and inflammatory tests (reviewed Yaksh, 1987).

Activation of all established spinal opioid receptor subtypes elicits analgesia (Pasternak, 1993). Activation of μ opioid receptors is considered the predominant receptor subtype responsible for eliciting spinal antinociception (reviewed Dickenson, 1993). I.t. administration of μ opioid receptor agonists such as morphine and sufentanil is presently the most efficacious and potent class of analgesic agents, eliciting antinociceptive actions in cutaneous thermal, mechanical, inflammatory and visceral chemical algesiometric tests (Schmauss and Yaksh, 1984; Porreca et al., 1987; Murray and Cowan, 1991). κ Opioid agonists are effective in modulating visceral and low intensity thermal and mechanical stimuli responses, whereas δ opioid agonists are potent in inhibiting a range of thermal, inflammatory and mechanical stimuli but are relatively ineffective in altering visceral nociception (Schmauss and Yaksh, 1984; Stewart and Hammond, 1993b; Porreca et al., 1987; Murray and Cowan, 1991). Table 3 displays

TABLE 3

Heterogeneity of opioid receptors and their ligands.

	μ	δ		κ
Endogenous ligands	β-endorphin dermorphin	met-enkephalin leu-enkephalin		dynorphins
Selective Agonists	DAMGO PLO17 Morphine	δ_1: DPDPE	δ_2: [D-Ala^2]deltophins [D-Ala^2, Cys^4]deltophin	U50488H U69893
Antagonists	naloxone β-FNA CTOP	nalxone ICI174864 naltrindole		naloxone nor-BNI
Effectors	↓ cyclic AMP ↑ K^+ channel	↓ cyclic AMP ↑ K^+ channel ↓ Ca^{2+} channel		↓ cyclic AMP ↓ Ca^{2+} channel ↑ IP_3

Summarized from Dickenson, 1991; Childers, 1991; Borsodi and Tóth, 1995.

highly selective agonists and antagonists for μ, δ and κ opioid receptors.

Spinal antinociceptive actions of morphine have been partially attributed to its ability to reduce C-fibre transmission following innocuous stimulation (Dickenson and Sullivan, 1986). In contrast, the same study reported that i.t. morphine had little effect on A-fibre-evoked responses. Subsequent electrophysiological studies demonstrated that i.t. administration of selective μ and δ opioid agonists produced dose-dependent inhibitions of C-fibre-evoked activity whilst A-fibre activity remained relatively unchanged (Dickenson et al., 1987). Others have demonstrated that i.t. administration of morphine dose-dependently depressed the nociceptive flexon reflex elicited by C-fibre afferents in the sural nerve of rats (Strimbu-Gozariu et al., 1993) which could be completely reversed by CTOP and nor-BNI (selective μ and κ opioid receptor antagonists, respectively) but only partially reversed by the δ opioid receptor antagonist naltrindole (Guirimand et al., 1994).

The spinal administration of morphine is an effective therapeutic agent in the control of pain, but its use can be limited by side effects such as urinary retention and respiratory depression (reviewed Cousins and Mather, 1984; Pasternak, 1993). Morphine produces each of these actions via activation of μ opioid receptors. This prompted interest in the development of new opioid analgesics that do not produce the adverse effects associated with activation of μ opioid receptors. The development of selective agonists and antagonists for δ opioid receptor subtypes has revealed antinociceptive effects associated with activation of both δ_1 and δ_2 receptor subtypes. [D-Pen^2,D-Pen^5]enkephalin (DPDPE), a δ_1 agonist, and [D-Ala^2,Glu^4]deltorphin, a δ_2

agonist, are highly selective peptidic δ opioid receptor agonists which induce antinociception following i.t. administration (Sofuoglu et al., 1991b; Mattia et al., 1992). More recently, a highly selective δ_2 opioid agonist [D-Ala^2, Cys^4]deltorphin (DELT) has been developed and shown to exhibit dose related-antinociceptive actions (Horan et al., 1992).

Concurrent interest has grown in δ opioid receptor subtypes from observations that there exists the possibility of synergistic actions between drugs as an alternative method for single drug therapy in providing pain relief (reviewed Solomon and Gebhart, 1994). Synergistic actions potentially provide the advantage of using lower doses of effective analgesic agents, such as morphine, with drug X. Combination of morphine with drug X sustains the same efficacy thus maintaining the desired analgesic effect, but limits the side effects associated with higher doses of morphine alone. Several studies have demonstrated that antinociception generated by i.t. opioid agonists acting at μ opioid receptors can be enhanced by coadministration of δ opioid receptor agonists (Heyman et al., 1989; Jiang et al., 1990; Malmberg and Yaksh, 1992; Porreca et al., 1992). The antinociceptive enhancement was found to be greater than additive, and represented a synergistic interaction between μ and δ opioid receptor agonists.

The development of selective agonists and antagonists for δ opioid receptor subtypes has provided insight into their involvement in μ/δ-induced antinociception. In rats, the i.t. administration of the δ_1 opioid receptor agonist DPDPE seemed more effective than the δ_2 receptor subtype agonist [D-Ala^2,Leu^5,Cys^6]enkephalin (DALCE) in modulating μ (morphine, [D-Ala^2,N-Me-Phe^4,Gly^{5}ol]enkephalin (DAMGO) and [N-

MePhe^3,D-Pro^4]morphiceptin (PLO17)) opioid antinociception in thermal threshold tests (Malmberg and Yaksh, 1992). In mice, both (D-Ala^2, D-Glu^4)deltorphin, a selective δ_2 opioid receptor agonist, and DPDPE are effective in potentiating μ opioid antinociception (Mattia et al., 1992). However, the use of selective δ opioid receptor antagonists has led to the conclusion that the δ_2 receptor subtype is responsible for the modulation of μ mediated antinociception in the mouse (Porreca et al., 1992).

It should be emphasized that antinociceptive synergy is not restricted to concurrent i.t. administration of opioid receptor agonists. Thus, synergy has been reported between i.t. administration of; (1) μ opioid receptor agonists and α_2 adrenergic receptor agonists (reviewed Solomon and Gebhart, 1994), (2) μ opioid receptor agonists and nonsteroidal antiinflammatory agents (Malmberg and Yaksh, 1993), and (3) opioid agonists and local anaesthetics (Maves and Gebhart, 1991).

2.4 Mechanisms of Opioid-Induced Antinociception

The spinal administration of agonists for each of the distinct classes of μ, δ, and κ opioid receptors reduces nociceptive responses. There appear to be two mechanisms by which opioids produce antinociception; hyperpolarization of transmission neurons and interneurons within the dorsal horn of the spinal cord, and inhibition of the release of neurotransmitters associated with pain transmission.

2.4.1 Presynaptic mechanisms

A presynaptic location for opioid receptors on the central terminals of small

diameter primary afferent neurons has been inferred from both *in vitro* and *in vivo* studies demonstrating an opioid inhibition of neurotransmitter release from the central terminals of primary afferent neurons. Opioid receptor agonists attenuate the release of substance P (Jessell and Iversen, 1977; Yaksh et al., 1980; Mudge et al., 1979) and excitatory amino acids (Malmberg and Yaksh, 1995) from the spinal cord. Activation of μ opioid receptors has been proposed to elicit analgesia by a presynaptic inhibition of C-fibre neurotransmitter release (Yaksh et al., 1980; Pang and Vasko, 1986). κ Receptor agonists depress firing of dorsal horn neurons but do not diminish release of spinal C-fibre transmitters (Go and Yaksh, 1987; Gross and MacDonald, 1987).

2.4.2 Postsynaptic mechanisms

Direct postsynaptic effects of opioids have been demonstrated where the excitatory effects of glutamate on dorsal horn neurons are attenuated by μ, δ and κ opioid receptor agonists (Fleetwood-Walker et al., 1988). Electrophysiological evidence has indicated that postsynaptic actions require higher doses of morphine than presynaptic effects based on comparative studies with and without intact presynaptic sites (reviewed Dickenson, 1995). Electrophysiological experiments have also demonstrated that opioids inhibit the firing of nociceptive neurons in the dorsal horn after iontophoretic administration (Dickenson et al., 1987; Einspahr and Piercey, 1980; Sullivan and Dickenson, 1991). Furthermore, opioids decrease excitatory amino acid- and substance P-evoked firing which is suggestive of a direct postsynaptic effect and consistent with a postsynaptic location of opioid receptors.

2.4.3 Cellular mechanisms

Opioid receptors share a commonality in their ability to couple to second messengers in that they are coupled to GTP-dependent G proteins (Childers, 1991; North et al., 1987). Stimulation of adenylate cyclase is mediated by G_s, while inhibition is mediated through G_i. Occupation of μ, δ or κ opioid receptors generally produces an inhibitory action on the associated cell. The G-protein α-subunit in its activated state (dissociated from the $\beta\gamma$ subunit) directly modulates a number of cellular functions such as adenylate cyclase, phospholipase C and ion conductance (Allende, 1988).

I.t. administration of pertussis toxin, which blocks activation of G_i and G_o proteins, inhibits opioid-induced analgesia implicating G-proteins in this action (Przewlocki et al., 1987). Activation of the three opioid receptors expressed from cloned cDNAs has been shown to elicit inhibition of adenylate cyclase activity in cultured cells (Uhl et al., 1994). This G-protein effector system, but not cyclic AMP, has been proposed to mediate opioid-induced inhibition of neurotransmitter release (Childers, 1991). Opioid-induced effects on adenylate cyclase have predominantly been implicated in models of tolerance and dependence. Cyclic AMP has been proposed to be involved in modulating neuropeptide synthesis including pro-enkephalin (Childers, 1991). Other opioid effector systems include phosphatidylinositol turnover and protein phosphorylation. It should be noted that opioids can exert effects independent of second messenger systems including direct coupling of opioid receptors to ion channels via G-proteins (North et al., 1987).

One cellular event that accounts for inhibitory effects of opioids on cellular

excitability and neurotransmitter release is the inhibition of voltage dependent Ca^{2+} channels (North, 1993). Activation of μ, δ and κ opioid receptors reduces Ca^{2+} currents in various preparations including dorsal root ganglion neurons (reviewed Satoh and Minami, 1995). This opioid-induced reduction in Ca^{2+} currents is blocked by pertussis toxin indicating the involvement of G_i and/or G_o proteins. Electrophysiological studies have demonstrated functional coupling of the three cloned opioid receptors with inwardly rectifying K^+ channels (Chen and Yu, 1994; Henry et al., 1995) and inhibition of N-type Ca^{2+} currents by activation of the cloned κ opioid receptor (Tallent et al., 1994).

Another cellular event that is thought to be important for opioids to reduce cellular excitability and inhibit neurotransmitter release is membrane hyperpolarization caused by an increase in K^+ conductance (reviewed North, 1993). Hyperpolarization of postsynaptic neurons due to an increase in K^+ conductance leads to an inhibition of Ca^{2+} entry during the action potential (North, 1993). The activation of μ and δ opioid receptors increases an inwardly rectifying K^+ conductance in various preparations. The κ opioid receptor also increases K^+ conductance in substantia gelatinosa neurons (Grudt and Williams, 1993). These increases in K^+ conductance are sensitive to pertussis toxin indicating mediation through inhibitory G-proteins (Tatsumi et al., 1990).

2.5 Excitatory Effects of Opioids

Activation of opioid receptors has generally been considered to produce inhibitory effects on neuronal activity (see section 2.4). However, excitatory actions of opioids also can occur. While some of these actions can be explained mechanistically in terms of

disinhibition (for example inhibition of GABAergic neurons), there are now many reports that demonstrate direct excitatory effects of opioids (reviewed Huang, 1995; Gintzler, 1995).

Such effects have been demonstrated in behavioural, electrophysiological, neurochemical and cell culture paradigms. In behavioural studies, paradoxical excitatory effects of morphine such as hyperalgesia have been shown in rats (Kayser et al., 1987) and humans (Wolff et al., 1940). In behavioural studies, systemic administration of morphine (3 μg/kg, i.v.) produced a hyperalgesic effect (Kayser et al., 1987), and caused itching and flare response (Oliveras et al., 1986). Conversely, low doses of naloxone can cause paradoxical analgesia in humans (Levine et al., 1979) and induces antinociception in both normal (Kraus and LeBars, 1986) and arthritic rats (Woolf, 1980; Kayser et al., 1988). It has also been reported that high doses of naloxone facilitated, whereas low doses reduced carrageenan-induced C-fibre-evoked responses (Stanfa et al., 1992).

Electrophysiological studies represent another paradigm where opioids produce excitatory actions. I.t. administration of morphine (Wiesenfeld-Hallin et al., 1990; Strimbu-Gozariu et al., 1993) or other μ agonists (Dickenson and Sullivan, 1986; Sullivan and Dickenson, 1988; 1991; Dickenson et al., 1987) at low concentrations produce a facilitory effect on C-fibre-evoked activity. Morphine was also shown to produce a biphasic effect on spontaneous discharges of C-fibre nociceptive neurons, where 100 nM morphine enhanced neuronal activity (Kellstein et al., 1990). I.t. administration of morphine produced a facilitation of reflex responses to C-fibre

activation before it exerted a depressive effect (Strimbu-Gozariu et al., 1993). Facilitory effects on flexor reflex after low doses (0.01 μg) of i.t. morphine and biphasic effects at higher doses (0.1-3 μg) have also been described (Wiesenfeld-Hallin et al., 1991; Xu and Wiesenfeld-Hallin, 1991). Opioid agonists can inhibit or excite glutamate-evoked responses of spinothalamic neurons (Willcockson et al., 1986) or spinal trigeminal slices (Chen and Huang, 1991) depending on the concentration of opioid agonist used.

Opioids exert dual effects on transmitter release. In the myenteric plexus of the guinea pig, μ, δ or κ selective opioid agonists, at concentrations below 10 nM, enhance electrically stimulated release of [met]enkephalin but inhibit release at higher concentrations (Xu et al., 1989; Gintzler and Xu, 1991). In the spinal cord, nanomolar concentrations of opioid agonists enhance the K^+-evoked release of substance P from whole cord slices (Mauborgne et al., 1987; Pohl et al., 1989). Furthermore, within rat spinal trigeminal nucleus caudalis slices, morphine produced multiphasic effects on the K^+-evoked release of substance P (Suarez-Roca et al., 1992). Stimulatory effects of morphine were seen at 100-300 nM, a low micromolar concentration (3 μM) suppressed release, and a higher micromolar concentration (30 μM) facilitated K^+-evoked release of substance P. More recently, it was reported that opioids enhanced K^+-evoked transmitter release from cultured dorsal root ganglion neurons (Suarez-Roca and Maixner, 1995), in addition to exhibiting the characteristic inhibition of release at higher doses. In this latter study, the excitatory effect of morphine was seen at 30 nM, whereas the inhibitory concentration of morphine occurred at 1 μM. The complex modulatory effects of morphine were naloxone reversible and Ca^{2+}-dependent. The studies cited above indicate

stimulatory effects of μ opioid receptors, however, stimulatory effects with δ opioid receptors may also occur. Morphine induced dual effects on the release of cholecystokinin from the spinal cord (Benoliel et al., 1991), although in this case stimulation is mediated by δ receptor activation, while inhibition is mediated by μ receptor activation (Benoliel et al., 1991; 1995). To some extent, the mechanisms by which opioids exhibit their dual actions have been attributed to activation of different opioid receptor subtypes (Suarez-Roca and Maixner, 1992; 1993).

Spinally mediated excitatory effects of opioids also have been demonstrated in cell culture preparations. In cultured dorsal root ganglion neurons, opioids produce complex effects on the action potential duration, with prolongation at low nanomolar concentrations (1-10 nM) of μ, δ and κ opioid receptor agonists, but a shortening at micromolar concentrations (Chen et al., 1988; Shen and Crain, 1989). These opioid-induced effects on action potential duration were proposed to be an explanation for the dual effects of opioids on transmitter release (see above). Similar dual effects on action potentials have also been observed in *in vitro* preparations as a low nanomolar dose of morphine enhances the Ca^{2+}-dependent component of action potentials in the nodose ganglion, while higher doses of morphine depressed the amplitude or duration of the Ca^{2+}-dependent spikes (Higashi et al., 1982). Recently, concentration-dependent dual regulation of μ, δ and κ opioid agonists on K^+ conductance in F11 cells (neuroblastoma x dorsal root ganglion neurons) was reported (Fan and Crain, 1995).

The second messengers implicated in opioid-induced excitatory effects in cultured dorsal root ganglion neurons include activation of G_s proteins and adenylate cyclase. In

dorsal root ganglion cells, the prolongation of action potential duration by μ and δ opioid receptor agonists is blocked by a cyclic AMP dependent protein kinase inhibitor (Chen et al., 1988) or in cells pretreated with cholera toxin A (Crain and Shen, 1990). A similar cholera toxin sensitivity was shown for opioid-induced transient increases in K^+ conductance in dorsal root ganglion neurons and F11 cells (neuroblastoma x dorsal root ganglion neuron) (Fan and Crain, 1995). Direct coupling of opioid receptors to G_s protein has been reported in F11 cells (Cruciani et al., 1993). Opioid-induced enhancement of [met]enkephalin release from the myenteric plexus is also abolished by cholera toxin A (Gintzler and Xu, 1991). Protein kinase C has also been implicated in excitatory effects of opioids; as an inhibitor of protein kinase C, but not protein kinase A, attenuates opioid-induced potentiation of glutamate-evoked responses in spinothalamic neurons (Chen and Huang, 1991). More recently, activation of protein kinase C has been demonstrated to increase the release of neuropeptides from sensory neurons (Barber and Vasko, 1994).

Ca^{2+} entry through voltage-sensitive Ca^{2+} channels has been implicated in the mechanism by which opioids produce spinal analgesia (Porzig, 1990). While most earlier studies examining the effects of opioids on Ca^{2+} currents demonstrated inhibition of Ca^{2+} entry into neurons (Moises et al., 1994 and citations therein), a number of recent studies have described mechanisms by which opioids may enhance Ca^{2+} entry into cells or increase intracellular levels of Ca^{2+}. Thus, studies utilizing Ca^{2+} imaging techniques have shown that opioids can increase intracellular Ca^{2+} levels in cultured neurons (Jin et al., 1992; Tang et al., 1994; Smart et al., 1994). In some cells this effect is mediated

by δ opioid receptor activation (Jin et al., 1992; Tang et al., 1994), but in other cells, it is mediated by μ opioid receptors (Smart et al., 1994). The second messenger system involved in the opioid-enhanced intracellular Ca^{2+} 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^{2+}-dependent and pertussis toxin-sensitive G protein-dependent increase in phosphoinositol levels (Smart et al., 1994).

3 ADENOSINE AND PAIN

There has been considerable interest in the role of adenosine in pain. Adenosine produces complex effects in modulating nociception, with complexity arising due to opposing effects seen peripherally versus centrally or between adenosine receptor subtypes. Activation of peripheral adenosine receptors induces pronociceptive (A_2 receptors) or antinociceptive (A_1 receptors) responses whereas central administration of adenosine analogues produces antinociception (reviewed Sawynok, 1995). In the human blister base preparation, adenosine produces algogenic effects by activating unmyelinated afferents (Bleehen and Keele, 1977). Intravenous administration (Sylven et al., 1986; 1988) or intracoronary injection (Lagerqvist et al., 1990) of adenosine elicits ischemic-like pain in humans. These pronociceptive actions of adenosine are attenuated by theophylline indicating that the effect is mediated by a cell surface adenosine receptor (Jonzon et al., 1989). Animal studies have confirmed that the pronociceptive action of adenosine following peripheral administration is the result of receptor activation. Adenosine A_2 receptor antagonists attenuate adenosine-induced hyperalgesia measured by

mechanical threshold tests (Taiwo and Levine, 1990). Another study examining pain behaviour induced by subcutaneous injection of formalin has demonstrated that the resulting first phase response was augmented by A_2 adenosine receptor activation (Karlsten et al., 1992). More recently, peripheral administration of exogenous adenosine was shown to augment the flinching behaviour associated with formalin through activation of A_2 rather than A_1 adenosine receptors (Doak and Sawynok, 1995). The same study, however, demonstrated that pain behaviour induced by subcutaneous formalin was shown to be either augmented or attenuated by selective A_1 and A_2 adenosine receptor antagonists, respectively. Others have implicated antinociceptive effects for peripheral A_1 adenosine receptors (Karlsten et al., 1992; Khasar et al., 1995). The analgesic effect attributed to peripheral A_1 adenosine receptors in this latter study is in contrast to that found in humans, where intradermal injection of adenosine induces pain through activation of A_1 receptors (Pappagallo et al., 1993; Gaspardone et al., 1995). The basis for this apparent contradiction of receptor subtypes involved in pronociceptive effects of adenosine has yet to be resolved. Pronociceptive actions of adenosine have been attributed to direct activation of sensory nociceptors (Dibner-Dunlap et al., 1993; Huang, 1995). Caffeine, an adenosine receptor antagonist, produces analgesia in both animals and humans, which may result from antagonism of peripheral A_2 adenosine receptors (Sawynok and Yaksh, 1993) as well as from interactions at supraspinal but not spinal sites (Sawynok et al., 1995).

Adenosine is now considered an important modulator of sensory transmission both in higher brain centres and within the spinal cord following noxious stimulation

(reviewed Sawynok and Sweeney, 1989; Salter et al., 1993). Recent clinical evidence has substantiated the hypothesis that adenosine is an important modulator of nociception. Systemic administration of adenosine alleviated spontaneous and stimulus-evoked pain in patients with peripheral neuropathic pain (Sollevi et al., 1995; Belfrage et al., 1995). Furthermore, adenosine infusion at low and non-hypotensive doses has antinociceptive properties in patients during anaesthesia (Sollevi, 1992). An antinociceptive effect of adenosine at a low and innocuous infusion dose (70 $\mu g.kg^{-1}.min^{-1}$) has also been demonstrated in awake healthy volunteers subjected to experimentally induced ischemic pain (Segerdahl et al., 1994). Similarly, intravenous infusion of adenosine has been shown to provide relief of allodynic pain in humans and may reduce the area of secondary hyperalgesia in healthy volunteers with experimentally induced skin trauma (Sollevi et al., 1995; Segerdahl et al., 1995). Furthermore, systemic adenosine infusion alleviated spontaneous and stimulus-evoked pain in patients with peripheral neuropathic pain (Belfrage et al., 1995). In this latter study, adenosine reduced both spontaneous pain of neuropathic origin and attenuated touch-evoked pain and/or hyperalgesia as assessed by von Frey filaments and pinprick, respectively.

Endogenous adenosine has been implicated as a mediator of nociceptive transmission in the analgesic responses that follow peripheral vibration, in that vibration-induced analgesia is thought to be partially mediated by the release of purines from large diameter sensory neurons (Salter and Henry, 1987; Salter et al., 1993; Li and Perl, 1994). A reduction in the firing of nociceptive neurons induced by vibration was potentiated by dipyridamole, an adenosine uptake blocker (Salter and Henry, 1987).

Caffeine can inhibit transcutaneous electrical nerve stimulation (TENS) induced analgesia (Marchand et al., 1995) supporting the hypothesis that adenosine is an important mediator involved in producing the analgesic response. Antinociceptive effects produced by spinal administration of adenosine analogues will be discussed in section 3.4.

3.1 Adenosine Receptor Classification

The first proposal that cell membrane adenosine receptors could be subdivided was based on whether adenosine could inhibit (A_1 subtype) or stimulate (A_2 subtype) adenylate cyclase (Van Calker et al., 1979). Subsequent biochemical, functional and receptor-cloning studies have provided supporting evidence for the existence of four adenosine receptors A_1, A_{2A}, A_{2B} and A_3 (reviewed Collis and Hourani, 1993; Fredholm et al., 1994). The majority of effects elicited by adenosine are mediated via an interaction with cell-surface receptors, although adenosine can inhibit adenylate cyclase via an intracellular P-site. Properties and characteristics of selective adenosine analogues for each receptor subtype are presented in Table 4.

3.2 Adenosine Receptors in the Spinal Cord

Tissue autoradiography has determined the distribution of A_1 and A_2 adenosine receptors in the rat spinal cord. Adenosine A_1 receptor binding was higher in dorsal compared to ventral laminae of the spinal cord, which are known terminal areas of primary nociceptive neurons; however, the occurrence of A_2 receptors was not significantly different between dorsal and ventral horns (Choca et al., 1987, 1988; Geiger

et al., 1984). Binding site densities for both A_1 and A_2 receptors were highest in the substantia gelatinosa, followed by lamina X, with low levels for the remaining binding sites displayed uniformly through the spinal cord. Autoradiograms demonstrated that A_1 receptors were localized in the superficial and deep layers of lamina II of the substantia gelatinosa whereas A_2 receptor density tended to be localized in the ventral portion of the substantia gelatinosa. Unilateral dorsal rhizotomy, hemitranssection and complete transection of the spinal cord as well as neonatal capsaicin failed to alter either A_1 or A_2 binding levels in the substantia gelatinosa (Geiger et al., 1984; Choca et al., 1988). However, kainic acid injected into the dorsal horn decreased both A_1 and A_2 binding. This suggests that adenosine receptors in the substantia gelatinosa of the spinal cord are located predominantly on intrinsic neurons and not on the terminals of either primary afferents or the descending pain modulating pathways. There have been reports of adenosine receptors on cultured dorsal root ganglion neurons raising the possibility of a presynaptic action on afferent terminals (Dolphin et al., 1986; MacDonald et al., 1986).

3.3 Biological Markers for Adenosine in the Spinal Cord

The localization of biological markers for adenosine containing neurons in the substantia gelatinosa of the spinal cord provides further evidence that endogenous adenosine plays an important physiological role in modulating the transmission of painful stimuli. The substantia gelatinosa contains high levels of 5'-nucleotidase (Scott, 1967), an enzyme responsible for the degradation of AMP to adenosine. High levels of

TABLE 4

Adenosine agonists and antagonists and their affinities at A_1 and A_2 receptors.

	A_1	A_{2a}	A_{2b}	A_3
Agonist Potency	CPA > R-PIA = CHA > NECA > CV1808 ≥ CGS21680	CGS21680 = NECA >> CPA	NECA > 2-CADO > R-PIA > CGS21680	APNEA > R-PIA = NECA > CGS21680
Antagonist potency	CPX > CPT > 8-PT	CGS15943 > DMPX > CPX > XCC	XCC >> CPX = 8-PT	unidentified
Affinity for adenosine	micromolar	nanomolar		micromolar
Transmitter release	inhibited	increased		inhibited

Summarized from Jacobson et al., 1992; Collis and Hourani, 1993; Stone, 1991; Fredholm et al., 1994.

adenosine-like immunoreactivity (Braas et al., 1986) as well as binding of radiolabeled nucleoside transport system inhibitors (Geiger and Nagy, 1984; 1985; Bisserbe et al., 1985; Geiger et al., 1985; Nagy et al., 1985) are present in the dorsal spinal cord. Immunoreactivity of adenosine deaminase, an enzyme that converts adenosine to the physiologically inactive compound inosine, was found to be highest in superficial dorsal horn regions compared to other regions of the cord (Geiger and Nagy, 1986; Nagy and Daddona, 1985; Nagy et al., 1984; 1985).

3.4 Adenosine in Spinal Mechanisms of Antinociception

The role for spinal adenosine systems as modulators of nociceptive input is supported by the observation that i.t. administration of adenosine receptor agonists induces methylxanthine-reversible antinociception in a wide variety of nociceptive tests. Thus, the i.t. administration of adenosine receptor agonists-induced antinociception in thermal nociceptive threshold tests (Post, 1984; DeLander and Hopkins, 1987; Sawynok et al., 1986; Fastbom et al., 1990; Karlsten et al., 1990; 1991), acetic acid writhing test (Sosnowski et al., 1989), inflammatory pain tests such as the formalin test (Malmberg and Yaksh, 1993; Poon and Sawynok, 1995) and carrageenan thermal hyperalgesia inflammatory test (Poon and Sawynok, unpublished). Adenosine receptor agonists also inhibit behaviour associated with putative nociceptive neurotransmitters such as substance P and excitatory amino acids as well as capsaicin following i.t. co-administration (Hunskaar et al., 1986, Doi et al., 1987; DeLander and Wahl, 1988). Recently, *in vivo* electrophysiological data has shown that spinal administration of adenosine analogues

inhibits C-fibre-evoked activity of acute and formalin-induced inflammatory nociception (Reeve and Dickenson, 1995).

An interesting clinical report also has contributed to the hypothesis that adenosine is an important modulator of spinal nociceptive transmission. Allodynia elicited by touch and vibration in a patient with intractable neurogenic pain was abolished by a single i.t. dose of R-PIA (Karlsten and Gordh, 1995). Low doses of adenosine analogues exhibiting mild thermal antinociception has dramatic effects in attenuating the nociceptive response in neurogenic pain models (Sosnowski and Yaksh, 1989; Yamamoto and Yaksh, 1991; Minimi et al., 1992).

Endogenous adenosine is considered to be an important modulator of nociceptive transmission. Tonic release of adenosine appears to regulate nociception in the spinal cord, as i.t. administration of adenosine receptor antagonists can induce hyperalgesia (Jurna, 1984; Sawynok et al., 1986). Furthermore, inhibition of adenosine kinase elicits antinociceptive effects in the tail flick thermal threshold test (DeLander and Keil, 1992) and the formalin test (Poon and Sawynok, 1995). Indeed, *in vivo* spinal cord superfusion and *in vitro* studies with spinal cord slices have demonstrated that adenosine kinase inhibition elevates the levels of endogenous adenosine (Golembioska et al., 1995; 1996).

While activation of both A_1 and A_2 adenosine receptors has been implicated in spinal nociception (reviewed Sawynok, 1991), studies using a wider range of agonists implicated A_1 receptors as the primary receptor subtype involved in antinociception (Karlsten et al., 1991). The adenosine analogues examined in nociceptive tests have primarily been CHA, R-PIA and NECA. These agonists for both A_1 and A_2 receptors

(Table 2) are active in producing antinociception in a variety of nociceptive tests and lead to the conclusion that both adenosine receptor subtypes can elicit analgesia (see Table 1 in Sawynok, 1991). More recent studies ulitizing the selective A_2 adenosine receptor agonists CV 1808 (DeLander and Wahl, 1988; Karlsten et al., 1991) and CGS 21680 (Poon and Sawynok, 1995) revealed that activation of A_2 adenosine receptors does not produce antinociception. Antinociceptive activity could only be produced at doses that correlated with the affinity for A_1 adenosine receptors (Karlsten et al., 1991).

3.5 Pharmacology of Adenosine-Induced Antinociception

Adenosine has long been recognized to inhibit the release of transmitters via activation of presynaptic receptors (Fredholm and Dunwiddie, 1988). It has been suggested that adenosine activates both pre- and postsynaptic receptors to alter cell excitability and to suppress the release of a diverse number of neuromodulators such as excitatory amino acids. If adenosine blocked the release of nociceptive transmitters such as glutamate the result would be manifested as an inhibition of nociceptive transmission. However, it remains controversial as to whether adenosine acts presynaptically to inhibit the release (from spinal cord neurons) of peptides associated with nociception. Adenosine analogues had no effect on K^+-evoked release of substance P from sensory afferent terminals (Vasko and Ono, 1990), although they inhibit electrically-evoked release of peptides from sensory primary afferent neurons (Santicioli et al., 1992). Adenosine and CHA inhibited the evoked release of CGRP from capsaicin-sensitive small diameter primary afferent neurons, while CGS21680 was ineffective, indicating that

activation of A_1 adenosine receptors mediates the presynaptic inhibition of CGRP release (Santicioli et al., 1993). Adenosine has been shown to inhibit Ca^{2+} conductances in cultured sensory neurons (Dolphin et al., 1986; MacDonald et al., 1986) and in spinal cord neurons (Sah, 1990). These observations provide one potential mechanism (inhibition of Ca^{2+} entry and neurotransmitter release) by which adenosine can inhibit noxious neurotransmitter release.

I.t. administration of adenosine analogues inhibits behaviourally-induced biting, licking and scratching syndrome precipitated by i.t. administration of substance P and excitatory amino acids (Doi et al., 1987) which is reversed by theophylline (DeLander and Wahl, 1988). Accordingly, the antinociceptive actions of adenosine have been suggested to result from a direct post-synaptic activation of adenosine receptors to inhibit the nociceptive transmission induced by such noxious mediators. This hypothesis is supported by the observation that the antinociceptive effects of i.t. adenosine analogues are not attenuated by pretreatment with i.t. capsaicin (Sawynok et al., 1991). Electrophysiological experiments demonstrate that adenosine causes a direct postsynaptic suppression of sensory transmission (Salter and Henry, 1985; Salter et al., 1993), which may occur via an increase in K^+ conductance that is G protein mediated (Trussell and Jackson, 1987) and involve activation of an ATP-sensitive K^+ channel (Salter et al., 1992). The antinociception produced by adenosine receptor agonists evaluated by the tail flick test was found to be mediated by the opening of ATP-sensitive K^+ channels (Ocaña and Baeyens, 1994). Adenosine inhibits synaptic transmission in neurons within the substantia gelatinosa as determined by whole cell recordings (Li and Perl, 1994) via

activation of a K^+ conductance in substantia gelatinosa neurons. Another mechanism by which adenosine may inhibit nociceptive transmission has been proposed whereby adenosine interacts directly with the binding of substance P to its receptor in the dorsal spinal cord (Stiller et al., 1991).

Inhibition of adenylate cyclase may be involved in spinal antinociception produced by CHA, as pretreatment with nonxanthine phosphodiesterase inhibitors, RO 20 1724 and rolipram reduces the antinociceptive effect (Sawynok and Reid, 1988). Subsequently, multiple second messenger systems for adenosine have been identified including stimulation of phosphatidylinositol (PI) turnover, potassium and calcium activation and cyclic GMP formation (Fredholm and Dunwiddie, 1988; Cooper and Caldwell, 1990). A_1 receptors are G-protein coupled and can act through effectors other than adenylate cyclase, including K^+ channels, Ca^{2+} channels, phospholipase A_2 or phospholipase C and guanylate cyclase (Olsson and Pearson, 1990). Indeed, G proteins have been implicated in the antinociceptive effects of i.t. adenosine analogues, as i.t. pretreatment with pertussis toxin, which ADP-ribosylates and inactivates G_i and G_o, inhibits antinociception (Sawynok and Reid, 1988).

4 ADENOSINE RELEASE IS A COMPONENT OF SPINAL OPIOID-INDUCED ANTINOCICEPTION

A number of mechanisms have been implicated in the spinal antinociception produced by morphine including inhibiting release of nociceptive transmitters, and hyperpolarization of postsynpatic neurons (see section 2.4). The release of adenosine

may also be involved in opioid-induced spinal antinociception (reviewed Sawynok and Sweeney, 1989). Several earlier observations had supported a possible role for adenosine in pharmacological effects of morphine at other sites. Thus, methylxanthines antagonized the inhibitory effects of morphine on electrically induced contractions in the guinea pig ileum (Sawynok and Jamandas, 1976; Ahlijanian and Takemori, 1985), and on release of acetylcholine from both the ileum (Sawynok and Jhamandas, 1976) and rat cortex (Jhamandas et al., 1978; Phillis et al., 1979; 1980). Furthermore, morphine was shown to release [^{3}H]purines from the cortex *in vivo* (Phillis et al., 1979, 1980; Jiang et al., 1980), and to increase depolarization-induced release of purines from brain slices *in vitro* (Fredholm and Vernet, 1978; Stone, 1981; Wu et al., 1982).

The involvement of adenosine in spinal antinociception by morphine was investigated initially as the result of a study which showed that systemic administration of aminophylline, an adenosine receptor antagonist, inhibited the spinal antinociceptive action of morphine in the tail flick test (Jurna, 1981). I.t. aminophylline also was shown to block antinociception produced by systemic injection of morphine, while the antinociceptive effects of i.t. morphine were attenuated by systemic aminophilline (Jurna, 1984). Subsequently, a number of studies have supported the hypothesis that adenosine is involved in opioid -induced antinociception by both neurochemical and behavioural paradigms. Thus, the i.t. administration of methylxanthine adenosine receptor antagonists attenuates the antinociceptive effects elicited by i.t. morphine (DeLander and Hopkins, 1986; DeLander et al., 1992; Sweeney et al., 1987; Yang et al., 1994), and i.t. selective opioid receptor agonists (DAMGO, [D-Pen^2,D-Pen^5]enkephalin (DPDPE),

and β-endorphin) (DeLander et al., 1992). These observations lead to the hypothesis that a component of the antinociceptive effects induced by spinal morphine is due to the release of adenosine from terminals within the spinal cord.

Neurochemical experiments have shown that morphine releases adenosine from the spinal cord both from synaptosomes (*in vitro*) and the intact spinal cord (*in vivo*) (Sweeney et al., 1987; 1989). Both paradigms demonstrated that release was receptor mediated. Synaptosomal studies found that opioid-evoked release of adenosine was Ca^{2+}-dependent, occurred from dorsal but not ventral spinal cord and was released as adenosine *per se* rather than a nucleotide that could be converted to adenosine by ecto nucleotidase enzymes (Sweeney et al., 1989). The evoked release of adenosine by morphine also was shown to originate from capsaicin-sensitive primary afferent neurons via a bidirectional nucleoside transporter (Sweeney et al., 1989; 1993). Originally, release from dorsal spinal cord synaptosomes was shown to occur at 10-100 μM morphine (Sweeney et al., 1987); but more recently, morphine has been shown to produce two phases of release (10 nM and 1-100 μM) in the presence of a partial depolarization produced by the addition of 6 mM K^+ (Cahill et al., 1993a). The second messenger system involved in morphine-evoked release of adenosine remains unclear, yet activation of G_i has been implicated, as pertussis toxin inhibits both morphine-evoked release of adenosine from synaptosomes and spinal cord superfusion (Sawynok et al., 1990), and the antinociceptive effects produced by i.t. morphine (Hoehn et al., 1988).

5 THESIS OBJECTIVES

Objective 1: To determine whether other agents which are involved in nociception and produce depolarization of nerve terminals, such as substance P, may augment morphine-evoked release of adenosine. Nanomolar concentrations of morphine evoke the release of adenosine from spinal cord synaptosomes in the presence of partially depolarizing conditions generated by an additional 6 mM K^+. Partial depolarization is accomplished by examining the effects of low concentrations of substance P and capsaicin, in combination with morphine, on adenosine release from spinal cord synpatosomes. Capsaicin is used as a potential depolarizing agent as capsaicin releases nucleotide(s) from spinal cord synaptosomes which is Ca^{2+}-dependent and originates from small diameter primary afferent neurons.

Objective 2: To determine the opioid receptor subtypes involved in the release of adenosine from rat dorsal spinal cord in neurochemical studies. This is accomplished by examining the effect of the selective μ opioid receptor agonists PLO17 (Chang et al., 1983), DAMGO (Handa et al., 1981) and morphine, the δ_1 opioid receptor agonist DPDPE (Mosberg et al., 1983), the δ_2 opioid receptor agonist [D-Ala^2,Cys^4]deltorphin (DELT) (Horan et al., 1992), and the κ opioid receptor agonist U50488H (Von Voigtlander et al., 1983) on the release of adenosine from synaptosomes prepared from the dorsal spinal cord.

Objective 3: To determine if a synergistic relationship exists between μ opioid and δ opioid receptor subtypes in the release of adenosine from the spinal cord. This is accomplished by determining whether low nanomolar concentrations of μ and δ opioid receptor agonists can act synergistically to evoke the release of adenosine from rat dorsal spinal cord synaptosomes above basal adenosine levels.

Objective 4: To implicate endogenous adenosine as a mediator of antinociception produced by i.t. injection of selective opioid receptor agonists. To this end, the methylxanthine adenosine antagonist, caffeine, is used to block nociceptive response latencies in the thermal threshold hot plate and tail flick tests. Caffeine was chosen as the adenosine receptor antagonist as it is a broad spectrum antagonist (blocking A1 and A2 receptors with comparable affinity) and is soluble in saline.

Objective 5: To determine which adenosine receptor subtype is activated subsequent to release by selective opioid receptor agonists. Comparative studies are performed using the selective adenosine A_1 receptor antagonist CPT and the relatively selective adenosine A_2 receptor antagonist DMPX. Opioid-induced antinociceptive effects are assessed following i.t. pretreatment with CPT and DMPX.

METHODS

1 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 behavioural experiments. They were maintained on a 12/12 hr light/dark cycle and were given food and water ad libitum. Experiments were carried out according to a protocol approved by the Animal Care Committee of Dalhousie University, Nova Scotia and deemed to be in accordance with the animal care guidelines and IASP guidelines on the use of animals in pain research.

2 NEUROCHEMICAL EXPERIMENTS

2.1 Preparation of Spinal Cord Synaptosomes

Release of adenosine from spinal cord synaptosomes was studied as previously described (Sweeney et al., 1987). The rats were decapitated and the spinal cord was removed rapidly by either vertebral laminectomy or by rapid hydrostatic pressure. The method of hydrostatic pressure was accomplished using a 20 ml syringe with an 18 gauge needle. Ice cold 0.32 M sucrose (pH 7.4 with 2-[4-(2-hydroxyethyl)-1-piperazine]-ethanesulfonic acid (HEPES)) was injected into the caudal end of the vertebral canal shearing the spinal cord free from its dorsal and ventral nerve roots and expelling it intact from the rostral end of the canal.

For the preparation of spinal cord synaptosomes, the dorsal spinal cord was

homogenized in 5 ml of ice cold HEPES-buffered sucrose using a teflon-glass homogenizer (0.25 mm clearance) followed by a 5 ml rinse of the homogenizing tube with sucrose. Homogenized tissue from two rats was combined (total volume of 20 ml) before being centrifuged at 1000 x g for 10 min at 4°C in a Sorvall centrifuge. Synaptosomes in the supernatant were kept on ice while the pellet was resuspended with ice-cold sucrose and centrifuged again at the same rate thus optimizing the synaptosomal yield. The synaptosomal suspensions were combined and centrifuged at 12,000 x g for 20 min yielding an enriched synaptosomal pellet. The resulting P_2 pellet contained the synaptosomes used in release studies. A schematic flow chart of the synaptosomal preparation is depicted in Figure 2.

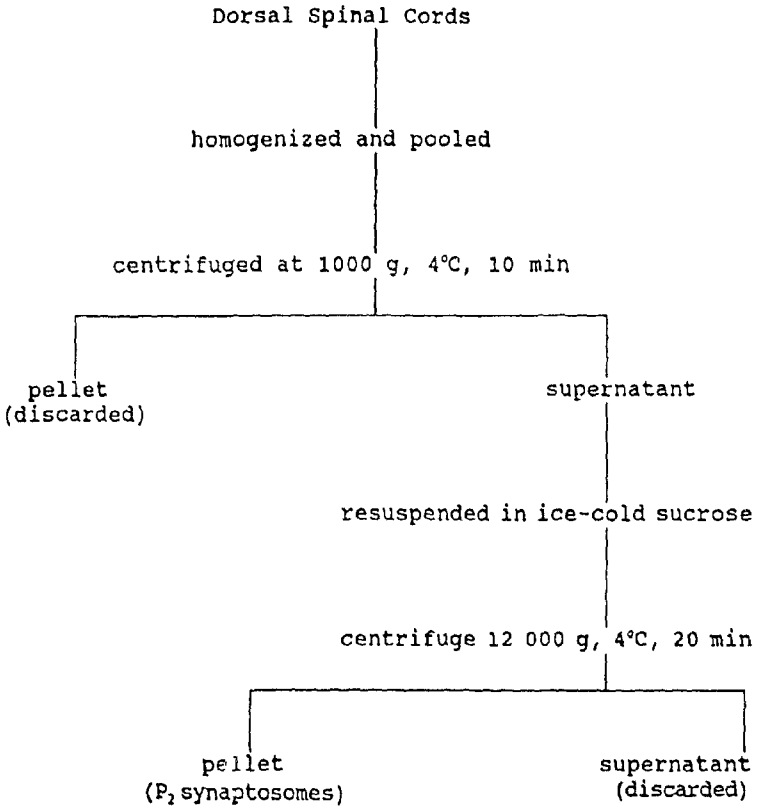
2.2 Release of Adenosine

Synaptosomes were resuspended in 5 ml of 37°C Krebs-Henseleit medium having the following composition (mM): NaCl 111, KCl 4.7, $CaCl_2$ 1.8, $NaHCO_3$ 26.2, NaH_2PO_4 1.2, $MgCl_2$ 1.2 and glucose 11 (pH adjusted to 7.4; continuously gassed with 95/5% mixture of O_2/CO_2). The synaptosomal suspension was incubated for 30 min at 37°C (warm water bath), and aerated with 95/5% O_2/CO_2 after the first 15 min. Following the preincubation in Krebs-Henseleit medium, synaptosomes were centrifuged at 3000 x g for 10 min at room temperature.

The synaptosomes were resuspended in 5-6 ml 37°C Krebs-Henseleit medium (protein concentration 1.5 - 2.5 mg/ml). Aliquots of the synaptosomal (P2 fraction) suspension (350 μl) were added to microfuge tubes containing the drugs to be

FIGURE 2

Schematic representation of the preparation of rat spinal cord synaptosomes.



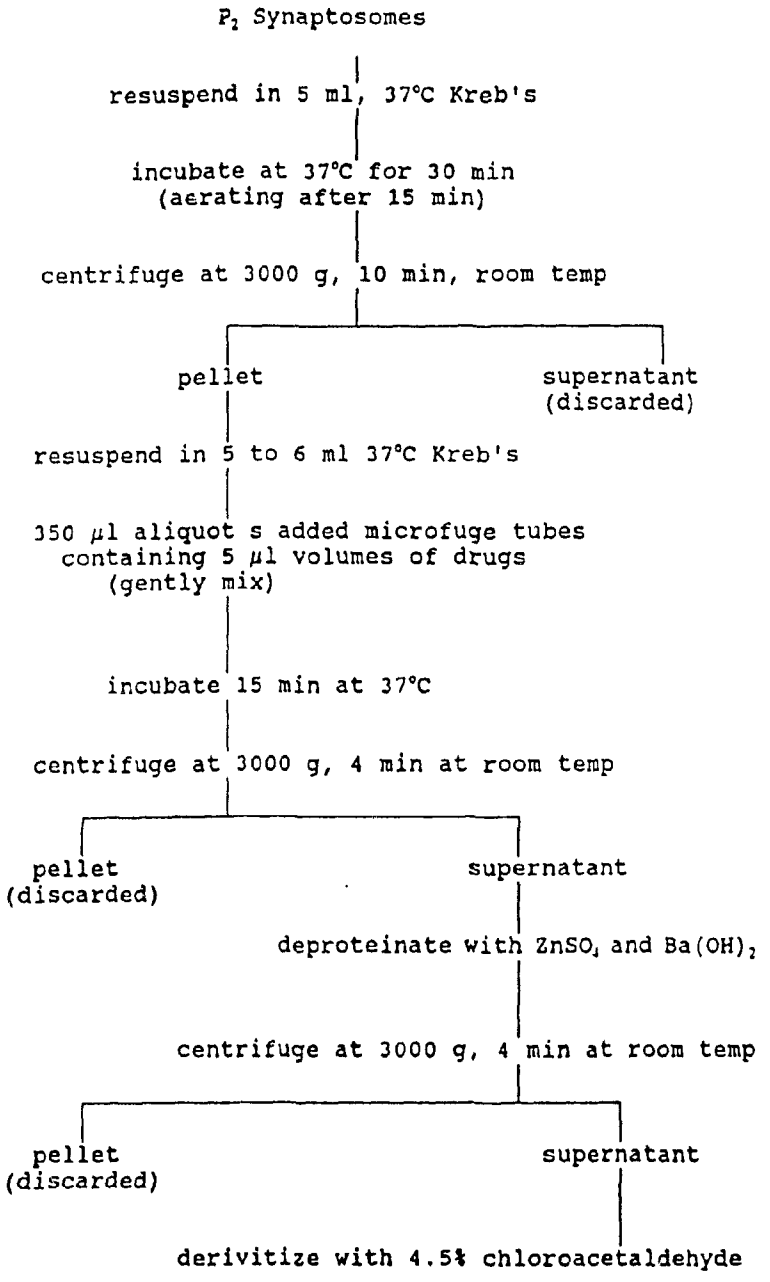
investigated (final volume of 365 μl). The contents of the tubes were gently mixed and incubated for 10 minutes at 37°C. In all cases, release was terminated by centrifugation at 3000 x g for 4 min. A 250 μl aliquot of supernatant was deproteinated with 125 μl 0.3 M $ZnSO_4$ and 125 μl 0.3 M $Ba(OH)_2$. The deproteinated supernatant (425 μl) was then derivatized with 75 μl 4.5% chloroacetaldehyde to form the etheno-derivative of adenosine by boiling the mixture for 20 min. Adenosine release was then quantitated by HPLC with fluorescence detection. Adenosine release was expressed as picomoles per milligram protein per 10 minutes (pmol/mg protein/10 min). A schematic representation of this procedure is shown in Figure 3.

Two control tubes containing only synaptosomes and Krebs medium were included in each experiment. One tube was centrifuged immediately before the 10 minute incubation (time 0 min) to determine the quantity of adenosine released during the preparation of the synaptosomes. The second tube was incubated for 10 min in the absence of drugs to provide an estimate of adenosine released in the absence of drugs (basal release). 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.

For Ca^{2+} free experiments, synaptosomes were prepared as above except the Krebs medium was Ca^{2+} free. Ca^{2+} was added back to synaptosomes during the drug incubation stage for controls only, thereby allowing comparisons to be made between

FIGURE 3

Schematic representation of adenosine release assay.



Ca^{2+} free conditions and Krebs with normal Ca^{2+} concentrations.

Experiments to determine whether release of adenosine originates as adenosine *per se* or a nucleotide that is converted to adenosine extracellularly were preformed by the addition of ecto-5'-nucleotidase inhibitors (α,β-methylene ADP and 5'GMP) at the drug incubation stage.

In all experiments an appropriate positive control of 24 mM K^+, 100 μM morphine or 10 nM morphine with 6 mM K^+ was included.

All experiments determining the effects of μ- and δ-opioid receptor agonist combinations were performed in the presence of a partial depolarization with K^+ (an additional 6 mM raising the total extracellular K^+ to 10.7 mM).

2.3 HPLC Detection

Samples were cooled and either analyzed immediately or stored in the refrigerator for no longer than 3 days before analysing. The quantitation of adenosine was performed by HPLC with fluorescence detection. Samples were aliquoted into 1 ml glass vials for automatic injection (100 μl) by the Waters WISP automatic injector model 712. The column used was a Waters reverse phase compression column. The mobile phase consisted of 50 mM acetate buffer (pH 4.5) containing 2 mM 1-octanesulfonic acid and approximately 15% acetonitrile, after degassing for a total time of 30 - 35 min. The retention time of adenosine was kept at 2 - 2.3 min providing sharp peaks which could be easily integrated. The flow rate was set at 0.8 ml/min for the Waters compression column on the Waters model 6000A solvent pump. Fluorescence detection was

accomplished by the Waters model 420AC fluorescence detector (excitation at 280 nm and a long pass emission filter at 399 nm) which was analyzed by computer integration, sensitivity 2 pmol per 100 μl injection). The Waters baseline program, baseline version 3.1, stored all the data and integrated the chromatographs by peak area or calculated peak height. Adenosine in the unknown samples was quantitated by direct comparison to a set of standards. Stock solutions of adenosine were prepared in millipore water and stored at -15°C. Standards were thawed and prepared for every experiment in Krebs-Henseleit medium and subjected to the same procedure for deproteination and derivitization as the unknowns to minimize the variability between sets of experiments. To ensure that the analysis was indeed of adenosine, a set of experiments were performed with adenosine deaminase. Samples were incubated with this enzyme during the drug incubation stage. Unknown samples containing the deaminase exhibited no peaks corresponding to adenosine on the chromatographs compared to adenosine standards or unknowns in the absence of adenosine deaminase, thus demonstrating that the assay was measuring adenosine.

2.4 Protein Analysis

Protein concentrations were determined by a modified version of Hartree (1972). Synaptosomal suspensions from each experiment were stored below 0°C prior to protein analysis. Samples were thawed and diluted to 1 in 30 providing concentrations of protein which were in the appropriate range for analysis. Absorbance was measured using the Beckman spectrophotometer. Concentrations of protein were quantitated by direct

comparison of unknown samples to standards prepared from bovine serum albumin. Adenosine was expressed as picomoles per milligram of protein per 10 min (pmol/mg protein/10 min).

2.5 Lactate Dehydrogenase Assay

Activity of lactate dehydrogenase (LDH) in spinal cord synaptosomes was measured according to the methods described by Wroblewski and LaDue (1955) providing a determination of the viability of the synaptosomes. A synaptosomal suspension of 100 μl was added to 2.8 ml phosphate buffer containing 100 mM NADH and $NADH_2$ at room temperature. The absorbance was calibrated with this suspension using a Beckman spectrophotometer before adding 100 μl of sodium pyruvate (22.7 M). The contents were mixed by inversion and the rate of change in absorbance was determined over a 2 min time interval at 340 nm. LDH was also measured following the addition of 100 μM morphine. The total LDH was determined following lysis of the synaptosomes with 10 μl Triton X (10% solution).

3 PRETREATMENT WITH INTRATHECAL NEUROTOXIN

Male Sprague-Dawley rats, 250 - 300 g, were obtained from Charles River, Quebec, Canada. Rats were anaesthetized with 3% halothane and mounted in the ear bars of a stereotaxic apparatus. Acute indwelling cannulas were surgically placed into the subarachnoid space as previously described by Sweeney et al. (1987). This was accomplished by making a small incision at the base of the brain and separating the

muscles overlaying the atlanto-occipitial membrane. A small hole was made in the membrane overlying the cisterna exposing the spinal cord and the cerebral spinal fluid. A saline filled cannula of PE 10 tubing was carefully advanced 7.5 cm down the spinal cord subarachnoid space to approximately the T12 region. At this point 20 μl of 60 μg capsaicin dissolved in 60% v/v DMSO in physiological saline or vehicle was injected, followed by a further 10 μl of saline to flush the contents of the cannula. The capsaicin was injected very slowly (over 7 to 10 min) to prevent tremors which can occur if it is administered too rapidly. This dose of capsaicin produces de-generation of small diameter primary afferent neurons (Palermo et al., 1981). Once the animal received the capsaicin the cannulas were gently removed. The muscles overlying the cisterna were stitched together followed by suturing of the skin to allow proper healing of the wound. Animals received isotonic solution subcutaneously to prevent dehydration during the first hours after surgery as well as intramuscular penicillin to decrease the possibility of infection. Animals were allowed to recover at least 7 days after surgery before being used in the adenosine release studies. Any animal that displayed signs of paralysis was excluded from the neurochemical experiments.

4 BEHAVIOURAL EXPERIMENTS

4.1 Intrathecal Injections.

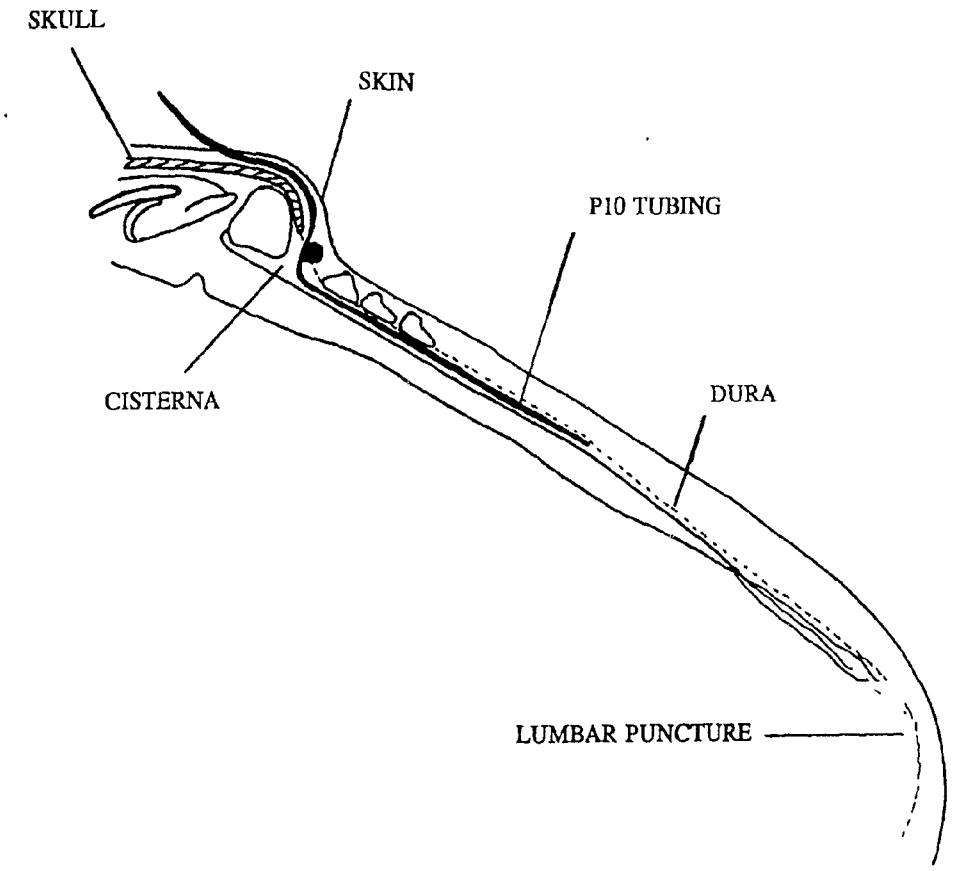
4.1.1 Lumbar puncture. Opioid agonists were injected directly into the subarachnoid space of rats (275-300 g) by lumbar puncture according to the method of Hylden and Wilcox (1980). Injections of 20 μl were made under halothane anaesthesia via a 30

gauge needle into an intravertebral space at the level of the 5th or 6th lumbar vertebra. A characteristic tail flick response confirmed entry into the subarachnoid space. Acute i.t. injections were used to obtain dose response relationships for each μ and δ agonist in thermal nociceptive tests.

4.1.2 Chronic Cannulation. Rats were implanted with chronic i.t. cannulas under halothane anaesthesia according to the modification of the method described by Yaksh and Rudy (1976). Briefly, a small opening was made in the atlanto-occipital membrane and a polyethylene (PE-10) catheter was advanced caudally (7.5 cm) to the lumbar spinal cord. Following surgery, rats were given penicillin G (PenlongXL^{R}) i.m. and 10 ml lactated Ringer's solution subcutaneous to promote recovery from surgery. Only animals exhibiting no motor deficits as the result of surgery were used for antinociception experiments. Experiments were commenced 7-10 days following surgery and animals were used for only one experiment. Chronic cannulas were implanted in animals used for studying the methylxanthine-sensitivity of selective opioid agonists. Animals implanted with chronic cannulas were used for antagonist experiments, as lumbar puncture injection of caffeine did not block morphine-induced antinociception. All opioid agonists and the adenosine antagonist, caffeine, were injected in a volume of 10 μl (cannula volume 8 μl) followed by 10 μl saline flush to ensure complete delivery of the drugs. For antagonist experiments, i.t. caffeine was injected 15 min prior to the i.t. administration of the opioid agonist tested. A schematic diagram of i.t. lumbar punctures versus chronic cannula placement is presented in Figure 4.

FIGURE 4

Placement of a chronically implanted cannula in the rat, and the site of i.t. lumbar puncture.



4.2 Antinociceptive Testing

Antinociception was quantitated using a constant temperature hot plate (50°C ± 0.5°C) and/or tail flick test. In the hot plate test, the response latency to a hindpaw lick was recorded (baseline 7-12 sec, cutoff 50 sec). In the tail flick test, the response latency to a tail flick was recorded (baseline 2-3 sec; cutoff 10 sec) on an automated tail flick apparatus (Ugo Basile, Italy). In the absence of a response, the animal was removed from the hot plate at 50 sec or from the tail flick apparatus 10 sec to avoid tissue injury, and assigned this latency.

4.3 Experimental Paradigm

4.3.1 Agonist studies. The first series of experiments determined the dose response and time course of μ and δ agonists on the hot plate response following administration by lumbar puncture. The following doses for each opioid agonist were examined in the hot plate test or tail flick test following i.t. administration: DAMGO (0.019 - 5.8 nmol; 0.1 - 3.0 μg), PLO17 (0.19 - 5.6 nmol; 0.1 - 3.0 μg), morphine (1.5 - 15 nmol; 1.0 - 10 μg), DPDPE (1.35 - 81.2 nmol; 1 - 60 μg), DELT (0.34 - 11.5 nmol; 0.3 - 10 μg) and U50488H (21.5 - 644 nmol; 10 - 30 μg).

Nociceptive testing for quantitation of thresholds for selective adenosine agonists were preformed on animals implanted with chronic i.t. cannulas. Rats were accommodated in plastic boxes which allowed access to the cannula for i.t. injections. These experiments were preformed to determine whether antinociceptive synergy occurs between A_1 and A_2 adenosine receptors.

4.3.2 Antagonist studies. Nociceptive testing was performed on animals implanted with i.t. cannulas. For evaluation of adenosine involvement in opioid antinociception, caffeine (103 - 515 nmol; 50 - 100 μg) or a saline control was injected i.t. 15 min prior to 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.

Evaluation of adenosine receptor subtypes involved in opioid-induced antinociception was performed with selective agonists and antagonists for A_1 and A_2 receptors. CHA and CGS21680 were selected as A_1 and A_2 receptor agonists, respectively; and CPT and DMPX as selective A_1 and A_2 adenosine antagonists, respectively. In addition to monitoring antinociception, rats were scored subjectively for motor effects at each time interval on a scale of 0 (no motor effects), 1 (some loss of muscle tone, lazy, dopey or staggering), 2 (hind limb extension while resting in cage but uprights when handled) or 3 (hind limb extension with rigidity and unable to upright for hind paw threshold evaluation). The evaluator of thermal threshold latencies and motor scores for adenosine ligands was blinded to both the dose and the agent each rat received during the experiment.

5 DRUGS

Drugs used in this study were obtained from the following sources: DPDPE (MW = 645.7) and PLO17 (MW = 535.6) (Penninsula Labs, Belmont, California), α,β-methylene ADP, 5'-GMP, capsaicin and DAMGO (MW = 513.6) (Sigma, St. Louis,

MO), morphine sulfate (MW = 334.4, British Drug Houses, Ontario, Canada), DELT (MW = 871, generous gift from Frank Porreca), U50488H (MW = 465.4, Upjohn, Kalamazoo, Mich, USA), CHA (MW = 349.4), CGS21680 (MW = 539.5), CPT (MW = 248.3) and DMPX (MW = 218.2) (RBI). All opioid drugs, CHA and CGS21680 were dissolved in physiological saline (0.9% NaCl w/v). Capsaicin used in neurotoxin experiments was dissolved in 60% DMSO in saline. DMPX and CPT were dissolved in 0.02% NaOH.

6 STATISTICS AND CALCULATION OF DATA

From the peak effect of the particular opioid agonist, dose-response curves were generated plotting percent MPE (maximum possible effect) vs. log dose. Response latency data from the hot plate measurements were converted to % MPE scores:

% MPE = (postdrug latency - baseline latency)/cutoff time - baseline latency) x 100.

Time effect and dose response data are presented as mean $\pm$ 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 used to show the effect of the adenosine antagonist refer to the dose producing a 50% reduction in the antinociceptive effect of the agonist used ($\sim ED_{75}$). ED_{50} and IC_{50} values were interpolated by the computer program INPLOT (Graph Pad). Statistical comparisons were made using analysis of variance followed by the Student Newman-Keuls test.

The statistical method used to evaluate synergistic interactions was the dose-addition model (Wessinger, 1986). By using an inactive dose of one drug (δ-opioid

receptor agonist) while eliciting a dose-related function for the other, a left-ward shift in the dose-response curve for the second drug indicates synergy (reviewed Solomon and Gebhart, 1994). This method was chosen over isobolographic analysis because an ED_{50} for the δ-opioid receptor agonists could not be obtained, as even micromolar concentrations do not release adenosine.

Statistical comparisons were made using analysis of variance with the Student Newman Keuls test for post hoc comparisons.

RESULTS

1 MORPHINE-INDUCED RELEASE OF ADENOSINE IS AUGMENTED BY DEPOLARIZING AGENTS OTHER THAN K^+

Micromolar concentrations of morphine evoke the release of adenosine from spinal cord synaptosomes. Following 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} M and 10^{-6} M morphine but not 10^{-7} M morphine (Figure 5; taken from Cahill et al., 1993a). The dose response curve for morphine-evoked release of adenosine in the presence of an elevated K^+ concentration is thus multiphasic. In this study, the addition of 6 mM K^+ was determined to be the optimum K^+ concentration when combined with 10 nM morphine to evoke the release of adenosine above basal levels (Figure 6).

It was of interest to determine whether other agents capable of producing neuronal depolarization and elevating intraneuronal Ca^{2+} (substance P and capsaicin) also could augment morphine-evoked release of adenosine. Substance P, alone, releases adenosine in a multiphasic manner, enhancing release above basal levels at 0.1-1 nM, and again at 1 μM (Figure 7). The extent of the adenosine released by substance P was comparable to that produced by maximum depolarization with K^+ (Cahill et al., 1993a). Two concentrations of substance P were selected, a submaximal concentration (0.1 nM) and the concentration at the trough (100 nM) of the dose-response curve, to be combined with multiple concentrations of morphine to determine whether these doses could enhance

FIGURE 5

Dose-related release of adenosine by the mu opioid agonist morphine 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 mean ± s.e.m. of the adenosine released above basal release values or above values obtained following the addition of 6 mM K^+ for n=6. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to basal release values, ++ $p<0.01$ compared to evoked adenosine release in the absence of the additional 6 mM K^+. Basal adenosine values 172 ± 7 ; 6 mM K^+ 180 ± 5 pmol/mg protein/15 min (data from Cahill et al., 1993a).

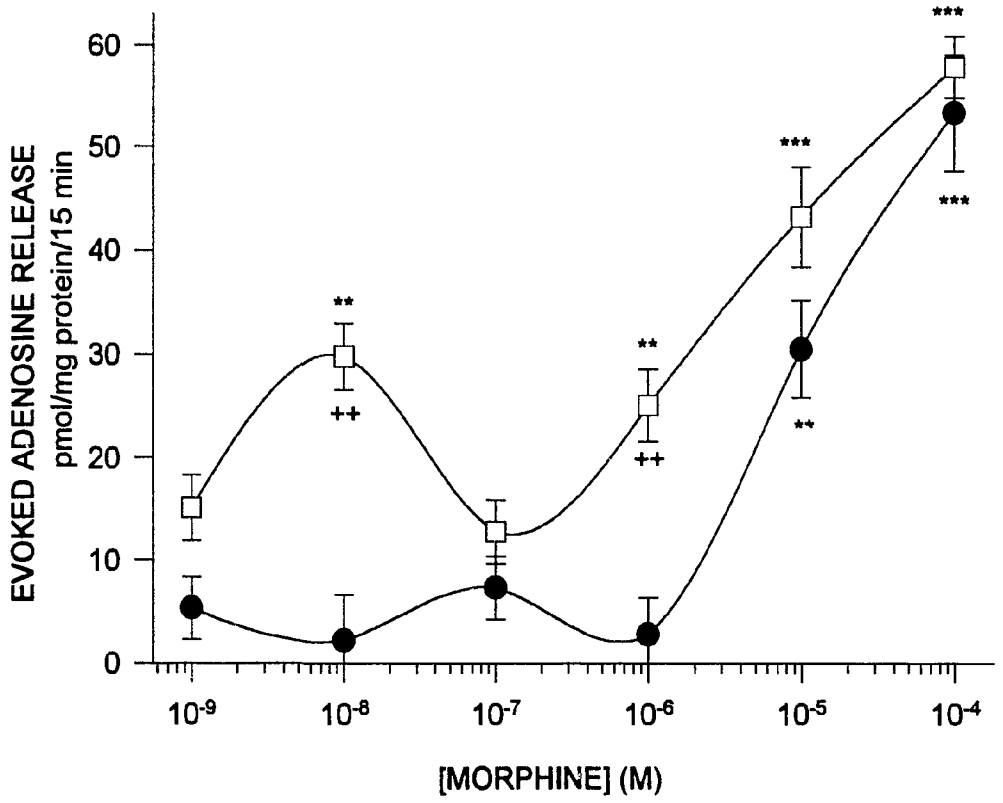


FIGURE 6

Dose-dependent effects of K^+ depolarization to evoke the release of adenosine in the presence of 10 nM morphine. Values represent mean ± s.e.m. for n=4. ++ $p<0.01$ compared to evoked adenosine by K^+. Basal values ranged from 186 - 210 pmol/mg protein/10 min.

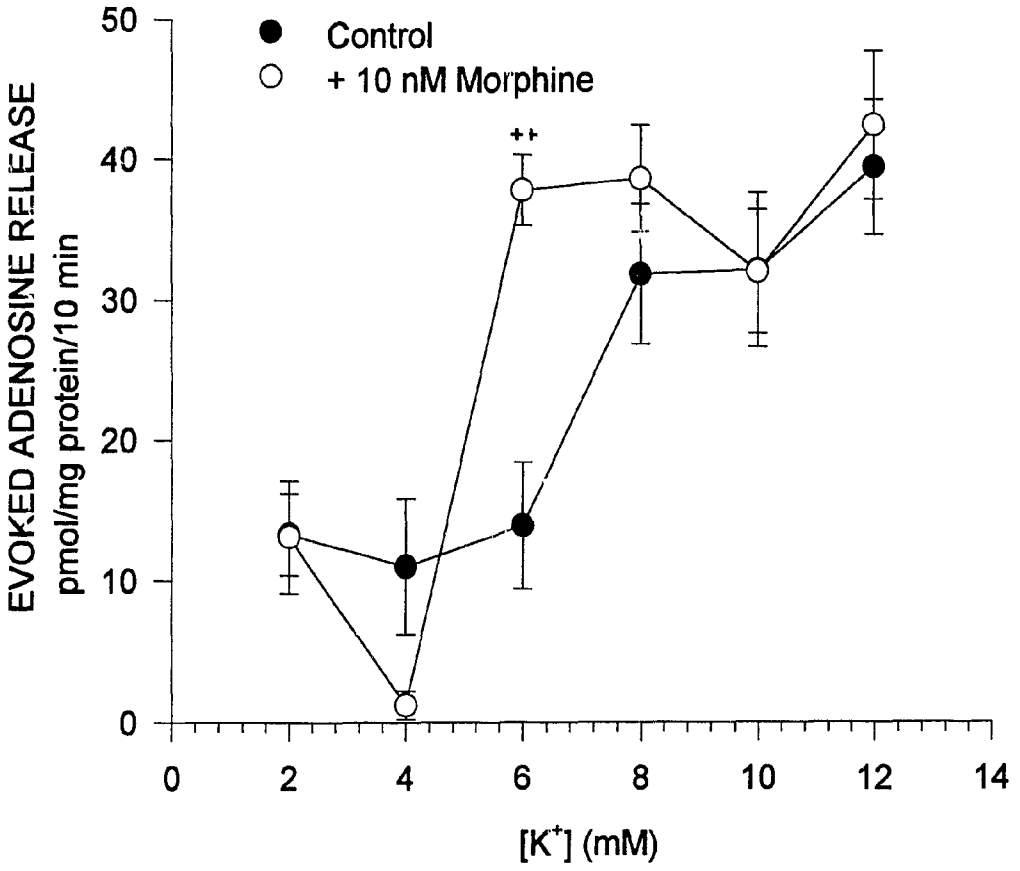
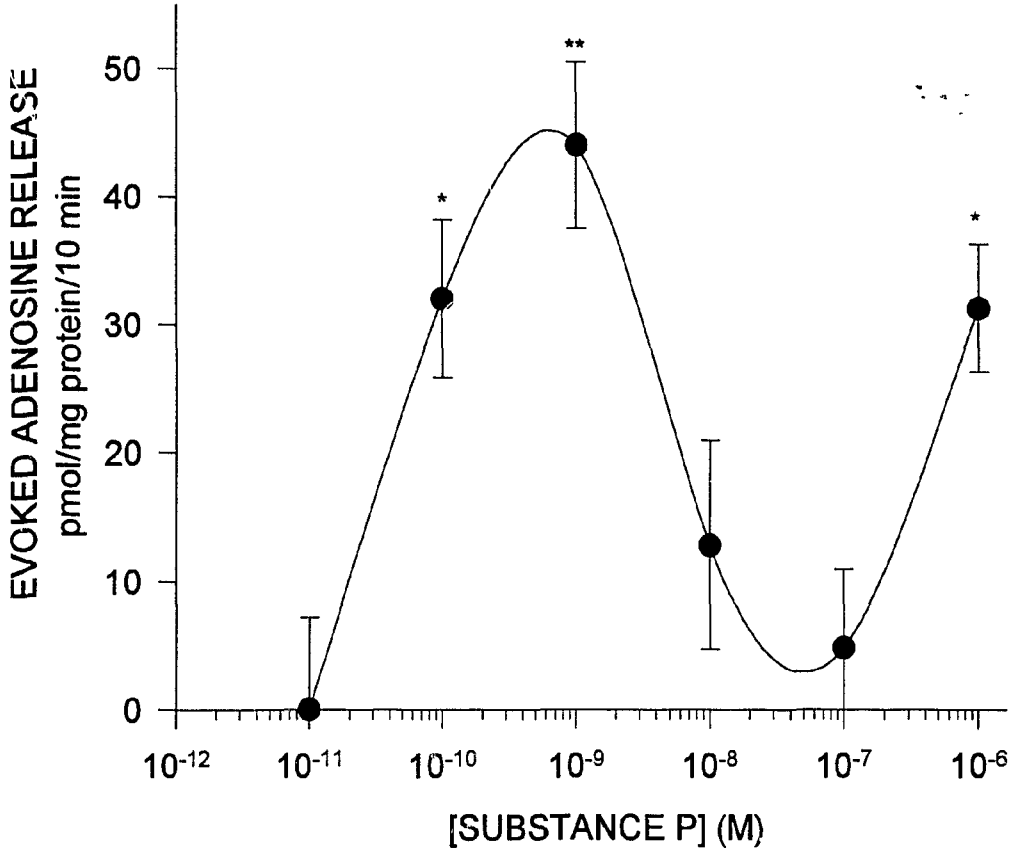


FIGURE 7

Dose-related release of adenosine by substance P from dorsal spinal cord synaptosomes. Values represent mean ± s.e.m. for n=5. * $p<0.05$, ** $p<0.01$ compared to basal release values. Basal adenosine 198 ± 12 pmol/mg protein/10 min.



opioid-induced release of adenosine. Substance P at 100 nM, but not at 0.1 nM, combined with morphine, facilitated release of adenosine at 10 nM, the same concentration of morphine which released adenosine in the presence of K^+ (Figure 8).

Morphine-evoked release of adenosine in the presence of a presumed partial depolarization generated by the addition of capsaicin also was examined. A previous study has demonstrated that micromolar concentrations (1-100 μM) of capsaicin release adenosine via a Ca^{2+}-dependent mechanism (Sweeney et al., 1989). Adenosine released by multiple concentrations of capsaicin (10^{-9} - 10^{-4} M) in combination with 10 nM morphine was not different from that produced by capsaicin alone (Figure 9). Thus, evoked release of adenosine by nanomolar concentrations of morphine could not be enhanced by the addition of capsaicin, unlike the addition of K^+ and substance P.

1.1 Characterization of Adenosine Released by Substance P and Morphine

Capsaicin is a neurotoxin which selectively de-generates small diameter primary afferent neurons following i.t. administration in adult rats (Palermo et al., 1981). Adult rats were pretreated with capsaicin or vehicle (60% DMSO in saline) 7-10 days before examining morphine- and substance P-evoked release of adenosine from both groups of animals. Substance P (1 nM) and substance P (100 nM) plus morphine (10 nM) evoked release of adenosine originates from capsaicin-sensitive nerve terminals, as release was significantly attenuated compared to release from synaptosomes prepared from vehicle treated rats (Figure 10A).

The release of adenosine by substance P (1 nM) and the combination of substance P

FIGURE 8

Morphine (10 nM) and substance P (100 nM) act synergistically to enhance the release of adenosine. Values represent mean ± s.e.m. of basal adenosine release for n=5. * $p<0.05$, ** $p<0.01$ compared to basal release values, + $p<0.05$ compared to release by 10 nM morphine. Basal values ranged from 190 - 245 ± 17 pmol/mg protein/10 min.

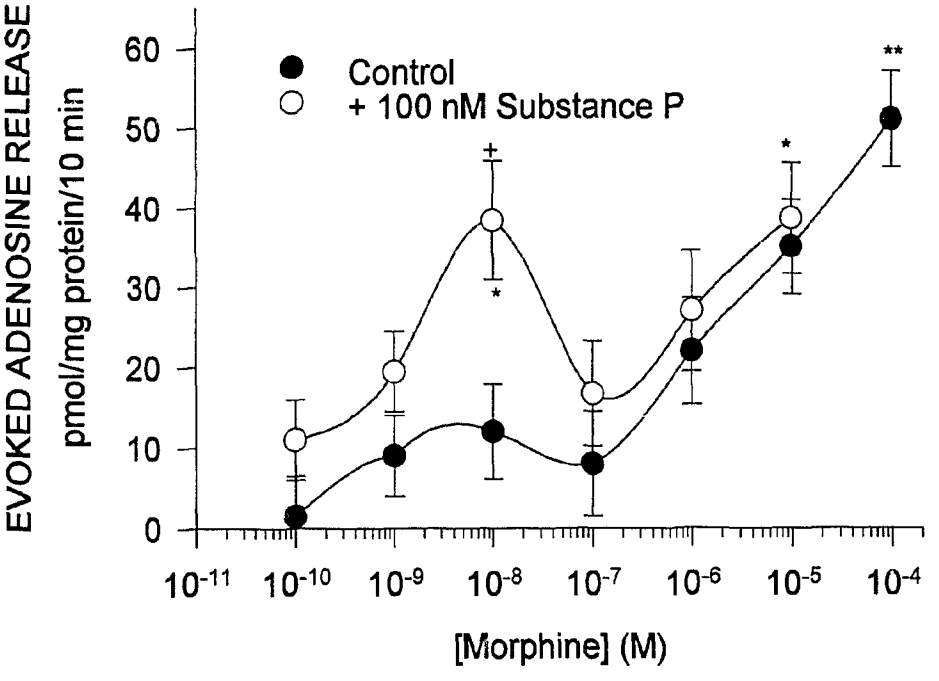
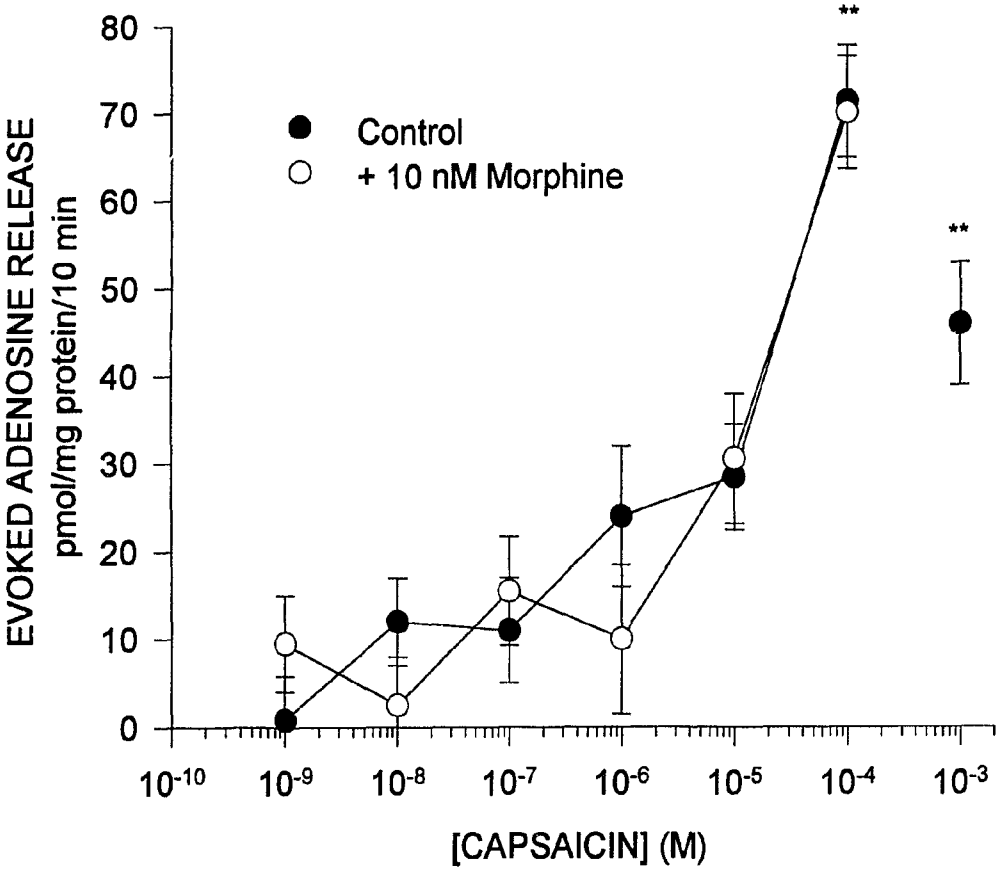


FIGURE 9

Capsaicin-evoked release of adenosine in the absence and presence of 10 nM morphine. Values represent mean ± s.e.m. of basal adenosine release for n=5. Basal values ranged from 198 - 238 ± 16 pmol/mg protein/10 min.



(100 nM) with morphine (10 nM) was found to be Ca^{2+}-dependent, as the evoked release of adenosine was significantly attenuated when synaptosomes were prepared and incubated in Ca^{2+}-free medium (Figure 10B). Substance P-evoked release of adenosine was significantly reduced in capsaicin versus vehicle treated animals (Figure 10A). Thus, adenosine release originated from small diameter primary afferent neurons. Basal adenosine levels in the presence of substance P and morphine were not different from basal values when incubated in Ca^{2+}-free medium or when synaptosomes were prepared from spinal cords of rats pretreated with i.t. capsaicin.

2 OPIOID RECEPTOR SUBTYPES INVOLVED IN THE RELEASE OF ADENOSINE FROM DORSAL SPINAL CORD SYNAPTOSOMES

Selective opioid receptor agonists were used to determine which opioid receptor subtype(s) is involved in adenosine release from dorsal spinal cord synaptosomes. The selective μ opioid agonist DAMGO had little effect on the release of adenosine alone, but the addition of 6 mM K^+ significantly augmented release of adenosine in a multiphasic manner similar to morphine (Figure 11A). Release of adenosine by 10^{-9} and 10^{-8} M DAMGO was particularly prominent. PLO17, another highly selective μ opioid agonist, significantly increased the release of adenosine alone at 10^{-7} M, and this release was further enhanced in the presence of an additional 6 mM K^+ at both nanomolar and micromolar concentrations (Figure 11B). The relative nanomolar and micromolar potencies of μ opioid agonists in the presence of 6 mM K^+ in releasing adenosine is presented in Figure 12. At nanomolar concentrations, the more selective μ opioid

FIGURE 10

Intrathecal pretreatment of capsaicin (panel A) and calcium dependency (panel B) of evoked adenosine release by substance P, morphine/K^+, and morphine/substance P. Values represent mean ± s.e.m. of the adenosine released above basal release values for n=5. Panel A: * $p<0.05$, ** $p<0.01$ compared to evoked release from synaptosomes prepared from vehicle treated animals. Panel B: * $p<0.05$, ** $p<0.01$ compared to evoked release under normal Ca^{2+} concentrations (1.8 mM). Basal values ranged from 200 - 245 ± 15 pmol/mg protein/10 min.

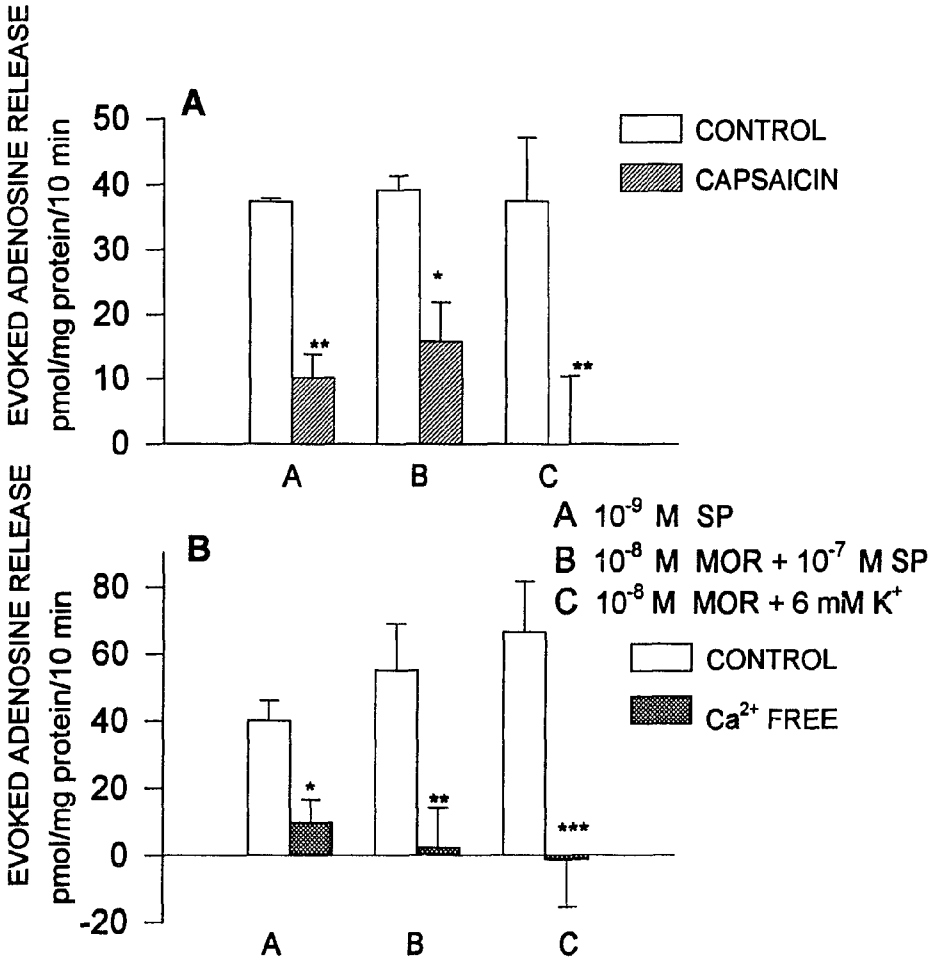


FIGURE 11

Dose-related release of adenosine by the selective μ opioid agonists (A) DAMGO and (B) PLO17 in the absence (●) or presence (O) of an additional 6 mM K^+ added to the Krebs-Henseleit medium (total concentration of 10.7 mM K^+). Values represent mean $\pm$ s.e.m. of the adenosine released by opioids above basal release values or above values obtained following the addition of 6 mM K^+ for n=6. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to basal release values, + $p<0.05$, ++ $p<0.01$, +++ $p<0.001$ compared to evoked adenosine release in the absence of the additional 6 mM K^+. A: basal 111 $\pm$ 7; 6 mM K^+ 112 $\pm$ 5 pmol/mg protein/10 min. B: basal 125 $\pm$ 8; 6 mM K^+ 132 $\pm$ 10 pmol/mg protein/10 min. In all cases, no significant change in the basal release of adenosine occurred as the result of the addition of this concentration of K^+.

FIGURE 11

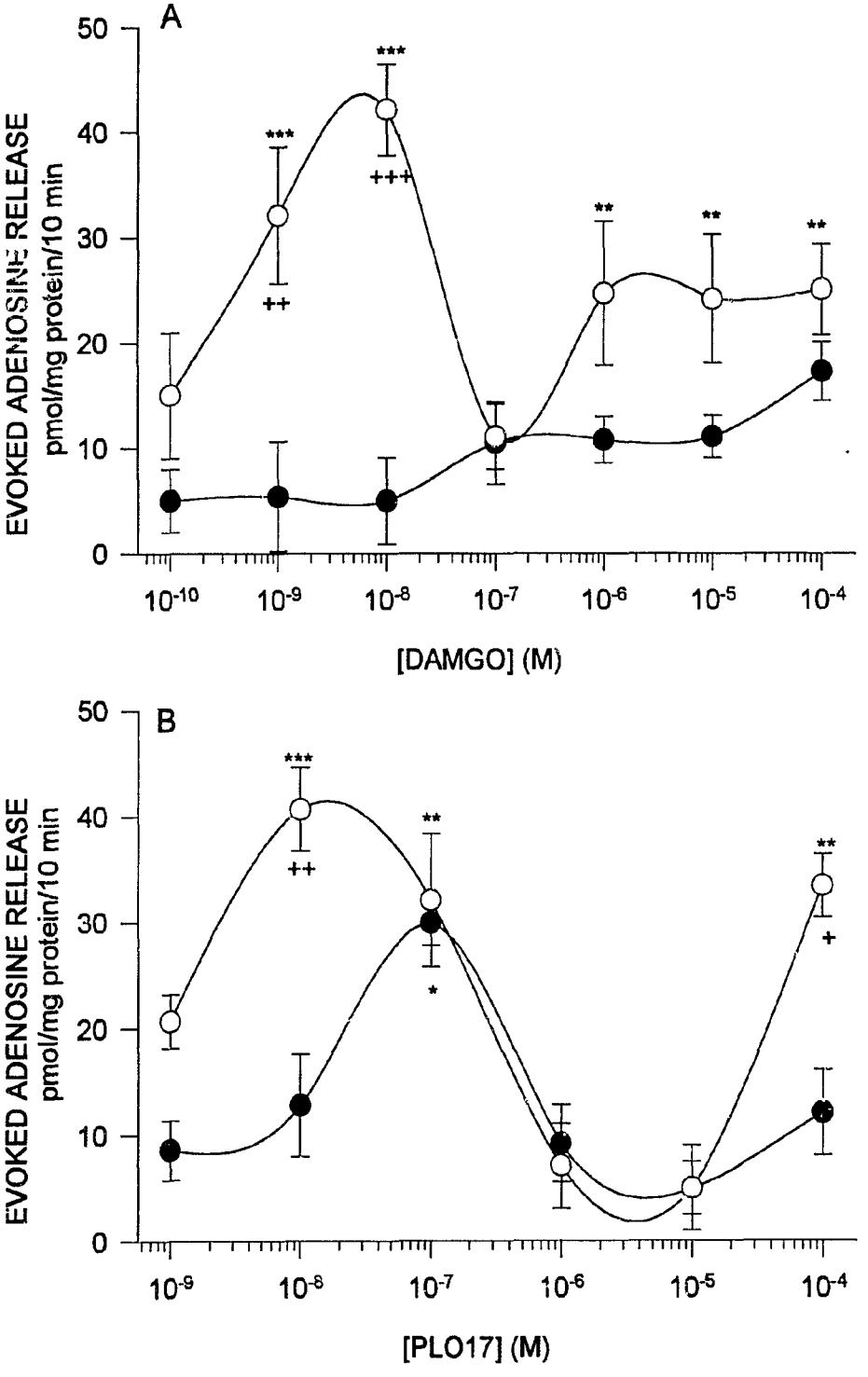
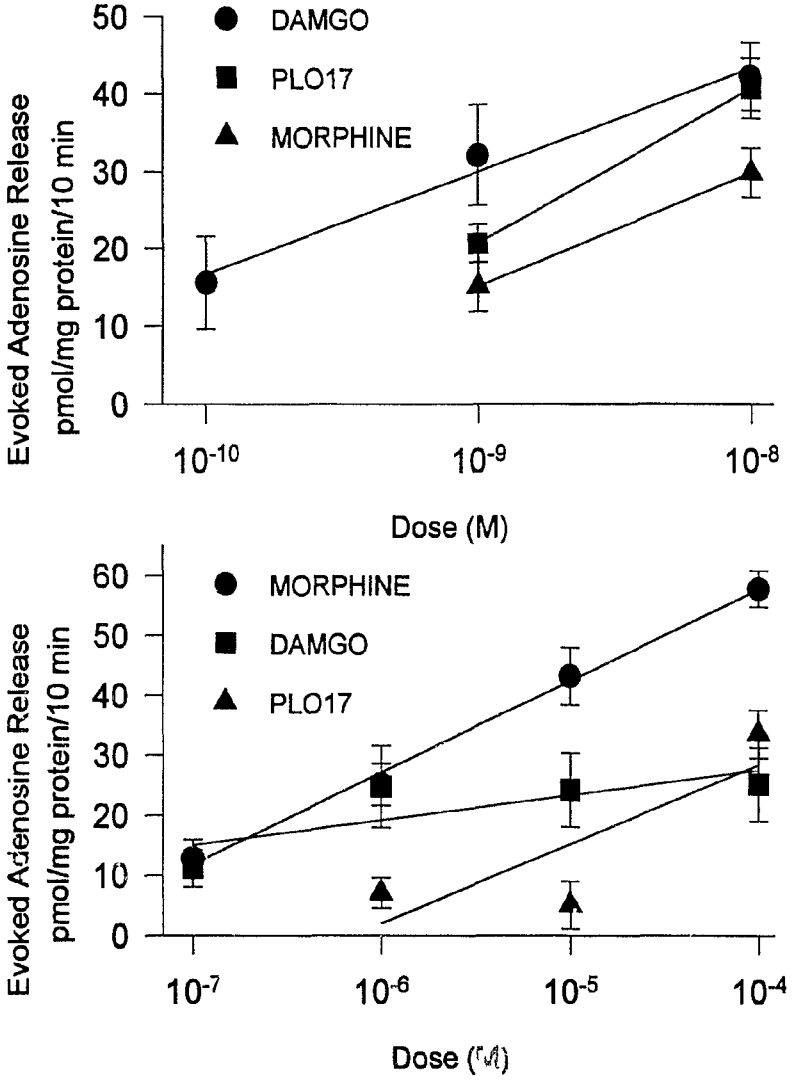

A
EVOKED ADENOSINE RELEASE
pmol/mg protein/10 min
50
40
30
20
10
0
***
***
+++
++
**
**
**
10-10
10-9
10-8
10-7
10-6
10-5
10-4
[DAMGO] (M)
B
EVOKED ADENOSINE RELEASE
pmol/mg protein/10 min
50
40
30
20
10
0
***
++
**
*
**
+
10-9
10-8
10-7
10-6
10-5
10-4
[PLO17] (M)

FIGURE 12

Comparison of nanomolar and micromolar potencies of the μ opioid agonists morphine, DAMGO and PLO17 in their ability to evoke the release of adenosine from dorsal spinal cord synaptosomes. Values represent mean $\pm$ s.e.m. for n=6.



ligands DAMGO and PLO17, appear more potent than morphine, but at micromolar concentrations, neither of the selective μ opioid agonists (PLO17 and DAMGO) evoked release of adenosine except in the presence of elevated K^+ concentrations.

The potential role of δ opioid receptors in releasing adenosine from the spinal cord was examined using the specific opioid agonists DPDPE (δ_1 agonist) and DELT (δ_2 agonist). DPDPE had no effect on the release of adenosine except at the highest dose (10^{-4} M) when synaptosomes were partially depolarized by the presence of an additional 6 mM K^+ (Figure 13A). DELT significantly increased the release of adenosine at 10^{-4} M; however, no additional enhancement on release was evident in the presence of an additional 6 mM K^+ (Figure 13B). Neither of the δ opioid agonists increased release of adenosine at nanomolar concentrations either with or without the addition of the 6 mM K^+.

The κ opioid agonist U50488H was used to examine the potential κ receptor involvement in release of adenosine from the spinal cord. U50488H had no effect on enhancing the release of adenosine in normal Krebs medium, although an enhancement of adenosine release at 10^{-5}-10^{-4} M U50488H occurred when synaptosomes were partially depolarized with 6 mM K^+ (Figure 14). Similarly, the κ opioid receptor agonist had no effect at nanomolar concentrations either in the absence or presence of partially depolarizing conditions generated by elevated K^+ concentrations.

FIGURE 13

Evoked release of adenosine by (A) DPDPE (δ1 agonist) or (B) DELT (δ2 agonist) in the absence (●) or presence (O) of an additional 6 mM K^+. Values represent means ± s.e.m. for n=6. * $p<0.05$, ** $p<0.01$ compared to basal release values. Basal adenosine values for A: 124 ± 5; 6 mM K^+ 126 ± 8 pmol/mg protein/10 min. B: 130 ± 7; 6 mM K^+ 121 ± 5 pmol/mg protein/10 min, C: 120 ± 10; 6 mM K^+ 132 ± 10 pmol/mg protein/10 min.

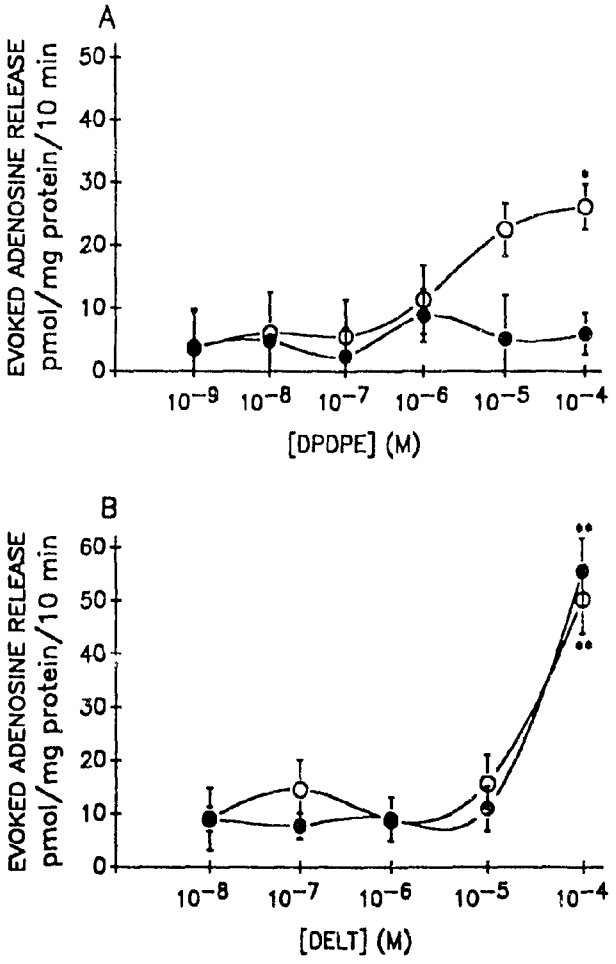
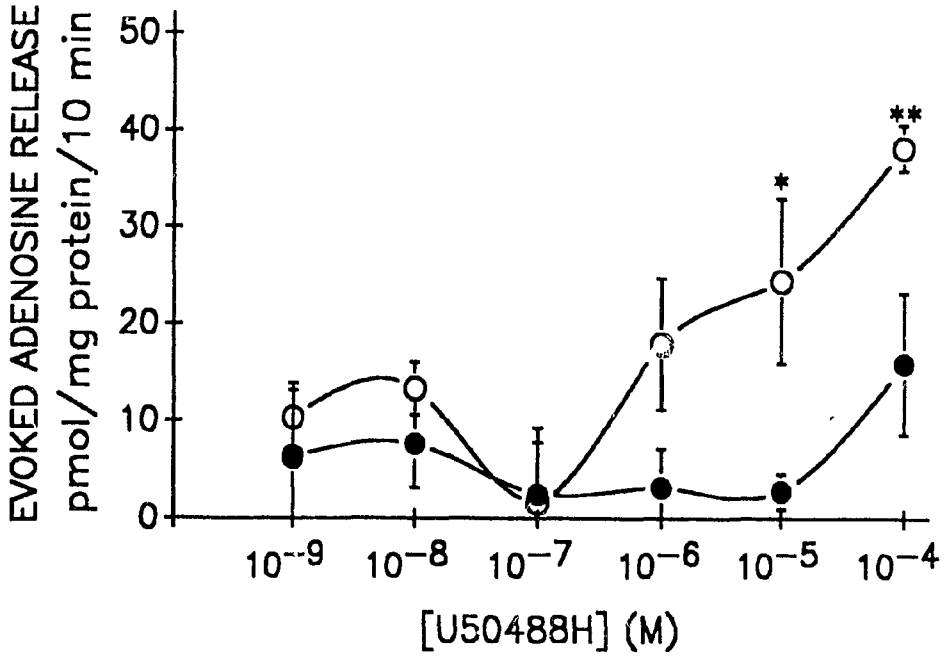


FIGURE 14

Evoked release of adenosine by U50488H (κ agonist) in the absence (●) or presence (O) of an additional 6 mM K^+. Values represent means ± s.e.m. for n=4. * $p<0.05$ compared to basal release values 120 ± 10; 6 mM K^+ 132 ± 10 pmol/mg protein/10 min.



2.1 Characterization of Adenosine Released by Opioid Agonists

2.1.1 Calcium Dependence

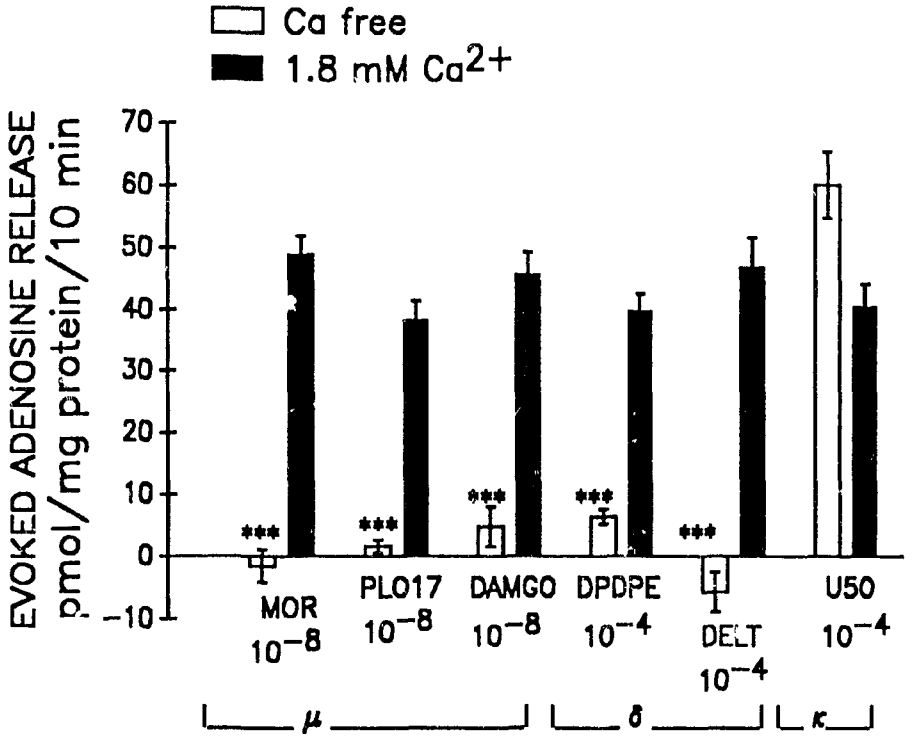
The evoked release of adenosine by nanomolar concentrations of the μ opioid agonists morphine, PLO17 and DAMGO, and micromolar concentrations of the δ opioid agonists was Ca^{2+}-dependent, as no significant release was seen above basal adenosine levels when synaptosomes were prepared and incubated in Ca^{2+}-free medium (Figure 15). In contrast, the κ opioid receptor agonist-evoked release of adenosine occurred via a Ca^{2+}-independent mechanism, as evoked release of adenosine by U50488H was still present and perhaps augmented in free Ca^{2+} (Figure 15). The addition of 1 mM EGTA (a Ca^{2+} chelator) in a separate experiment did not alter this observation (data not shown).

2.1.2 Adenosine vs Nucleotide

The release of adenosine by nanomolar concentrations of the μ opioid receptor agonists PLO17, DAMGO and morphine was further characterized by determining whether adenosine released by these agents represents adenosine *per se* or arises from a nucleotide that is converted to adenosine extracellularly. Synaptosomes were incubated with and without α,β-methylene ADP and 5'-GMP. These agents inhibit ecto-5'-nucleotidase activity, the enzyme necessary for conversion of adenosine monophosphate to adenosine. The degree of inhibition of this enzyme was determined by calculating the conversion of 5'-AMP (1 μM) to adenosine in the absence and presence of the inhibitors. Enzyme activity was inhibited 80 $\pm$ 6% by α,β-methylene ADP and 5'-GMP (n=4). There was no difference in the amount of adenosine released by each μ opioid agonist

FIGURE 15

Release of adenosine by the μ opioid agonists morphine (MOR), PLO17 and DAMGO, the δ opioid agonists DPDPE and DELT, and the κ agonist U50488H (U50) in the absence and presence of 1.8 mM Ca^{2+}. Synaptosomes were prepared in Ca^{2+} free medium and the normal concentration of Ca^{2+} in Krebs was added back at the drug incubation stage. Values represent mean $\pm$ s.e.m. for n=4. *** $p<0.001$. Basal values ranged between 199-220 pmol/mg protein/10 min. Release of adenosine by each opioid agonist was examined in the presence of the additional 6 mM K^+.



in the absence and presence of these ecto-5'-nucleotidase inhibitors. This indicated that the adenosine release evoked by μ opioid receptor agonists originates from the cell as adenosine *per se* rather than as nucleotide(s) which is converted to adenosine by ecto-5'-nucleotidase enzymes (Figure 16).

2.1.3 Capsaicin Pretreatment

The release of adenosine from spinal cord synaptosomes by selective μ opioid receptor agonists in the presence of an additional 6 mM K^+ was completely absent in rats pretreated with i.t. capsaicin (Figure 17). Evoked release of adenosine by 100 μM DELT was not significantly different in control versus capsaicin treated rats; however, evoked release by 100 μM DPDPE was significantly inhibited. U50488H also releases adenosine from a capsaicin-sensitive source.

2.2 Synergy Between μ and δ Opioid Receptor Agonists

Subnanomolar concentrations of morphine (10^{-10}, 10^{-11} M) in the presence of an additional 6 mM K^+ had little effect on the release of adenosine from spinal cord synaptosomes (Figure 18). Following the addition of the δ_1 opioid receptor agonist DPDPE (10^{-7} M), which by itself did not alter the release of adenosine, a modest enhancement of the release of adenosine occurred at 10^{-10} and 10^{-9} M morphine (Figure 18). Subnanomolar concentrations of the selective μ opioid receptor agonist DAMGO had little effect on the release of adenosine. The addition of DPDPE significantly augmented the release of adenosine, as DAMGO now released adenosine at 10^{-10} M, and

FIGURE 16

Release of adenosine by nanomolar concentrations of the μ 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 mean $\pm$ s.e.m. for n=4.

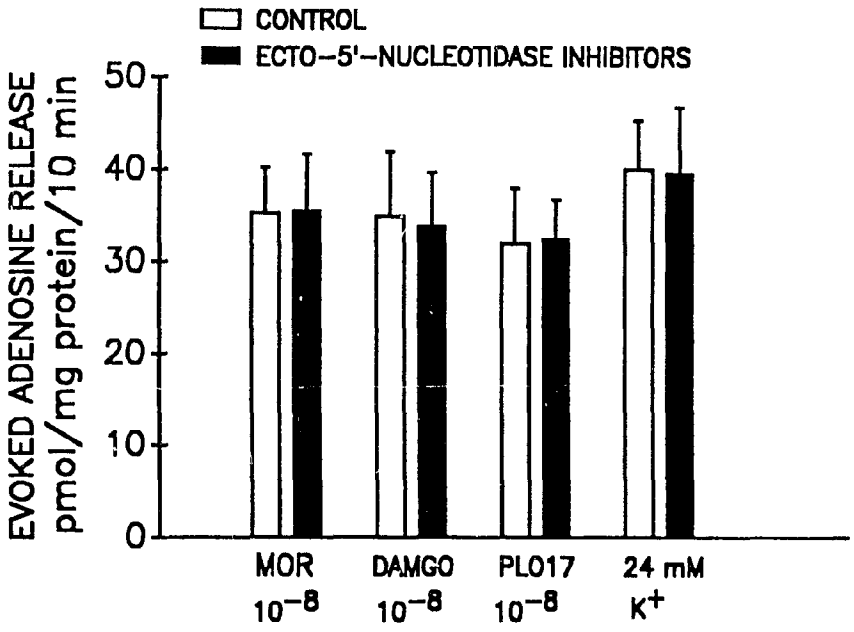


FIGURE 17

Effect of lesioning small diameter primary afferent neurons with capsaicin on opioid-evoked release of adenosine from dorsal spinal cord synatosomes, in the presence of an additional 6 mM K^+. Control values were obtained from rats receiving a vehicle of 60% DMSO in saline. Values represent mean ± s.e.m. for n=4 per group. * $p<0.05$, *** $p<0.001$ compared to adenosine release from control treated animals. Basal adenosine values 190 ± 16 for vehicle and 176 ± 25 pmol/mg protein/10 min for capsaicin treated.

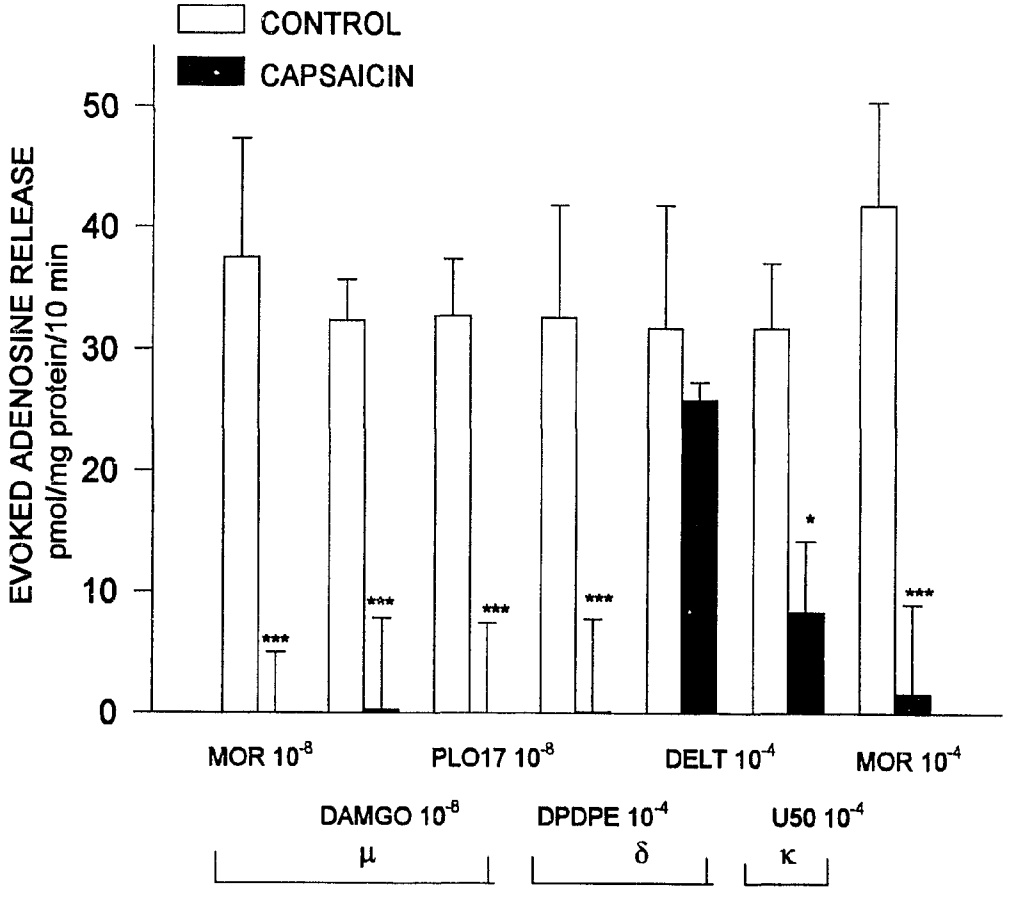
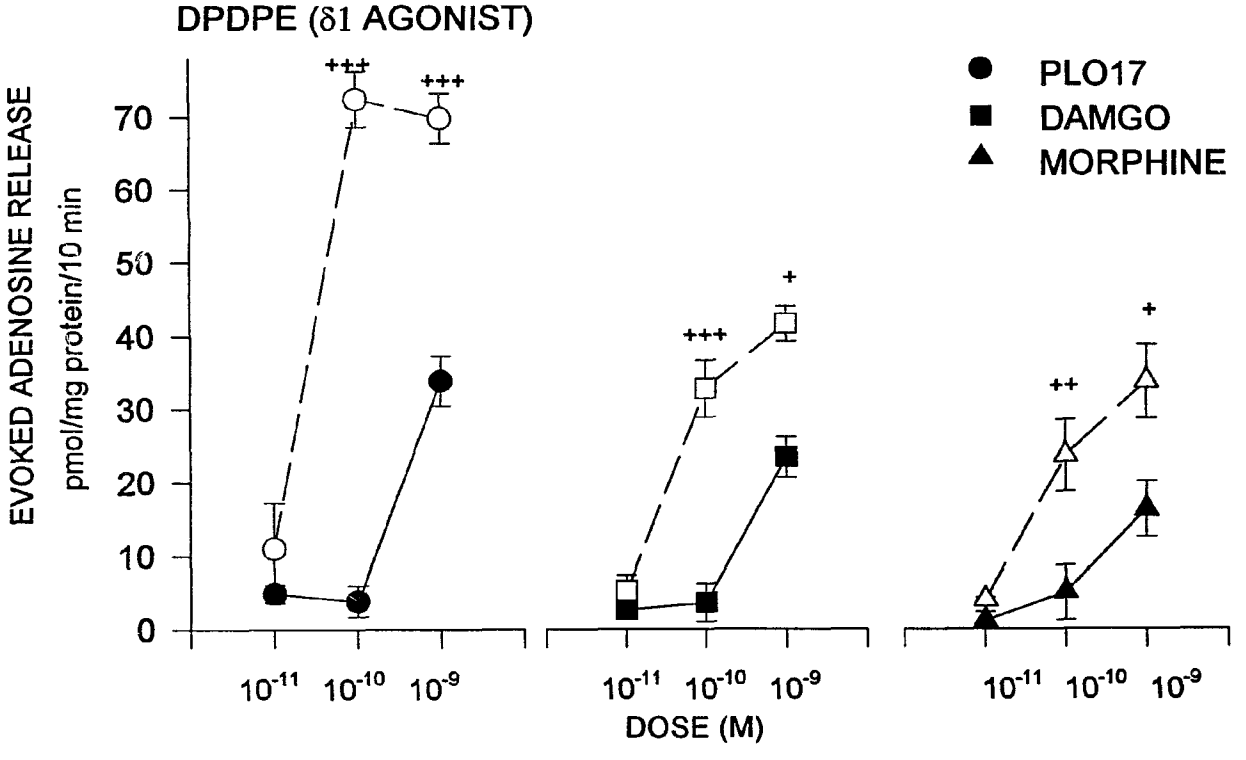


FIGURE 18

Dose-dependent release of adenosine by μ opioid agonists with an inactive dose of δ_1-opioid receptor agonist DPDPE (100 nM). Values represent mean $\pm$ s.e.m. for n=4. + $p<0.05$, ++ $p<0.01$ and +++ $p<0.001$ compared to evoked adenosine release by μ opioid agonists. Basal values ranged from 189- 223 pmol/mg protein/ 10 min.



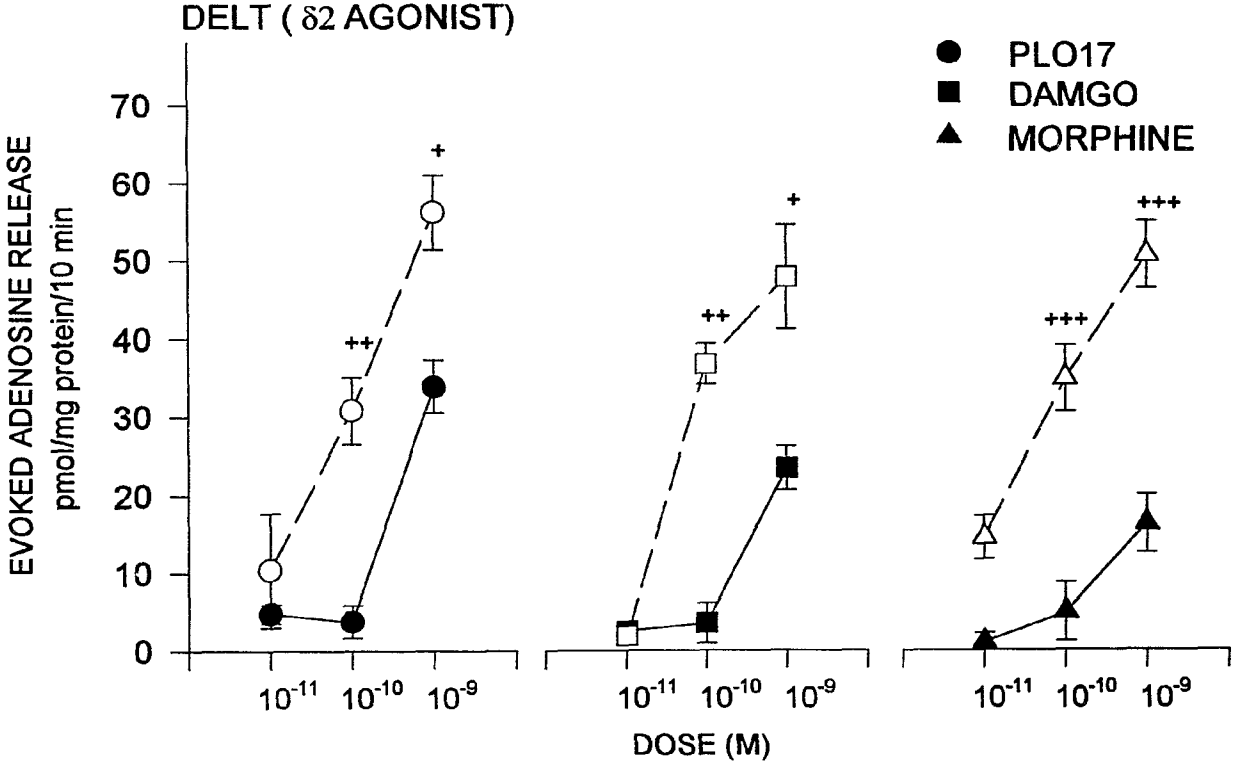
there was an augmentation of release at 10^{-9} M (Figure 18). Subnanomolar concentrations of the selective μ opioid receptor agonist PLO17 likewise did not release adenosine. DPDPE (10^{-7} M) released adenosine in combination with 10^{-10} M PLO17 and enhanced 10^{-9} M PLO17 (Figure 18).

The δ_2 opioid receptor agonist DELT, when combined with morphine, produced a greater degree of enhancement of adenosine release compared to DPDPE with morphine. DAMGO enhanced the release of adenosine when combined with DELT in a similar manner to morphine. DELT produced only a slight enhancement of PLO17 mediated release of adenosine. The evoked release of adenosine by each μ agonist in combination with 10^{-7} M DELT is demonstrated in Figure 19.

Characterization of the evoked release of adenosine by nanomolar concentrations of DPDPE and DAMGO was addressed by determining whether release occurred via a Ca^{2+}-dependent mechanism. Evoked release of adenosine by the combination of 10^{-8} M DPDPE and DAMGO was not different than basal adenosine levels when synaptosomes were incubated in Ca^{2+} free medium (data not shown). Further experiments demonstrated that this release involved activation of N-type voltage dependent Ca^{2+}, channels as evoked release was blocked by ω-conotoxin GVIA (data not shown).

FIGURE 19

Dose-dependent release of adenosine by μ opioid agonists with an inactive dose of δ_2-opioid receptor agonist DELT (100 nM). Values represent means $\pm$ S.E.M. for n=4. + $p<0.05$, ++ $p<0.01$ and +++ $p<0.001$ compared to evoked adenosine release by μ opioid agonists. Basal values ranged from 210-230 pmol/mg protein/10 min.



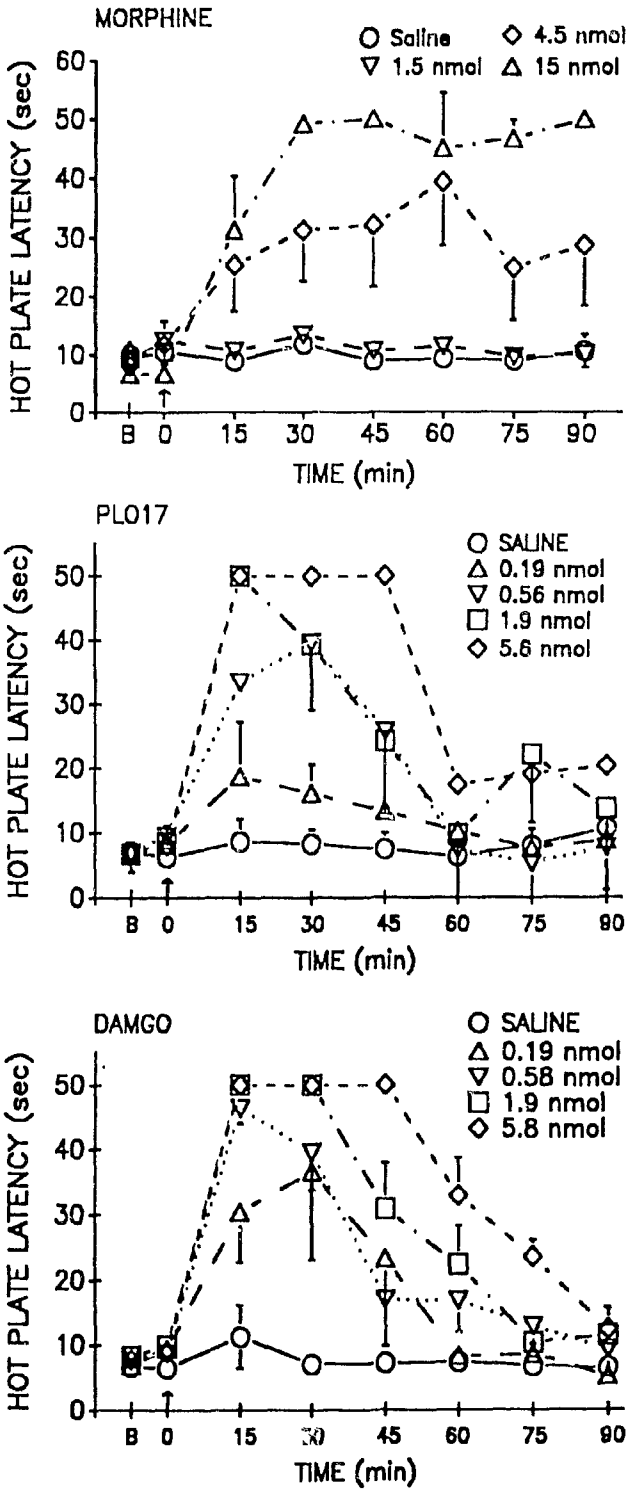
3 SPINAL ANTINOCICEPTION BY SELECTIVE OPIOID AGONISTS

The antinociceptive effects of selective opioid receptor agonists were evaluated in the hot plate thermal threshold test following i.t. administration by acute lumbar puncture under brief halothane anaesthesia. Antinociceptive effects of i.v. injection of each μ opioid receptor agonist morphine, PLO17 and DAMGO are shown in Figure 20. The i.t. administration of each μ opioid agonist produces a dose-dependent increase in hot plate latency; between 1.5 - 15 nmol (1 - 10 μg) for morphine, 0.19 - 5.6 nmol (0.1 - 3.0 μg) for PLO17 and 0.19 - 5.8 nmol (0.1 - 3.0 μg) for DAMGO. These agents differed with regard to their duration of action and onset of antinociceptive effects. The more selective μ opioid agonists DAMGO and PLO17 exhibited a more rapid onset of analgesic action compared to morphine but were shorter in their duration of action. Thus, PLO17 and DAMGO produced their greatest effect at the first postdrug measurement (15 min) and this was diminished at 45 - 75 min, while morphine antinociception lasted throughout the test period of 90 min (Figure 20). Some hind limb rigidity was apparent after i.t. administration of PLO17 (5.6 nmol) or DAMGO (5.8 nmol), but rats would upright when handled and could elicit the endpoint hindpaw lick response after placement onto the hotplate. However, no severe changes in motor function were observed except for hindlimb rigidity at high doses (9.3 nmol = 5 μg) of DAMGO and PLO17; this type of impairment of motor function was not present at the doses presented. Administration of each μ opioid agonist produced a calming effect on the rats, i.e. they no longer exhibited nervous reactions to cage opening, handling or sudden noise. The dose response curves for each μ agonist were plotted as the peak

FIGURE 20

Dose- and time-related antinociceptive effects of μ opioid receptor agonists morphine, PLO17 and DAMGO administered by lumbar puncture i.t. injections. B indicates baseline latencies determined at 15 min intervals prior to the i.t. injection of drug. Each line on the graph represents the mean $\pm$ s.e.m. for n=5 in the hot plate test.

FIGURE 20


MORPHINE
Saline
4.5 nmol
1.5 nmol
15 nmol
HOT PLATE LATENCY (sec)
TIME (min)
PLO17
SALINE
0.19 nmol
0.56 nmol
1.9 nmol
5.6 nmol
HOT PLATE LATENCY (sec)
TIME (min)
DAMGO
SALINE
0.19 nmol
0.58 nmol
1.9 nmol
5.8 nmol
HOT PLATE LATENCY (sec)
TIME (min)

effect for each dose examined (Figure 23). EC_{50} values were interpolated (or extrapolated for DAMGO) from the dose response curves and are presented in table 5.

The antinociceptive effects of the δ opioid agonists DPDPE and DELT in the hot plate test are presented in figure 21. Both agonists produced a dose-dependent increase in hot plate latency between 1.35 - 81.2 nmol (1 - 60 μg) for DPDPE and 0.34 - 11.5 nmol (1 - 10 μg) for DELT. EC_{50} values were interpolated from the dose response curves and presented in Table 5. Both δ 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, and no changes in behavioural state were observed.

The antinociceptive effects of U50488H also were evaluated in the hot plate test following i.t. administration by lumbar puncture (Figure 22). The highest dose of U50488H (644 nmol = 300 μg) produced an apparent antinociception, but evaluation was considered unreliable as the rats were quite agitated and jumped frequently rather than manifesting a hind paw lick.

The relative potencies of the μ, δ and κ opioid receptor agonists are depicted in figure 23. Peak analgesic effects of each agonist were converted to %MPE and presented as a function of dose. The antinociceptive potency of the opioid agonists is as follows: DAMGO, PLO17 > DELT, morphine > DPDPE > U50488H.

FIGURE 21

Dose- and time-related antinociceptive effects of δ opioid receptor agonists DPDPE (δ_1 selective) and DELT (δ_2 selective) administered by lumbar puncture i.t. injection. B indicates baseline latencies determined at 15 min intervals prior to the i.t. injection of drug. Each line on the graph represents the mean ± s.e.m. for n=5 in the hot plate test.

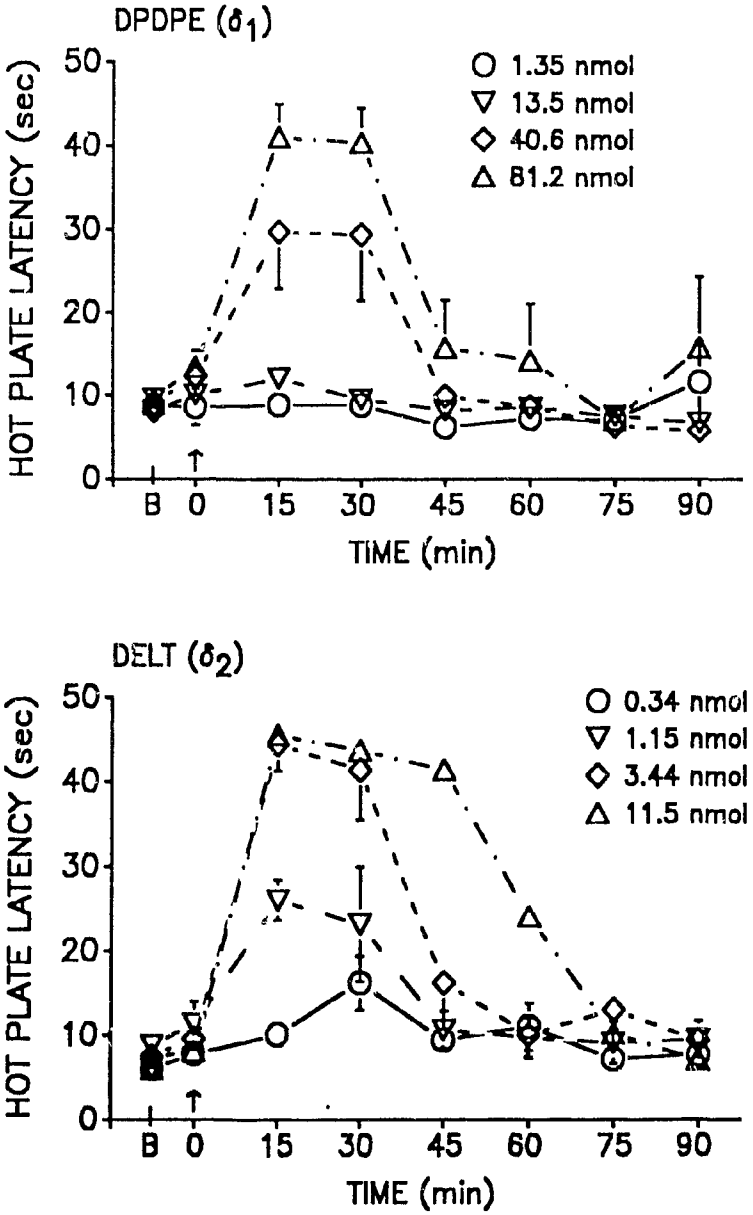


FIGURE 22

Dose- and time-related antinociceptive effects of κ opioid receptor agonist U50488H administered by lumbar puncture i.t. injection. B indicates baseline latencies determined at 15 min intervals prior to the i.t. injection of drug. Each line on the graph represents the mean and s.e.m. for n=3.

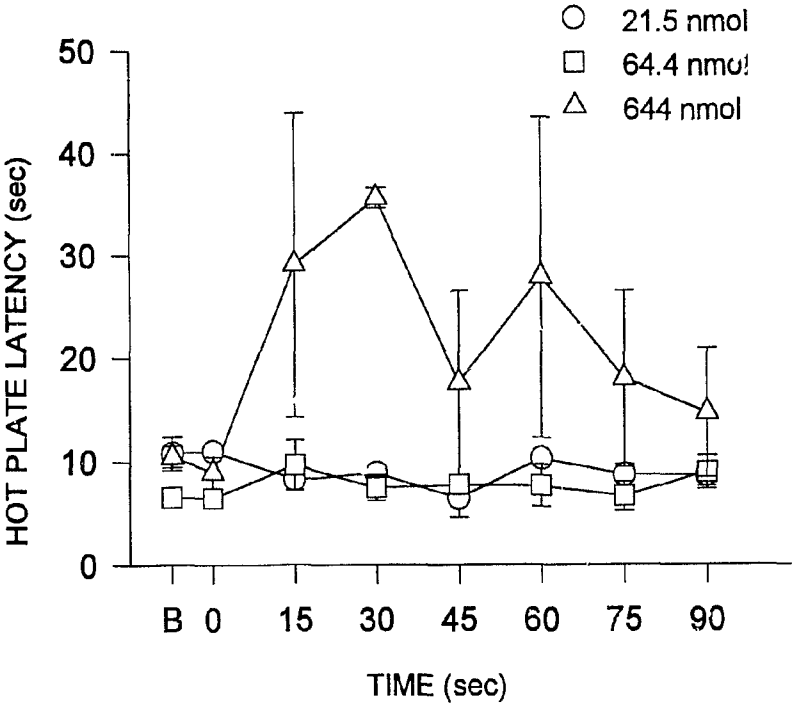


FIGURE 23

Dose response curves for lumbar puncture i.t. injection of μ opioid receptor agonists DAMGO, PLO17 and morphine, the δ opioid receptor agonists DPDPE and DELT and the κ opioid agonist U50488H in the hot plate test. EC_{50} values for the dose response curves are presented in Table 5. Each point on the graph represents the mean $\pm$ s.e.m. for n=3-5 derived from latencies in the hot plate test.

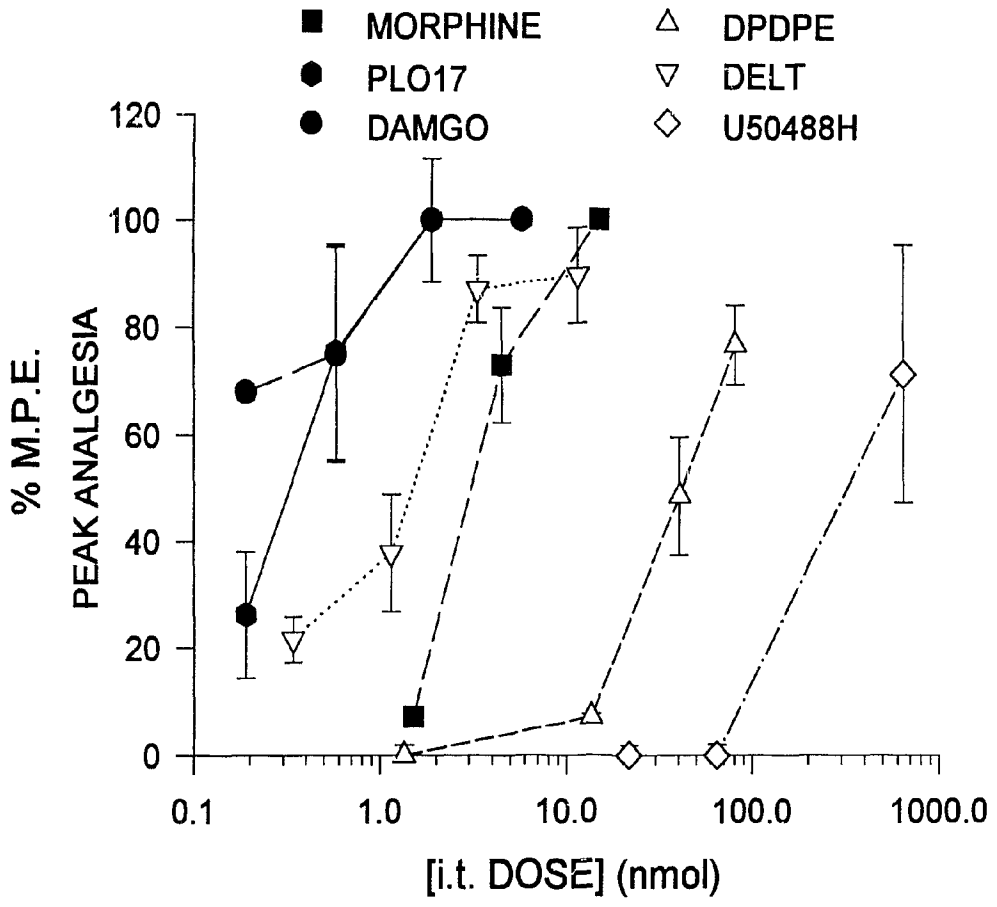


TABLE 5

Effects of i.t. administered μ and δ agonists. The EC_{50} values given are expressed with confidence intervals (95%) with upper and lower limits in parentheses. EC_{50} values were calculated from dose response curves in figure 23.

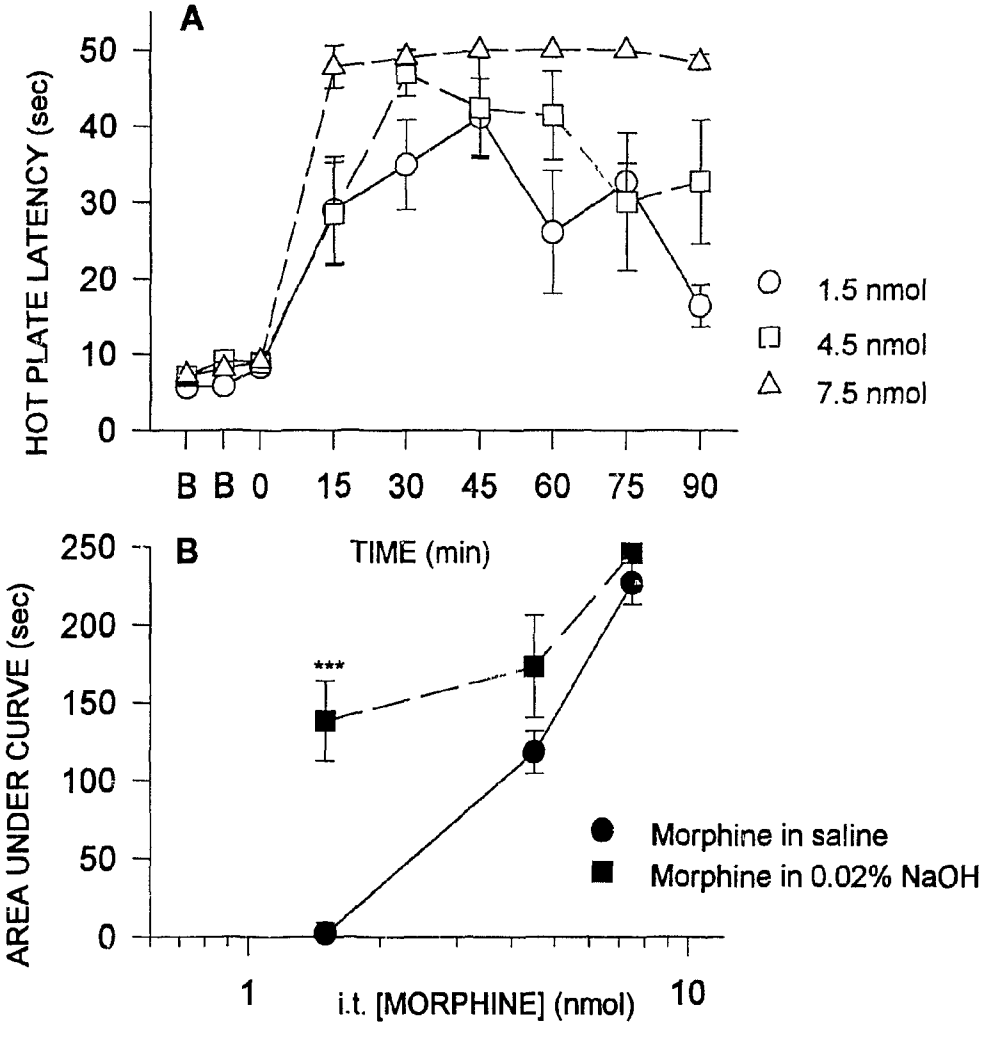
	EC_{50} values (nmol)	(μg)
Morphine	3.9 (1.112 - 6.690)	2.62
DAMGO	0.097 (0.084 - 0.110)	0.05
PLO17	0.22 (0.091 - 0.349)	0.12
DPDPE	68.7 (42.99 - 94.41)	50.75
DELT	1.78 (1.523 - 2.037)	1.55

4 METHYLXANTHINE-SENSITIVITY OF SPINAL ANTINOCICEPTION INDUCED BY μ AND δ AGONISTS

Previous experiments had demonstrated that i.t. pretreatment with 8-phenyltheophylline (3 - 10 μg) dose-dependently antagonized the antinociceptive effects produced by i.t. morphine, where i.t. injection was made via chronically implanted cannulas (Sweeney et al., 1987). Initial experiments to determine the methylxanthine-sensitive component of opioid-induced antinociception were performed in rats by i.t. lumbar puncture. Morphine and the adenosine receptor antagonist, 8-phenyltheophylline, were co-administered by a single injection between vertebrae L5 and L6 during brief halothane anaesthesia as performed for the agonist studies described above. The antinociceptive effect elicited by the combination of i.t. 8-phenyltheophylline (10 μg) and morphine (7.5 nmol) was not different than i.t. morphine alone (data not shown). I.t. administration of 8-phenyltheophylline by lumbar puncture 15 min prior to the i.t. administration of morphine also did not attenuate morphine-induced antinociception. The antinociceptive effects of i.t. morphine administered by lumbar puncture in the presence of 0.02% NaOH (vehicle for 8-phenyltheophylline) were compared to morphine in saline (Figure 24). The presence of NaOH appeared to shorten the onset of morphine-induced antinociception (Figure 24A) and to increase the potency of morphine (Figure 24B). Due to the effects of the vehicle on morphine-induced antinociception, caffeine was selected as an alternative adenosine receptor antagonist because caffeine is soluble in saline. However, i.t. administration of caffeine by lumbar puncture also did not antagonize the antinociception elicited by i.t. morphine when co-injected, administered

FIGURE 24

Panel A: Dose- and time-related antinociceptive effects of morphine in the presence of 0.02% NaOH administered by lumbar puncture i.t. injection. Panel B compares the dose-response relationship of morphine-induced antinociception in saline vs NaOH vehicle. Each point in both panels represents mean ± s.e.m. for n=4 or 5. *** $p<0.001$ compared to the antinociceptive index of morphine in saline.



15 min prior to morphine, or administered 10 min post morphine injection. Varying the dose of caffeine (50 - 100 μg) and the volume (10 - 40 μl) of injection did not reveal antagonism. Thus, i.t. administration by lumbar punctures was abandoned and antagonist experiments were performed on rats implanted with chronic i.t. cannulas. I.t. administration of caffeine through the chronically implanted cannula dose-dependently 103 - 515 nmol (20 - 100 μg), attenuated the antinociceptive effect of morphine (7.5 nmol) (Figure 25).

The methylxanthine-sensitivity of morphine-induced antinociception was observed in rats implanted with chronic cannulas but not in rats where the i.t. administration was made via a lumbar puncture. It was considered important to determine whether (a) the presence of the chronic cannula or (b) the site of injection generated the methylxanthine-sensitivity of morphine-induced antinociception. I.t. chronic cannulas terminate at the lower thoracic spinal cord, whereas an i.t. lumbar puncture is made below the spinal cord between vertebrae L5 and L6 (see Figure 4). I.t. caffeine administration by lumbar puncture did not attenuate morphine-induced antinociception produced by i.t. lumbar puncture morphine administration in rats with chronically implanted i.t. cannulas (Figures 26). Caffeine administration through the chronic implanted cannula antagonized the antinociceptive effects of i.t. morphine (either lumbar puncture or through chronic cannula) (Figure 26).

I.t. administration by lumbar puncture of methylene blue dye (20 μl) in rats with and without a chronic cannula was performed to test the distribution of drugs after injection. In both situations, the dye was detected at the level of the lumbar enlargement

FIGURE 25

Time course comparison of the adenosine receptor antagonist, caffeine, on morphine-induced antinociception by lumbar puncture (panel A) vs cannula injection (panel B). Panel A represents threshold latencies of morphine or caffeine co-administered with morphine after lumbar puncture (L.P.) i.t. injection. Panel B: represents caffeine dose-dependent antagonism of morphine-induced antinociception by i.t. administration through chronically implanted cannulas. Values represent mean $\pm$ s.e.m. for n=6 for both panels. ** $p<0.01$ compared to morphine antinociceptive index.

FIGURE 25

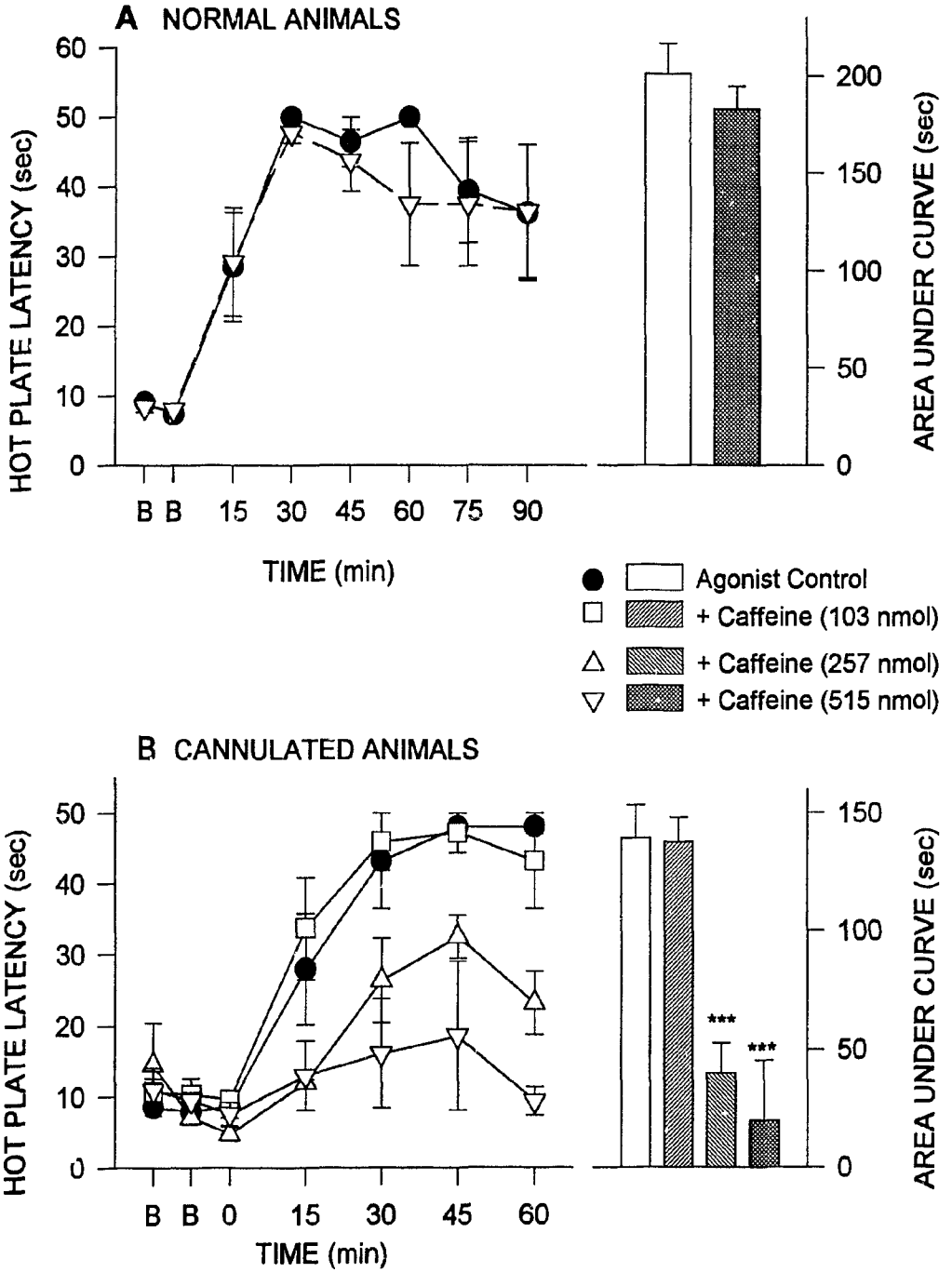
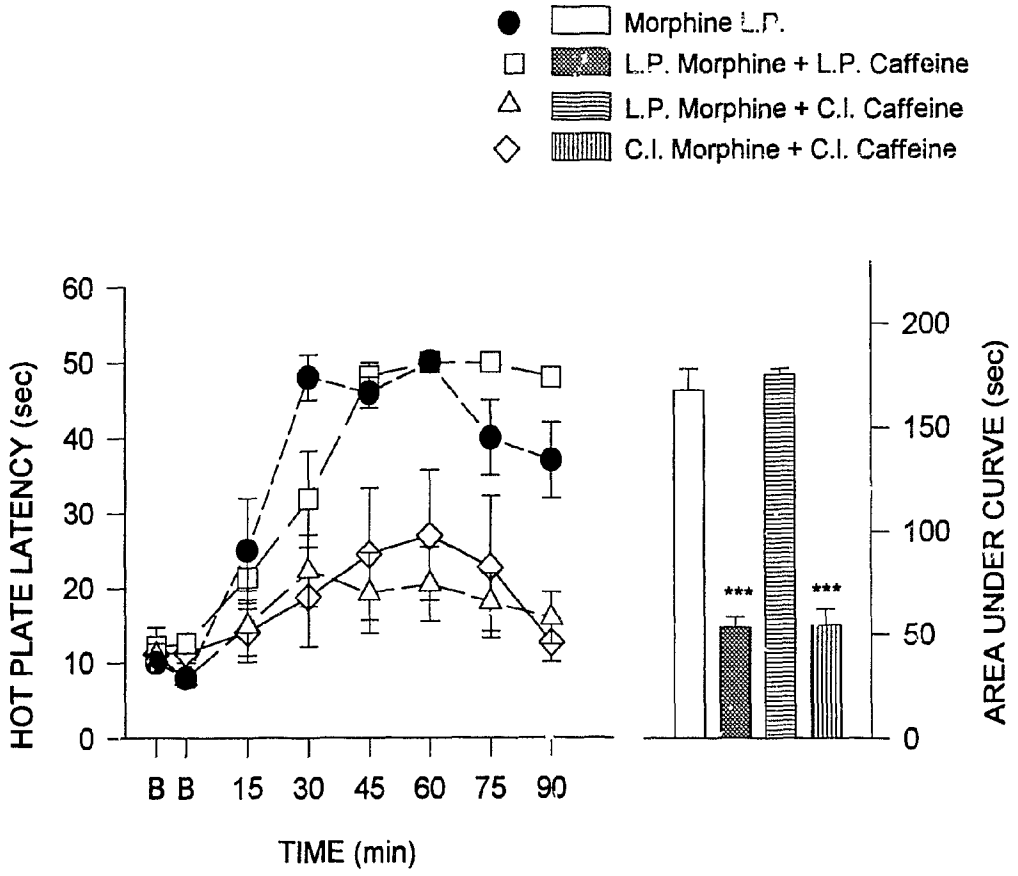

A NORMAL ANIMALS
HOT PLATE LATENCY (sec)
AREA UNDER CURVE (sec)
TIME (min)
B B 15 30 45 60 75 90
Agonist Control
+ Caffeine (103 nmol)
+ Caffeine (257 nmol)
+ Caffeine (515 nmol)
B CANNULATED ANIMALS
HOT PLATE LATENCY (sec)
AREA UNDER CURVE (sec)
TIME (min)
B B 0 15 30 45 60
***
***

FIGURE 26

Comparison of the effects of caffeine (515 nmol) administered by either i.t. lumbar puncture (L.P.) or through a chronic implanted cannula (C.I.) on morphine (7.5 nmol) induced antinociception. Values represent means ± s.e.m. for n=6. *** $p<0.001$ compared to the antinociceptive index of morphine by i.t. lumbar puncture.



in the spinal canal. Thus, the precision of the lumbar puncture was substantiated and the volume of injection was justified in that the agent reached the targeted levels of the spinal cord.

This series of experiments demonstrates that methylxanthine-sensitivity of opioid-induced antinociception is only exhibited when caffeine is administered by i.t. injection at the level of the lumbar spinal cord via chronically implanted cannulas.

4.1 Effect of Caffeine on Opioid-Induced Antinociception in the Spinal Cord

Rats implanted with chronic i.t. cannulas were used to determine the role of adenosine in the antinociceptive effect elicited by an EC_{75} dose of selective opioid receptor agonists. Caffeine dose-dependently (103 - 515 nmol = 20 - 100 μg) attenuated the antinociceptive effect of both selective μ agonists (DAMGO 0.58 nmol = 0.3 μg, PLO17 0.56 nmol = 0.3 μg) in the hot plate (Figure 27) and the tail flick (Figure 28) thermal threshold tests. Caffeine attenuated the peak analgesic response and appeared to shortened the duration of action of each ligand. Caffeine, alone, had no effect on hot plate baseline latencies and produced no overt behavioural effects at any of the doses used (data not shown). The degree of antagonism is depicted by area under the curve values for each agonist with increasing doses of caffeine for hot plate (Figure 27) and tail flick (Figure 28) tests. IC_{50} values for caffeine in blocking μ opioid receptor actions in the hot plate test were determined to be 58 μg (morphine), 82 μg (DAMGO) and 75 μg (PLO17), indicating that caffeine had a similar potency in antagonizing each μ opioid receptor agonist.

FIGURE 27

Dose-dependent antagonism by caffeine of the antinociceptive effect of selective μ opioid agonists DAMGO and PLO17 in the hot plate test. I.t. caffeine was administered at the second baseline time point 15 min prior to the i.t. administration of DAMGO or PLO17. B indicates baseline latencies determined at 15 min intervals. The data are expressed as the mean $\pm$ s.e.m. for the latency in the hot plate test of n=5. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to the antinociceptive index of each agonist alone.

FIGURE 27

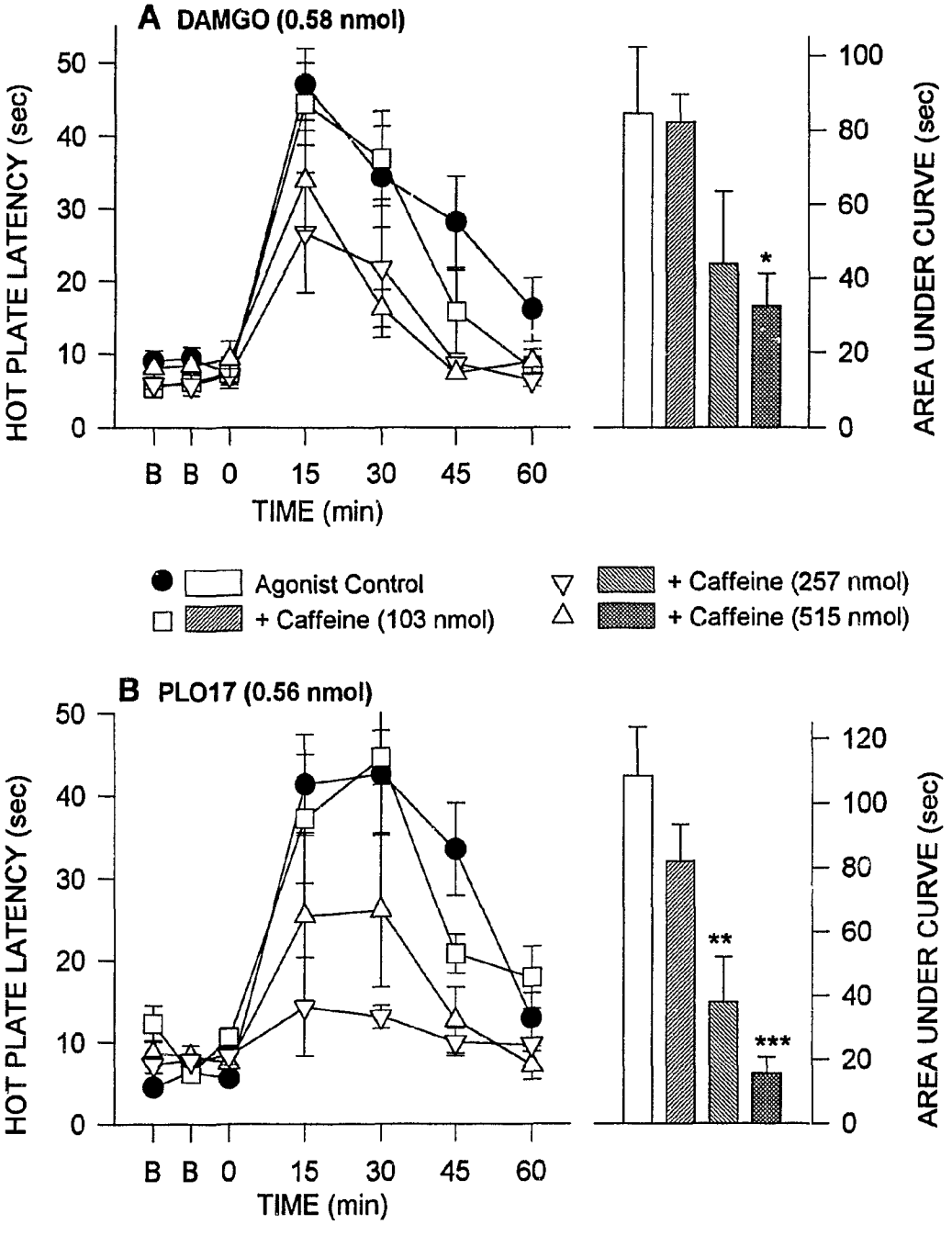
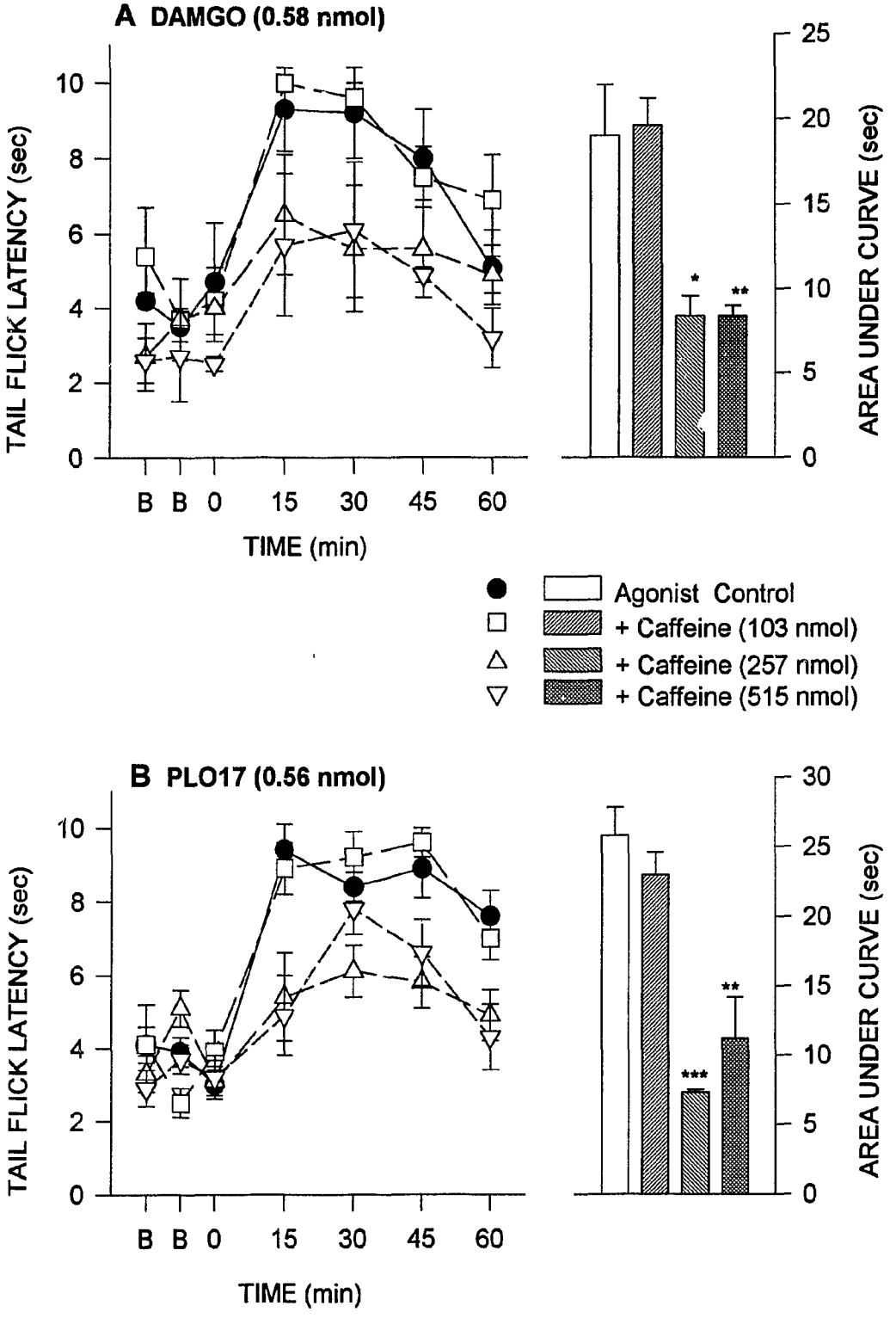

A DAMGO (0.58 nmol)
HOT PLATE LATENCY (sec)
AREA UNDER CURVE (sec)
TIME (min)
B B 0 15 30 45 60
0 10 20 30 40 50
0 20 40 60 80 100
*
Agonist Control
+ Caffeine (103 nmol)
+ Caffeine (257 nmol)
+ Caffeine (515 nmol)
B PLO17 (0.56 nmol)
HOT PLATE LATENCY (sec)
AREA UNDER CURVE (sec)
TIME (min)
B B 0 15 30 45 60
0 10 20 30 40 50
0 20 40 60 80 100 120
**
***

FIGURE 28

Dose-dependent antagonism by caffeine of the antinociceptive effect of selective μ opioid agonists DAMGO and PLO17 in the tail flick test. I.t. caffeine was administered at the second baseline time point 15 min prior to the i.t. administration of DAMGO or PLO17. B indicates baseline latencies determined at 15 min intervals. The data are expressed as the mean $\pm$ s.e.m. for the latency in the tail flick test of n=5. * $p<0.05$, ** $p<0.01$, ** $p<0.001$ compared to the antinociceptive index of each agonist alone.

FIGURE 28


A DAMGO (0.58 nmol)
TAIL FLICK LATENCY (sec)
AREA UNDER CURVE (sec)
TIME (min)
B B 0 15 30 45 60
Agonist Control
+ Caffeine (103 nmol)
+ Caffeine (257 nmol)
+ Caffeine (515 nmol)
B PLO17 (0.56 nmol)
TAIL FLICK LATENCY (sec)
AREA UNDER CURVE (sec)
TIME (min)
B B 0 15 30 45 60

In contrast to the antagonism of μ opioid-induced antinociception, i.t. pretreatment with caffeine did not attenuate the antinociceptive effects elicited by the δ opioid agonists DPDPE and DELT in the hot plate test (Figure 29). Interestingly, caffeine ($\geq$ 515 nmol) produced a significant enhancement of δ opioid mediated antinociception and prolonged the antinociceptive response. A similar enhanced antinociception was observed for DPDPE with caffeine in the tail flick test (Figure 30). The doses of caffeine which augmented the antinociceptive effects of δ opioid agonists had no significant effect in either the hot plate latency or tail flick tests nor did they produce any overt behavioural effects (data not shown).

In all adenosine antagonist experiments, the selected doses of μ and δ opioid receptor agonists, administered via chronic cannulas, produced a similar degree of antinociception to that observed in the dose response studies where agonists were administered by i.t. lumbar puncture. The only exception was DELT, and this may be due to the use of two different supplies of DELT in the respective experiments.

4.2 Adenosine Receptor Subtypes Involved in Spinal Opioid-Induced Antinociception

Spinal opioid-induced antinociception was evaluated in the hot plate thermal threshold test following i.t. pretreatment with the adenosine receptor antagonists 8-cyclopentyl-1,3-dimethylxanthine (CPT, A_1 receptor, 180 fold selectivity) and 3,7-dimethyl-1-propargylxanthine (DMPX, A_2 receptor, 57 fold selectivity) (Jacobson et al., 1992). All experiments with selective adenosine receptor antagonists were performed in

FIGURE 29

Dose-dependent effects of caffeine on the antinociceptive effect of δ opioid agonists DPDPE and DELT in the hot plate test. I.t. caffeine was administered at the second baseline time point 15 min prior to the i.t. administration of DPDPE or DELT. B indicates baseline latencies determined at 15 min intervals. The data are expressed as the mean ± s.e.m. for the latency in the hot plate test of n=5. ** $p<0.01$ compared to area under the curve for each agonist alone.

FIGURE 29

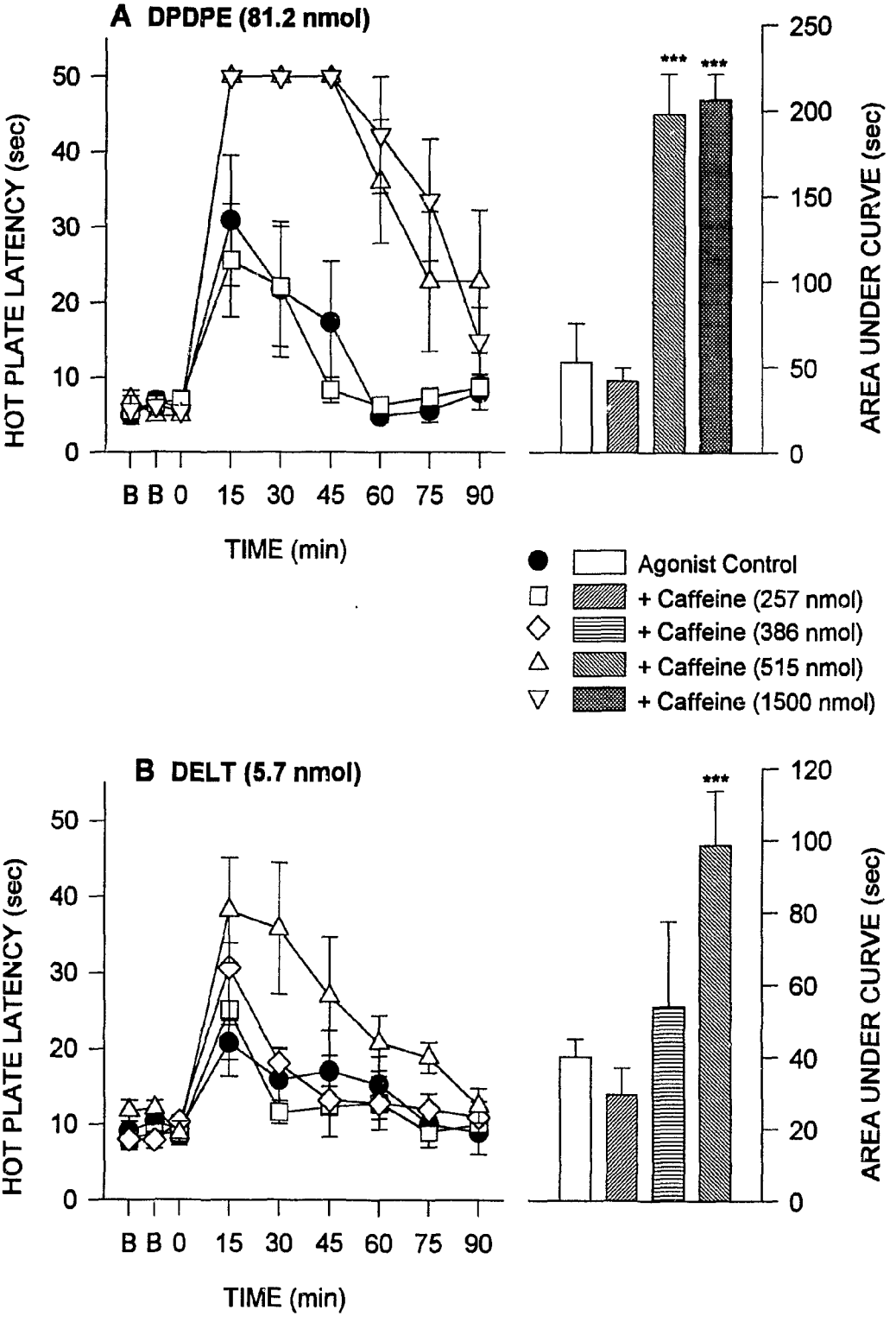
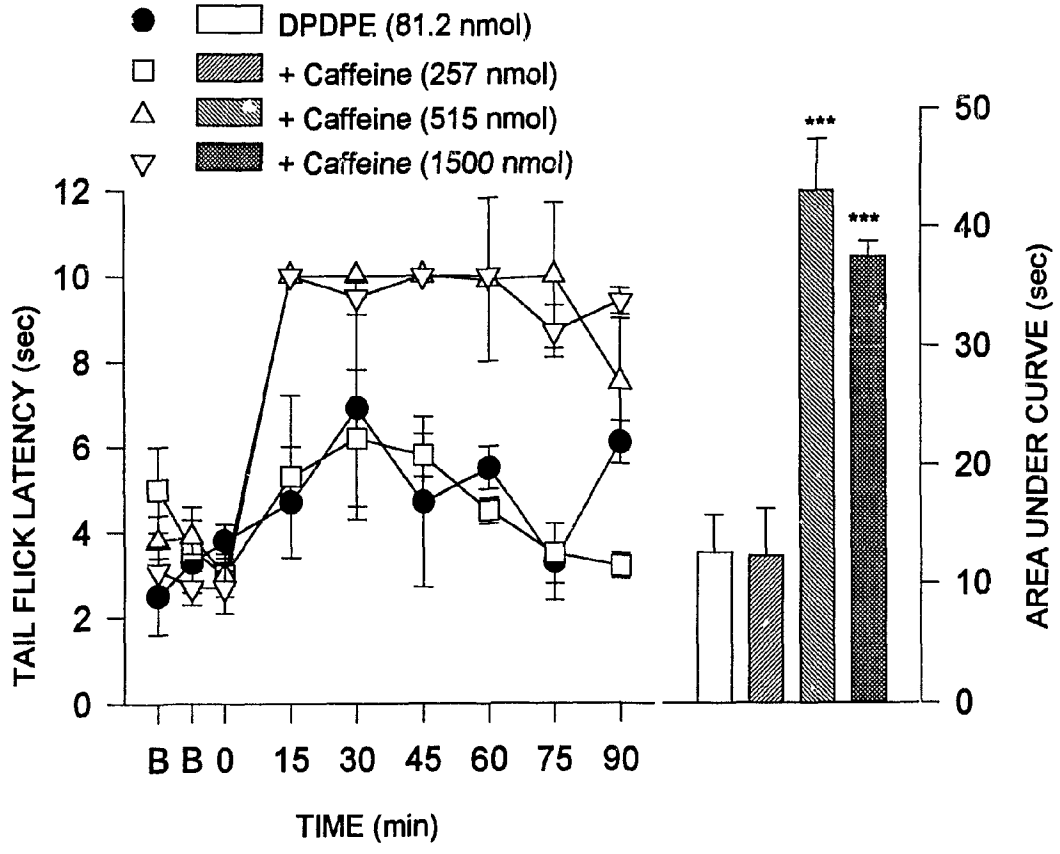

A DPDPE (81.2 nmol)
HOT PLATE LATENCY (sec)
0
10
20
30
40
50
B B 0 15 30 45 60 75 90
TIME (min)
AREA UNDER CURVE (sec)
0
50
100
150
200
250
***
***
Agonist Control
+ Caffeine (257 nmol)
+ Caffeine (386 nmol)
+ Caffeine (515 nmol)
+ Caffeine (1500 nmol)
B DELT (5.7 nmol)
HOT PLATE LATENCY (sec)
0
10
20
30
40
50
B B 0 15 30 45 60 75 90
TIME (min)
AREA UNDER CURVE (sec)
0
20
40
60
80
100
120
***

FIGURE 30

Dose-dependent augmentation by caffeine of the antinociceptive effect of selective δ opioid agonist DPDPE in the tail flick test. I.t. caffeine was administered at the second baseline time point 15 min prior to the i.t. administration of DPDPE. B indicates baseline latencies determined at 15 min intervals. The data are expressed as the mean ± s.e.m. for the latency in the tail flick test of n=5. ** $p<0.01$ compared to area under the curve for DPDPE alone.



rats with chronically implanted i.t. catheters. I.t. CPT dose-dependently antagonized the antinociceptive effects of morphine (Figure 31). A dose of 4.5 nmol rather than 7.5 nmol morphine was chosen in this study, as the vehicle for CPT was 0.02% NaOH in saline. Previous studies described in section 3.0 illustrated the potential for the morphine-induced antinociceptive effects to be shifted to the left by the NaOH vehicle, hence the dose of morphine was lowered to diminish the risk of achieving cut off antinociceptive values. Intrathecal DMPX (dissolved in 5% DMSO in saline) 275 nmol (60 μg) had no effect on morphine-induced antinociception (Figure 32). The peak analgesic effect produced by morphine at the 30 min time point appeared to be attenuated by the presence of DMPX, however this was not statistically significant.

In subsequent experiments, the combination of similar low doses of CPT and DMPX was administered by i.t. co-injection 15 min prior to morphine to evaluate whether activation of both A_1 and A_2 adenosine receptors might be involved in eliciting methylxanthine-sensitivity of opioid-induced antinociception. Thus, caffeine, which is a nonselective adenosine receptor antagonist, may block both A_1 and A_2 adenosine receptors in antagonizing μ opioid-induced antinociception. Antinociception elicited by morphine (1.5 nmol) was attenuated by the combination of CPT and DMPX; however, antinociception induced by higher doses of morphine was unaffected by the presence of the low dose of CPT and DMPX (Figure 33).

In contrast to the antagonism of morphine-induced antinociception, i.t. CPT (240 nmol = 60 μg) did not attenuate the antinociceptive effects elicited by DAMGO (Figure 34). I.t. pretreatment with DMPX had no effect on the antinociceptive actions elicited

FIGURE 31

Dose-dependent antagonism by CPT (A_1 adenosine receptor antagonist) of the antinociceptive effects of morphine. I.t. CPT was administered 15 min prior to opioid agonist at the second baseline latency time point. B indicates baseline latencies determined at 15 min intervals. The data are expressed as the mean ± s.e.m. for the latency in the hot plate test of n=5. * $p<0.05$, *** $p<0.001$ compared to the antinociceptive index for morphine alone.

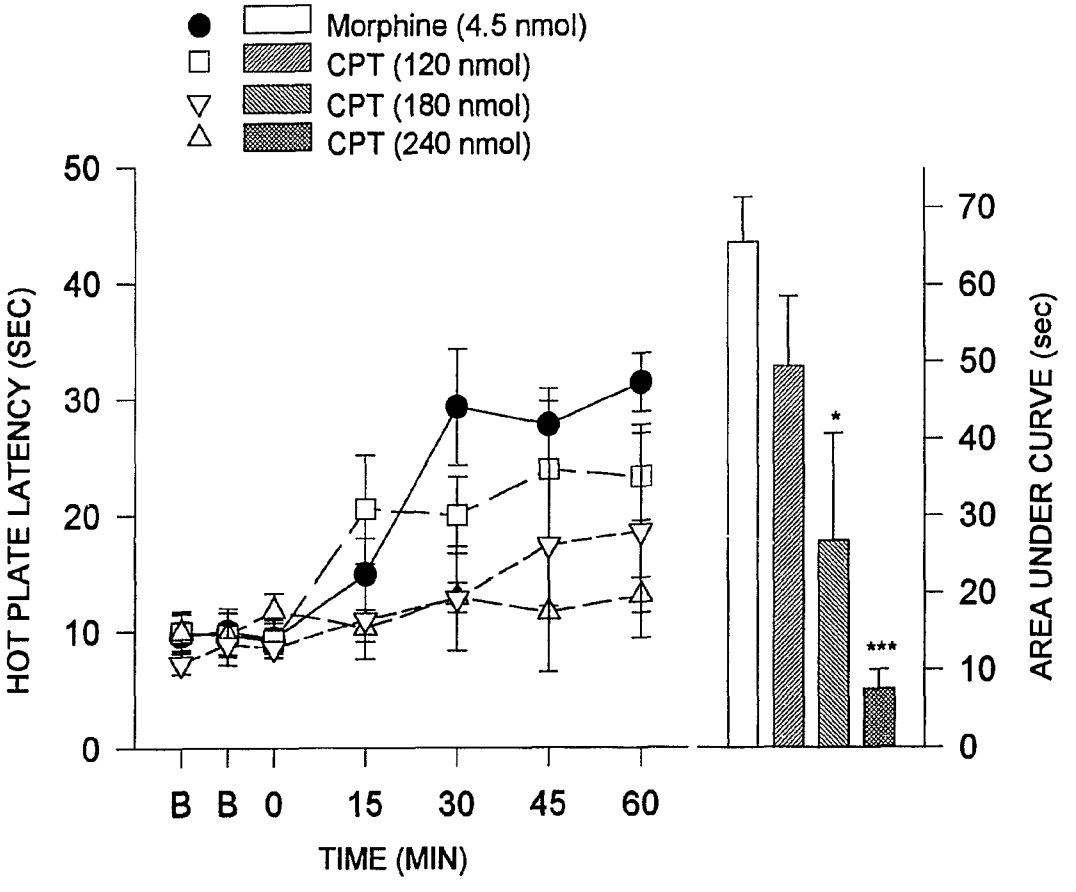


FIGURE 32

Nonsignificant effect by DMPX (A_2 adenosine receptor antagonist) on morphine-induced antinociception. I.t. DMPX was administered 15 min prior to morphine at the second baseline latency time point. B indicates baseline latencies determined at 15 min intervals. The data are expressed as the mean ± s.e.m. for the latency in the hot plate test of n=5. *** $p<0.001$ compared to the antinociceptive index for morphine alone.

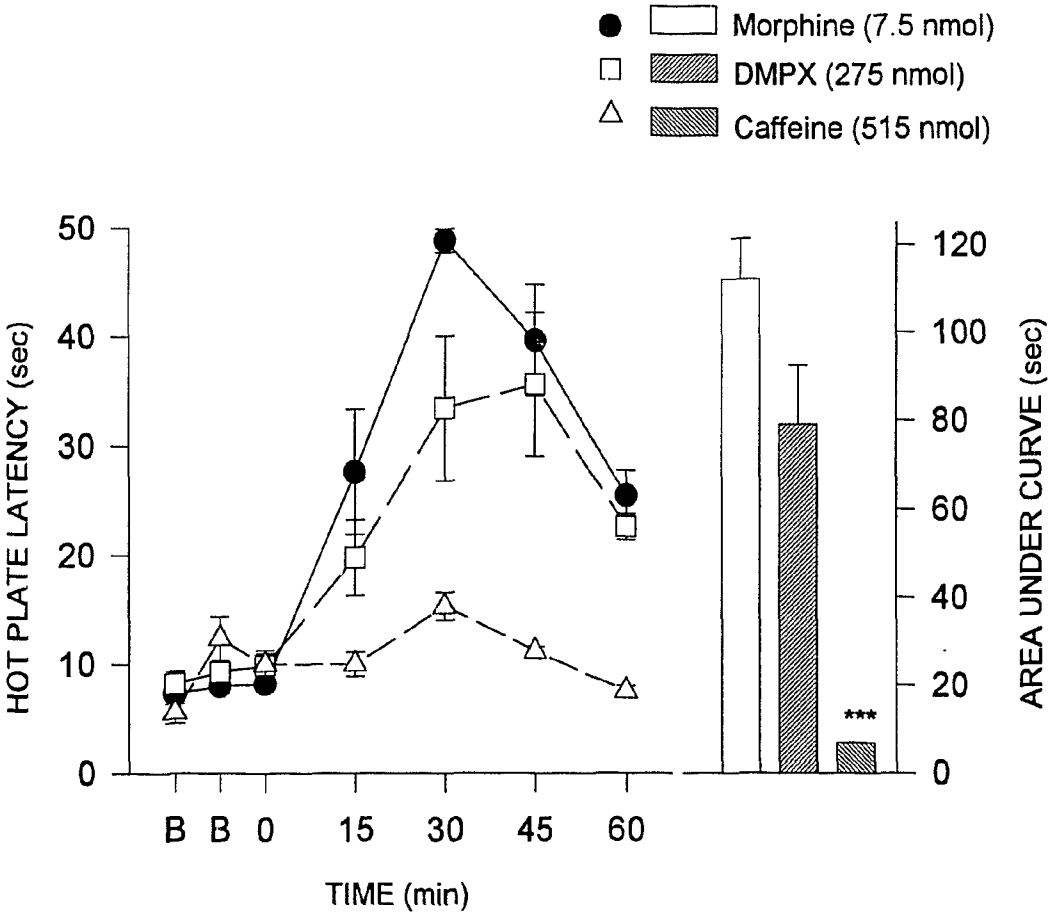
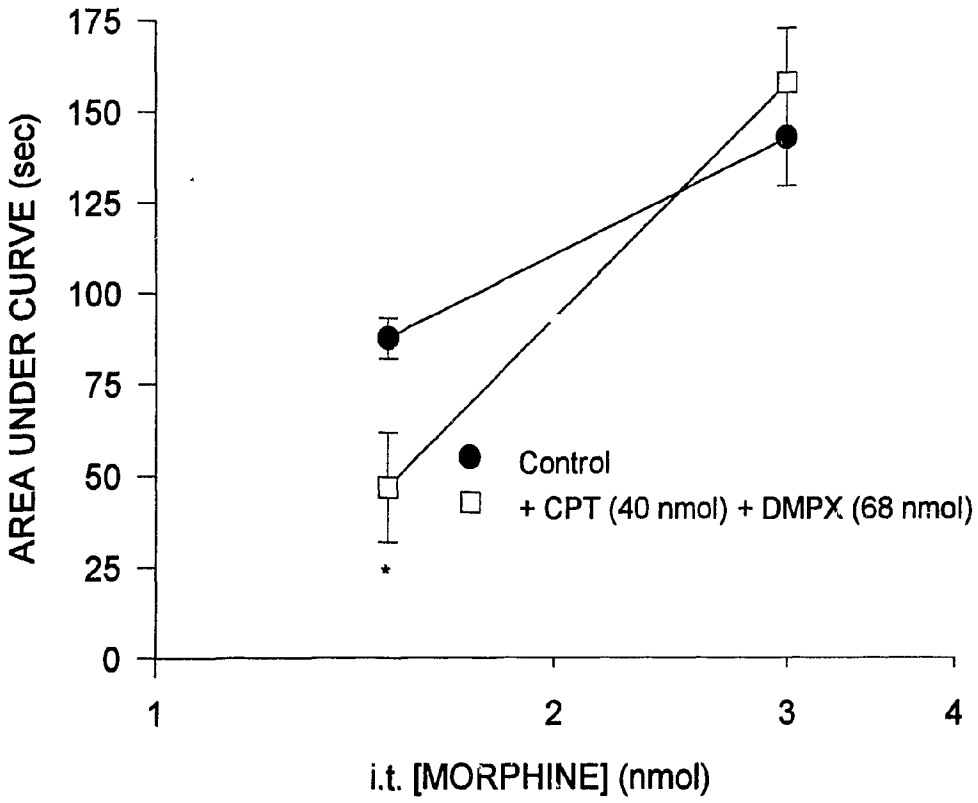


FIGURE 33

Effects of i.t. CPT (40 nmol) and DMPX (68 nmol) co-administered 15 min prior to opioid agonist morphine. The data are expressed as the mean area under the curve for a 90 min time course ± s.e.m. for the latency in the hot plate test of n=5.



by the selective μ opioid agonist (Figure 34). The most effective antagonist was the non-selective adenosine receptor antagonist, caffeine, which attenuated DAMGO-induced antinociceptive effects as illustrated in earlier experiments. I.t. co-injection of CPT (40 nmol = 10 μg) and DMPX (68 nmol = 15 μg) antagonized the antinociceptive effects elicited by 0.19 nmol (0.1 μg) and 0.58 nmol (0.3 μg) DAMGO but not 1.9 nmol (1.0 μg) DAMGO (Figure 35A). Experiments determining the methylxanthine-sensitive component of DAMGO-induced antinociception with caffeine demonstrated similar results, in that i.t. caffeine (515 nmol = 100 μg) attenuated the antinociceptive effects elicited by 0.58 nmol (0.3 μg) DAMGO (Figure 27) but not 1.9 nmol (1.0 μg) DAMGO (Figure 35B). A summary of the effects of CPT and DMPX on μ opioid-induced antinociception is illustrated in table 6.

I.t. CPT (120, 240 nmol), DMPX (275 nmol) or the combination of CPT (40 nmol) and DMPX (68 nmol) had no effect on DPDPE-induced antinociception (Figure 36). I.t. caffeine (515 nmol) augmented the antinociceptive effects produced by the δ opioid agonist as demonstrated in figure 29.

5 ANTINOCICEPTION PRODUCED BY A_1 AND A_2 ADENOSINE RECEPTOR AGONISTS

Results from the previous set of experiments examining effects of selective adenosine receptor antagonists on opioid-induced antinociception suggested that a possible interaction between A_1 and A_2 adenosine receptor subtypes may exist. The antinociceptive effects of i.t. administration (via chronic implanted cannulas) of the

FIGURE 34

The effects of CPT (panel A) or DMPX (panel B) on DAMGO-induced antinociception. Each adenosine antagonist was administered 15 min prior to opioid agonist at the second baseline latency time point. B indicates baseline latencies determined at 15 min intervals. The data are expressed as the mean $\pm$ s.e.m. for the latency in the hot plate test of n=5, * $p<0.05$ compared to the area under the curve value for DAMGO alone.

FIGURE 34

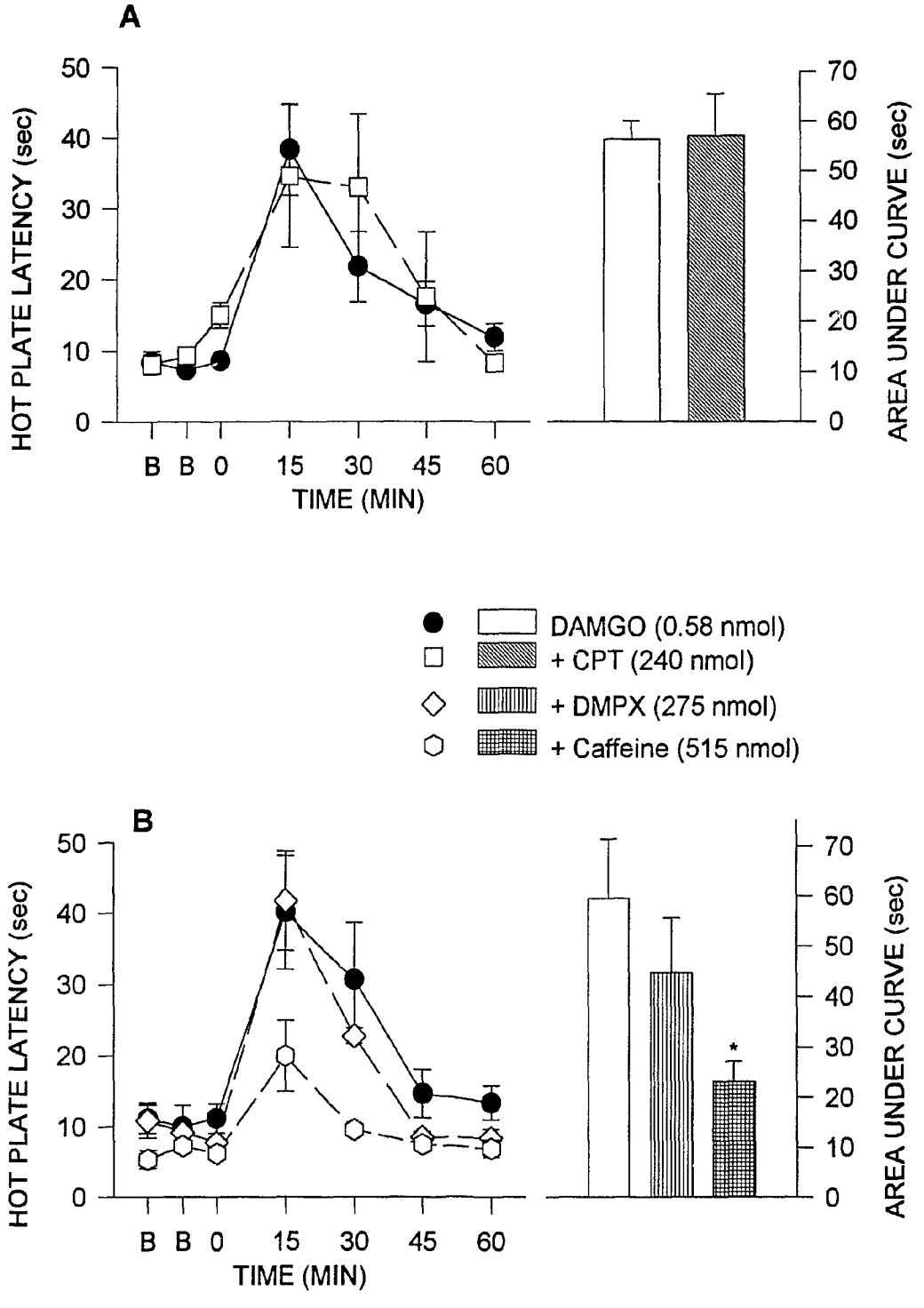

A
HOT PLATE LATENCY (sec)
50
40
30
20
10
0
B B 0 15 30 45 60
TIME (MIN)
70
60
50
40
30
20
10
0
AREA UNDER CURVE (sec)
DAMGO (0.58 nmol)
+ CPT (240 nmol)
+ DMPX (275 nmol)
+ Caffeine (515 nmol)
B
HOT PLATE LATENCY (sec)
50
40
30
20
10
0
B B 0 15 30 45 60
TIME (MIN)
70
60
50
40
30
20
10
0
AREA UNDER CURVE (sec)
*

FIGURE 35

Antagonism of i.t. DAMGO-induced antinociception by caffeine or the combination CPT (40 nmol) and DMPX (68 nmol). Adenosine antagonists were co-administered 15 min prior to opioid at the second baseline latency time point. B indicates baseline latencies determined at 15 min intervals. The data are expressed as the mean area under the curve for a 60 min time course ± s.e.m. for the latency in the hot plate test of n=5, * $p<0.05$, ** $p<0.01$ compared to area under the curve for the respective dose of DAMGO in each panel.

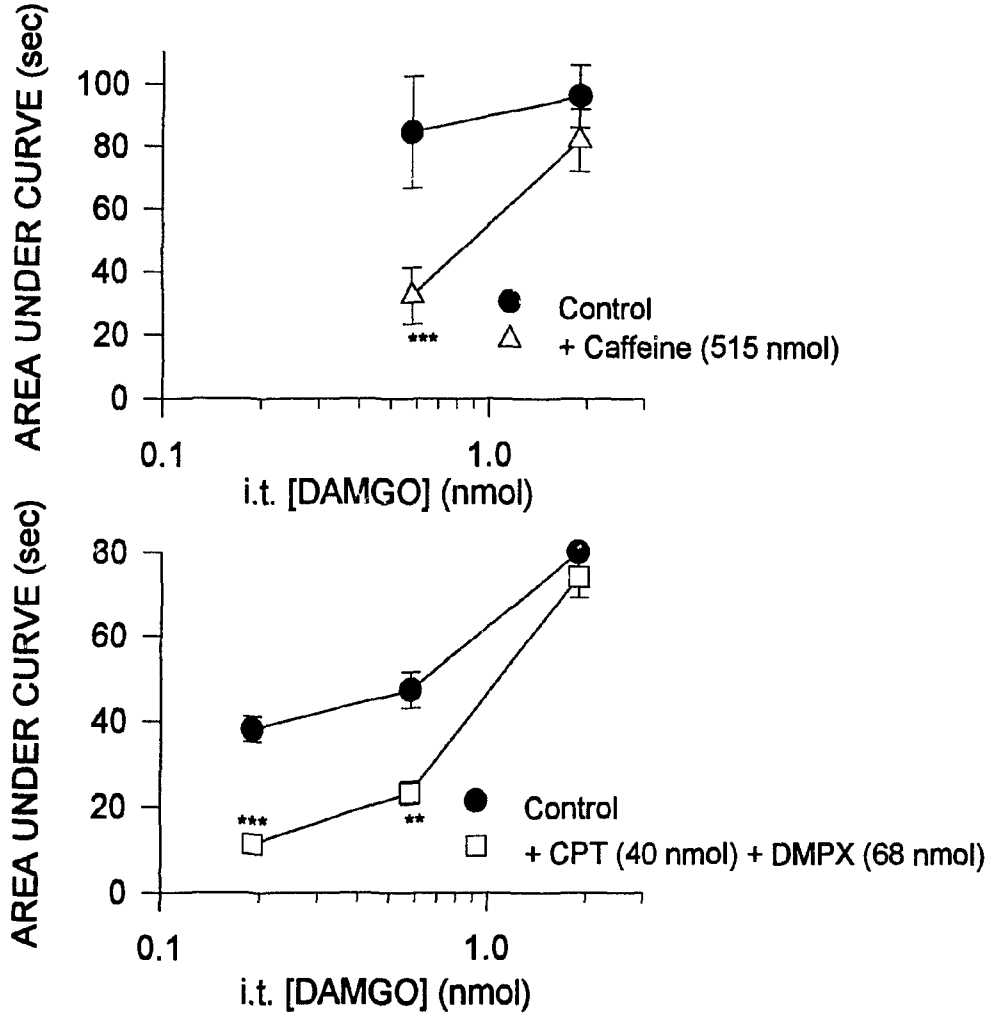


TABLE 6

Summary of effects of adenosine receptor antagonists on opioid-induced antinociception.

	Morphine		DAMGO	
	Low Dose < 4.5 nmol	High Dose ≥ 4.5 nmol	Low Dose ≤ 0.58 nmol	High Dose > 0.58 nmol
CPT (240 nmol)		↓↓↓	↔	↔
DMPX (275 nmol)		↔	↔	↔
CPT (40 nmol) + **DMPX** (68 nmol)	↓	↔	↓↓↓	↔
Caffeine (515 nmol)		↓↓↓	↓↓↓	↔

$\geq$ 70 % reduction = ↓↓↓, $\geq$ 30 % reduction = ↓, and no effect = ↔.

TABLE 7

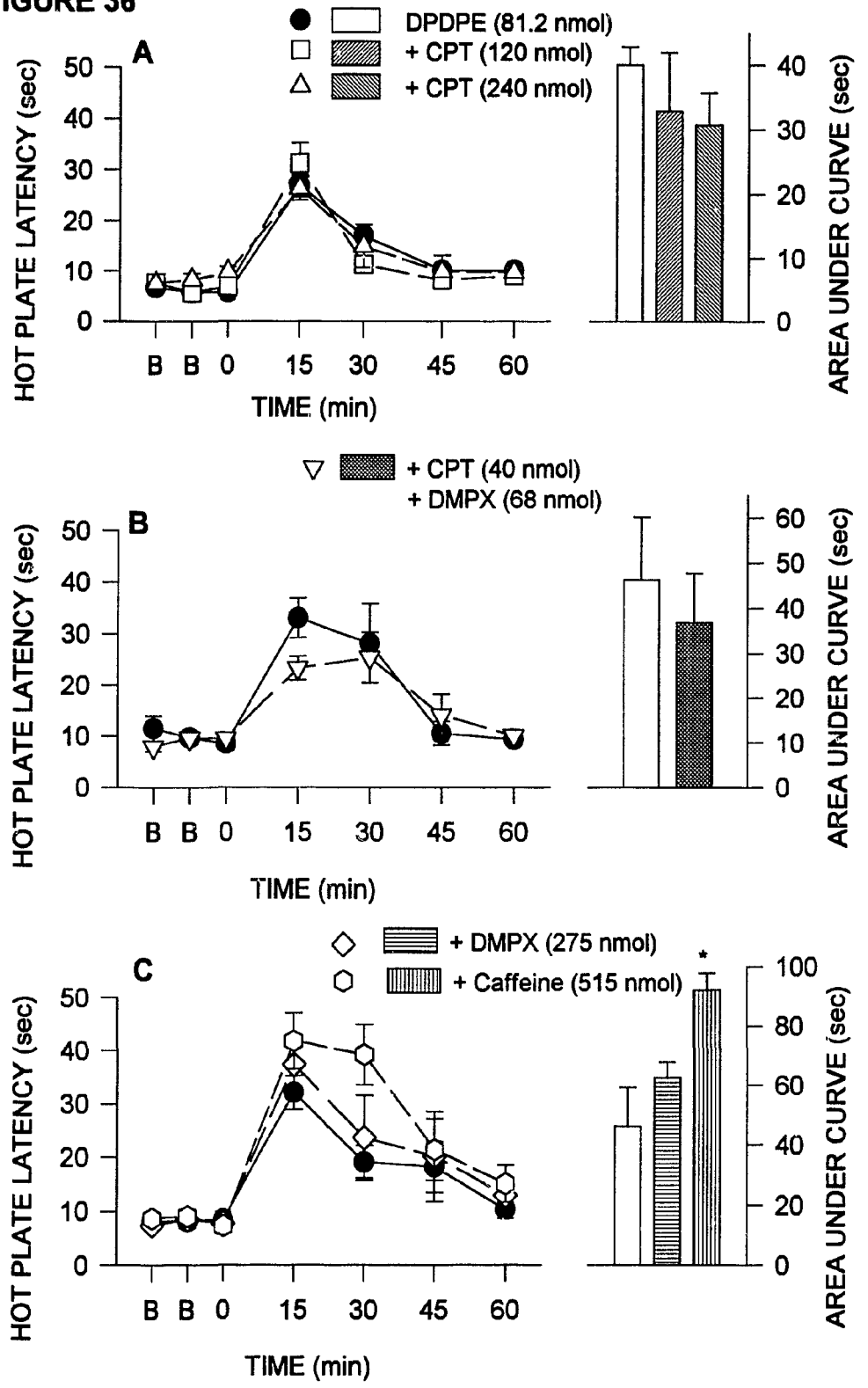
Area under the curve values for multiple combinations of adenosine receptor agonists CHA and CGS21680. Values were calculated from response latencies in the hot plate test of a 90 min time course. Values represent mean ± s.e.m. for n=4-5 per group.

			CGS 21680		
			0.56 nmol	***5.6 nmol***	***18.6 nmol***
			9.0 ± 2.0	4.1 ± 3.6	42.3 ± 5.1
CHA	***0.29 nmol***	44.7 ± 10.5	31.7 ± 3.6	17.0 ± 5.9	38.6 ± 9.2
	0.86 nmol	40.6 ± 15.0	12.9 ± 4.8	42.6 ± 8.3	43.0 ± 7.7

FIGURE 36

Effects of graded doses of i.t. CPT and/or DMPX administered 15 min prior to the opioid agonist DPDPE. B indicates baseline latencies determined at 15 min intervals prior to the i.t. injection of drug. The data are expressed as the mean $\pm$ s.e.m. for the latency in the hot plate test of n=5.

FIGURE 36


A
DPDPE (81.2 nmol)
+ CPT (120 nmol)
+ CPT (240 nmol)
HOT PLATE LATENCY (sec)
AREA UNDER CURVE (sec)
TIME (min)
B
+ CPT (40 nmol)
+ DMPX (68 nmol)
HOT PLATE LATENCY (sec)
AREA UNDER CURVE (sec)
TIME (min)
C
+ DMPX (275 nmol)
+ Caffeine (515 nmol)
*
HOT PLATE LATENCY (sec)
AREA UNDER CURVE (sec)
TIME (min)

selective adenosine receptor agonists N^6-cyclohexyladenosine (CHA, A_1 receptor) and 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine (CGS21680, A_2 receptor) are presented in figure 37. Both adenosine receptor agonists dose-dependently induce modest antinociceptive effects in the hot plate test. Similar antinociceptive profiles were demonstrated in the tail flick test (data not shown). Both adenosine agonists at higher doses (CGS21680 18.5 nmol = 10 μg, CHA 1.7 nmol = 3 μg) tended to induce motor incoordination manifested as hindlimb rigidity and a flattening of body posture similar to that produced by high doses of μ opioid receptor agonists. These effects were more prominent in animals receiving i.t. CHA. Animals injected with doses greater than 0.86 nmol CHA also appeared nervous and reacted to any change in background noise with a startled response.

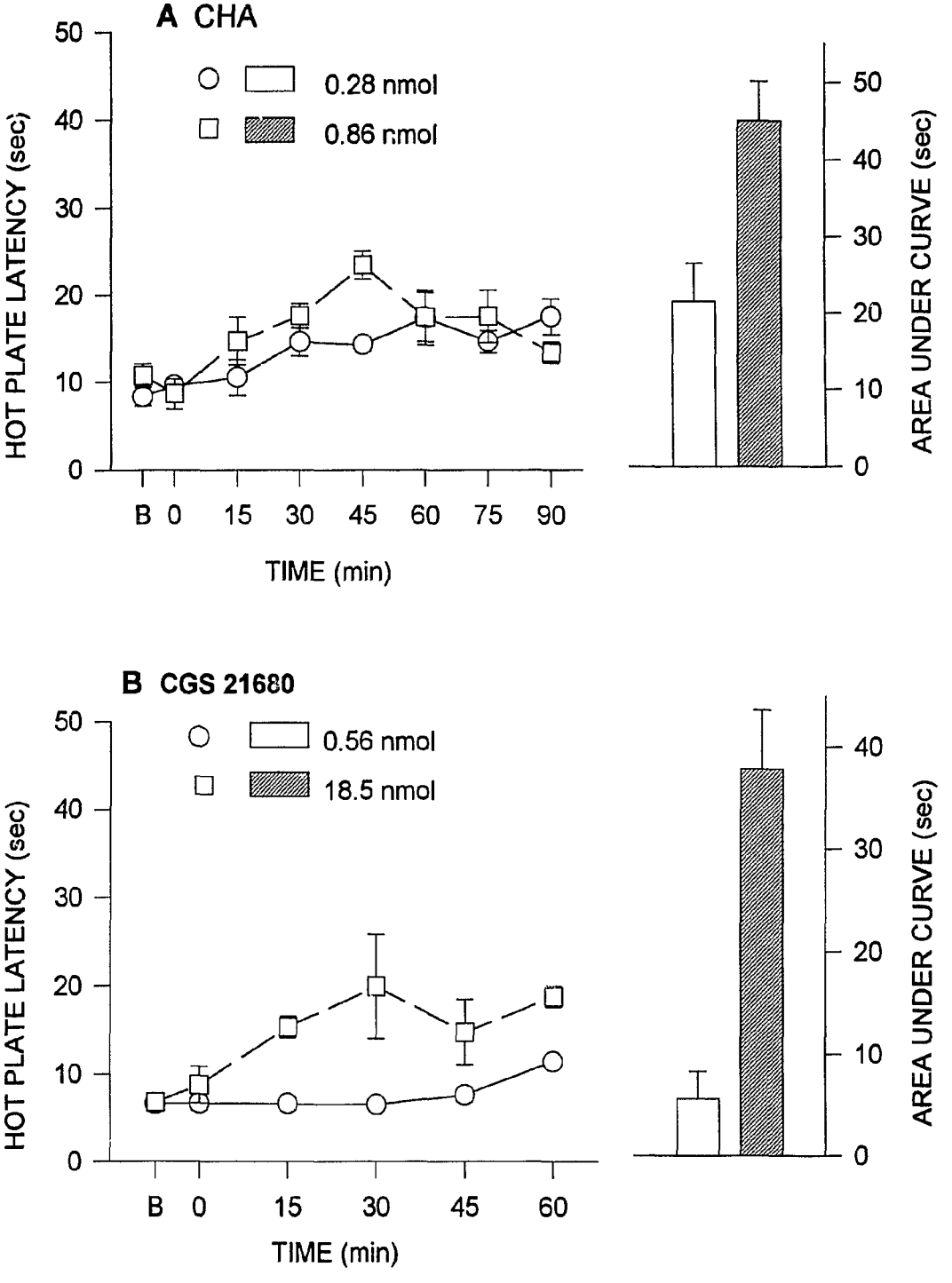
Possible antinociceptive synergy between adenosine receptor agonists was investigated by i.t. co-administration of multiple doses of each agonist. The antinociceptive effects of i.t. co-administration of CHA (0.29 and 0.86 nmol) and CGS21680 (0.56, 5.6 and 18.6 nmol) are represented as area under the curve values (90 min time course) in table 7. No combination examined produced an augmented antinociceptive effect, change the duration, the onset of action or the peak antinociceptive response compared to the antinociception of each agonist alone.

Antagonism by selective adenosine receptor antagonists of adenosine receptor agonist-induced antinociception and motor impairment was performed. This series of experiments was executed in a blind manner, as the motor scoring was subjective. The motor impairment induced by CHA was partially blocked by A_1 but not A_2 adenosine

FIGURE 37

Dose- and time- related antinociceptive effects of adenosine receptor agonists CHA and CGS21680 administered by i.t. injections via chronic implanted cannulas. B indicates baseline latencies determined at 15 min intervals prior to 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.

FIGURE 37


A CHA
0.28 nmol
0.86 nmol
HOT PLATE LATENCY (sec)
AREA UNDER CURVE (sec)
TIME (min)
B 0 15 30 45 60 75 90
B CGS 21680
0.56 nmol
18.5 nmol
B 0 15 30 45 60

receptor antagonists (data not shown). The motor effects associated with i.t. CGS21680 were less pronounced than CHA and was not attenuated by either selective antagonist (data not shown).

DISCUSSION

The primary focus of this research was (1) to determine whether depolarizing agents other than K^+ augment morphine-evoked release of adenosine, (2) to determine the primary opioid receptor subtypes (μ vs δ vs κ) involved in the spinal release of adenosine using both behavioural and neurochemical approaches, (3) to determine potential synergistic interactions between μ and δ opioid receptor subtypes in neurochemical paradigms, and (4) to characterize cell surface adenosine receptors mediating the methylxanthine-sensitive component of spinal antinociception induced by i.t. morphine and more selective agonists for μ and δ opioid receptors.

1 SUBSTANCE P AND MORPHINE

Previous studies have demonstrated that morphine releases adenosine from the dorsal spinal cord in two distinct phases (nanomolar and micromolar) (Cahill et al., 1993a). The nanomolar component of morphine-evoked release of adenosine is only revealed in the presence of an additional 6 mM K^+. The addition of 6 mM K^+ is not sufficient to evoke the release of adenosine above basal values. It is presumed that the elevated K^+ concentration creates a partial depolarization which allows morphine to exhibit nanomolar activity. This study demonstrated that 6 mM is the optimum K^+ concentration in combination with morphine to evoke adenosine release. Depolarization produced by a high nanomolar concentration of substance P (an ineffective dose), but not capsaicin at any dose, augmented morphine-evoked release of adenosine.

The requirement of slightly elevated K^+ concentrations to release adenosine at

nanomolar concentrations of morphine is of interest. It is plausible that this observation has physiological significance in that morphine is a more effective analgesic during pain states where K^+ is elevated due to tissue injury. It is well known that enhanced opioid antinociception occurs during inflammatory conditions. Intrathecal μ, δ and κ opioid agonists exhibit increased antinociceptive potency on carrageenan induced C-fibre-evoked responses compared to normal animals (Stanfa et al., 1992). Antinociceptive actions of morphine (s.c.) are greater in inflamed vs noninflamed paw following an unilateral injection of Freund's adjuvant (Barthó et al., 1990). The same investigators demonstrated that this difference in the potency of morphine was absent in adult rats pretreated with capsaicin. Thus, capsaicin-sensitive C-fibre afferents are essential for the increased antinociceptive effect of morphine in the inflamed tissue.

Substance P is a neuropeptide present in primary afferent neurons associated with the transfer of painful or nociceptive stimuli from the periphery to the central nervous system, and is released from primary afferent terminals by noxious stimulation (reviewed Levine et al., 1993; Regoli et al., 1994). Exogenous substance P elicits nociceptive responses following i.t. administration (Moochhala and Sawynok, 1984; Yashpal et al., 1982), while i.t. administration of substance P antagonizes the antinociceptive effects of morphine via activation of neurokinin receptors (Sawynok et al., 1984). These observations are consistent with pain facilitory effects of substance P. There exists also a number of earlier studies that have reported antinociceptive effects of centrally administered substance P (Malick and Goldstein, 1978; Mohrland and Gebhart, 1979; Stewart et al., 1976; Oehme et al., 1980). Additionally, intraperitoneal administration

of substance P-induced antinociception in the hot plate test which was antagonized by naloxone in mice (Hall and Stewart, 1983). Intracerebroventricular administration of substance P produces an increase in threshold responses that is blocked by naloxone and a peptidase inhibitor (Naranjo et al., 1982) suggesting that substance P releases endogenous opioids. Spinal antinociceptive effects of substance P have also been demonstrated, whereby i.t. injection of substance P produces antinociceptive effects in thermal threshold tail flick tests in rats which is abolished by i.t. naloxone (Doi and Jurna, 1981; Yashpal and Henry, 1982). This further supports a release of endogenous opioids by substance P. Biochemical studies have demonstrated directly that substance P can release endogenous opioids from a supraspinal site via a Ca^{2+}-dependent mechanism (Naranjo et al., 1986; Iadarola et al., 1986).

More recently, the i.t. administration of certain doses of substance P has been shown to produce antinociceptive effects that can be antagonized by both intravenous naloxone and intraperitoneal caffeine (Yashpal and Henry, 1992). The authors hypothesized that substance P releases endogenous opioids to elicit this analgesic effect, and a subsequent endogenous release of adenosine further mediates the antinociceptive response.

The present study demonstrates that substance P, alone, releases adenosine from spinal cord synaptosomes. Release occurs in a biphasic manner, and originates from a capsaicin-sensitive source. A similar bell shaped dose-response relationship for substance P has been reported in behavioural paradigms where only low doses produce analgesia (Frederickson et al., 1978; Hall and Stewart, 1983). Whether the release of adenosine

is due to a direct effect of substance P on the synaptosomes or an indirect effect of substance P via the release of endogenous opioids remains to be determined.

It is plausible that substance P could activate neurokinin receptors directly on presynaptic nerve terminals to release adenosine. Thus, it has been demonstrated that (a) substance P can change presynaptic terminal excitability following application in the cat spinal cord (Randic et al., 1982), (b) electrophysiological studies have reported that substance P selectively modulated C-fibre-evoked discharges of dorsal horn nociceptive neurons in rats (Kellstein et al., 1990), and (c) substance P depolarizes sensory neurons directly (Spigelman and Puil, 1991). Substance P may thus function as a neuromodulator of C-fibre afferent mediated nociception. Excitatory effects of exogenous substance P have been observed in spinal sensory neurons and are thought to exert many effects on sensory neurons at multiple sites within the trigeminal system of the spinal cord (Spigelman and Puil, 1991). Electrophysiological studies have demonstrated that neurons of trigeminal nucleus slices exhibit dose-dependent depolarization responses to bath applications of substance P (10^{-8} M) (Spigelman and Puil, 1988; 1990). However, autoradiographic binding studies do not support this hypothesis in that substance P binding is not reduced by dorsal rhizotomy (Yashpal et al., 1991) or by capsaicin treatment (Helke et al., 1986) suggesting that substance P receptors are not present on presynaptic nerve terminals.

A recent study has demonstrated the co-existence of μ, δ and κ opioid receptor mRNAs with the mRNA of preprotackykinin A, a precursor of substance P, in dorsal root ganglion neurons (Minami et al., 1995). The expression of μ opioid receptor

mRNA occurred in approximately 90% of substance P-containing neurons. Furthermore, co-localization of μ opioid receptor-like and substance P-like immunoreactivity in axon terminals within the superficial layers of the dorsal spinal cord of the rat has also been demonstrated (Ding et al., 1995). This provides justification for the hypothesis that substance P can modulate opioid mediated effects on these presynaptic terminals. However, in a synaptosomal preparation, this requires appropriate juxtaposition of synaptosomes containing opioids with those containing adenosine.

Substance P-evoked release of adenosine and substance P augmentation of morphine-evoked release of adenosine occurs via a Ca^{2+}-dependent mechanism. Substance P produces an elevation in intracellular Ca^{2+} by mobilizing its release from intracellular stores (Womack et al., 1988), as well as increasing Ca^{2+} influx through voltage-gated Ca^{2+} channels (Womack et al., 1989). However, substance P can also elevate intraneuronal [Ca^{2+}] by releasing Ca^{2+} from intracellular stores independent of its influx (Womack et al., 1988). Capsaicin depolarizes primary afferent neurons and increases intracellular [Ca^{2+}] through activation of a ligand gated non-selective cation channel which can be blocked by ruthenium red (Dray et al., 1990). Previous studies have demonstrated that capsaicin evokes the release of a nucleotide(s) from spinal cord synatosomes that is converted to adenosine extracellularly (Cahill et al., 1993b). This release was dose-dependently blocked by ruthenium red but not L- or N-type voltage dependent Ca^{2+} channel blockers (Cahill et al., 1993b). It was surprising that capsaicin did not augment morphine-evoked release of adenosine. Sensory neurons contain capsaicin sensitive Ca^{2+} uptake sites that regulate the release of neuropeptides (Dray et

al., 1990; Holzer, 1991). Due to the narrow concentration window that stimulates the release of adenosine for K^+ and substance P, perhaps an effective concentration of capsaicin which augmented morphine-evoked release of adenosine was overlooked. It is also possible that voltage dependent Ca^{2+} channels are an integral step in inducing the release of adenosine; substance P has been shown to activate these channels (Regoli et al., 1994) and morphine activated Ca^{2+} channels in cultured neurons (Fields et al., 1995).

The ability of substance P to enhance release of adenosine by morphine can be hypothesized to have a physiological significance. It has been well established that the potency of morphine is shifted to the left in the presence of noxious stimulation. Thus, in tonic pain states where C-fibres are continually stimulated, morphine antinociception is augmented (Bartho et al., 1990) compared to thermal threshold tests. This study provides a plausible mechanism by which morphine is more effective under certain pain states; substance P enhances morphine-evoked release of adenosine which is known to be an inhibitory neuromodulator of nociceptive transmission.

2 OPIOID RECEPTOR ACTIVATION RELEASES ADENOSINE FROM SPINAL CORD SYNAPTOSOMES

Nanomolar concentrations of morphine in the presence of elevated K^+ concentrations can release adenosine from spinal cord synaptosomes. This phenomenon is exhibited by the selective μ opioid agonists DAMGO and PLO17, but not by either δ opioid agonists DPDPE and DELT or the κ opioid agonist U50488H. This profile of

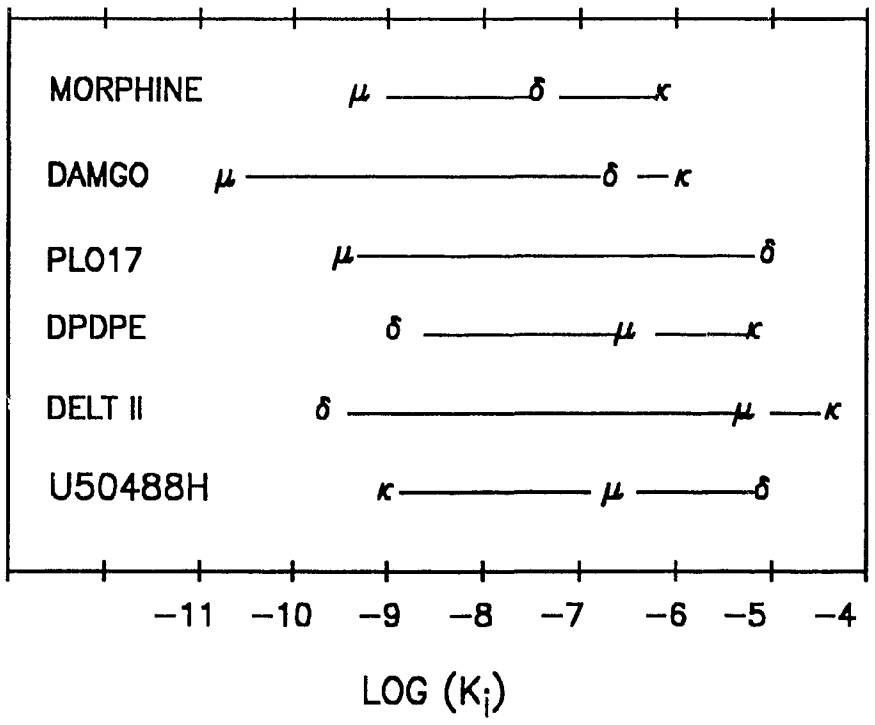
activity indicates that adenosine released at nanomolar concentrations of morphine is mediated through activation of the μ opioid receptor subtype. Such neurochemical data is consistent with the observation that methylxanthines inhibit spinal antinociception produced by μ opioid receptor agonists (DeLander et al., 1992). Furthermore, nucleoside transport inhibitors attenuated μ but not δ opioid-induced antinociception in mice (Keil and DeLander, 1995).

The relative potency for nanomolar concentrations of μ opioid receptor agonists to evoke the release of adenosine from synaptosomes partially depolarized with elevated K^+ concentrations was determined to be DAMGO > PLO17 > morphine. This result suggests that the opioid receptor agonists with high affinity for μ opioid receptor subtypes are more effective at evoking the release of adenosine than is morphine. The relative micromolar potency of μ opioid receptor selective agonists to induce adenosine release (morphine > PLO17, DAMGO) is opposite to that seen with nanomolar concentrations. Thus, morphine is more active at micromolar concentrations than the more selective μ ligands DAMGO and PLO17 even in the presence of a partial depolarization. The micromolar component of activity for μ opioid receptor agonist-evoked release of adenosine may involve activation of multiple opioid receptors, as all of the selective opioid agonists for μ, δ, and κ receptors release adenosine at 100 μM (Figure 38). Alternatively, evoked release of adenosine by high micromolar concentrations of δ and κ opioid receptor agonists may be due to a loss in their selectivity and act at μ opioid receptors (Figure 38).

The difference in potency of micromolar concentrations of morphine compared

FIGURE 38

Binding affinities for opioid receptor agonists. Comprised from (Chang et al., 1983; Erspamer et al., 1989; Goldstein, 1987).



to the more selective μ opioid receptor agonists may be the result of morphine acting at both δ and μ opioid receptors in this concentration range. Thus, micromolar concentrations of morphine act at both μ and δ opioid receptors producing synergistic or additive effects to evoke the release of adenosine. Occupancy of δ opioid receptors by DELT and DPDPE does not have a further significant effect on enhancing adenosine released by micromolar concentrations of morphine. Certainly, μ/δ synergy is expressed at the synaptosomal level, as 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.

Morphine-induced antinociception (Heyman et al., 1989; Jiang et al., 1990; Malmberg and Yaksh, 1992), and morphine-induced C-fibre depression (Guirimand et al., 1994) have been shown to be attenuated by both μ and δ opioid receptor antagonists. Other studies have demonstrated that effects elicited by morphine can be attenuated by both μ and δ opioid receptor antagonists.

The degree of enhancement of adenosine release by each μ opioid receptor agonist varied when combined with the δ opioid receptor ligands DELT (δ_2) and DPDPE (δ_1). Inactive nanomolar concentrations of both δ_1 and δ_2 opioid receptor subtypes produce synergistic effects with μ opioid receptor agonists to release adenosine. However, the δ_1 opioid agonist appears more potent than the δ_2 opioid agonist in producing the μ/δ synergy when combined with the most selective μ opioid agonist PLO17. Ligand binding studies indicate that the degree of selectivity for PLO17 is >600 fold, for DAMGO is 130 fold and for morphine is 60 fold greater for μ-opioid receptors compared to δ (Chang

et al., 1983; James and Goldstein, 1984; Goldstein, 1987) (Figure 38). Less of a difference was exhibited when DPDPE (δ_1) was combined with the least selective μ opioid receptor agonist, morphine. Consistent with this data are the results of a study by Malmberg and Yaksh (1992) demonstrating that the magnitude of augmentation of DPDPE with μ opioid receptor agonists in a thermal threshold test following i.t. administration in rats was PLO17 > DAMGO > morphine, whereas no differences in augmentation of opioid-induced antinociception produced by PLO17, DAMGO and morphine with activation of δ_2 opioid receptors was observed.

There are many recent studies providing evidence for synergistic spinal antinociceptive interactions between μ and δ opioid ligands, thus demonstrating that complex interactions between μ and δ opioid receptors in more integrated systems utilized in behavioural studies exist. Concurrent i.t. administration of DPDPE and μ opioid receptor agonists (morphine, PLO17 or DAMGO) produced synergistic antinociceptive actions in both the thermal and pressure threshold tests (Sutters et al., 1990; Malmberg and Yaksh, 1992; Mattia et al., 1992; Miaskowski et al., 1992). Furthermore, an electrophysiological study demonstrated that i.t. DAMGO and DPDPE produced synergistic suppressive effects on noxious evoked activity of wide dynamic range neurons within the dorsal spinal cord of cats (Omote et al., 1990). Antinociceptive synergy is exhibited between adenosine analogues and δ opioid receptor agonists but only additive effects are produced between adenosine analogues and μ opioid receptor agonists (DeLander and Keil, 1994). This latter study suggested that synergistic effects were not observed between μ opioid agonists and adenosine agonists because antinociception

induced by activation of μ opioids is partially mediated by the release of adenosine.

A variety of mechanisms by which μ and δ opioid agonists elicit spinal synergistic antinociceptive effects have been proposed. These include both pharmacokinetic and pharmacodynamic domains. Thus, drug A may change the kinetics, such as altered clearance, of drug B causing an increase in the levels of drug B at the effector site hence prolonging its antinociceptive effect. Drug A and drug B may act on the same primary afferent neuron through a common second messenger system with a common effector such as K^+ ionic conductance (North, 1993). Others have suggested that allosteric interactions exist between μ and δ opioid receptors that result in increased agonist affinity (Rothman and Westfall, 1982). The existence of a physically coupled μ/δ receptor complex in the spinal cord has been proposed on the basis of autoradiographic studies (Schoffelmeer et al., 1990), biochemical studies (Schoffelmeer et al., 1990; 1992) and *in vivo* studies (Heyman et al., 1989; Jiang et al., 1990; Malmberg and Yaksh, 1992). Finally, activation of separate anatomical sites such as pre- and post-synaptic elements may magnify the effects produced by either drug. Hence, functional interactions between receptors also can occur due to activation at different sites within the cascade of nociceptive integration (Malmberg and Yaksh, 1992; Traynor and Elliott, 1993).

The mechanism by which the release of adenosine following μ opioid receptor activation is enhanced by δ opioid receptor activation is not clear. As reviewed in the introduction, there exists differences in the distribution between μ and δ opioid binding sites in the rat lumbar spinal cord (Morris and Herz, 1987). On the basis of distinct distribution, functional interaction at different anatomical sites is unlikely to contribute

to interactions observed at the synaptosomal level in the current study. If activation of μ and δ receptors independently evoked the release of adenosine, one would expect additivity rather than synergy. The current study is supportive of the pharmacodynamic hypothesis, whereby activation of μ and δ opioid receptors evokes the release of adenosine in a supra-additive manner from dorsal spinal cord synaptosomes via a common second messenger system. Independent activation of μ and δ opioid receptors by selective ligands produces an augmented response (adenosine release) with lower fractional receptor occupancy. Although the second messenger system involved in opioid-evoked release of adenosine is not completely understood, μ/δ-evoked release of adenosine from spinal cord synaptosomes is Ca^{2+}-dependent and involves activation of a N-type voltage sensitive Ca^{2+} channel, reflecting the properties of morphine (Cahill et al., 1993a). Nanomolar concentrations of μ, and micromolar concentrations of δ, opioid receptor agonists release adenosine *per se* from spinal cord synaptosomes and it is likely that the combination of δ with μ opioid receptor agonists has characteristics similar to each agonist alone in mediating the release of adenosine. Both μ and δ opioid receptors have been identified on presynaptic afferent nerve terminals (Fields et al., 1980; Besse et al., 1991) and coexist on the cell body of dorsal root ganglion neurons (Shen and Crain, 1989). This provides anatomical support for a direct interaction between μ and δ opioid receptors in spinal cord synaptosomes.

3 EXCITATORY EFFECTS OF SPINAL OPIOIDS

Morphine evokes the release of adenosine in a multiphasic manner, where release

is exhibited by nanomolar concentrations, inhibited by low micromolar concentrations and finally release is evoked at high micromolar concentrations. The ability of opioids to release adenosine from dorsal spinal cord synaptosomes represents an excitatory action of opioids. Although opioids are generally considered to produce inhibitory actions on cellular function, a growing number of studies have reported excitatory effects of low concentrations of opioids in behavioural, neurochemical and electrophysiological paradigms (see section 2.5 of Introduction).

One possible mechanism that could explain how μ opioid receptor agonists produce such complex multiphasic effects on the release of adenosine may be that they produce concentration-dependent activation of different opioid receptor subtypes on presynaptic nerve terminals. The paradoxical excitatory effects of morphine depicted by the release of transmitters/modulators from sensory afferents also may result from activation of a different opioid receptor subtype than that which induces inhibitory actions. Morphine was shown to have multiphasic effects on K^+-evoked release from the spinal trigeminal nucleus slices in a manner where morphine enhanced release at nanomolar concentrations (100 nM), inhibited at low micromolar concentrations (3 μM), and finally facilitated release again at high micromolar concentrations (Suarez-Roca et al., 1992). The different modulatory phases of morphine on K^+-evoked release of substance P were attributed to activation of different opioid receptors (Suarez-Roca and Maixner, 1992). Thus, antagonism of μ opioid receptors by β-FNA and δ opioid receptors by ICI 174,864, respectively, inhibited the facilitory effects of morphine (100 nM) or suppressed the inhibitory effects of morphine (3 μM) on K^+-evoked substance P

release (Suarez-Roca and Maixner, 1992). Similarly, selective δ and μ opioid receptor agonists inhibit or facilitate, respectively, K^+-evoked release of substance P (Mauborgne et al., 1987; Pohl et al., 1989). Thus, it is plausible that the inhibitory phase of adenosine released by DAMGO, PLO17 and morphine seen at 10^{-7} M may result from activation of δ opioid receptors. Selective opioid antagonists on morphine-evoked release of adenosine may resolve this question. It should be noted that an inhibitory effect of morphine on adenosine release was not seen in this paradigm as release was examined above basal levels rather than determining the modulatory effects of morphine on K^+-evoked adenosine release.

The requirement for higher nanomolar concentrations of these μ opioid receptor agonists to produce a δ receptor mediated effect is consistent with the binding affinities of these ligands to opioid receptors (Wood et al., 1981). It has been well established that all three opioid receptor subtypes are localized on primary afferent neurons (Fields et al., 1980), and that morphine activates μ, δ and κ opioid receptors in a concentration-dependent manner (see Figure 38).

Dual excitatory and inhibitory effects of opioid receptor agonists have also been observed in cultured cell preparations (Shen and Crain, 1989; Fields et al., 1995). While these studies demonstrate dual excitatory and inhibitory effects of opioids, the effects are not discriminated by selective agonists, as μ, δ and κ ligands produce each effect.

Three possible mechanisms of bimodal regulation of opioids on K^+ conductance have been proposed by Fan and Crain (1995) but extend to all dual effects seen by opioids. (1) Each opioid receptor subtype may have two subtypes coupling through

cholera toxin- and pertussis toxin-sensitive transduction systems. (2) Dose-dependent effects of opioids may be mediated by the same receptor subtype coupled to both transduction systems through different G-proteins (G_i and G_s). β_2 Adrenergic receptors (Okamoto et al., 1991) and α_2 adrenergic receptors (Eason et al., 1992) are coupled to both G_s and G_i. The net effect on adenylate cyclase depends on the state of the receptor, resulting in either excitatory or inhibitory action. Direct coupling to both G_s and G_i proteins has been demonstrated (Cruciani et al., 1993). (3) Each opioid receptor may be coupled to only one G-protein but can have both cholera- and pertussis toxin-sensitive effects (Lustig et al., 1993).

It has been suggested that inhibitory and facilitory effects of opioids on neuronal activity may be mediated by different mechanisms as the result of activating different opioid receptor subtypes which are coupled to different second messenger systems or ion channels (Chen et al., 1988; Gintzler and Xu, 1991; Shen and Crain, 1989; Xu et al., 1989; Cruciani et al., 1993). Thus, low concentrations of μ opioid receptor agonists act at the μ site which in turn activates an intracellular process that is overcome or altered by the activation of another opioid receptor subtype. As agonists lose their selectivity for their respective receptor, activation of another opioid receptor results in the generation of a different second messenger system cascade that leads to inhibition rather than stimulation. Another possible explanation which has been proposed for morphine producing multiphasic effects is an indirect mechanism (Suarez-Roca et al., 1992). That is, μ opioid receptor agonists could release endogenous opioids and/or antagonists (e.g. cholecystokinin) that modulate release of peptides from primary afferent neurons (Faris

et al., 1983; Glass et al., 1986). It is unlikely that such a mechanism occurs in a synaptosomal preparation and therefore is an unlikely explanation to account for the excitatory effects of evoked release of adenosine by μ opioid receptors agonists in this study.

The current study shows that the release of adenosine by both μ opioid (nanomolar) and δ opioid (micromolar) agonists release adenosine via a Ca^{2+}-dependent mechanism (release by the κ opioid agonist is Ca^{2+}-independent). Nanomolar concentrations of μ agonists release adenosine *per se*, rather than a nucleotide, that originates from capsaicin-sensitive small diameter primary afferent neurons. 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^{2+} dependent mechanism (Sweeney et al., 1989; Cahill et al., 1993a). Furthermore, spinal release of adenosine by high micromolar concentrations of morphine is capsaicin-sensitive implicating a primary afferent source for this release (Sweeney et al., 1989).

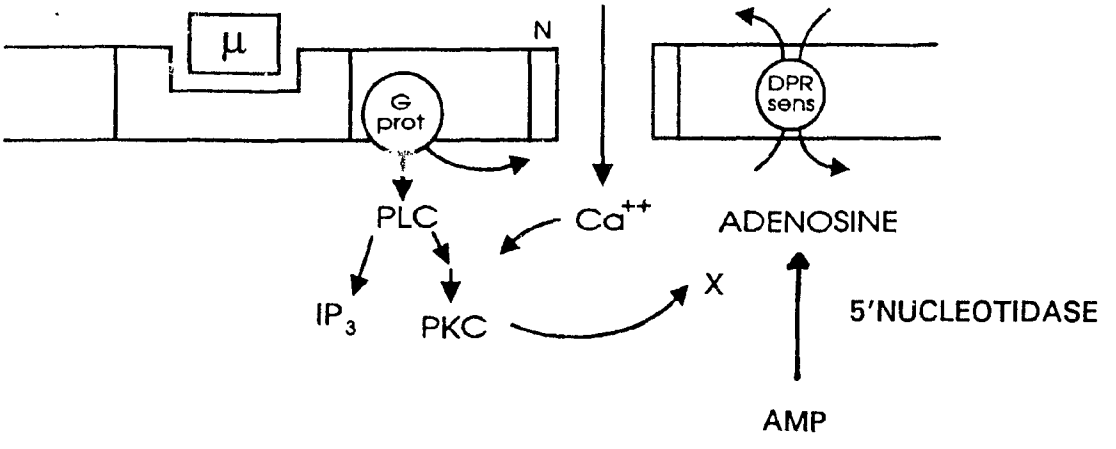
Morphine-induced release of adenosine involves activation of ω-conotoxin sensitive voltage dependent Ca^{2+} channels (Cahill et al., 1993a). The current study demonstrates that synergistic interactions between μ and δ opioid agonists to evoke the release of adenosine also involves activation of N-type voltage dependent Ca^{2+} channels. N-type voltage dependent Ca^{2+} channels which are blocked by ω-conotoxin GVIA have been identified on rat sensory neurons (Scroggs and Fox, 1991). A recent study has demonstrated that cloned μ opioid receptors are coupled to ω-conotoxin sensitive Ca^{2+} currents in NG108-25 cells (Morikawa et al., 1995).

While most earlier studies examining the effects of opioids on Ca^{2+} currents have demonstrated inhibition of Ca^{2+} entry into neurons (Moises et al., 1994), a number of recent studies have described mechanisms by which opioids may enhance Ca^{2+} entry into cells or Ca^{2+} intracellular levels. Thus, studies utilizing Ca^{2+} imaging techniques have shown that opioids can increase intracellular Ca^{2+} levels in cultured neurons (Jin et al., 1992; Tang et al., 1994) and augment K^+-induced elevation in intracellular Ca^{2+}; an effects that was dependent on extracellular Ca^{2+} (Fields et al., 1995). In some cells these effects are mediated by δ opioid receptor activation (Jin et al., 1992; Tang et al., 1994), but in other cells, they are mediated by μ opioid receptors (Smart et al., 1994).

The second messenger system involved in the opioid enhancement of intracellular Ca^{2+} 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^{2+}-dependent and pertussis toxin-sensitive G protein-dependent increase in phosphoinositol levels (Smart et al., 1994). Morphine-evoked release of adenosine is both Ca^{2+}-dependent (Sweeney et al., 1989) and 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 mediated by the protein kinase C second messenger system. A proposed model by which μ opioid receptor activation increases the release of adenosine from spinal cord synaptosomes is presented in figure 39. A recent study in another biological system has demonstrated that adenosine released from cardiac tissue during hypoxic conditions occurred via activation of protein kinase C (Minamino et al.,

FIGURE 39

Proposed mechanism for opioid-evoked release of adenosine via stimulation of protein kinase C. DPR sens: dipyridamole sensitive; PLC: phospholipase C; PKC protein kinase C; IP_3: inositol trisphosphate; N: N-type voltage dependent Ca^{2+} channel.



1995), and protein kinase C can increase nucleotidase activity (). While stimulatory effects of opioids in cultured dorsal root ganglion cells and the myenteric plexus have been attributed to stimulation of cyclic AMP (Crain and Shen, 1990; Gintzler, 1995), this effect does not appear to be involved in adenosine release from synaptosomes (Nicholson et al., 1991).

Interestingly, substance P-evoked release of adenosine may also involve protein kinase C activation. Substance P triggers an increase in phosphoinositol metabolism resulting in the production of IP_3 (Koizumi et al., 1992) which can then stimulate the translocation and activation of protein kinase C.

4 ADENOSINE MEDIATES SPINAL OPIOID ANTINOCICEPTION

In the current study, each selective agonist for μ and δ opioid receptors resulted in a dose-dependent elevation in thermal nociceptive threshold. The duration of effect produced by each agent was variable, with morphine having the longest analgesic action of all of the opioid receptor agonists tested and DPDPE having the shortest duration of action. The order of potency for these agonists was DAMGO, PLO17 > DELT, morphine > DPDPE > U50488H. This is similar to the order of potency of the μ and δ agonists in our synaptosomal release study as well as that already reported for spinal administration of opioid receptor agonists in the hot plate test (DAMGO > PLO17 > morphine > DPDPE) (Malmberg and Yaksh, 1992). The true pharmacological efficacy of the opioid receptor agonists could not be determined due to limitations created by ethical considerations imposed by a 50 sec cutoff in the hot plate test.

Antinociceptive effects of U50488H were determined in the hot plate thermal threshold test. The resulting latencies produced by i.t. U50488H were not different from base line latencies except at the highest dose tested (300 μg or 644 nmol). At this dose some animals appeared to experience antinociception; however, most rats exhibited increased activity including jumping and slapping of their paws on the hot plate indicating that the expression of the endpoint had changed. Thus, the latencies recorded implicate the induction of antinociception but this inference is probably due to the increase the rat's activity on the hot plate rather than true antinociceptive effects. Previous studies have demonstrated a lack of analgesic efficacy for U50488H in thermal threshold tests (Schmauss and Yaksh, 1984); κ opioid agonists are known to be more active in non-thermal (pressure and chemical) tests (Millan, 1986).

Caffeine was selected as the adenosine antagonist rather than more selective adenosine receptor antagonists because they require solvent vehicles which appeared to modify the antinociceptive effects elicited by morphine. Most methylxanthines are not soluble in saline but require organic solvents and/or weak acids or bases. A recent study has demonstrated that low pH modifies G protein coupling in NG108-15 cells resulting in an increase in opioid agonist efficacy due to a decreased inactivation of G proteins (Selley et al., 1993). Both ethanol and DMSO are highly lipophilic agents and can alter distribution of drugs. Furthermore, DMSO blocks C-fibre conduction and increases nociceptive latencies (Evans et al., 1993). Ethanol also has been reported to increase extracellular adenosine by inhibition of adenosine uptake via the nucleoside transporter (Nagy et al., 1990).

In this study, the methylxanthine-sensitivity of opioid-induced antinociception was not observed in animals receiving an i.t. injection by lumbar puncture. However, caffeine antagonized the antinociceptive effects of morphine following i.t. administration through a chronically implanted catheter. It is generally believed that systemic morphine acts at the spinal level not only by direct mechanisms but also by indirect mechanisms involving supraspinal structures. However, it is presumed that the methylxanthine component of antinociception measured by thermal threshold nociceptive tests is assessing the spinal component of antinociception induced by i.t. morphine in these experiments. Previous studies have shown that intrathecal radiolabelled morphine fails to diffuse towards the brain in any significant quantities; no evidence of diffusion could be detected more than 4-5 cm rostrally at least one hour after i.t. morphine (Yaksh and Rudy, 1977; Nishio et al., 1989). In this study, the precision of the i.t. injection by lumbar puncture was evaluated by administration of a 20 μl volume of dye. This also confirmed that this volume of injection was sufficient to diffuse to the lumbar enlargement of the spinal cord.

It is unlikely that the difference in methylxanthine-sensitivity between lumbar puncture and cannula injection results from the presence of the cannula in this group of animals. It is possible that provocation of central inflammation occurs in animals implanted with chronic cannulas, but caffeine administered by lumbar puncture in these animals does not block morphine-induced antinociception. Thus, the possible development of inflammation does not account for the difference in methylxanthine-sensitivity of morphine-induced antinociception.

The lack of methylxanthine-sensitivity of opioid-induced antinociception for lumbar puncture compared to that of chronic cannula injection may result from a difference of the site of injection into the subarachnoid space. Lumbar puncture injection delivers drugs into the subarachnoid space below the spinal cord, whereas cannula injection delivers the drugs at the T12 (just prior to the lumbar enlargement). Although morphine spreads to active sites to induce antinociception following both methods of i.t. administration, the distribution of caffeine may be more limited due to differences in the kinetics between these two agents. Morphine is not very lipid soluble compared to caffeine. It is also possible that caffeine could not block the antinociceptive effects of morphine following i.t. lumbar puncture administration because the exposure of rats to halothane. It has been demonstrated that halothane significantly enhances i.t. morphine-induced antinociception in the formalin test depending on the length of exposure to halothane (O'Connor and Abram, 1994). Furthermore, halothane reduces the release of adenosine in cardiac tissue (Buljubasic et al., 1993). In light of this, halothane did not appear to augment morphine-induced antinociception, but the effects of halothane on morphine-evoked release of adenosine were not determined.

The i.t. pretreatment with caffeine (via chronically implanted cannulas) antagonized the antinociceptive effects induced by μ but not δ opioid receptor agonists. This suggests that only the release of adenosine which occurs at nanomolar concentrations of μ opioid receptor agonists is relevant to the expression of behavioural actions, and that spinal adenosine release is not uniformly involved in all opioid-mediated actions. DeLander et al. (1992) demonstrated that i.t. administration of methylxanthines

antagonized opioid-induced antinociception but not opioid-induced inhibitory effects on gastric emptying or gastrointestinal propulsion. In this study, caffeine dose-dependently attenuates the antinociceptive effects of PLO17, DAMGO and morphine in both the tail flick and hot plate thermal threshold tests, thus implicating adenosine as a mediator of opioid-induced antinociception in phasic pain tests. While the methylxanthine-sensitivity of antinociception produced by combinations of μ and δ opioid receptor agonists was not determined, the present results suggest that the behavioral effects of such combinations should be reduced by methylxanthines.

In rats, the A_3 receptor is almost insensitive to many methylxanthines, including caffeine, whereas A_1 and A_{2A} receptors are likely to be the major targets of methylxanthines. Thus, antagonism of opioid-induced antinociception by caffeine is likely to be mediated by blockade of either A_1 and/or A_2 adenosine receptors. Attempts to characterize which adenosine receptor was important in eliciting this effect were made by pretreating animals with the selective adenosine receptor antagonists CPT and DMPX. Intrathecal CPT, but not DMPX, dose-dependently attenuated the antinociceptive effects induced by i.t. morphine. This implies that activation of A_1 adenosine receptors rather than A_2 adenosine receptors by endogenously released adenosine is important in spinal morphine-induced antinociception. When determining involvement of adenosine receptor subtypes in the antinociceptive actions elicited by DAMGO, neither i.t. pretreatment with CPT nor DMPX significantly modified the response latencies compared to DAMGO alone. However, concomitant administration of low dose CPT and DMPX antagonized the antinociceptive effect induced by low (but not high) doses of DAMGO. Neither

caffeine nor the combination of CPT and DMPX blocked the antinociceptive effects of high doses of DAMGO.

It is of interest that the adenosine receptor involvement in the action of morphine compared to DAMGO appears to be dissimilar. CPT antagonizes the antinociceptive effects induced by morphine but does not attenuate DAMGO-induced antinociception. However, the combination of CPT and DMPX antagonises the antinociceptive effects produced by low doses of DAMGO. Similarly, caffeine antagonizes the antinociceptive effects of morphine but only the effects produced by lower doses of DAMGO. One possible explanation for the differences seen between low and high doses of DAMGO compared to morphine may be due to a difference in their mechanism of action. Thus, the antinociceptive effects produced by low doses of DAMGO is mediated by adenosine release, whereas higher doses activate another mechanism (such as inhibition of postsynaptic neurons) which has a greater role in mediating the antinociceptive effects. Thus, other mechanisms involved in the antinociception produced by high doses of DAMGO masks the methylxanthine-sensitive component. The methylxanthine-sensitivity of morphine-induced antinociception may result from enhanced adenosine release due to synergistic interactions between opioid receptor subtypes.

Neither of the selective adenosine receptor antagonists, CPT and DMPX, alone had any effect on DAMGO-induced antinociception, whereas concurrent administration of CPT and DMPX attenuated the response, prompting the investigation of synergistic effects between A_1 and A_2 adenosine receptors. Previous studies had suggested that synergistic effects between adenosine receptor agonists existed in locomotor studies.

Thus, i.p. administration of 8-(3-chlorostyryl)caffeine (A_{2a} antagonist) and CPX (A_1 antagonist), both at nonstimulating doses, increased locomotor activity (Jacobson et al., 1993). Synergistic depressant effects on locomotor activity have been demonstrated between CHA (A_1) and APEC (A_2) adenosine agonists (Nikodijevic et al., 1991). However, no antinociceptive synergy was detected in the current study following i.t. administration of multiple combinations of CHA and CGS21680. Furthermore, i.t. coadministration of the adenosine agonists CPA and CGS21680 resulted in subadditive interaction (DeLander and Keil, 1994).

Research to date has supported the hypothesis that the A_1 adenosine receptor subtype is primarily responsible for spinal antinociception. Sawynok and colleagues (1986) reported a rank order of potency for i.t. adenosine analogues (L-PIA > CHA > NECA > CADO) determined by thermal threshold testing which was consistent with A_1 > A_2 adenosine receptors. Antinociception resulting from i.t. administration of adenosine analogues also was assessed in a neurogenic pain model induced by i.t. strychnine (Sosnowski et al., 1989). In this latter study, PIA, NECA and CHA produced similar potencies. Antinociception produced by adenosine analogues correlates with their affinity for A_1 adenosine receptors (Karlsten et al., 1991). While A_2 adenosine receptors have been implicated in eliciting spinal antinociception, this conclusion was based on the antinociceptive effects produced by NECA (an adenosine agonist with similar affinities for A_1 and A_2 receptors). Another study demonstrated that CV1808, a selective A_2 adenosine receptor agonist, was inactive in nociceptive tests (DeLander and Wahl, 1988). Recently, electrophysiological data on C-fibre-evoked activity in the spinal cord also has

implicated A_1 receptors in suppressing C-fibre-evoked activity, windup and post discharge depolarization (Reeve and Dickenson, 1995). The current study extends the evidence for a role of adenosine in the control of nociception in the spinal cord and in opioid-induced antinociceptive effects, and supports the importance of the A_1 receptors in these effects.

Studies on opioid dependence have suggested changes in A_1 adenosine receptors may contribute to this process. Chronic morphine treatment, with either intraperitoneal or intracerebroventricular administration, caused down regulation of spinal adenosine A_1 receptors in rats (Tao and Liu, 1992; Tao et al., 1995). A decrease in the number of binding sites (B_{max}) with no change in the affinity (K_d) of [^{3}H]CHA for the A_1 receptor was observed. This latter study demonstrated that rats were not only tolerant to morphine but also to CPA. This is in contrast to an earlier study where adenosine analogue-induced antinociception was not different in mice made tolerant to morphine (Ahiijanian and Takemori, 1986). The difference between these two groups may have resulted from different methods of morphine tolerance induction. Other studies have demonstrated that adenosine A_1 receptor agonists inhibit the expression of morphine withdrawal (Dionyssopoulos et al., 1992; Germany et al., 1990), where CHA significantly reduced the incidence of behavioral effects associated with naloxone-precipitated withdrawal. CHA was also shown to suppress the development of morphine tolerance assessed by antinociceptive effects in the hot plate test (Germany et al., 1990).

The present study revealed the interesting observation that antinociception produced by δ opioid receptor activation was augmented by pretreatment with the adenosine receptor antagonist 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 δ agonist-induced effects is due to species, (rats vs mice), or to a difference in protocol is not clear; however, i.t. injections were made by lumbar puncture compared to i.t. injection through cannulas in the current study. More recently, DeLander and Keil (1994) showed that i.t. adenosine agonists produced antinociceptive synergy when combined with δ opioid agonists but only additivity with μ opioid agonists. It was argued that this observation supported a μ opioid receptor- but not a δ opioid receptor-mediated release of adenosine. Furthermore, antinociception induced by μ opioid receptor agonists was inhibited by i.t. pretreatment with nucleoside transport inhibitors but antinociception induced by δ ligands was not affected (Keil and DeLander, 1995). This provides further *in vivo* evidence that μ but not δ opioid receptor activation is responsible for the release of adenosine following i.t. morphine.

Some studies have reported an augmentation of opioid-induced antinociception with methylxanthines. Thus, following intracerebroventricular 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). Although the doses of caffeine that augmented antinociception by δ opioid agonists were the same as those which antagonized morphine and μ opioid-induced antinociception in the current study, the augmentation of δ opioid-induced antinociception by caffeine is

probably not mediated through antagonism of adenosine receptors, as the effect could not be reproduced by selective adenosine receptor antagonists CPT and DMPX. High doses of methylxanthines have pharmacological actions other than adenosine receptor blockade, including inhibition of cyclic AMP phosphodiesterase (reviewed 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.

δ Opioid receptor subtypes (δ_1 and δ_2) regulate nociceptive transmission at the spinal level. The existence of distinct and functionally significant δ receptor subtypes at the spinal level in rats is based on the antinociceptive effect of selective δ antagonists in behavioral tests (Sofuoglu et al., 1991b; 1993; Malmberg and Yaksh, 1992; Mattia et al.,1992; Stewart and Hammond, 1993a), and by the lack of antinociceptive cross-tolerance following i.t. administration of δ subtype selective agonists in mice (Sofuoglu et al., 1991a). The present study provides additional evidence that the δ opioid receptor is important in modulating nociceptive input, as the δ_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 6-10 fold more potent following intracerebroventricular 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 δ opioid receptor subtype agonists in the carrageenan-induced model of thermal hyperalgesia in rats demonstrated that both δ_1 and δ_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 δ_2 agonist DELT at doses 0.34 - 11.5 nmol. The present study demonstrates that in rats, DELT is approximately 10 times more potent than DPDPE following the i.t. route of administration, hence the Cys^4- derivative appears to be a more potent analogue than the Glu^4 deltorphin derivative, with no motor impairment at analgesic doses.

5 SUMMARY AND CONCLUSIONS

In summary, this study has demonstrated: **(1) The opioid receptor involved in adenosine release from the rat spinal cord appears to be a μ receptor subtype** with little evidence for a selective involvement of δ or κ receptor subtypes. **(2) Inactive doses of either DELT or DPDPE act synergistically when combined with subnanomolar doses of morphine, PLO17 or DAMGO to enhance the release of adenosine from spinal cord synaptosomes**, indicating that μ/δ synergy is expressed at the synaptosomal level as well as in more integrated systems utilized in behavioral studies. This release occurs at much lower doses of opioid receptor agonists than previously reported and thus may be one of the mechanisms contributing to the phenomenon of spinal antinociceptive synergy elicited by μ and δ opioid agonists. The concomitant use of multiple drugs that act synergistically to produce the same degree of analgesia, potentially limits the side effects associated with single drug therapy. The current study provides neurochemical

evidence that supraadditive interactions exist between μ and δ opioid receptors to evoke the release of adenosine from dorsal spinal cord synaptosomes. **(3) Substance P enhances the ability of morphine to release adenosine.** The combination of substance P and morphine evokes the release of adenosine which can then act as an inhibitory neuromodulator of pain transmission. The physiological significance of this observation may correlate with an increase in opioid potency in tonic pain tests. Substance P is released from C-fibre primary afferent neurons which then can act synergistically with morphine to enhance adenosine release. Adenosine released by activation of opioid receptors may be an important component of antinociceptive effects of morphine in inflammatory pain syndromes. **(4) Behavioral experiments substantiate neurochemical data in that only the μ opioid agonists are attenuated by adenosine receptor antagonists.** Caffeine augmentation of δ opioid-induced antinociception may be a novel approach to enhancing the efficacy of δ opioid analgesics. Caffeine is an analgesic adjuvant in many non-steroidal antiinflammatory analgesic formulations. **(5) Activation of A_1 rather than A_2 adenosine receptors mediates μ opioid-induced antinociception.** The lack of attenuation, by pretreatment with selective adenosine receptor antagonists, of antinociception induced by high doses of opioids correlates with only low concentrations of opioids evoking the release of adenosine.

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. In humans, systemic administration of morphine produces analgesia

when nanomolar concentrations of morphine are achieved in the cerebrospinal fluid (Moore et al., 1984; Neumann et al., 1982). This study has demonstrated that nanomolar concentrations of μ opioid agonists release adenosine from small diameter primary afferent neurons. Thus, clinical doses of morphine may release adenosine which can then act at postsynaptic A_1 purinergic receptors to inhibit the transmission of noxious information to higher brain centers.

The mechanism by which opioids evoke the release of adenosine has yet to be determined. Released adenosine originates from capsaicin-sensitive small diameter primary afferent neurons as adenosine *per se* which then exits the cell via a dipyridamole-sensitive bidirectional carrier system. This release is Ca^{2+}-dependent and involves activation of N-type Ca^{2+} channels. It is plausible that opioid-induced adenosine release may involve stimulation of phospholipase C and IP_3 accumulation or protein kinase C activation. Studies have demonstrated that opioid-induced effects in other paradigms involve these second messenger systems, but the hypothesis that activation of protein kinase C is an important mechanism in the release of adenosine has yet to be proven.

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