AN ABSTRACT OF THE THESIS OF

Gary J. Keil II for the degree of Doctor of Philosophy in Pharmacy presented on June 12, 1995.

Title: Modulation of Sensory Afferent Processing by Endogenous Spinal Adenosine.

Abstract approved: Redacted for Privacy

Gary E. DeLander

The objective of these investigations was to continue characterization of spinal adenosine and opioid systems which modulate acute nociceptive neurotransmission. Adenosine receptor agonists interact additively with *mu* but synergistically with *delta* receptor-selective opioid agonists to induce antinociception. These data suggest *mu*, but not *delta*, opioid agonists evoke adenosine release to induce antinociception.

Although studies suggest an endogenous spinal adenosine system is present modulating sensory input, we were interested to determine if manipulations of endogenous adenosine could modulate nociceptive input, as well as effect opioid-mediated antinociception. Antinociception induced following adenosine kinase inhibition was reversed by adenosine receptor antagonists and generally interacted in an additive manner with *mu*, but synergistically with *delta*, opioid receptor-selective agonists. These results suggest adenosine kinase inhibition facilitates endogenous adenosine release which interacts with opioid agonists in a manner similar to exogenously administered adenosine agonists. Inhibition of adenosine deaminase had little effect on opioid-mediated antinociception. Because opioid-evoked adenosine release has been suggested to be

mediated by the nucleoside transport system(s) [NTS(s)], effects of opioid-mediated antinociception by NTS(s) inhibitors were investigated. NTS(s) inhibitor pretreatment decreased, while post-treatment increased, *mu* opioid receptor-mediated antinociception. NTS(s) inhibition, therefore, appears to decrease *mu* opioid receptor-mediated effects, possibly by blocking evoked adenosine release, but can also potentiate *mu* opioid receptor-mediated effects by blocking adenosine reuptake. NTS(s) inhibitors, regardless of administration timing, enhanced *delta* opioid receptor-mediated antinociception suggesting NTS(s) inhibitors increase endogenous adenosine levels which synergize with *delta* opioid receptor agonists.

Finally, these studies investigated potential nociceptive behavior following spinal adenosine receptor antagonist administration. Caudally-directed biting and scratching was observed following spinal adenosine receptor antagonist administration. Moreover, tactile allodynia was also observed following administration of spinal adenosine receptor antagonists. Inhibition of a spinal adenosine "tone" appears, therefore, to disinhibit certain excitatory neurotransmitter systems which manifests in nociceptive behaviors.

This thesis summarizes our current understanding of endogenous spinal purinergic systems regulating sensory afferent input. The results of our investigations support a significant role for adenosine in future opioid and non-opioid strategies to control pain.

Modulation of Sensory Afferent Processing by Endogenous Spinal Adenosine

by

Gary J. Keil II

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Complete June 12, 1995
Commencement June 1996

Doctor of Philosophy thesis of Gary J. Keil II presented on June 12, 1995

APPROVED:

Redacted for Privacy

Major Professor, representing Pharmacy

Redacted for Privacy

Dean of College of Pharmacy

Redacted for Privacy

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Redacted for Privacy

Gary J. Keil II, Author

DEDICATION

This thesis, my life, my love and happiness are all dedicated to my beautiful wife, Michelle. To say you're the "wind beneath my wings" wouldn't be quite correct - we've flown together, supported each other, pulled and pushed. No matter what we've done - we've done it together. We've gone from young kids to more experienced kids; we've built our friendship into love and our love into "buddiness". Without you I wouldn't be all that I am. Thank you for your love, encouragement and friendship.

All of my life is also dedicated to my beautiful daughter, Jordan Alexis. You are, indeed, from angels and are made with the perfect combination of rainbow chunks, butterfly kisses and power hugs.

ACKNOWLEDGEMENTS

I would first like to acknowledge the support and guidance of my major professor, Dr. Gary DeLander. Gary has proven wholeheartedly that true teaching goes way beyond any textbook or lab bench. He has taught me not only the finest scientific methods and ideologies, but has taught me much about life in general. His styles have made a major impact not only on my science but on my personal life as well. For all he has done over the past 5 years, I thank him. For all the stress I may have caused him, I hope I caused at least as much joy.

I would also like to acknowledge the helpful insights and suggestions from Drs. Ge Zhang, Tom Murray and Paul Franklin. Ge spent many an hour with me discussing the wonderful world of adenosine. I thank her for all her helpful information and suggestions. Dr. Murray has provided me much information on the rapidly changing field of neuropharmacology. His guidance and suggestions have provided an impetus for many parts of this thesis. Dr. Franklin survived a year of being my "surrogate" while my mentor was on sabbatical -- help with publications, experiments and life, in general, was heartily taken.

A major part of my understanding of isobolographic analyses came directly from Dr. Sandy Roerig. Her assistance (and statistical program) -- and "grilling" before my prelims -- helped set the stage for a large chunk of my thesis work.

Support in the laboratory and the pharmacy office -- namely, Mollie Roth, Marty Knittel, Carol Roberts (and the rest of the office crew) -- also was gladly taken. A big handshake also goes to Dean Richard Ovhall for all his support and encouragement.

Lastly, but of course not the least, I'd like to acknowledge the support and camaraderie of my fellow graduate students -- I can only hope I've given back a part of the awesome kinship they've given me over the last five years. Thanks, guys.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF TABLES

ABBREVIATIONS / DEFINITIONS

Purinergics

P_1 receptors	Receptors primarily activated by nucleosides (e.g., adenosine).
P_2 receptors	Receptors primarily activated by nucleotides [e.g., ATP (adenosine 5'-triphosphate) and ADP (adenosine 5'-diphosphate)].
Adenosine	Non-selective agonist at A_1 and A_2 adenosine (P_1) receptors.
CPA	A_1-selective receptor agonist.
CGS 21680	Putative A_{2a}-selective receptor agonist.
NECA	Non-selective A_1/A_2 receptor agonist.
Theophylline	Non-selective A_1/A_2 receptor antagonist.
8-*p*SPTheo	Non-selective A_1/A_2 receptor antagonist.
DPCPX	A_1-selective receptor antagonist.
5'-NH_2dAdo	Selective inhibitor of adenosine kinase.
dCF	Selective inhibitor of adenosine deaminase.
NBMPR, DPR, dilazep	Nucleoside transport system(s) [NTS(s)] inhibitors.
AK	Adenosine kinase - catalyzes the phosphorylation of adenosine to AMP.
ADA	Adenosine deaminase - catalyzes the deamination of adenosine into inosine and NH_3.
NTS(s)	Nucleoside transport system(s) - bi-directional transporters involved in influx/efflux of nucleosides (e.g., adenosine).
5'-N	5'-Nucleotidase - catalyzes the dephosphorylation of AMP into adenosine and inorganic phosphate.
SAHH	s-Adenosylhomocysteine hydrolase - bi-directionally catalyzes the interconversion of adenosine and homocysteine into s-Adenosylhomocysteine (s-AdoHcy).

ABBREVIATIONS / DEFINITIONS (Continued)

Opioids

morphine	Prototypical opioid receptor agonist, partially selective at *mu* opioid receptor types.
DAMGO PL017	*Mu*-selective opioid receptor agonists.
DPDPE/DSLET DADL/Delt II	*Delta*-selective opioid receptor agonists.
U50488	*Kappa*-selective opioid receptor agonist.

Excitatory amino acid/Tachykinins

EAA	Excitatory amino acid.
NMDA AMPA kainic acid	Selective agonists at EAA receptors.
MK-801 AP7	NMDA-selective receptor antagonists.
CNQX	AMPA-selective receptor antagonist.
kynurenate	Non-selective EAA receptor antagonist.
substance P	Neurokinin/tachykinin receptor agonist.
spantide	Neurokinin/tachykinin receptor antagonist.

MODULATION OF SENSORY AFFERENT PROCESSING BY ENDOGENOUS SPINAL ADENOSINE

CHAPTER I

INTRODUCTION

Neural systems involved in the detection and transmission of sensory information from the periphery into the central nervous system are known as the somatic senses. In general, somatic senses can be classified into two physiological systems: 1) those detecting non-noxious, "low threshold" signals; and 2) those detecting noxious or potentially harmful, "high threshold" signals (Besson and Chaouch, 1987). The general makeup of these systems are similar in that peripheral sensory information is conducted via primary afferent neurons into the dorsal horn of the spinal cord. The somatic senses differ, however, in the specific type of neuron transmitting information, sites of primary afferent synapses in the dorsal spinal cord, and neurotransmitters released from neurons. These differences enable an organism to detect, differentiate and evaluate a wide range of noxious and innocuous stimuli (Woolf, 1994). The multiple mechanisms behind the detection, transmission and evaluation of these signals provide multiple sites for pharmacologic manipulation of sensory transmission. A more complete understanding of these processes offers special insights into the physiological and pathophysiological events which occur during sensory afferent input, in addition to helping researchers develop novel strategies for therapeutic intervention.

One physiological purpose of the somatic senses is to warn an organism that damaging or potentially damaging stimuli are present. Alterations in the generation or

transmission of stimuli which serve no physiological purpose are termed "pathologic". Examples of pathologic processes include hyperalgesia, in which increases in the sensitivity or activity of nociceptive neuronal systems occur, or allodynia, in which non-noxious stimuli activate nociceptive neuronal systems (Merskey and Bogduk, 1994). Events such as chronic noxious input, inflammation or nerve injury can induce changes in the neurophysiology or neuroanatomy of normal somatic systems (reviewed, Dray *et al.*, 1994). An example of one of these changes is the central sprouting of myelinated afferents following peripheral nerve injury (Woolf *et al.*, 1992). These novel synaptic connections can lead to activation of central neuronal systems that are normally not activated in innocuous or noxious conditions. Prevention, or even reversal, of these potentially irreversible changes may depend upon a critical "window" of opportunity. Synaptic rearrangements (Woolf *et al.*, 1992; McLachlan *et al.*, 1993) or increased synaptic activities ("wind up") of central neurons following uninterrupted primary afferent input (Mendell, 1966; Dickenson and Sullivan, 1987) may be altered by early, but not late, pharmacologic intervention (Dickenson, 1994). A crucial site in the convergence and integration of sensory information, as well as pathologic changes in certain chronic pain conditions, is the dorsal horn of the spinal cord.

Sensory afferent processing and the spinal cord

Innocuous stimuli are generally conducted via large diameter, myelinated primary afferents (A_β-fibers). These fibers pass into spinal areas through the dorsal root, proceed medially past the lateral margin of the spinal cord and terminate in deep laminae (laminae III - VI) of the dorsal horn of the spinal cord (reviewed, Besson and Chaouch, 1987). Noxious stimuli are largely conducted via small diameter unmyelinated (C-fibers) or

lightly myelinated (A_δ-fibers) primary afferents, also through dorsal roots. These neural tracts branch in the dorsolateral tract of Lissauer and terminate in either superficial laminae [lamina I or laminae II/III (substantia gelatinosa)] or deeper laminae (laminae V or X) (Kerr and Wilson, 1978) of the dorsal horn. Excitation of central secondary neurons in specific laminae is, therefore, partially determined by the type(s) of primary afferents activated.

The spinal cord plays a crucial role in not only the immediate relay of sensory transmission, but in short- and long-term plasticity that accompanies certain pathological pain states. As discussed above, the dorsal horn of the spinal cord is the termination point for neurons relaying non-noxious and noxious peripheral sensory information. Additionally, polysynaptic segmental transmission (reviewed, Evans, 1989) and non-synaptic transmission (Duggan *et al.*, 1990; reviewed, Urban *et al.*, 1994) occurs at spinal sites. Multiple actions and interactions between excitatory neurotransmitter systems relay sensory information into and out of the spinal dorsal horn. As will be discussed, multiple inhibitory systems also exist at spinal sites which regulate excitatory sensory transmission. The dorsal horn, therefore, is the first central level in which somatic pathways are extensively regulated. Spinal excitatory and modulatory mechanisms have thus been termed the "gate" controlling the ultimate relay of sensory information to supraspinal sites (Melzack and Wall, 1965).

Early data by Curtis *et al.* (1959) indicated glutamate might be an excitatory neurotransmitter in the spinal cord. Recently, both small and large sensory fibers containing glutamate (and aspartate) have been demonstrated (Battaglia and Rustioni, 1988; Maxwell *et al.*, 1990; Clements *et al.*, 1991; Merighi *et al.*, 1991). It is now

appreciated that activation of either large-diameter non-nociceptive or small-diameter nociceptive primary afferents result in the release of glutamate in the dorsal horn of the spinal cord (reviewed Evans, 1989). Activation of a subpopulation of excitatory amino acid (EAA) receptors known as "non-NMDA" receptors (see below) is involved in the majority of primary afferent neurotransmission from either small or large diameter primary afferents (Dickenson and Sullivan, 1990; Evans, 1989).

Observations that up to 90% of primary afferent neurons containing glutamate also contain the peptide, substance P, (Battaglia and Rustioni, 1988; Merighi *et al.*, 1991) suggest synaptic transmission of excitatory information is mediated by both excitatory amino acids (EAAs) and neuropeptides. Differential release of excitatory amino acids and substance P, however, are seen under certain circumstances (Merighi *et al.*, 1991) demonstrating a complex interplay between excitatory neurotransmitter systems in the relay of sensory information from the periphery to the central nervous system (Wilcox, 1991; Zeiglgansberger and Tölle, 1993; Yaksh and Malmberg, 1994).

The overall complexity of excitatory neurotransmission is multiplied by recent discoveries of several additional peptides thought to mediate and modulate acute and chronic sensory afferent input. Additional excitatory neurotransmitters implicated in sensory afferent transmission include nitric oxide (NO), calcitonin gene-related peptide (CGRP), bombesin, vasoactive intestinal peptide (VIP), cholecystokinin (CCK) and somatostatin (reviewed, Yaksh and Malmberg, 1994). Potential interactions between these excitatory systems can lead to pronounced increases in the activation of central projection neurons (Woolf and Wiesenfeld-Hallin, 1986; Mjellem-Joly *et al.*, 1991).

Multiple inhibitory systems modulate excitatory neurotransmission at spinal sites. Agonists at γ-aminobutyric acid (GABA) receptors (Hwang and Wilcox, 1989; Aran and Hammond, 1991; Malcangio *et al.*, 1992) induce antinociception in certain animal models, although the clinical efficacy in humans has not been substantiated (reviewed, Dellemijn and Fields, 1994). Cannabinoid (Yaksh, 1981) and cholinergic (Yaksh *et al.*, 1985; Gordh *et al.*, 1989) receptor agonists also induce antinociception, but their clinical potential is not understood. The nucleoside, adenosine, induces antinociception in animal models and human pain states and may be involved in antinociception induced by other modulatory systems (see below). 5-hydroxytryptamine (5-HT; serotonin) also induces antinociception (see Basbaum and Fields, 1978). 5-HT, however, apparently induces antinociception via interactions with certain receptor subtypes [5-HT_{1B}, 5-HT_{1C} or 5-HT_{1s} (Murphy and Zemlan, 1990; Danzebrink and Gebhart, 1991; Alhaider and Wilcox, 1993), 5-HT_2 (Solomon and Gebhart, 1988) or 5-HT_3 (Glaum *et al.*, 1990; Alhaider *et al*, 1991)] but nociception with others [5-HT_{1A} (Solomon and Gebhart, 1988; Murphy and Zemlan, 1990)]. Two additional inhibitory systems, opioids (see below) and α-adrenergics (Reddy *et al.*, 1980; reviewed, Yaksh, 1985), have been successfully used in human pain states. While α-adrenergic agonists have clinical efficacy, long-term studies are needed to fully assess their overall therapeutic application. Opioids, on the other hand, have been used to alleviate pain and suffering as long as there has been written history. Although opioids have been utilized for many centuries and remain the "cornerstone" in the treatment of various pain states, not all pain-related phenomena are opioid-sensitive and opioids are not without limiting side effects. Alternative analgesic non-opioid analgesics or pharmacological agents which enhance opioid-mediated effects,

therefore, would significantly add to our therapeutic armamentarium in the treatment of various clinical pain states.

The role of opioids in analgesia

As mentioned previously, opioid analgesics are the "cornerstone" in the management of acute, moderate to severe pain (AHCPR, 1992) and moderate to severe pain in cancer (AHCPR, 1994). The neuropharmacological actions of opioid systems are among the best understood of the multiple endogenous systems mediating analgesia due largely to their vast usage in multiple pain states (Mansour *et al.*, 1988). In addition, development of selective receptor agonists and antagonists (Mansour *et al.*, 1988), elucidation of the distribution of the three endogenous opioid peptide precursors (Khachaturian *et al.*, 1985), and the recent cloning and expression of at least three opioid receptor gene products (Uhl *et al.*, 1994) have enabled significant advances in our understanding of the distribution of endogenous opioid systems and the mechanisms of opioid analgesic compounds. An extensive amount of literature generated recently from these studies supports the hypothesis by Kosterlitz and his colleagues (Lord *et al.*, 1977) that endogenous opioid peptides or exogenously administered opioids interact with at least three distinct membrane-bound receptor subtypes: *mu*, *delta*, and *kappa*. A fourth receptor subtype, the *epsilon* receptor, may represent an additional opioid-receptor gene subfamily (Uhl *et al.*, 1994), but additional studies are needed to prove this possibility.

Endogenous or exogenously administered opioid receptor agonists elicit analgesic effects via interactions with pertussis toxin-sensitive G-protein coupled receptors (reviewed Uhl *et al.*, 1994). In general, activation of opioid receptors is associated with an inhibition of nerve cell firing rate (postsynaptic hyperpolarization) or an inhibition of

neurotransmitter release (presynaptic inhibition). These actions appear linked to increases in specific membrane potassium conductances (for *mu* and *delta* opioid receptors) or decreases in calcium conductances (*kappa* opioid receptors) (North, 1986). In contrast to inhibitory effects commonly seen following opioid receptor activation, opioid receptor agonists have been reported to induce excitatory effects in a number of neuronal types and increased release of some neurotransmitters (Crain and Shen, 1990). These seemingly paradoxical effects of opioids, however, occur at different opioid concentrations (nM - excitatory vs. μM - inhibitory) with different sensitivities to cholera and pertussis toxins. Dual effects of opioids may explain mechanisms behind opioid-mediated inhibition of excitatory neurotransmitter release and opioid-stimulated release of inhibitory neurotransmitters.

Activation of peripheral, spinal or supraspinal opioid receptors may each contribute to opioid-induced analgesia. In the periphery, local opioid receptor agonist administration (Levine and Taiwo, 1989; Stein *et al.*, 1989; Dalsgaard *et al.*, 1994; Hong and Abbott, 1995) or inhibition of the breakdown of endogenous opioids (Maldonado *et al.*, 1994) decrease experimentally-induced pain behavior or modulate afferent fiber activities of visceral neurons (Bałkowiec *et al.*, 1994). Peripheral injection of the opioid receptor antagonist, naloxone (NLX), significantly increases paw edema following the subplantar injection of carrageenan or saline although it did not alter the behavioral correlates of pain (Planas *et al.*, 1995). These results illustrate endogenous opioids may modulate physiological responses (microvascular leakage) in inflamed tissues (Planas *et al.*, 1995) as well as nociceptive processes.

In the spinal cord, opioid receptors are found both pre- and post-synaptically to primary afferent neuron terminals (LaMotte *et al.*, 1976; Gamse *et al.*, 1979). Higher levels of opioid receptors are found dorsally than ventrally (Morris and Herz, 1987) and opioid precursor peptides are concentrated in the substantia gelatinosa (Mansour *et al.*, 1988) of the dorsal horn. *Mu* and *delta*, but not *kappa*, opioid receptor activation inhibits electrically-evoked release of SP from primary afferents (Go and Yaksh, 1987). Opioid receptor agonists also inhibit activation of wide dynamic range (WDR) neurons following noxious stimulation (Fleetwood-Walker *et al.*, 1988; Hope *et al.*, 1990). These anatomical and neurophysiological data support the hypothesis that significant modulation of sensory afferent transmission occurs by spinal opioid actions (Yaksh and Noueihed, 1985).

Multiple supraspinal loci regulate the analgesic effects of opioids as well. Opioid receptors are found in many areas of the telencephalon, diencephalon, mesencephalon and midbrain (reviewed, Mansour *et al.*, 1988; Yaksh and Malmberg, 1994) as are precursor peptides for endogenous opioids (Khachaturian *et al.*, 1985; Mansour *et al.*, 1988). Although found in many areas of the brain, significant regional heterogeneity is observed for both opioid receptors and opioid peptides. Of particular importance to this thesis is the activation of descending neuronal systems by supraspinal opioid receptor agonists. Originally outlined as a three-tiered pain control system (Basbaum and Fields, 1984), opioid receptor activation modulates neuronal activity in the periaqueductal gray (PAG), various subregions of the rostral ventral medulla [nucleus raphe magnus (NRM); nucleus reticularis magnocellularis (Rmc); and nucleus reticularis paragigantocellularis lateralis (Rpgl)], and descending bulbospinal neurons via the dorsolateral funiculus. Ultimately,

activation of these descending bulbospinal neurons release inhibitory neurotransmitters including 5-HT (Basbaum and Fields, 1984), norepinephrine (NE) and acetylcholine (ACh) (reviewed, Yaksh and Malmberg, 1994). Secondary release of inhibitory neurotransmitters from intrinsic spinal neurons by these descending systems include GABA (Basbaum and Fields, 1984) and enkephalin (Fields *et al.*, 1991). Release of inhibitory neurotransmitters such as adenosine and Neuropeptide Y from primary afferent neurons has also been suggested (reviewed, Yaksh and Malmberg, 1994).

While each of these sites are independently involved in modulating pain neurotransmission, the antinociceptive actions following the systemic administration of opioid receptor agonists may involve cooperation between these sites. Support of this hypothesis are the findings that morphine given both supraspinally (intracerebroventricularly; i.c.v.) and spinally (intrathecally; i.t.) interact in a multiplicative (i.e., supra-additive) manner (Yeung and Rudy, 1980; Roerig *et al.*, 1984; 1991; Wigdor and Wilcox, 1987; Miyamoto *et al.*, 1991).

In addition to interactions between neuroanatomical sites, opioid receptor-induced antinociception may be modulated in a supra-additive manner by other analgesic compounds. Direct i.t. administration of opioids with local anesthetics (Kaneko *et al.*, 1994), α_2-adrenergic agonists (Monasky *et al.*, 1990; Ossipov *et al.*, 1990a,b; Plummer *et al.*, 1992; Malmberg and Yaksh, 1993), the cyclooxygenase inhibitor, ketorolac (Malmberg and Yaksh, 1993), and in our laboratory adenosine, induces supra-additive antinociceptive actions.

While not conclusive, supra-additive interactions between neurotransmitter systems imply activation of independent systems to achieve a common physiological or

pharmacological effect. A number of mechanisms may underlie interactions between neurotransmitter systems including pharmacokinetic interactions, pharmacodynamic interactions and functional interactions (Solomon and Gebhart, 1994). Regardless of the mechanisms behind positive interactions between neurotransmitter systems, possible increases in efficacy or reduced dosage requirements following coadministration of compounds has profound clinical implications.

Although opioids are effectively used in the treatment of pains from multiple etiologies, opioid-refractive pain states continue to be a clinical problem. Central pain, defined by the International Association for the Study of Pain (IASP) as pain caused by a lesion or dysfunction in the central nervous system (Merskey and Bogduk, 1994), neurogenic pain or neuropathic pain are known to be opioid-refractive (Boivie, 1994). The apparent reduced efficacy of opioids in these pain states may be due to specific pathophysiologic changes (Boivie, 1994) or alterations in the neural mechanisms of opioid-mediated analgesia (Dougherty and Lenz, 1994).

Characterization of the interactions between opioid and non-opioid analgesic systems may provide alternate analgesic paradigms for increased efficacy and decreased toxicity in pain management. One system directly involved in the modulation of sensory afferent input, as well as opioid-mediated effects, is the purinergic system.

Purinergic neuronal activities

Drury and Szent-Gyorgyi (1929a,b) first reported the physiological effects of adenosine by documenting the effects of brain, myocardial, kidney and spleen extracts on cardiovascular function. Since that time, purinergic compounds including the nucleoside, adenosine, and nucleotides, adenosine 5'-triphosphate (ATP), adenosine 5'-

diphosphate (ADP), and adenosine 5'-monophosphate (AMP), have been implicated in a wide variety of physiological processes (reviewed, Cushing and Mustafa, 1991). The possibility that adenosine or ATP might be involved in synaptic transmission of sensory information was suggested by Holton and coworkers while studying antidromic vasodilation following electrical stimulation of peripheral nerves (Holton and Holton, 1954; Holton, 1959). In these studies, adenine nucleotides released from peripheral nerves were demonstrated to mediate peripheral vasodilation. Importantly, the suggestion of a similar release occurring from central termini of these neurons was also proposed. Studies demonstrating ATP acts as a synaptic mediator at primary afferent terminals were subsequently investigated by Galindo *et al.* (1967) and Fyffe and Perl (1984). Fyffe and Perl (1984) demonstrated *in vivo* iontophoretic application of ATP selectively excites proprioceptive spinal neurons. The role of ATP in non-noxious sensory neurotransmission has also been investigated by other laboratories and has recently been reviewed (Salter *et al.*, 1993). Adenine nucleotides are now known to exert multiple effects via interactions with cell surface P_2 purinoceptors (reviewed, Fredholm, *et al.*, 1994 and Zimmermann, 1994). Although not well characterized, the synaptic transmission of non-noxious stimuli by adenine nucleotides is likely mediated via interactions with these receptors.

An important relationship exists between the generally excitatory actions of purinergic nucleotides and inhibitory nucleosides. Enzymes which degrade ATP to adenosine are found at sites of ATP release, as are receptors activated by adenosine (P_1 purinoceptors, see below). Thus, extracellular metabolism of ATP may serve a dual physiological function: Termination of the excitatory effects of nucleotides and the

generation of inhibitory nucleoside metabolites (see Newby, 1984). Exercise, hypoxia and ischemia induce adenosine release as part of an "energy supply/balance" mechanism (reviewed, Bruns, 1991). Released adenosine subsequently exerts local effects decreasing any excitatory events which might further compromise tissue. Moreover, activation of specific neurons are also a source of ATP. For example, adenine nucleotides are co-stored with various neurotransmitters in central and peripheral nerves (reviewed, Westfall *et al.*, 1991). Activation of adrenergic and cholinergic nerves may induce the release of adenine nucleotides. Activation of P_2 purinoceptors, as well as P_1 purinoceptors via nucleoside metabolites, could possibly occur following activation of adrenergic and cholinergic neuron activation.

Although adenosine generated by adenine nucleotide breakdown may induce physiological effects, not all nucleotide-mediated effects are physiologically-antagonized by adenosine nor is nucleotide-derived adenosine thought to be a significant source of adenosine in the central nervous system (see Fredholm *et al.*, 1991). Direct adenosine release from nerve terminals has also been demonstrated (reviewed, Sawynok and Sweeney, 1989). This release may or may not be related to the increased stressors on neuronal systems. Indeed, inhibitory compounds, such as opioids (see below), have been shown to induce adenosine release. Thus, extracellular adenosine can be derived from a number of physiological processes. One physiological process involving adenosine release which has recently received attention is the modulation of sensory afferent input.

Role of adenosine in modulating nociceptive input

Adenosine or adenosine receptor agonists induce antinociception following systemic (Vapaatalo *et al.*, 1975; Holmgren *et al.*, 1983; Ahlijanian and Takemori,

1985), intracerebroventricular (i.c.v.) (Yarbrough and McGuffin-Clineschemidt, 1981; Mantegazza *et al.*, 1984; Herrick-Davis *et al.*, 1989) or intrathecal (i.t.) (Post, 1984; Holmgren *et al.*, 1986; Sawynok *et al.*, 1986; DeLander and Hopkins 1986, 1987a,b) routes of administration. Systemically, adenosine receptor agonists inhibit nociceptive behaviors in traditional animal models of pain including the tail flick (Ahlijanian and Takemori, 1985; Holmgren *et al.*, 1983, 1986; Woolfolk and Holtzman, 1993), hot plate (Vapaatalo *et al.*, 1975; Crawley *et al.*, 1981; Holmgren *et al.*, 1986), and acetic acid stretching (Ahlijanian and Takemori, 1985; Herrick-Davis *et al.*, 1989) assays. Additionally, systemic adenosine receptor agonists induce antinociception in less traditional nociceptive assays. The local hyperalgesic effect of subcutaneous formalin (but see below) (Karlsten *et al.*, 1992), mechanical hyperalgesia induced in streptozotocin-diabetic animals (Ahlgren and Levine, 1993), and supraspinally integrated nociceptive threshold in awake rats (Paalzow, 1994) are all inhibited following systemic adenosine receptor agonist administration. Recently, a report in human patients with neuropathic pain (Sollevi *et al.*, 1995) demonstrated almost complete abolition of tactile allodynia and spontaneous pain following intravenous adenosine infusion.

I.c.v. or supraspinally administered adenosine receptor agonists have also been reported to induce antinociception, although the effects following this route of administration are less clear. Antinociception is seen in the hot plate (Yarbrough and McGuffin-Clineschmidt, 1981; Contrearas *et al.*, 1990) and the acetic acid stretching assay (Herrick-Davis *et al.*, 1989) in mice. No antinociception is seen in either the mouse (Yarbrough and McGuffin-Clineschmidt, 1981) or rat (Mantegazza *et al.*, 1984; Holmgren *et al.*, 1986) tail flick assays, however.

The primary site of adenosine-induced antinociception is thought to be spinal. Adenosine analogs (i.t.) induce antinociception in thermal nociceptive tests (i.e., tail flick, tail immersion and hot plate tests) in mice (Post, 1984; DeLander and Hopkins, 1986, 1987a,b; Karlsten *et al.*, 1991) and rats (Juna, 1984; Holmgren *et al.*, 1986; Sosnowski *et al.*, 1989; Fastbom *et al.*, 1990; Karlsten *et al.*, 1990). Significant inhibition of acetic acid-induced stretches (Sosnowski *et al.*, 1989) or caudally-directed biting and scratching behavior induced by i.t. EAAs or SP (DeLander and Wahl, 1988; but see Hunskaar *et al.*, 1986) or capsaicin (Hunskaar *et al.*, 1986) is seen following spinal administration of adenosine analogs. In addition, spinally administered adenosine analogs induce antinociception in less "traditional" models of nociception including the second phase of the formalin test (Malmberg and Yaksh, 1993), thermal hyperalgesia following nerve compression (Yamamoto and Yaksh, 1991), and strychnine-(Sosnowski and Yaksh, 1989; Sosnowski *et al.*, 1989) or prostaglandin $F_{2\alpha}$-(Minami *et al.*, 1992a,b) induced allodynia. As will be discussed below, spinal adenosine release is also involved in the actions of other antinociceptive agents. Spinal adenosine-mediated inhibition of nociceptive neurotransmission may also be involved in non-pharmacologically-induced antinociception (Salter and Henry, 1987). Spinal adenosine receptor activation, therefore, is considered a potentially significant mechanism to inhibit nociceptive processes with wide-ranging etiologies.

Adenosine-induced actions are mediated via P_1 purinoceptors (reviewed, Fredholm *et al.*, 1994). Multiple types of P_1 purinoceptors were suggested following observations that adenosine receptor agonists induce inhibitory and excitatory effects (Van Calker *et al.*, 1979). Moreover, rank order potencies of selective adenosine receptor agonists were

different depending on the tissue and assay system utilized (reviewed, Burnstock, 1991). Two classes of P_1 purinoceptors, A_1 and A_2, were initially proposed by van Calker *et al.* (1979), but four P_1 purinoceptors are currently proposed (Fredholm *et al.*, 1994). In the spinal cord, both A_1 and A_2 receptors have been demonstrated using binding techniques (Murray and Cheney, 1982; Geiger *et al.*, 1984; Bruns *et al.*, 1986; Choca *et al.*, 1987, 1988) or autoradiography (Goodman and Synder, 1982; Lee and Reddington, 1986; Choca *et al.*, 1988). Highest levels of agonist binding for both A_1 and A_2 receptors are found in the substantia gelatinosa, moderate levels in lamina X, and diffuse non-laminar binding throughout other areas of the dorsal and ventral horns. Because unilateral dorsal rhizotomy, hemitransection and complete transection of the spinal cord fail to alter radiolabeled agonist binding, while unilateral microinjections of kainic acid decrease binding approximately 40%, receptors found in the substantia gelatinosa are thought to be predominantly located on intrinsic neurons (Choca *et al.*, 1988). High levels of A_1 receptor mRNA are found in dorsal laminae (Reppert *et al.*, 1991) indicating A_1 adenosine receptors are synthesized in cell soma of dorsal horn neurons. The presence of adenosine receptors in the superficial dorsal horn support involvement of adenosine in the modulation of sensory afferent input.

Potential limiting side effects of adenosine analogs

The potential clinical utility of adenosine analogs administered systemically, spinally or supraspinally adenosine analogs may be hampered by side effects. Although systemic adenosine induces antinociception (discussed above), pro-nociceptive or algogenic effects also occur. In conscious human volunteers, adenosine induces a variety of pain reactions (reviewed, Sollevi, 1991). In animal models, peripheral adenosine

induces nociceptive behaviors (Collier *et al.*, 1966) and induces hyperalgesia directly (Taiwo and Levine, 1990, 1991). Subcutaneous adenosine may also enhance the algesic effect of formalin (Karlsten *et al.*, 1992). Activity of unmyelinated afferents measured electrophysiologically (Moteiro and Ribeiro, 1987; Runold *et al.*, 1987) are also increased by peripheral adenosine, demonstrating neurophysiological correlates to behavioral studies.

Spinally, adenosine receptor agonists generally do not induce nociception or hyperalgesia (reviewed, Sawynok and Sweeney, 1989), nor is chronic administration associated with neuropathological changes at the electron microscopic level (Karlsten *et al.*, 1993). A recent study by Behbehani and Dollberg-Stolik (1994), however, has demonstrated adenosine receptor agonists increase the duration of spinal dorsal horn neuronal responses to noxious stimulation in rats following partial sciatic nerve ligation (Behbehani and Dollberg-Stolik, 1994). Adenosine receptor agonists did not effect the basal activity or receptive field sizes of control or nerve ligated rats in the absence of stimulation, however. More studies will be required to verify and clarify these results, but they indicate the physiological responses to pharmacologic agents may be altered in certain disease states. Significant motor effects including hind limb flaccidity or paralysis, however, have been reported by multiple laboratories (reviewed, Sawynok and Sweeney, 1989) following spinal administration of adenosine receptor agonists. These motor effects may be associated with the actions of adenosine analogs on ventral motor nuclei, as adenosine receptor agonists hyperpolarize ventral as well as dorsal spinal roots (Phillis and Kirkpatrick, 1978). Although some investigators have suggested motor effects may partially explain antinociceptive actions of adenosine receptor agonists

(Herrick-Davis *et al.*, 1988), others have shown distinct pharmacological mechanisms for antinociception and motor effects (DeLander and Hopkins, 1987a; Karlsten *et al.*, 1990).

Lastly, adenosine analogs induce a wide spectrum of central side effects which may lessen their clinical acceptance. Adenosine receptor agonists induce sedation and hypothermia, effect food intake and taste perception, and effect cognitive behavior (reviewed, Barraco, 1991). Because adenosine has heterogeneous effects in physiological processes, strategies for selectively activating adenosine receptors resulting in therapeutic effects without concurrent unwanted side effects, would be desirable. Characterization of the relative interdependence and independence of adenosine and other antinociceptive systems might allow for combination therapies in which result in supra-additive antinociceptive effects.

Spinal adenosine involvement in opioid, noradrenergic and serotonergic antinociceptive systems

Opioids

Original observations by Ho *et al.* (1972; 1973) that methylxanthines inhibit opioid-mediated antinociception were the first to demonstrate an "adenosine link" in the analgesic actions of opioids. Because opioids were known to exert some of their actions via inhibition of the formation of cyclic 3',5'-adenosine monophosphate (cAMP) (Chou *et al.*, 1971), the antagonistic actions of methylxanthines were hypothesized to occur via inhibition of the enzyme responsible for degrading cAMP into 5'-adenosine monophosphate and inorganic phosphate [phosphodiesterase (PDE)] (Sutherland and Rall, 1958; Butcher and Sutherland, 1962). Also supporting their hypothesis were observations in the same studies (Ho *et al.*, 1972; 1973) that exogenous cAMP inhibited

both opioid-induced antinociception and the development of opioid tolerance. Recent experiments with non-xanthine, selective PDE inhibitors (Nicholson *et al.*, 1991a,b) further support opioid-induced inhibition of cAMP production as one mechanism of opioid-mediated antinociception. Observations that the concentrations of methylxanthines required to antagonize adenosine receptors are much lower than those needed for PDE inhibition (reviewed, Sawynok and Yaksh, 1993), however, suggested methylxanthine-mediated inhibition of opioid-induced antinociception might involve antagonism of adenosine receptors.

Much evidence now exists supporting evoked adenosine release as an integral mechanism of certain opioid-induced effects. Systemic administration of the adenosine receptor antagonist, aminophylline, blocks the antinociceptive actions of systemic (Jurna, 1981) or i.t. morphine (Jurna, 1984). Intrathecal adenosine receptor antagonists block antinociception by systemic (Jurna, 1984), intrathecal (DeLander and Hopkins, 1986; Sweeney *et al.*, 1987b; DeLander *et al.*, 1992) or supraspinal (DeLander and Hopkins, 1986; DeLander and Wahl, 1989; Sawynok *et al.*, 1991; Sweeney *et al.*, 1991) opioids. These studies indicate opioids administered by any route evoke the release of spinal adenosine as one mechanism of antinociception. Moreover, inhibition of spinal nucleoside transport system(s) significantly potentiates supraspinal opioid-mediated antinociception (DeLander and Hopkins, 1987a), indicating manipulations which alter spinal adenosine clearance can also effect opioid-induced antinociception.

Numerous biochemical studies support behavioral results suggesting opioids evoke adenosine release. Spinal adenosine release has been quantitated *in vivo* following supraspinally- (Sweeney *et al.*, 1991) or spinally-administered opioids (Sweeney *et al.*,

1987a; 1988). Opioid-evoked adenosine release is apparently mediated via a nucleoside transporter (Sweeney *et al.*, 1993), is linked to activation of ω-conotoxin-sensitive N-type Ca^{+2} channels (Cahill *et al.*, 1993) and occurs from small-diameter primary afferent nerve terminals (Sweeney *et al.*, 1989). Interestingly, opioids do not have significant motor side effects (Yaksh and Noueihed, 1985). *In vitro*, opioids have been shown to induce adenosine release from dorsal but not ventral horn synaptosomes (Sweeney *et al.*, 1987a; 1989; 1993; Cahill *et al.*, 1993). Sawynok and Sweeney (1989) hypothesize the lack of motor effects by opioids is due to the absence of adenosine release from ventral spinal neurons. Selective dorsal release of adenosine, therefore, might prove to be a powerful alternative strategy in pain management (see below).

The involvement of spinal adenosine is apparently not uniform for all opioid receptor agonists. *In vivo*, i.t. theophylline differentially inhibits antinociception induced by i.t. or i.c.v. administered opioid receptor agonists depending on the opioid receptor subtype activated (DeLander *et al.*, 1992). *In vitro*, differential release of adenosine from spinal cord synaptosomes by selective opioid receptor agonists has also been suggested (Cahill *et al.*, 1992; Dr. Jana Sawynok - personal communication). As will be discussed, combinations of antinociceptive agents with distinct pharmacologies can interact in supra-additive fashions. Analysis of opioid systems which induce adenosine release versus adenosine-independent opioid systems will continue to provide insights toward opioid pharmacology as well as potential combination therapeutic strategies.

Recently, interactions between adenosine and opioids have been suggested to occur at the cellular level. Activation of *mu* or *delta* opioid receptors activates increases in K^+ fluxes (North, 1989) putatively via a subtype of K^+ channels known as ATP-

sensitive potassium channels (K_{ATP}) (Ocaña *et al.*, 1990; Wild *et al.*, 1991). At spinal sites, K_{ATP} channels have been isolated in the dorsal horn (Yamashita *et al.*, 1994), and Salter *et al.* (1992) report adenosine mediates an inhibitory postsynaptic potential via activation of K^+ currents through K_{ATP} channels. Based on behavioral investigations, Welch and Dunlow (1993) propose that antinociception induced by K_{ATP} channel openers and opioid receptor agonists are mediated via a final common mechanism, K_{ATP} channels.

Noradrenergics

Activation of α_2-adrenoceptors induces antinociception following systemic, spinal and supraspinal agonist administration (reviewed, Yaksh, 1985; Pertovaara, 1993). Direct spinal effects of α_2-adrenoceptor agonists include presynaptic inhibition of SP release from primary afferent nerve terminals and postsynaptic hyperpolarization of spinal dorsal horn neurones. *In vitro*, NE induces the release of a nucleotide which is subsequently converted to adenosine (Sweeney *et al.*, 1987b). The exact origin of this nucleotide is unclear. NE-evoked nucleotide release is not reduced by pretreatment by the selective neurotoxins, 5,7-dihydroxytryptamine, 6-hydroxydopamine or capsaicin, which destroy 5-HT, NE and small-diameter primary afferents, respectively (Sawynok *et al.*, 1991). Unlike morphine and 5-HT (see below), NE evokes nucleotide release *in vitro* in comparable levels from dorsal and ventral synaptosomes (Sweeney *et al.*, 1989).

The significance of adenosine in NE-induced nucleotide release is not clear because no NE-evoked adenosine is detected *in vivo* (Sweeney *et al.*, 1990). Similarly, antinociceptive actions of NE are not blocked by i.t. adenosine receptor antagonists (DeLander and Hopkins, 1987b; Sweeney *et al.*, 1987b). Finally, adenosine appears to potentiate the antinociceptive actions of NE (DeLander and Hopkins, 1987b; Aran and

Proudfit, 1990), indicating spinal adenosine and noradrenergic systems work independently from each other to inhibit nociceptive neurotransmission.

Serotonergics

As discussed above, activation of specific 5-HT receptor subtypes in the spinal cord induces either anti- or pro-nociceptive behavior following spinal administration. Also discussed is the proposal that descending bulbospinal serotonergic neurons are an endogenous pain control system (reviewed, Basbaum and Fields, 1984 and Besson and Chaouch, 1987). Similar to NE, 5-HT induces the release of a nucleotide which is subsequently converted to adenosine (Sweeney *et al.*, 1988; 1990). Unlike NE, however, adenosine receptor antagonists significantly inhibit 5-HT-mediated antinociception (DeLander and Hopkins, 1987b). Moreover, a sub-antinociceptive dose of an adenosine receptor agonist did not enhance 5-HT (DeLander and Hopkins, 1987b). Thus 5-HT induces the spinal release of a nucleotide, subsequently converted to adenosine, as one mechanism to induce antinociception. Characterization of specific spinal 5-HT receptor subtype(s) which might induce adenosine release has not been determined.

In summary, selected spinal systems modulating sensory input may cause the release of spinal adenosine in the induction of antinociception. Coactivation of these systems by adenosine receptor agonists would be expected in an additive manner. Anti-nociceptive systems acting independent of spinal adenosine release may interact supra-additively with spinal adenosine receptor agonists. Evaluation of the relative role of spinal adenosine in these systems has been largely dependent upon examining actions and interactions of exogenously-administered adenosine analogs.

Systems regulating endogenous adenosine

Although detailed experiments show exogenous administration of adenosine or adenosine receptor agonists induces antinociception, there is little conclusive evidence for the existence and involvement of an endogenous purinergic system in sensory processing. One way of investigating the possible significance of an endogenous system in physiological processes is to look at effects following manipulations of the regulatory pathways governing the system of interest.

Intra- and extra-cellular adenosine concentrations are tightly regulated (reviewed, Schrader, 1983; 1991). The activities of these regulatory systems show marked regional heterogeneity (Arch and Newsholme, 1978; Phillips and Newsholme, 1979) which are likely related to the various physiological effects of endogenous adenosine (Arch and Newsholme, 1978). In addition, these systems are dynamic. Alterations in the activities of these systems are seen developmentally (Brosh *et al.*, 1990) and in certain disease states such as spontaneous hypertension (Davies *et al.*, 1987), immunodeficiency (Seegmiller, 1985) or following axotomy (Nacimiento and Kreutzberg, 1990).

In general, these systems can be classified into systems which are involved in the generation [5'-nucleotidase (5'-N), S-Adenosylhomocysteine (S-AdoHcy) hydrolase (SAHH), and unspecific nucleotide phosphatases] or clearance of adenosine [adenosine kinase (AK), adenosine deaminase (ADA) and SAHH]. In addition to these systems, adenosine levels are controlled by the nucleoside transport systems [NTS(s)] regulating the efflux and influx of adenosine (reviewed, Plagemann and Wohlhueter, 1980). Although these systems have been extensively studied in physiological systems throughout the body, full characterization of these systems in neuronal processes is not yet complete.

Nonetheless, significant advances in our understanding of the actions and interactions of these systems in nervous tissues have been made. General summaries of these systems are presented in the following sections.

Adenosine Deaminase

Adenosine deaminase (ADA) catalyzes deamination of adenosine to the less pharmacologically active compound, inosine (reviewed, Geiger *et al.*, 1991). ADA-catalyzed effects show marked heterogeneity in humans (van der Weyden and Kelley, 1976) with highest activity in the thymus, spleen, gastrointestinal tract, pancreas and cerebellum; with moderate activity detected in the testes, appendix, liver and spinal cord (van der Weyden and Kelley, 1976; reviewed, Geiger *et al.*, 1991). Historically, ADA has been considered an intracellular enzyme although extracellular (ecto-ADA) ADA activity has been suggested (Franco *et al.*, 1986). Although highly tissue-dependent, intracellular adenosine levels are generally lower than the K_m (affinity) of ADA for adenosine (Arch and Newsholme, 1978; Phillips and Newsholme, 1979). The relative contribution of ADA in adenosine clearance is considered low under these conditions. ADA, however, displays high maximal enzymatic activity when adenosine levels are elevated (Arch and Newsholme, 1978; Phillips and Newsholme, 1979). Selective inhibitors of ADA include deoxycoformycin (dCF) (reviewed, Klohs and Kraker, 1992) and *erythro*-9-(2-hydroxy-3nonyl) adenine (EHNA) (Agarwal *et al.*, 1977). The characteristics of these inhibitors, however, vary widely. Although inhibition of ADA by dCF is virtually irreversible (Rogler-Brown *et al.*, 1978), dCF is a poor permeant of the NTS(s) (Rogler-Brown and Parks, 1980). Accumulation of dCF into tissues correlates with ADA content (Chassin *et al.*, 1979) and may explain the tissue-dependent

effects of this compound. EHNA is considered a "semi-tight" binding, reversible ADA inhibitor (Agarwal *et al.*, 1977) which permeates cells via the NTS(s) much more readily than dCF. EHNA, however, is less specific than dCF at inhibiting the various pathways involved in purine ribonucleotide synthesis (Henderson *et al.*, 1977).

Adenosine Kinase

Adenosine kinase (AK) catalyzes phosphorylation of adenosine to 5'-AMP and is thought to be primarily a cytosolic enzyme (Ho *et al.*, 1968; Shimizu *et al.*, 1972; Palella *et al.*, 1980; Yamada *et al.*, 1980). AK is considered the primary metabolic pathway for adenosine at or near normal physiological levels. Evidence for a prominent role of AK in regulating levels of endogenous adenosine are observations that the majority of exogenously administered adenosine or adenosine analogs are detected intracellularly as corresponding nucleotides (Santos *et al.*, 1968; Shimizu *et al.*, 1972; Winn *et al.*, 1980). Inhibition of adenosine kinase significantly reduces the uptake (see NTS(s) section below) of [^{3}H]adenosine (Davies and Hambley, 1986) demonstrating adenosine "trapping" in cells is adenosine kinase-dependent. Additionally, kinetic studies (Arch and Newsholme, 1978; Phillips and Newsholme, 1979) demonstrate AK is active at much lower adenosine levels than those needed for activation of additional adenosine systems (see Chapter II and General Discussion). $MgATP^{2-}$ is the substrate for AK but the overall activity of AK depends upon pH, as well as Mg^{2+} ion, adenosine, and AMP or ADP concentrations (Palella *et al.*, 1980). Selective inhibitors of AK have been synthesized and include compounds such as 5'-amino 5'-deoxyadenosine (5'-NH_2dAdo) (Miller *et al.*, 1979), and the compounds tubercidin, 5-iodotubercidin and 5'-deoxy 5-iodotubercidin (Davies, 1985). Activity of AK is effected by a number of compounds,

however, previously thought to be adenosine receptor-selective (Lin *et al.*, 1988). Because of the kinetic profile of AK, inhibition of AK would be expected to induce significant effects on multiple physiological systems. For example, effects on the cardiovascular system (Davies *et al.*, 1984; 1986) and brain (Zhang and Murray, 1991; Zhang *et al.*, 1993; Pak *et al.*, 1994) have been observed following AK inhibition.

S-Adenosylhomocysteine hydrolase

S-Adenosylhomocysteine hydrolase (SAHH) catalyzes the reversible hydrolysis of S-AdoHcy into homocysteine and adenosine. When the concentration of substrates exceeds micromolar levels, the reaction favors synthesis of S-AdoHcy (Ueland, 1982). Therefore, SAHH activity can increase or decrease adenosine formation depending on intracellular conditions. The bidirectional nature of SAHH has recently been utilized as a means to index free intracellular adenosine levels in the heart (Deussen *et al.*, 1988; Borst *et al.*, 1992) following the exogenous administration of *l*-homo-cysteine. Additionally, S-AdoHcy is the endproduct and inhibitor of S-adenosylmethionine (S-AdoMet)-dependent transmethylation reactions (reviewed, Refsum and Ueland, 1990). Inhibition of SAHH or increased formation of S-AdoHcy (Schatz *et al.*, 1981), therefore, can have significant effects on protein methylation-dependent reactions. Adenosine analogs can serve as substrates of SAHH leading to the S-AdoHcy analog, but inhibition or inactivation of SAHH is seen with adenosine analogs (Refsum and Ueland, 1990). These actions noted above underlie both the therapeutic (e.g., antiviral) and toxic effects of agents having effects on S-AdoMet metabolism. Many nucleoside analogs inhibit SAHH, including adenosine dialdehyde, neplanocin A and C-c^3 Ado (reviewed, Ueland, 1982; De Clercq, 1987).

5'-Nucleotidase

5'-Nucleotidase (5'-N), although originally thought to be only an intracellular enzyme, is both an endoenzyme (i.e., its catalytic site is exposed to the intracellular space) and an ectoenzyme (i.e., the catalytic site is exposed extracellularly). 5'-N, therefore, can hydrolyses intracellular nucleotides or those released from nearby cells (see Arch and Newsholme, 1978; Nacimiento and Kreutzberg, 1990). Basal extracellular adenosine can be derived from tonically-released nucleotides (Sweeney *et al.*, 1987b). 5'-N, therefore, may influence not only excitatory mechanisms of nucleotides, but inhibitory actions of nucleoside degradation products. Ecto-5'-N can be inhibited by α,β-methylene ADP (AOPCP) and 5'-guanosine monophosphate (Pons *et al.*, 1980; MacDonald and White, 1985).

Nucleoside transport system(s)

In addition to adenosine forming and clearance systems, extra- and intra-cellular levels of adenosine are governed by the nucleoside transport system(s) [NTS(s)]. Two general classes of transport systems are presently known. Sodium-dependent transport against cellular concentration gradients are termed "active" NTS(s), while bidirectional, sodium-independent transporters are known as "passive" (reviewed, Hertz, 1991).

Nucleoside transport by either mechanism allows nucleosides to enter the nucleotide pool or be cleared by intracellular enzymes (reviewed, Schrader, 1991). As discussed above (see Adenosine Kinase section), these nucleosides are readily phosphorylated by adenosine kinase, forming membrane-impermeable nucleotides. Importantly, phosphorylation keeps concentration gradients of extra- to intra-cellular levels of nucleosides relatively high. Transport of nucleosides and the subsequent

phosphorylation, deamination or formation of s-AdoHcy, is collectively termed "uptake". Nucleoside uptake is dependent upon, but distinct from, simple nucleoside transport via the NTS(s) (see Patterson *et al.*, 1981 for review). As the majority of nucleoside transport in the central nervous system is believed to be via the bidirectional, facilitated diffusion transport system(s) (reviewed, Plagemann and Wohlhueter, 1980 and Patterson *et al.*, 1981), alterations of secondary events (phosphorylation, deamination, etc.) would be expected to alter nucleoside uptake rates. Indeed, reduced uptake of adenine nucleosides is seen following administration of inhibitors of adenosine kinase (Davies and Hambley, 1986). This reduced uptake is theorized to be mediated by decreases in the extracellular:intracellular concentration gradient that is maintained by phosphorylation of the intracellular nucleosides.

Subtypes of equilibrative NTS(s) have been identified and can be distinguished by their differential sensitivities to inhibition by certain NTS(s) inhibitors (see references in Hammond, 1991). The so called nitrobenzylthioinosine (NBMPR)-sensitive NTS(s) are bidirectional systems sensitive to inhibition by NBMPR. Other NTS(s) inhibitors, including dipyridamole (DPR), dilazep and hexobendide, may inhibit both NBMPR-sensitive and -insensitive transporters (reviewed, Deckert *et al.*, 1988). Inhibition of these bidirectional systems can have varying effects on intra- and extra-cellular adenosine levels depending upon which process these inhibitors affect. Inhibition of adenosine efflux would be expected to *decrease*, while inhibition of adenosine uptake would *increase*, extracellular adenosine levels. In general, the pharmacological spectrum of these NTS(s) inhibitors is thought to be mediated largely via inhibition of adenosine reuptake (Newby, 1986; reviewed, Deckert *et al.*, 1988).

Spinal adenosine systems

Although much work has been done characterizing central adenosine regulatory systems *in vitro*, spinal adenosine systems are not well characterized. Results from investigations which have begun defining these systems are summarized below.

Adenosine is a ubiquitous compound. Despite this, antibodies recognizing a conjugated form of adenosine demonstrate heterogeneous staining patterns throughout the brain and spinal cord (Braas *et al.*, 1986). In the cord, immunoreactivity was highest in the substantia gelatinosa and ventral motor nuclei. Staining intensity was also found in the trigeminal nerve and trigeminal nucleus in the brainstem (the cervical correlates of primary afferent neurons and substantia gelatinosa, respectively).

Of the adenosine clearing systems, only the regional spinal distribution of only ADA has been investigated. ADA-immunoreactivity is higher in dorsal than ventral horn (Geiger and Nagy, 1986) and is present in dorsal roots and Lissauers tract, as well as the substantia gelatinosa and the trigeminal nucleus (Nagy and Daddona, 1985). Treatment with the neurotoxin capsaicin greatly reduced ADA-immunoreactive neurons in the dorsal roots and Lissauers tract and virtually eliminated the positively staining neurons in lamina IIo (but not laminae I). These studies indicate ADA is present in capsaicin-sensitive, small-diameter primary afferents as well as descending fibers, interneurons or capsaicin-insensitive myelinated fibers. In the same study (Nagy and Daddona, 1985) the presence of spinal 5'-N was also demonstrated. In agreement with a study by Scott (1967), the substantia gelatinosa is a rich source of 5'-N, and most dorsal root ganglion neurons exhibit some degree of 5'-N staining (Nagy and Daddona, 1985). Interestingly, the lowest levels of 5'-N staining were found in those neurons staining highest for ADA.

Similarly, ADA-immunoreactive neurons were among those containing the lowest density of 5'-N staining. These results indicate that neurons generating and metabolizing adenosine might be distinctly different neurophysiologically, as well as anatomically. While the regional distribution of adenosine kinase is unknown, activity of spinal adenosine kinase has been shown to be lower or similar to other brain regions (Davies and Hambley, 1986). Interplay between the adenosine clearing (ADA and AK) and adenosine forming (5'-N) systems in the spinal cord is unclear at the present time, although these systems may interact in a complex manner.

Regional binding of radiolabeled inhibitors of the NTS(s) is also found in the spinal cord. Distribution of [^{3}H]NBMPR binding is highest in the substantia gelatinosa and trigeminal nucleus (Geiger and Nagy, 1985). Neonatal pretreatment with capsaicin reduced binding in dorsal roots by 35% indicating that a large population (approximately 65%) of binding sites are on central (descending neurons or interneurons) or capsaicin-insensitive neurons. Localization of NBMPR-insensitive or Na^+-dependent, concentrative nucleoside transport systems, however, are presently unknown.

Objectives

Opioid analgesics remain the "gold standard" for treating multiple pain states. Because opioid analgesics are not overtly effective in all pain states, however, alternate analgesic therapies or agents which expand the spectrum of available analgesics is desirable. Recent characterizations of the mechanisms of opioid-mediated analgesia, and advances in our understanding of other spinal modulatory systems with opioid analgesics, have occurred. This thesis was begun to further characterize the potential significance of adenosine, and endogenous purinergic systems, in the modulation of nociceptive input

with and without coadministration of opioids. To characterize this potential role of adenosine, the following objectives were established:

> **Objective I:** To characterize interactions between spinal opioid and adenosine systems to induce antinociception.
>
> **Objective II:** To characterize if inhibitors of systems regulating endogenous adenosine levels result in antinociception. In addition, to identify if these inhibitors also effect opioid-induced antinociception via alterations of endogenous adenosine neurotransmission.
>
> **Objective III:** To investigate if inhibition of spinal purinergic "tone" facilitate nociceptive behavior.

Results from experiments conducted while addressing these objectives are divided into the following chapters:

Objective I: ***Chapter II. Analyses of interactions between opioid and adenosine agonists.***

Objective II: ***Chapter III. Effects of adenosine kinase and adenosine deaminase inhibition: Induction of antinociception and effects on adenosine- and opioid-induced antinociception.***

Chapter IV. Effects of nucleoside transport inhibition: Induction of antinociception and effects on adenosine- and opioid-induced antinociception.

Objective III: ***Chapter V. Induction of nociceptive behavior following spinal adenosine receptor antagonism.***

General methods

Animals

Male, Swiss-Webster mice (Simonsen, Gilroy, CA) weighing 20–30 g were used for all experiments. Mice were housed in groups of 5 in a temperature-controlled room with a 12 h light/dark schedule. Food and water were available *ad libitum* until time of experiments. Animals were tested in the same building as housing and were allowed to

acclimate to the testing room for at least one hour before each experiment. All studies were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee of Oregon State University (Corvallis, OR).

Assay for antinociception -- tail flick assay

Antinociception was determined using the radiant heat tail flick assay of D'Amour and Smith (1941). Control latencies were approximately 2.5 to 3 s. An 8 s cutoff time was used to prevent tissue damage. Antinociception was expressed as a function of maximum percent effect (%MPE) using the formula:

$$\%MPE = 100 \text{ X } [(\text{test - control latency})/(8 \text{ s - control latency})]$$

where test is the latency time (seconds) for mice to withdraw tails from the radiant heat source following administration of drug(s).

Assay for nociception -- caudally-directed biting and scratching behavior

Potential nociceptive effects of various agents were evaluated by quantitating caudally-directed biting and scratching behavior following intrathecal administration. Mice were injected compounds (i.t.) using modifications of the method of Hylden and Wilcox (1980) that allows administration through intact skin. Animals were placed immediately in a glass observation chamber. The number of scratches made using the hind limbs and the number of caudally-directed licks or bites were counted commencing with the animals placement into the chamber. Behaviors induced by excitatory amino acid agonists or substance P were quantitated over a standard 1 min period (see Wilcox, 1988 for details). Potential behaviors induced by other agents were quantitated until the behaviors subsided and returned to control values (normal grooming behavior).

Assay for antinociception -- inhibition of excitatory amino acid/substance P induced biting and scratching

Caudally-directed biting and scratching behavior was assessed as described above. Mice were injected (i.t.) with the excitatory amino acids, N-methyl-D-aspartate (NMDA), (*S*) α-amino-3-hydroxy-5-5-methylisoxazole-4-propionate (AMPA) or kainic acid, the tachykinin, substance P, or other putative nociceptive compounds at doses of each agent which induce between 80-100 behaviors during the 1 min observation period.

Drugs

Drugs were purchased from the following sources: Adenosine hemisulfate, theophylline (anhydrous), dilazep, kainic acid, 5'-amino 5'-deoxyadenosine (5'-NH_2dAdo), and [D-Ala^2,D-Leu^5] enkephalin (DADL) - Sigma Chemical Co. (St. Louis, MO); morphine sulfate (M.S.) - Mallinckrodt Chemical Works (St. Louis, MO); [D-Ala^2,N-MePhe^4,Gly-ol] enkephalin (DAMGO) and spantide - Bachem California (Torrance, CA); [D-Pen^2-D-Pen^5] enkephalin (DPDPE) and substance P - Cambridge Research Biochemicals, Wilmington, DE; [D-Ala^2] deltorphin II (Delt II) and [D-Ser^2]-Leucine enkephalin-Thr (DSLET) - Bachem Bioscience Inc., Philadelphia, PA; trans-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]benzene-acetamidemethanesulfonate hydrate (U50,488), *erythro*-9-(2-hydroxy-3nonyl) adenine (EHNA), 2-hydroxypropyl β-cyclodextrin (HPCD), dipyridamole (DPR), s-(4-nitrobenzyl)-6-thioinosine (NBMPR), (DPCPX), N^6-cyclopentyl adenosine (CPA), 2-*p*-(2-Carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine hydrochloride (CGS 21680) and 8-*p*-Sulfophenyl theophylline (8*p*SPTheo) - Research Biochemicals Inc., Natick, MA; kynurenate and N-methyl-D-aspartic acid (NMDA) - Aldrich Chemical Co. (Milwaukee, WI); 5'-(N-ethyl)-

carboxamido adenosine (NECA) - Boehringer Mannheim (Mannheim, W. Germany); AMPA and CNQX - Tocris Neuramin (St. Bristol, UK); (PLO17) - Peninsula Laboratories Inc. (San Carlos, CA). Deoxycoformycin (dCF) was a generous gift from Parke Davis Pharmaceutical Research Division (Warner-Lambert Co. Ann Arbor, MI), MK-801 from Merck, Sharp and Dohme (B. Clineschmidt, West Point, PA) and 2-amino-7-phosphonoheptanoic acid (AP7) - Searle Research and Development (P. Contreras, St. Louis, MO). All dosages are expressed as the salt form.

CHAPTER II

ANALYSES OF INTERACTIONS BETWEEN OPIOID AND ADENOSINE AGONISTS

Abstract

Intrathecal administration of adenosine or adenosine analogs induces antinociception. In addition, opioid-induced antinociception is mediated, in part, by spinal adenosine release. Recent investigations from our laboratory suggest the significance of adenosine in opioid-mediated actions varies between different pharmacologic effects of opioid agonists and may vary after selective activation of different opioid receptors. A series of investigations using isobolographic analysis was designed to examine the functional significance of adenosine in antinociception induced by opioid receptor-selective agonists in the mouse tail-flick assay. Combinations of A_1 or A_2 adenosine receptor-selective agonists were coadministered in a constant dose ratio with *mu*, *delta* or *kappa* opioid receptor-selective agonist. Additive interactions were observed for adenosine agonists coadministered with *mu* opioid receptor selective agonists. Synergism was generally observed after coadministration of adenosine receptor agonists with agonists selective for either $delta_1$ or $delta_2$ opioid receptors. A synergistic interaction was also observed after coadministration of an A_1 adenosine receptor agonist with a *kappa* opioid receptor agonist. Observations reported with *mu* opioid receptor selective agonists are consistent with earlier reports demonstrating opioid-mediated adenosine release as one mechanism os opioid-induced antinociception. Results with combinations of adenosine agonists and *delta* or *kappa* opioid receptor agonists suggest a more complex functional interaction between spinal adenosine and *delta* or *kappa* opioid systems.

Introduction

Involvement of spinal adenosine as a mediator of opioid actions is demonstrated by inhibition of opioid-induced antinociception by adenosine receptor antagonists (DeLander and Hopkins, 1986; Sweeney *et al.*, 1987a; DeLander and Wahl, 1989; Sawynok *et al.*, 1991). In addition, opioid-stimulated release of adenosine is reported *in vitro* (Sweeney *et al.*, 1987a, 1989) and *in vivo* (Sweeney *et al.*, 1987b; 1991) from spinal sites. Adenosine involvement in antinociception at spinal sites is also supported by adenosine and adenosine agonist (i.t.)-mediated inhibition of responses to noxious stimuli in tail-flick, hot-plate, stretching and substance P or excitatory amino acid assays (see Sawynok and Sweeney, 1989 for review).

Recent studies suggest adenosine involvement in opioid-induced antinociception does not extend to all opioid-mediated actions (DeLander *et al.*, 1992) Similarly, the significance of adenosine in opioid-induced antinociception may vary depending upon which opioid receptor(s) is activated. Coadministration of methylxanthines (i.t.) with morphine (i.t. or i.c.v.) typically shifts dose-response curves for morphine to the right, but methylxanthine-mediated antagonism of various selective opioid receptor agonists yields results that are qualitatively different. Recent investigations reveal interactions of theophylline with *mu* selective opioid receptor agonists result in a parallel shift of dose-response curves, whereas interactions of theophylline with *delta* opioid receptor selective ligands show nonparallel shifts of dose-response curves (DeLander *et al.*, 1992). These results suggest adenosine/opioid interactions are not homogeneous and prompted our laboratory to investigate more specifically the nature of adenosine interactions with opioid receptor-selective agonists. Preliminary investigations by Cahill *et al.* (1992) suggest

opioid-evoked adenosine release be heterogenous and might occur with some, but not all, opioid receptor agonists.

In the present investigations, we found heterogenous interactions between adenosine receptor agonists and *mu*, *delta* and *kappa* selective opioid receptor agonists. Coadministration of adenosine receptor agonists with *mu* opioid receptor selective agonists resulted in additive interactions, whereas interactions between adenosine receptor agonists and *delta* or *kappa* opioid receptor-selective agonists were multiplicative. These observations are important in discerning the functional significance of adenosine as a mediator of opioid actions induced via interactions at specific opioid receptors.

Materials and methods

Assay for antinociception

Antinociception was determined using the tail flick assay of D'Amour and Smith (1941) as described (see General Methods).

Experimental protocol

Opioid or adenosine agonists were administered i.t. alone initially to determine ED_{50} values for antinociception and time to peak effect. All agonists were found to reach peak effect 10 min after i.t. injection. Subsequent experiments involved coadministration (i.t.) of selective opioid receptor agonists with selective adenosine receptor agonists. Dose-response curves for constant dose ratios of selective opioid and adenosine receptor agonists were determined int the tail flick assay 10 min after coinjection (i.t.). Constant dose ratios were normalized based upon a comparison of ED_{50} values for each drug administered alone, such that a 1:1 constant dose ratio represented coadministration of

two agonists in a ratio proportional to their respective ED_{50} values. Antinociception induced by an "ED_{50} DOSE" of each agonist to be tested was confirmed each day before beginning experiments involving coadministration of agonists. As noted under "Results," apparent seasonal variation made it necessary to adjust the ED_{50} dose for CPA.

Analysis of interactions

Interactions between agonists were evaluated using isobolographic methods (Lowe, 1953, 1957). Isobolograms were constructed by plotting ED_{50} value of adenosine agonists on the abscissae and ED_{50} value for opioid agonists on the ordinates. The line connecting these two points represented the theoretical additive line for all dose ratio combinations of agonists. The dose-response curve resulting from coadministration of agonists in a 1:1 constant dose ratio was used to determine the total combination dose required to produce 50% MPE. Individual ED_{50} values for each agonist were resolved from the combination dose required to cause 50% MPE and were plotted on the isobologram as the experimental combination dose. A 1:1 dose ratio, therefore, represents the administration of equieffective doses for each agonist proportional to the ED_{50} value. For example, as show in Fig. II-1B, the individual ED_{50} values for antinociception induced by morphine sulfate (102 pmol) and CGS 21680 (5080 pmol) are plotted on their respective axes. Morphine sulfate was approximately 50-fold more potent than CGS 21680. Combination doses of 20/2500, 25/1250 and 12.5/625 pmol for morphine sulfate and CGS 21680, respectively, were administered to groups of mice to determine an ED_{50} value for the combination. The experimental ED_{50} value for the combination yielded individual ED_{50} values of 43.6 and 2180 pmol for morphine sulfate and CGS 21680, respectively, and were plotted on the respective axes.

Experimental points were compared to theoretical additive values as defined by the theoretical additive line. Total fraction values (see Table II-1) are useful in predicting the nature of agonist interactions. Total fraction values are determined by comparing experimental dose combinations to dose combinations predicted theoretically to add together to cause a specific level of effect. Briefly, additive dose combinations of agonists are defined by the theoretical additive line according to the following equation: $Z_1/Z_1^* + Z_2/Z_2^* = 1$. Z_1 and Z_2 are the dose of dug 1 and 2, respectively, administered in combination; Z_1^* and Z_2^* are the doses of drug 1 and 2 respectively, required to elicit a specific effect (e.g. ED_{50} values) when administered separately. Using the example above, ED_{50} values for morphine sulfate (102 pmol) and CGS 21680 (5080 pmol) are Z_1^* and Z_2^*, respectively. If the values Z_1^* and Z_2^* are assigned relative potency values of 1 ("normalized"), the resulting combination ED_{50} values (Z_1 and Z_2) can be expressed as fractions of Z_1^* and Z_2^*. Using the combination doses above, the fraction for morphine sulfate is 43.6/102 = .43 and 2180/5080 = .43 for CGS 21680. Addition of the two fractions gives a total fraction value of 0.86 (see Table II-1). Total fractions values less than 1 imply a multiplicative interaction because smaller doses of each drug were required to cause a specific degree of effect than was predicted by a purely additive interaction. Experimentally, total fractions values of 0.5 or less commonly correspond to a statistically significant multiplicative interaction.

Statistical analysis to show whether experimental points are significantly different from theoretical additive values has ben explored by several investigators. We used methods of analysis described by Tallarida *et al.* (1989) and Roerig *et al.* (1988) in determining the nature of agonist interactions. Both of these methods have been

rigorously tested [Roerig and Fujimoto (1989); Roerig *et al.* (1991); Tallarida (1992)] and, as discussed by Roerig *et al.* (1991), we find that both methods commonly reach similar conclusions. One significant difference between these methods of analysis is that the method described by Roerig *et al.* (1988) required parallel dose-response curves for analysis of drug interactions, whereas the method of Tallarida *et al.* (1989, pp. 955-956) allows for analysis when dose-response curves are not parallel. The method of Tallarida *et al.* (1989) was used as our primary statistical testing method because some pairs of agonists had nonparallel dose-response curves (see under "Results"). The method of Roerig *et al.* (1988) was used to confirm analyses for those pairs of agonists that had parallel dose-response curves. Experimental points were considered to be significantly different from additivity if the total fraction was significantly less than 1 and antagonistic if the total fraction was significantly greater the 1. Interactions were considered different from a theoretical additive interactions at a P value $< .05$.

The experimental point of each figure is plotted with its 95% CI. It is important to note, however, that statistical significance is based on variance [S.E.M. for the method described by Tallarida *et al.* (1989) and fiducial limits for the method described by Roerig *et al.* (1988)]. Although variance for each method is directly related to CI, a simple visual inspection of isobolograms may sometimes imply conclusions that are not consistent with actual statistical analyses.

Drugs and drug administration

Drugs were administered using the method of Hylden and Wilcox (1980) with modifications that allow direct injections through intact skin. All drugs were prepared in saline with the exception of CGS 21680 which was prepared in 0.1 N NaOH. CGS

21680 vehicle had no effect on control tail-flick latencies of antinociception induced by opioid agonists. The combination of CGS 21680 and U50,488 could not be tested, however, because the vehicle caused precipitation of U50,488 from solution. Drug solutions were prepared to the total injection volume was 5 μl.

Results

Interactions of adenosine agonists with mu opioid receptor-selective agonists

Morphine has been used widely as a *mu* opioid receptor ligand, but was selected for our initial investigation because of its potential to interact with multiple types of opioid receptors. Dose-response curves resulting from coadministration of a normalized 1:1 constant dose ratio of morphine with CPA, an A_1 adenosine receptor-selective agonist, or CGS 21680, an A_2 adenosine receptor-selective agonist, were determined. Isobolographic analysis of morphine with CPA (Fig. II-1A) indicated a multiplicative interaction as determined by the method of Tallarida *et al.* (1989), but an additive interaction as determined by the method of Roerig *et al.* (1988). The interaction of morphine with CGS 21680 (Fig. II-1B) was additive by both methods of analysis. As noted (Materials and Methods), analysis of interactions by the method of Roerig *et al.* (1988) requires that dose-response curves be parallel and was not appropriate for all drug combinations used.

Multiplicative interactions were not observed when either CPA (Fig. II-2A) or CGS 21680 (Fig. II-2B) were coadministered (i.t.) with DAMGO, a *mu* opioid receptor-

Fig. II-1. Isobolograms for antinociception induced by coadministration of adenosine agonists with morphine i.t. Antinociception was determined after coadministration (i.t.) of morphine and CPA (A) or CGS 21680 (CGS) (B) in a 1:1 constant dose ratio. The solid line connects the individual ED_{50} values for adenosine agonist and morphine sulfate injected i.t. alone and represents the theoretical additive interaction between agonists. Experimental ED_{50} values for each agonist when combinations were administered i.t. are indicated by the closed circles (●). The 95% CI for the experimental point is shown as a line extending toward the theoretical additive point for the 1:1 dose ratio. * Values significantly different from theoretical additive values ($P < .05$) indicating a synergistic interaction.

Fig. II-1.

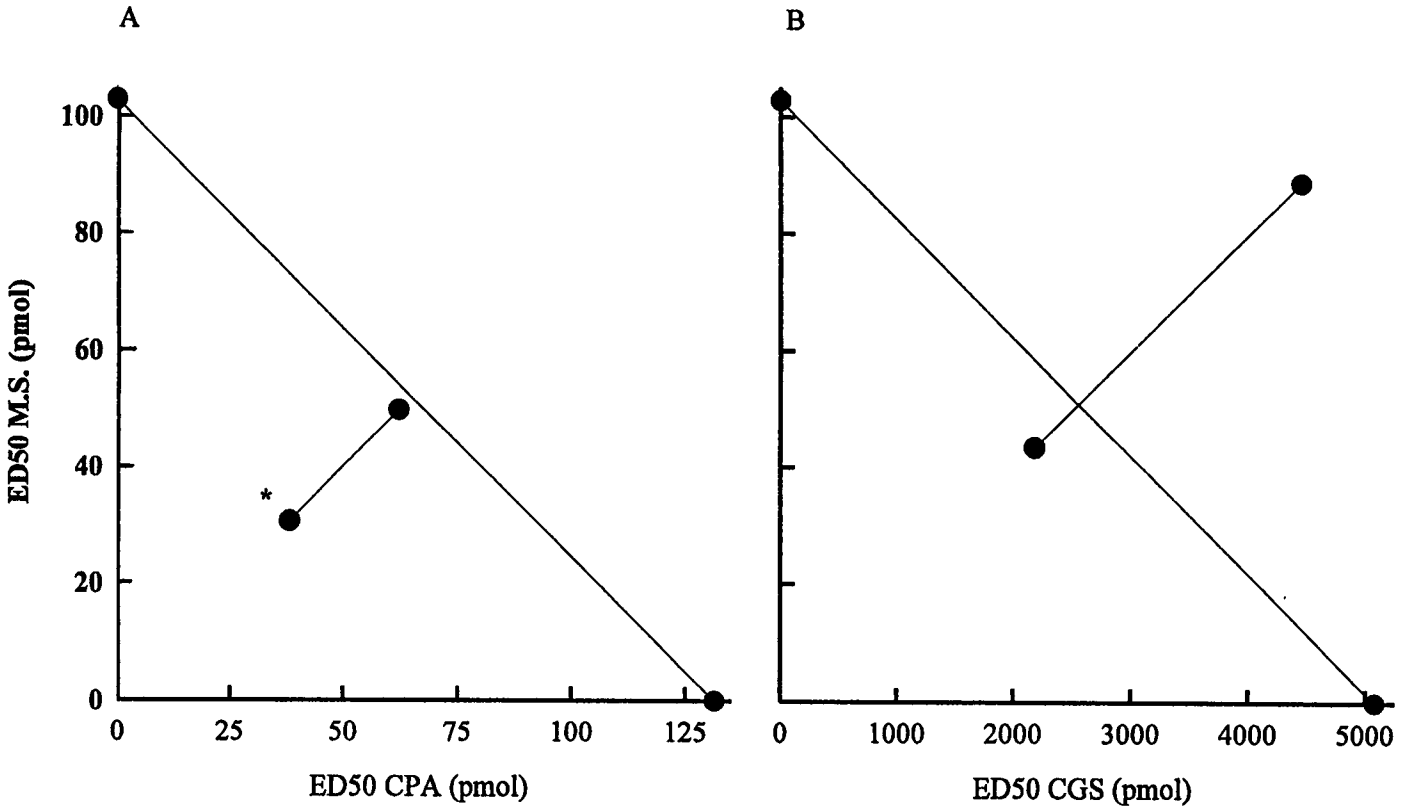
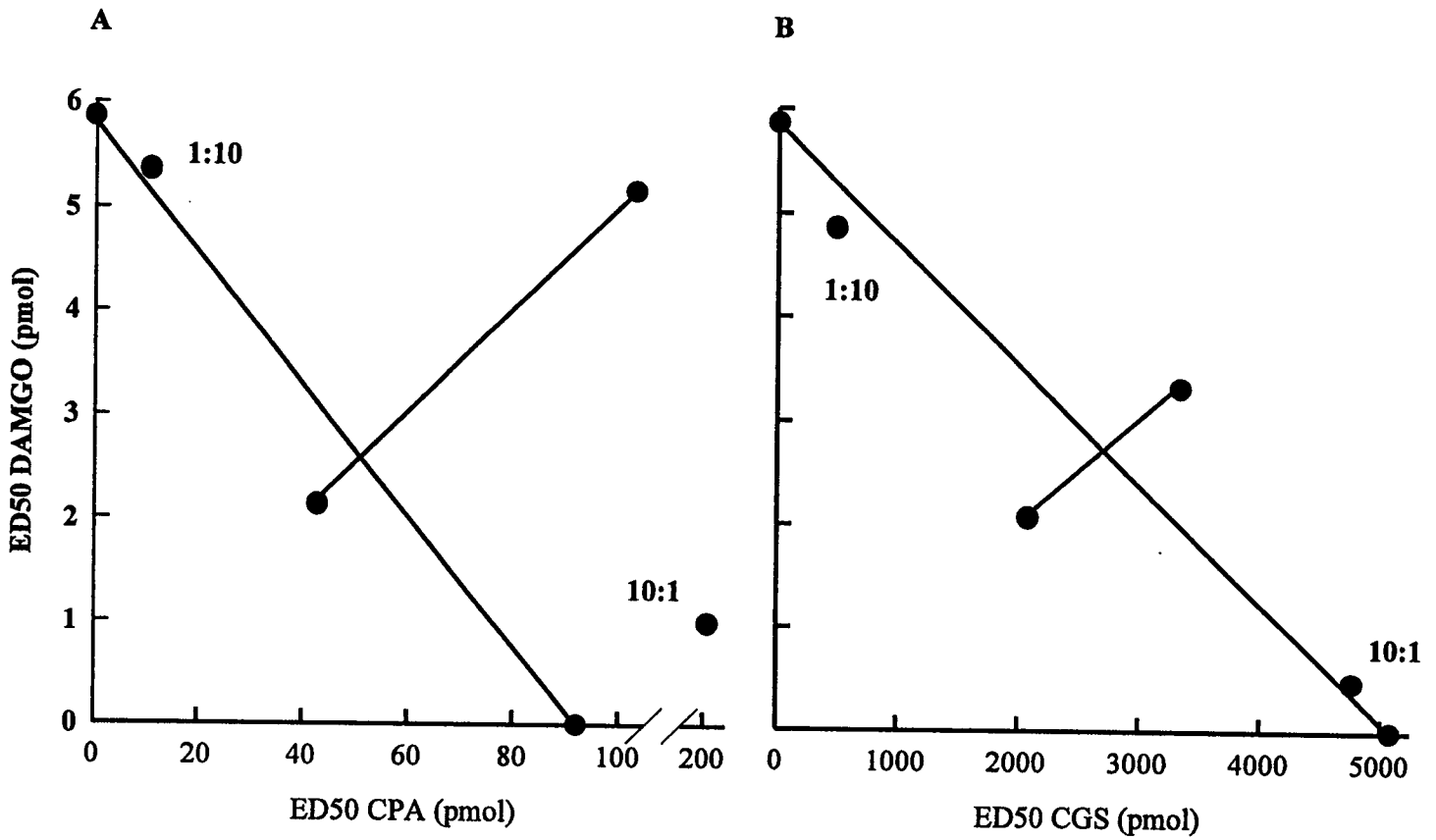

A
B
100
80
60
40
20
0
ED50 M.S. (pmol)
*
0
25
50
75
100
125
ED50 CPA (pmol)
0
1000
2000
3000
4000
5000
ED50 CGS (pmol)

Fig. II-2. Isobolograms for antinociception induced by coadministration of adenosine agonists with DAMGO i.t. Administration was determined after coadministration (i.t.) of DAMGO and CPA (A) or CGS 21680 (CGS) (B) in 1:1, 1:10 and 10:1 constant dose ratios. The solid line connects the individual ED_{50} values for adenosine agonist and DAMGO injected i.t. alone and represents the theoretical additive interaction between agonists. Experimental ED_{50} values for each agonist when combinations were administered i.t. are indicated by the closed circles (●). The 95% CI for the experimental point is shown as a line extending toward the theoretical additive point for the 1:1 dose ratio; confidence intervals for the 1:10 and 10:1 ratios are not shown for clarity.

Fig. II-2.


A
B
ED50 DAMGO (pmol)
1:10
10:1
0
1
2
3
4
5
6
0
20
40
60
80
100
200
ED50 CPA (pmol)
1:10
10:1
0
1000
2000
3000
4000
5000
ED50 CGS (pmol)

selective agonist, in a constant dose ratio of 1:1. Although a constant dose ratio of 1:1 was assumed to be most likely to reveal synergistic interactions, two additional constant-dose ratios (1:10 and 10:1) were investigated (Fig. II-2). Each experiment failed to reveal a significant degree of synergism. Similarly, interactions observed after adenosine agonist coadministration (i.t.) with a second *mu* opioid receptor-selective agonist, PLO17, in a 1:1 constant dose ratio did not reveal synergism (Fig. II-3).

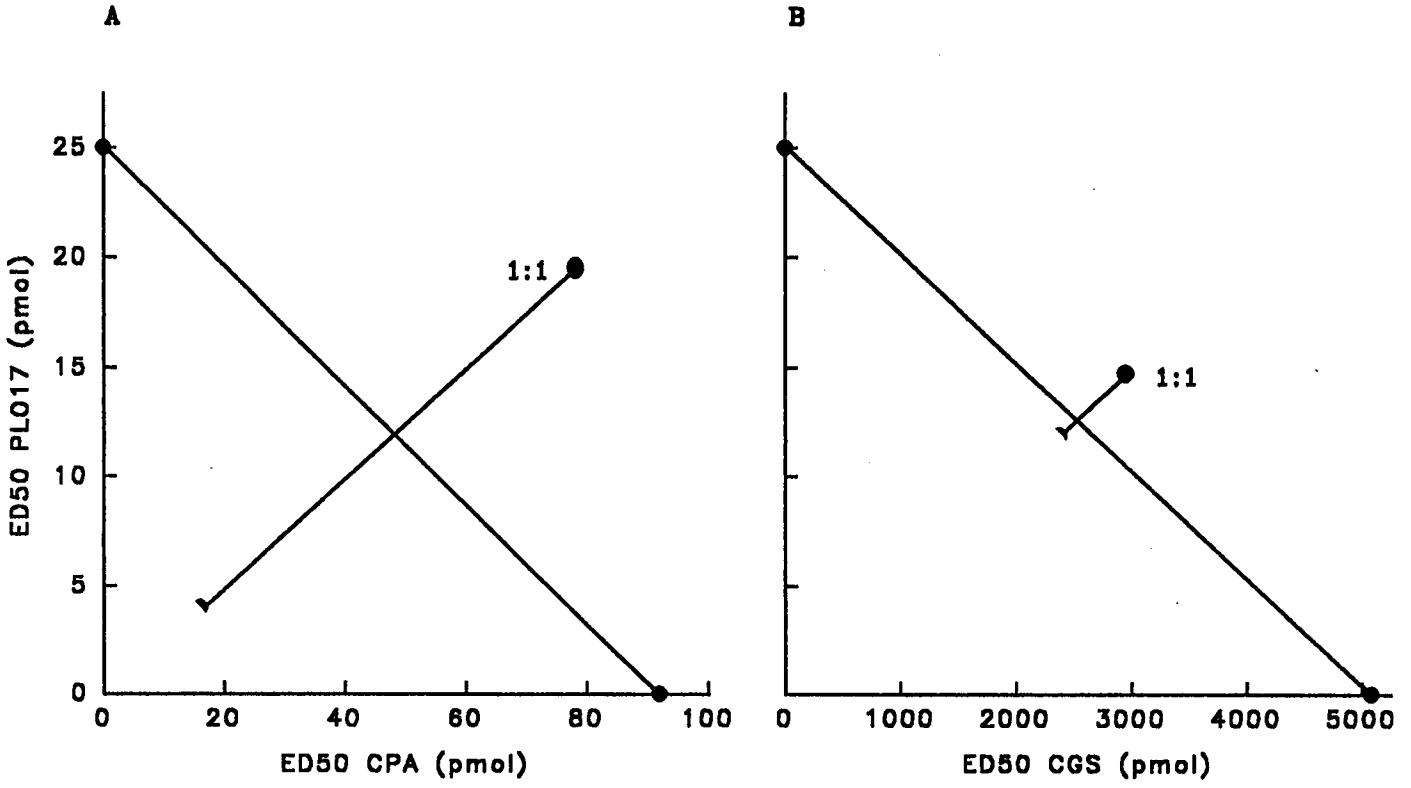
Experiments using DAMGO and PLO17 were conducted several months before the remaining investigations described below and there was apparently a seasonal variation in the control ED_{50} value for CPA over this time period. Therefore, experiments involving CPA with DAMGO or PLO17 are based on an ED_{50} value for CPA of 92 pmol (95% CI, 53-160 pmol) and all subsequent experiments are based on an ED_{50} value for CPA of 131 pmol. Interactions of CPA with DAMGO were re-examined based on the new ED_{50} value for CPA to ensure our interpretation of the interaction was not changed. Isobolographic analysis of DAMGO and old (92 pmol) or new (131 pmol) CPA ED_{50} values, in a 1:1 constant dose ratio, each showed additive interactions (total fractions 0.83 and 0.82, respectively). the nature of interactions between adenosine and *mu* opioid receptor agonists was independent of the method of statistical analysis used.

Interactions of adenosine agonists with delta opioid receptor-selective agonists

Previous investigations suggest the existence of multiple *delta* opioid receptor subtypes (Jiang *et al.*, 1991; Sofuoglu *et al.*, 1991). DPDPE and DADL are proposed to have selectivity for $delta_1$ opioid receptors, whereas Delt II and DSLET are thought to have greater selectivity for $delta_2$ opioid receptors. In the present investigations,

Fig. II-3. Isobolograms for antinociception induced by coadministration of adenosine agonists with PLO17 i.t. Antinociception was determined after coadministration (i.t.) of PLO17 and CPA (A) or CGS 21680 (CGS) (B) in a 1:1 constant dose ratio. The solid line connects the individual ED_{50} values for adenosine agonist and PLO17 injected i.t. alone and represents the theoretical additive interaction between agonists. Experimental ED_{50} values for each agonist when combinations were administered i.t. are indicated by the closed circles (●). The 95% CI for the experimental point is shown as a line extending toward the theoretical additive point for the 1:1 dose ratio.

Fig. II-3.


A
B
25
20
15
10
5
0
ED50 PL017 (pmol)
1:1
0
20
40
60
80
100
ED50 CPA (pmol)
1:1
0
1000
2000
3000
4000
5000
ED50 CGS (pmol)

synergism was observed for interactions between CPA and each *delta* opioid receptor-selective ligand (Fig. II-4 A through D). Results obtained from experiments using CGS 21680, however, were mixed (Fig. II-5 A through D). Coadministration of Delt II or DSLET with CGS 21680 revealed synergistic interactions, whereas coadministration of DPDPE or DADL with CGS 21680 revealed additive interactions.

Dose-response curves for DADL, DSLET and Delt II were not parallel to the dose-response curve for CPA and, therefore, were analyzed only by the method described by Tallarida *et al.* (1989). The nature of interactions between all other agonist combinations was identical by both methods of statistical analysis.

Interactions of adenosine agonists with kappa opioid receptor-selective agonists

Potential involvement of adenosine as a mediator of *kappa* opioid receptor-mediated antinociception (i.t.) in the tail-flick assay has not been reported. In our investigations, antinociception induced by U50,488 was not significantly antagonized by coadministration (i.t.) of theophylline (up to 222 nmol), an adenosine receptor antagonist (Fig. II-6A). In the hot plate assay, coadministration of theophylline (222 nmol) with U50,488 (i.t.) causes a nonparallel shift of the U50,488 dose-response curve to the right (data not shown). Coadministration (i.t.) of U50,488 with CPA in a 1:1 constant dose ratio resulted in a multiplicative interaction (Fig. II-6B). Interpretation of these observations was complicated by adverse behavioral effects that resulted when these agonists were coadministered. Investigations of U50,488 with CGS 21680 were not performed because the CGS 21680 vehicle precipitated U50,488 from solution.

Fig. II-4. Isobolograms for antinociception induced by coadministration of CPA with *delta* opioid receptor-selective agonists i.t. Antinociception was determined after coadministration (i.t.) of CPA with DPDPE (A), DADL (B), Delt II (C) or DSLET (D) in a 1:1 constant dose ratio. The solid line connects the individual ED_{50} values for CPA and *delta* opioid receptor-selective agonists injected i.t. alone and represents the theoretical additive interaction between agonists. Experimental ED_{50} values for each agonist when combinations were administered i.t. are indicated by the closed circles (●). The 95% CI for the experimental point is shown as a line extending toward the theoretical additive point for the 1:1 dose ratio. * Values significantly different from theoretical additive values ($P < .05$) indicating a synergistic interaction.

Fig. II-4.

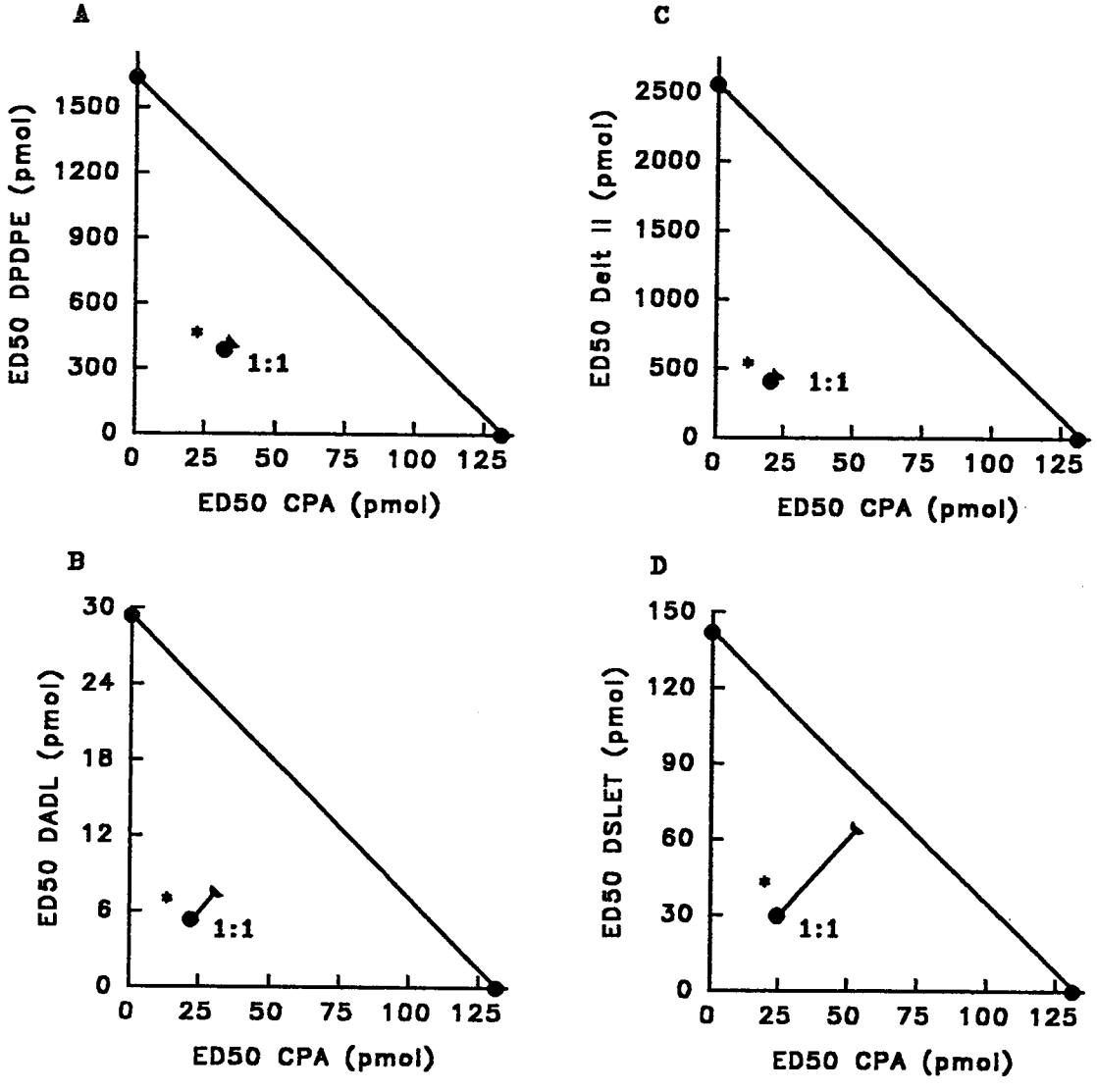

A
ED50 DPDPE (pmol)
1500
1200
900
600
300
0
1:1
0 25 50 75 100 125
ED50 CPA (pmol)
C
ED50 Delt II (pmol)
2500
2000
1500
1000
500
0
1:1
0 25 50 75 100 125
ED50 CPA (pmol)
B
ED50 DADL (pmol)
30
24
18
12
6
0
1:1
0 25 50 75 100 125
ED50 CPA (pmol)
D
ED50 DSLET (pmol)
150
120
90
60
30
0
1:1
0 25 50 75 100 125
ED50 CPA (pmol)

Fig. II-5. Isobolograms for antinociception induced by coadministration of CGS 21680 with *delta* opioid receptor-selective agonists i.t. Antinociception was determined after coadministration (i.t.) of CGS 21680 (CGS) with DPDPE (A), DADL (B), Delt II (C) or DSLET (D) in a 1:1 constant dose ratio. The solid line connects the individual ED_{50} values for CGS and *delta* opioid receptor-selective agonists injected i.t. alone and represents the theoretical additive interaction between agonists. Experimental ED_{50} values for each agonist when combinations were administered i.t. are indicated by the closed circles (●). The 95% CI for the experimental point is shown as a line extending toward the theoretical additive point for the 1:1 dose ratio. * Values significantly different from theoretical additive values ($P < .05$) indicating a synergistic interaction.

Fig. II-5.

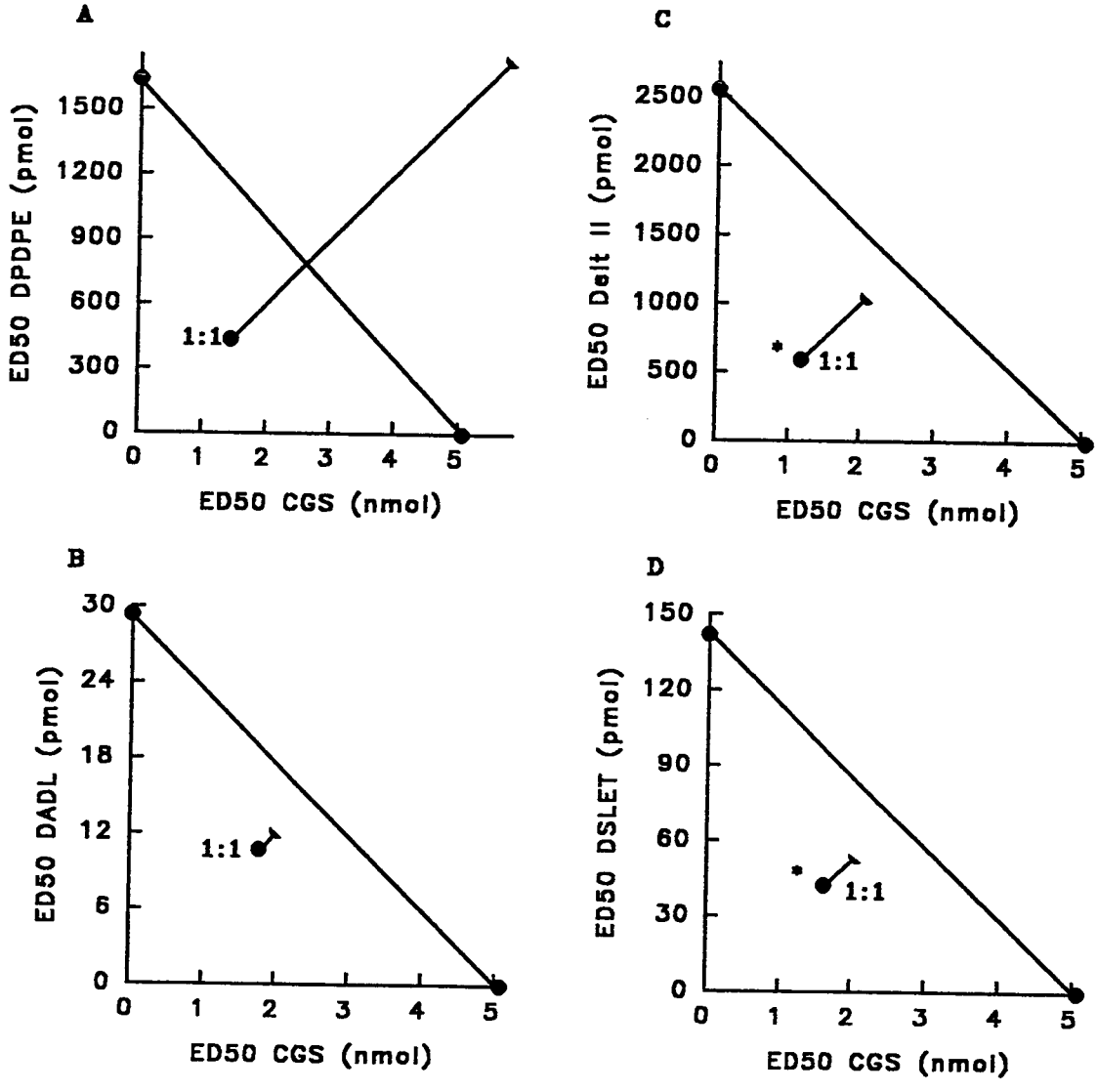
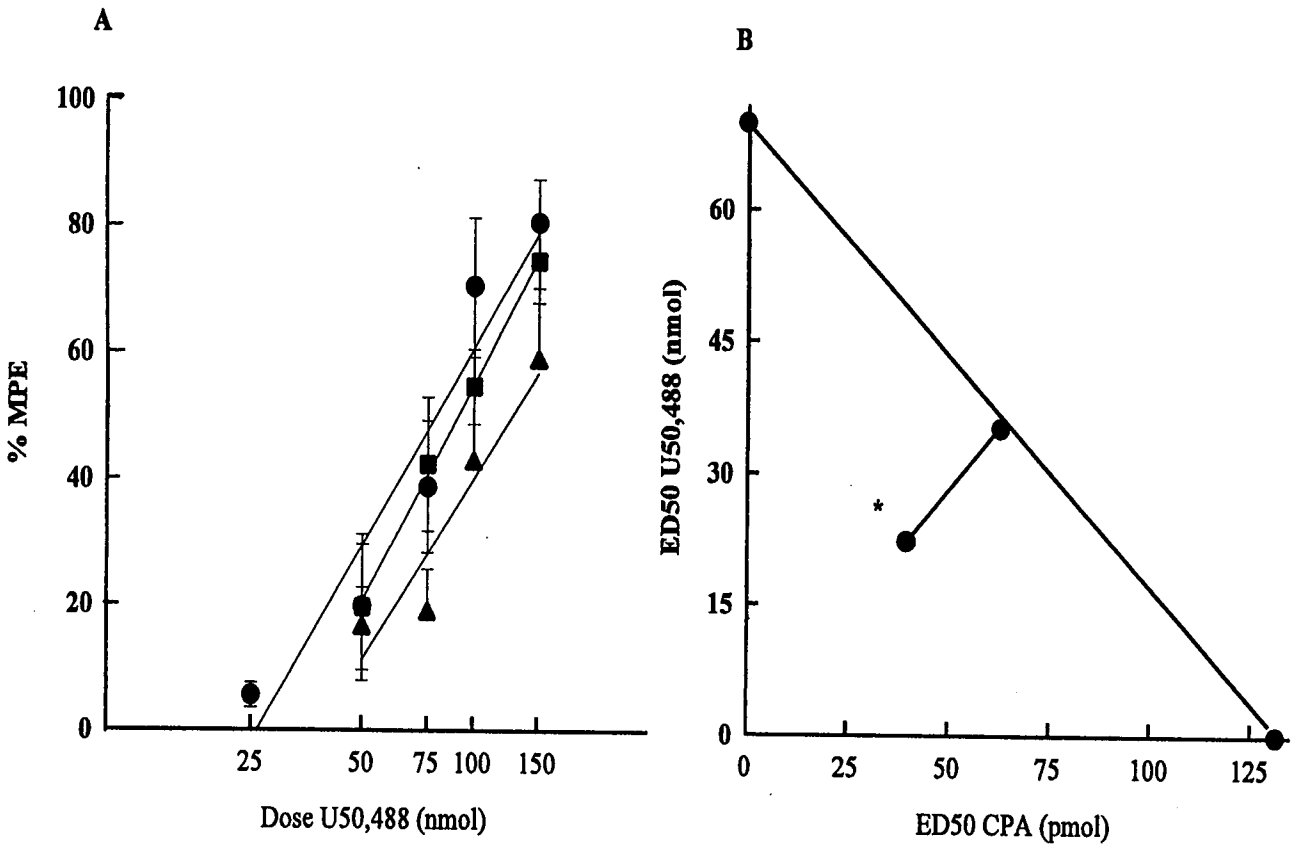

A
ED50 DPDPE (pmol)
1500
1200
900
600
300
0
1:1
0 1 2 3 4 5
ED50 CGS (nmol)
C
ED50 Delt II (pmol)
2500
2000
1500
1000
500
0
*
1:1
0 1 2 3 4 5
ED50 CGS (nmol)
B
ED50 DADL (pmol)
30
24
18
12
6
0
1:1
0 1 2 3 4 5
ED50 CGS (nmol)
D
ED50 DSLET (pmol)
150
120
90
60
30
0
*
1:1
0 1 2 3 4 5
ED50 CGS (nmol)

Fig. II-6. Interactions between adenosine receptor ligands and U50,488. A, dose-response curves for antinociception induced by U50,488 were determined using the tail-flick assay in the absence (●) and presence of theophylline [111 nmol (■) or 222 nmol (▲), respectively] coadministered i.t. B, antinociception was determined after coadministration (i.t.) of U50,488 and CPA in a 1:1 constant dose ratio. The solid line connects the individual ED_{50} values for CGS and *delta* opioid receptor-selective agonists injected i.t. alone and represents the theoretical additive interaction between agonists. Experimental ED_{50} values for each agonist when combinations were administered i.t. are indicated by the closed circles (●). The 95% CI for the experimental point is shown as a line extending toward the theoretical additive point for the 1:1 dose ratio.

Fig. II-6.


A
100
80
60
40
20
0
% MPE
25
50
75
100
150
Dose U50,488 (nmol)
B
60
45
30
15
0
ED50 U50,488 (nmol)
*
0
25
50
75
100
125
ED50 CPA (pmol)

Interactions between adenosine receptor-selective agonists

Coadministration (i.t.) of the two adenosine agonists resulted in a sub-additive interaction (Fig. II-7). Coadministration of CPA and CGS 21680 in a 1:1 constant dose ratio showed a significant tendency toward antagonism (total fraction 1.47).

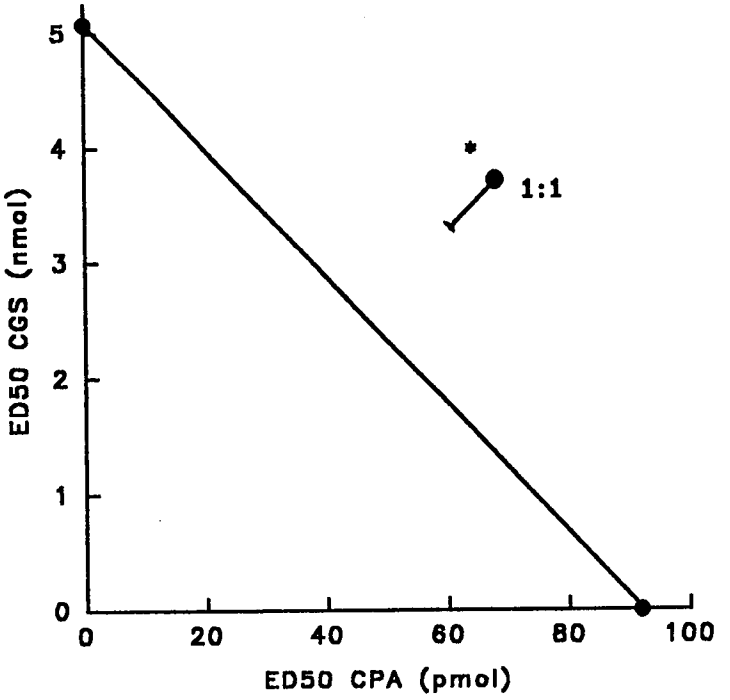
Discussion

Much evidence exists demonstrating the role of spinal adenosine in antinociception (Sawynok and Sweeney, 1989 for review). Adenosine appears to mediate, at least in part, antinociception induced by opioids administered systemically, i.c.v. and i.t. Recent investigations suggest adenosine does not mediate all actions induced by opioids, however, and adenosine involvement in opioid-induced antinociception may not be identical for all types of opioid receptors (DeLander *et al.*, 1992).

Although morphine has been used by investigators as a *mu* opioid receptor-selective agonist, it was selected for our initial investigations because of its potential to interact with multiple types of opioid receptors. Antinociception induced by i.t. injections of morphine alone and adenosine agonists alone was compared to antinociception observed after coadministration of a 1:1 constant dose ratio of morphine with an adenosine agonist. Isobolographic analyses of morphine interactions with adenosine agonists revealed mixed results. A synergistic interaction was observed for morphine with CPA, an A_1 adenosine receptor agonist, when determined by the method described by Tallarida *et al.* (1989) for nonparallel dose-response curves, but an additive interaction was determined when using the method of Roerig *et al.* (1988) for parallel dose-response curves. The interaction between morphine and CGS 21680 was additive

Fig. II-7. Isobologram for antinociception induced by coadministration of CPA and CGS 21680. Antinociception was determined after coadministration (i.t.) of CPA and CGS 21680 (CGS) in a 1:1 constant dose ratio. The solid line connects the individual ED_{50} values for CPA and CGS injected i.t. alone and represents the theoretical additive interaction between agonists. Experimental ED_{50} values for each agonist when combinations were administered i.t. are indicated by the closed circles (●). The 95% CI for the experimental point is shown as a line extending toward the theoretical additive point for the 1:1 dose ratio. * Values significantly different from theoretical additive values ($P < .05$) indicating an antagonistic interaction.

Fig. II-7.



regardless of analysis procedure. The mixed nature of these results prompted us to investigate adenosine/opioid agonist interactions with selective opioid receptor agonists. Isobolographic analyses of experiments involving coadministration (i.t.) of adenosine agonists and agonists selective for *mu* opioid receptors (*i.e.*, DAMGO and PLO17) did not reveal interactions significantly different than additivity. Classically, multiplicative interactions between drugs causing the same effect suggest distinct mechanisms of action. Additive pharmacologic interactions, as observed for adenosine receptor and *mu* opioid receptor-selective agonists, do not exclude the possibility of each agent acting by independent mechanisms, but additive interactions are consistent with common mechanisms of action. Therefore, additive interactions between *mu* opioid receptor-selective agonists and adenosine receptor agonists were consistent with the hypothesis that adenosine is a significant mediator of antinociception induced by *mu* opioid receptor agonists at spinal sites. These results were also consistent with earlier *in vitro* reports (Sweeney *et al.*, 1987a; 1989) and *in vivo* (Sweeney *et al.*, 1987b; 1989; 1991) demonstrating morphine-induced release of adenosine.

Experiments utilizing combinations of selective adenosine receptor agonists and selective *delta* opioid receptor agonists yielded varied results. Multiplicative interactions were observed for 1:1 constant dose ratio combinations of selective agonists at A_1/*delta*$_1$, A_1/*delta*$_2$ and A_2/*delta*$_2$ adenosine opioid receptors, respectively. Although experiments using A_2 adenosine and *delta*$_1$ opioid receptor-selective agonists demonstrated a tendency toward synergism, this interaction was not significantly different from additivity. Overall, these data support synergistic interactions between adenosine receptor and *delta* opioid receptor-selective agonists for antinociception and are indicative of independent

mechanisms of action to achieve a common endpoint. An earlier report describes a significant, but nonparallel shift of the dose-response curve for DPDPE when coadministered with theophylline (DeLander *et al.*, 1992). This earlier observation coupled with the present results suggests adenosine release may indirectly mediate antinociception induced by *delta* opioid receptor-selective agonists. These experiments do support a functional interaction between *delta* opioid- and adenosine-mediated antinociception at spinal sites.

Coadministration of an A_1 adenosine receptor-selective agonist with U50,488, an agonist selective for *kappa* opioid receptors, also revealed multiplicative interactions. In contrast to earlier investigations with *mu* or *delta* receptor-selective agonists, theophylline, an adenosine receptor antagonist, failed to antagonize antinociception induced by U50,488 in the tail-flick assay. The failure of theophylline to inhibit U50,488-induced antinociception and the apparent synergistic interaction observed when U50,488 and CPA were coadministered (i.t.) were complicated by behavioral effects caused when the two agents were administered together. Additional investigations will be required to clarify the significance of these observations.

The significance of additive interactions observed between adenosine agonists selective for A_2 receptor subtypes and either *mu* or *delta*$_1$ opioid receptor selective agonists is unclear at this time. Adenosine A_1 and A_2 receptors have been localized at spinal sites (Choca *et al.*, 1988), but conflicting results with respect to the importance of different adenosine receptor subtypes in spinal antinociception (Sawynok *et al.*, 1986; DeLander and Hopkins, 1987a; Aran and Proudfit, 1990; Karlsten *et al.*, 1991) have been observed. Although both A_1 and A_2 selective agonists induce antinociception after

i.t. administration, we and others (Karlsten *et al.*, 1990) consistently find A_2 receptor-selective agonists have more profound motor effects than A_1 agonists. The failure of A_2 adenosine receptor-selective agonists to demonstrate consistently synergism is possibly due to greater difficulty in the interpretation of behavioral tests after administration of A_2-selective agonists. In contrast to our observations, Aran and Proudfit (1990) have suggested A_2 adenosine receptors are responsible for synergism observed after spinal co-administration of noradrenergic agonists and adenosine agonists. Results from Aran and Proudfit (1990) may be complicated by their use of NECA, an agonist with equal affinity at A_1 and A_2 adenosine receptors, to assess A_2 adenosine receptor involvement.

An alternative hypothesis to explain the absence of synergism for interactions between A_2 adenosine and *delta*$_1$ opioid receptor-selective agonists may be that CPA and CGS 21680 induce antinociception via interactions at a common receptor. Significantly greater doses of CGS 21680, as compared to CPA, are required to induce antinociception and one could hypothesize that this is due to interactions at a common receptor at which CGS 21680 has lesser intrinsic activity. The hypothesis that these two agonists induce antinociception via interactions at a common receptor could explain our observation of the isobolographic analysis revealing an antagonistic interaction between CPA and CGS 21680 after coadministration (i.t.) in a 1:1 constant dose ratio. Clearly, additional experiments are needed to test this hypothesis. The ability to verify this possibility continues to be hampered by the absence of highly selective, water-soluble antagonists for adenosine receptor subtypes.

In addition to the relative involvement of either A_1 or A_2 receptors in producing synergistic interactions with opioid agonists, our investigations demonstrated a differential

involvement in the opioid receptor subtypes involved. Synergistic interactions were noted between adenosine agonists and morphine, and either *delta*- or *kappa*-selective agonists but not *mu*-selective agonists. Results from the present experiments are similar to those of Roerig and Fujimoto (1989). Those authors saw similar interactions between opioid agonists and the *alpha* adrenergic agonist, clonidine; synergism between clonidine and morphine or DPDPE, and additive interactions between clonidine and DAMGO. The interaction between clonidine and U50,488 was found to be additive in nature. The "magnitude" of synergism (see below) was also similar to our results: The rank order of total fraction values (in ascending order) in their studies was DPDPE > morphine > DAMGO ≥ U50,488 = 0 (additivity). Our results are also in agreement with the observations of Ossipov *et al.* (1990a,b) of synergistic interactions after i.t. administration of clonidine with morphine, U69,593 (*kappa*-selective receptor agonist) and DPDPE. Although their studies did not include selective *mu* agonists, the possibility of additive interactions between clonidine and *mu* opioid agonists can not be excluded. Characterization of the interactions between adenosine and other antinociceptive systems will enhance our knowledge of this complex set of systems. A recent finding in our laboratory of an additive interaction between CPA and norepinephrine (total fraction = 0.80; data not shown) indicates, preliminarily, a final common pathway of adenosinergic and $alpha_2$ adrenergic antinociceptive systems.

The relative magnitude of interactions between the adenosine agonists and opioid receptor subtype-selective agonists also appears to be heterogenous. Our studies, and those of Roerig and Fujimoto (1989) and Ossipov *et al.* (1990a,b), indicate synergistic interactions are most profound with adenosine or clonidine with *delta* opioid agonists.

The relative magnitude, if measured by the total fraction values, in our studies yield an ascending rank ordering of: *delta* ≥ morphine > *kappa* > *mu* = 0 (additivity). This in agreement with the degree of shift of the dose-response curves for the opioid agonists in constant dose ratios with clonidine [Roerig and Fujimoto (1989), Ossipov *et al.* (1990a,b)]. The relative magnitude of interactions between antinociceptive systems may indicate the degree of redundancy (or lack of redundancy for synergistic interactions) between antinociceptive systems. Taken as such, the intrinsic involvement of spinal adenosine in the antinociception induced after i.t. administration is much less for agonists selective at opioid *delta* and *kappa* receptors than for *mu*-selective agonists. The farther the total fraction is from unity, the greater the relative interaction between the antinociceptive systems. Total fractions might, therefore, be a relative indicator of how redundant, or similar, two systems are in producing similar behavioral effects.

Our investigations continue to support a role for adenosine in spinal systems modulating nociceptive neurotransmission. As suggested recently (DeLander *et al.*, 1992), the significance of adenosine in opioid-induced antinociception does not appear to be homogeneous. Additive interactions observed between *mu* opioid receptor-selective and adenosine receptor agonists were consistent with previous demonstrations of morphine- or DAMGO-induced adenosine release (reviewed by Sawynok *et al.*, 1989; Cahill *et al.*, 1992). If spinal adenosine release is a significant part of antinociception induced by *mu* agonists, the administration of adenosine receptor agonists would compensate additively for the reduced amount of *mu* agonist administered. Synergism observed after coadministration of adenosine receptor and either *delta* or *kappa* opioid receptor agonists, however, suggest each may induce antinociception independently.

Whether the mechanisms governing synergistic or additive interactions are determined at the neuronal level by actions on distinct nociceptive or antinociceptive neuronal pathways or at the molecular level by the activation (or inhibition) of distinct or redundant second messenger systems will require additional investigations.

TABLE II-1. Antinociception induced by coadministration (i.t.) of adenosine and opioid receptor agonists in a normalized 1:1 constant dose ratio in mice.

	ED_{50} Agonist Alone (95% CI)[a,b]	ED_{50} Adenosine Agonist (95% CI)	ED_{50} Opioid Agonist (95% CI)	Total Fraction[c]
CPA	131 (118-146)			
MS	102 (48-219)	38.2 (23.4-62.2)	30.5 (18.7-49.8)	0.59*
DAMGO[d]	5.9 (4.-7.2)	42.8 (17.7-103)	2.1 (0.9-5.2)	0.83
PLO17	25.0 (19.2-32.0)	78 (16-385)	19.5 (4.0-96.2)	1.63
DPDPE	1640 (1180-2280)	32.3 (29.0-36.0)	387 (374-432)	0.48*
DADL	29.4 (16.8-51.6)	22.2 (15.3-32.0)	5.3 (3.7-7.7)	0.37*
Delt II	2560 (1720-3810)	20.5 (17.4-24.1)	410 (348-481)	0.32*
DSLET	142 (103-195)	24.7 (11.3-54.3)	29.7 (13.5-65.2)	0.40*
U50,488	69880 (44000-110000)	39.6 (25.0-62.8)	22190 (14009-35150)	0.62*
CGS	5080 (3130-8250)			
MS[e]		2180 (1070-4450)	43.6 (21.4-88.9)	0.85
DAMGO		2080 (1303-3333)	2.1 (1.3-3.3)	0.76
PLO17		2950 (2380-3660)	14.8 (11.9-18.3)	1.17
DPDPE		1456 (366-5790)	437 (110-1740)	0.55
DADL		1780 (1570-2030)	10.7 (9.4-12.2)	0.72
Delt II		1190 (670-2112)	594 (335-1056)	0.47*
DSLET		1630 (1280-2090)	42.5 (33.2-54.2)	0.62*
U50,488[f]				

[a] All doses are expressed in pmol.

[b] From procedure 8, Tallarida and Murray, 1981.

[c] As described by Tallarida *et al.* (1989), see under "Materials and methods".

[d] doses indicated and total fraction indicated for DAMGO and PLO17 are shown assuming an ED_{50} value for CPA of 92 pmol (see under "Materials and methods"); results do not differ from experiments performed assuming an ED_{50} value of 131 pmol.

[e] ED_{50} values for opioid agonists listed above.

[f] No data because interactions between CGS 21680 and U50,488 could not be performed due to incompatibility in solution.

* Indicates a multiplicative interaction. Experimental ED_{50} values significantly different from theoretical additivity ($P < .05$).

CHAPTER III

EFFECTS OF ADENOSINE KINASE AND ADENOSINE DEAMINASE INHIBITION: INDUCTION OF ANTINOCICEPTION AND EFFECTS ON ADENOSINE- AND OPIOID-INDUCED ANTINOCICEPTION

Abstract

Endogenous purinergic systems mediating antinociception, and their interactions with opioids, were characterized following intrathecal (i.t.) administration of inhibitors of adenosine clearance in mice. 5'-amino 5'-deoxyadenosine (5'-NH_2dAdo), an inhibitor of adenosine kinase, but not deoxycoformycin (dCF) or *erythro*-9-(2-hydroxy-3nonyl) adenine (EHNA), inhibitors of adenosine deaminase, induced significant antinociception after i.t. injection. 5'-NH_2dAdo-induced antinociception was dose-dependently inhibited by theophylline. Both 5'-NH_2dAdo and dCF, however, significantly enhanced and prolonged exogenous adenosine-induced antinociception; dCF effects being quantitatively greater than 5'-NH_2dAdo. Coadministration of dCF did not alter opioid agonist antinociceptive dose-response curves while 5'-NH_2dAdo shifted these curves leftward. Isobolographic analysis of antinociception following coadministration (i.t.) of 5'-NH_2dAdo with opioids revealed additive interactions with *mu* and synergistic interactions with *delta* opioid receptor-selective agonists. These results confirm different physiologic roles for adenosine kinase and adenosine deaminase in spinal purinergic systems. 5'-NH_2dAdo interactions with opioid receptor selective agonists demonstrate significant, but heterogeneous interactions between endogenous adenosine and opioid spinal systems mediating antinociception which parallel interactions seen between exogenously administered adenosine analogs and opioid receptor agonists.

Introduction

Adenosine and adenosine analogs have been observed to induce antinociception after systemic, intracerebroventricular (i.c.v.; Yarbrough and McGuffin-Clineschmidt, 1981), or intrathecal (i.t.; DeLander and Hopkins, 1986; 1987a,b) administration. In addition, release of adenosine from spinal sites may participate in opioid-induced antinociception (reviewed, Sawynok and Sweeney, 1989). The extent of spinal adenosine involvement in opioid-induced antinociception, however, appears complex (DeLander and Hopkins, 1986; Chapter II) and may not extend to all opioid-mediated effects (DeLander *et al.*, 1992).

Antinociception observed following intrathecal (i.t.) opioid administration is mediated by actions at spinal sites (Yaksh and Noueihed, 1985). Involvement of spinal adenosine in spinally-mediated actions of opioid agonists has been demonstrated by behavioral studies (DeLander *et al.*, 1992), and by *in vivo* (Sweeney *et al.*, 1987a; 1989) and *in vitro* adenosine release studies (Sweeney *et al.*, 1989; 1993). The involvement of spinal adenosine, however, is apparently not uniform for all opioid receptor agonists. I.t. theophylline differentially inhibits antinociception induced by i.t. or i.c.v. administered opioid receptor agonists depending on the opioid receptor subtype activated (DeLander *et al.*, 1992). Moreover, adenosine receptor agonists interact differently with selective opioid receptor agonists to induce antinociception (Chapter II). *In vitro*, differential release of adenosine from spinal cord synaptosomes by selective opioid receptor agonists has also been suggested (Cahill *et al.*, 1992).

Although many studies have implied a role for spinal purinergic systems in antinociception (Braas *et al.*, 1986; Geiger and Nagy, 1986; Nagy and Daddona, 1985;

Choca *et al.*, 1988; Sosnowski *et al.*, 1989), the antinociceptive effects of endogenous adenosine have not been investigated. The purpose of this study was to investigate the antinociceptive potential following inhibition of spinal adenosine kinase and adenosine deaminase. As discussed above, anatomic evidence consistent with spinal involvement of endogenous adenosine in antinociception includes localization of adenosine receptors (Choca *et al.*, 1987; Lee and Reddington, 1986), adenosine-containing neurons and axon terminals (Braas *et al.*, 1986), adenosine deaminase (Geiger and Nagy, 1986) and adenosine transporter sites (Geiger and Nagy, 1985) in the substantia gelatinosa.

Characterization of how adenosine steady state levels are controlled is key to a full understanding of the role of adenosine in antinociception and possibly other adenosine actions in the central nervous system. Regulation of adenosine levels is thought to primarily involve 1) dephosphorylation of AMP to adenosine by 5'-nucleotidase, 2) deamination of adenosine to the less active compound inosine by adenosine deaminase, 3) phosphorylation of adenosine to AMP by adenosine kinase, and 4) nucleoside transporters (for review see Geiger *et al.*, 1991). Contribution of adenosine deaminase and nucleoside transporter systems in the control of adenosine levels in the central nervous system have been investigated in a wide variety of biological processes (Geiger *et al.*, 1991), but the potential role of adenosine kinase has received less attention.

Adenosine deaminase has greater activity in dorsal regions of spinal cord (Geiger and Nagy, 1986) and is present in small diameter primary afferent nerve terminals (Nagy and Daddona, 1985), suggesting a role for adenosine deaminase in nociceptive processing in the cord. The potential role of adenosine deaminase in regulating endogenous

adenosine, therefore, exists since this enzyme is concentrated in areas known to be involved in the transmission and modulation of sensory afferent input. An earlier study in brain (Yarbrough and McGuffin-Clineschmidt, 1981), however, was unable to demonstrate antinociception following inhibition of adenosine deaminase.

The spinal distribution of adenosine kinase has not been characterized, although the relative activity of adenosine kinase in the cord has been reported to be equal or slightly lower than other brain regions (Davies and Hambley, 1986). Investigations by Murray and coworkers (Zhang and Murray, 1991; Zhang *et al.*, 1993) have revealed a critical role for adenosine kinase, relative to adenosine deaminase, in physiological processes modulated by endogenous adenosine. Similarly, studies by Davies *et al.* (1984; 1986) and Pak *et al.* (1994) support an important role for adenosine kinase in the regulation of physiological adenosine concentrations.

Antinociceptive effects, as well as effects on opioid(i.t.)-induced antinociception, following inhibition of these enzymes were evaluated in the present investigations. Different effects on opioid agonist-induced antinociception were seen following inhibition of adenosine kinase and adenosine deaminase. Although adenosine deaminase appeared to be an important enzyme in the clearance of large amounts of exogenously administered adenosine, adenosine kinase inhibition induced antinociception in the absence of exogenously administered adenosine. Effects induced following adenosine kinase inhibition are apparently mediated via activation of extracellular adenosine receptors as adenosine receptor antagonism reversed this affect. Antinociception observed following coadministration of an inhibitor of adenosine kinase and selective opioid receptor agonists parallel earlier observations using combinations of adenosine receptor agonists with

selective opioid receptor agonists (Chapter II). The nature of adenosine/opioid interactions observed were found to be heterogeneous and dependent upon specifically which type of opioid receptor type is activated.

Materials and methods

Assay for antinociception

Antinociception was determined using the tail flick assay of D'Amour and Smith (1941) as described (see General Methods).

Experimental protocol

Behavioral and antinociceptive effects of spinal opioids were evaluated in the presence or absence of inhibitors of adenosine metabolism. Individual time- and dose-response curves for each agent were determined in groups of 7-10 mice. Individual ED_{50} values and 95% confidence intervals (95% C.I.) were determined at peak effect with the use of a computer (Tallarida and Murray, 1981; procedure #08). Time-response curves were expressed as area under the %MPE curve (AUC) determined by Simpson's rule (Tallarida and Murray, 1981; procedure #26).

Potential antinociception induced by 5'-NH_2dAdo and dCF were evaluated in the mouse tail flick assay following i.t. administration. Time-dependent effects were evaluated at times 5, 10, 15, 30, 60 and 90 min for both compounds; effects of i.t. dCF were evaluated out to 6 hours.

Interaction studies

Two techniques were employed to examine antinociception induced by interactions between opioid agonists and inhibitors of adenosine metabolism: 1) inhibitor-induced changes in opioid agonist dose and time courses (AUC); and 2) isobolographic analysis at time of peak drug effects.

Dose and time course (AUC) analysis

Mice were treated with normal saline or inhibitors of adenosine metabolism at various time points to investigate the effects of adenosine kinase or adenosine deaminase inhibition on opioid agonist-induced antinociception. Opioid agonist dose-response curves were investigated in the absence or presence of adenosine kinase or adenosine deaminase inhibitors to determine if coadministration of adenosine metabolism inhibitors effect agonist-induced antinociception. To investigate if adenosine kinase or adenosine deaminase inhibition effect the time course characteristics of opioid receptor agonists, the antinociception following administration of ED_{50} doses of opioid receptor agonists was quantified by calculating the AUC. Interactions were considered supra-additive if AUC values for pretreated mice were significantly greater than the sum of AUC values for the inhibitor of adenosine metabolism and opioid receptor agonist when administered alone, using Student's *t*-test (significant at $P < 0.05$).

Isobolographic analysis

Isobolographic analysis was performed as described above (Chapter II, Materials and methods).

Drugs and drug administration

All drugs were diluted in normal saline and injected intrathecally (i.t.) in a total volume of 5 μl using modifications of methods described by Hylden and Wilcox (1980). For pretreatment studies involving adenosine metabolism inhibitors, multiple i.t. injections were necessary. Neither drug vehicle nor drug administration protocols had significant effects on control values. Solubility limitations prevented higher doses of dCF or *erythro*-9-(2-hydroxy-3nonyl) adenine (EHNA) (> 200 nmol/5 μl = 40 mM) from being investigated.

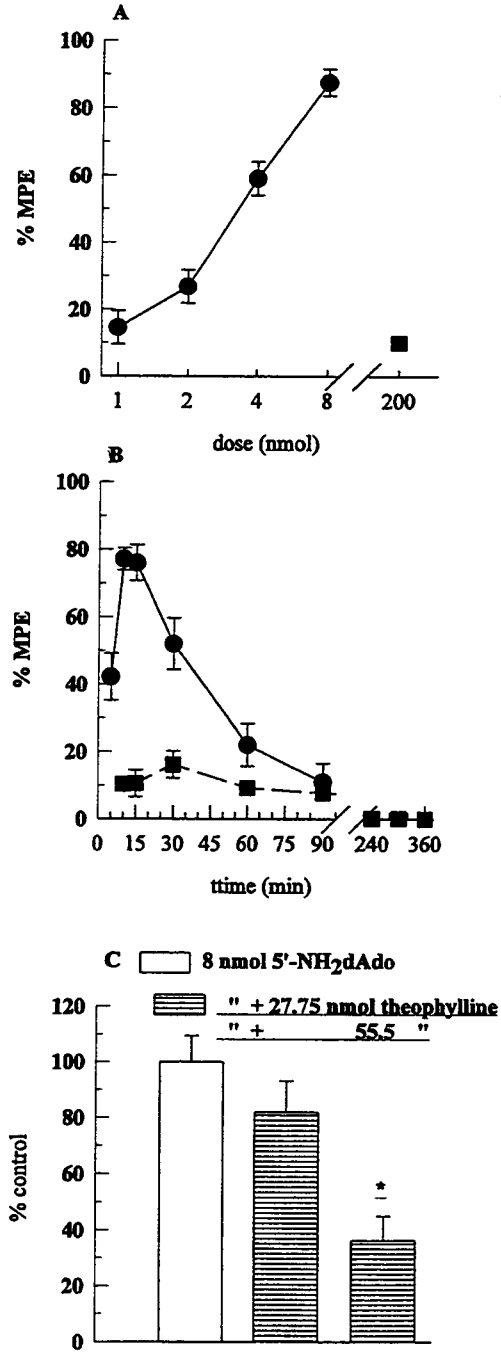
Results

Antinociception induced by individual agents following i.t. administration

Time- and dose-dependent antinociception [Fig. III-1A; ED_{50} 3.9 (2.0-4.6 - 95% C.I.) nmol] was induced by 5'-NH_2dAdo (i.t.), an adenosine kinase inhibitor (Miller *et al.*, 1979). Peak effect was observed at 10 min (Fig. III-1B) with latencies returning to baseline values by 60 min. 5'-NH_2dAdo-induced antinociception was dose-dependently inhibited by the adenosine receptor antagonist, theophylline (Fig. III-1C). dCF, a tight-binding inhibitor of adenosine deaminase (reviewed, Klohs and Kraker, 1992), demonstrated a small (20% MPE), transient effect at 5 min, but was generally without effect at the highest dose tested (200 nmol) out to 360 min (Fig. III-1A and B). Limited studies of the antinociceptive effect of EHNA, a "semi-tight-binding" inhibitor of adenosine deaminase (Agarwal *et al.*, 1977), were also conducted. EHNA (200 nmol) produced limited, short-lasting antinociception at 1, 2 and 5 min following i.t. administration (maximal MPE = 20% at 2 min; data not shown). While significantly

Fig. III-1. Antinociception induced following inhibition of spinal adenosine kinase or adenosine deaminase. A) Mice (N = 7 - 10) were administered (i.t.) graded doses of the adenosine kinase or adenosine deaminase inhibitors, 5'-NH_2dAdo (●) and dCF (■), respectively, and antinociception evaluated in the tail flick assay at 10 min. B) Mice were administered 8 nmol 5'-NH_2dAdo (●) or 200 nmol dCF (■) and antinociception evaluated at the times indicated in the tail flick assay. C) To evaluate the possible involvement in endogenous adenosine in 5'-NH_2dAdo-mediated antinociception, graded doses of the adenosine receptor antagonist, theophylline, was coadministered with 8 nmol 5'-NH_2dAdo and anti-nociception evaluated 10 min later in the tail flick assay. * $P <$ .05 Dunnett's Test.

Fig. III-1.


A
100
80
60
40
20
0
% MPE
1
2
4
8
200
dose (nmol)
B
100
80
60
40
20
0
% MPE
0 15 30 45 60 75 90 240 360
ttime (min)
C
8 nmol 5'-NH2dAdo
" + 27.75 nmol theophylline
" + 55.5 "
120
100
80
60
40
20
0
% control
*

greater than control values, antinociception induced by EHNA was complicated by immediate (< 30 s) hind limb flaccidity in almost every animal tested. Hindlimb flaccidity lasted approximately 5 min and all animals recovered completely within 20 min after administration. Additional behavioral effects induced by EHNA included a late-onset, whole body shaking and caudally-directed biting and scratching (10 to 15 min after EHNA administration). These behavioral and motor effects precluded the use of EHNA in subsequent investigations.

Dose- and time-dependent antinociception was seen following opioid agonist administration (i.t.) (Figs. III-2 and III-3; Table III-1). Effects induced by morphine, DAMGO, deltorphin II, and U50,488 were all maximal at 10 min and nonsignificant by 60 min. Exogenous administration of adenosine (i.t.) also induced time- and dose-dependent antinociception [ED_{50} 110 (95-127 - 95% C.I.) nmol; data not shown] which peaked at 5 min and returned to baseline by 15 min (Fig. III-5).

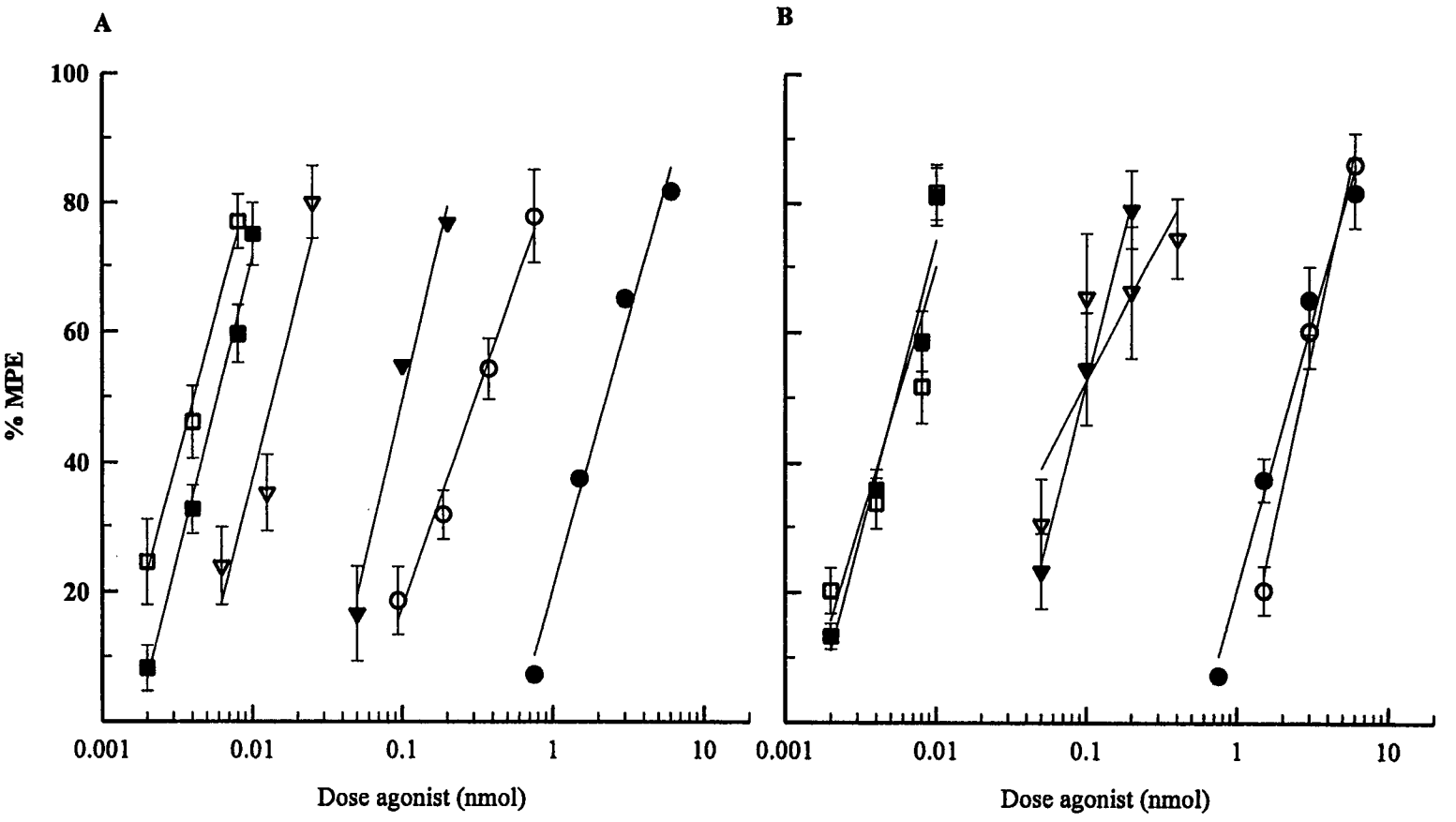
Effects of adenosine kinase or adenosine deaminase inhibition on opioid-induced antinociception

Dose-dependent antinociception induced by morphine and deltorphin II were significantly potentiated by coadministration with 5'-NH_2dAdo (1 nmol) as evidenced by the significant shift to the left of the agonist dose-response curves (Fig. III-2A). Antinociception induced by DAMGO was not significantly effected by coadministration of 5'-NH_2dAdo (Fig. III-2A). No shifts in morphine, DAMGO or deltorphin II dose-response curves were seen following coadministration with 200 nmol dCF (Fig. III-2B).

Potential interactions between 5'-NH_2dAdo or dCF and opioid receptor selective agonists were studied further by examining the time course of opioid-induced

Fig. III-2. Dose-response curves for opioid receptor agonists in the presence of inhibitors of adenosine kinase and adenosine deaminase. Dose-response curves for opioid receptor agonists were evaluated in the tail flick assay following coadministration with saline (closed symbols) or either (A) 5'-NH_2dAdo (1 nmol) or (B) dCF (200 nmol) (open symbols). Preliminary studies demonstrated all agonists were at peak effect within 10 min following i.t. administration. A) A small but nonsignificant leftward shift for DAMGO (■) was observed in the presence of 5'-NH_2dAdo. A significant leftward shift of morphine (▼) and deltorphin II (●) dose-response curves, however, were seen in the presence of 5'-NH_2dAdo. B) Dose-response curves for morphine (▼), DAMGO (■), or deltorphin II (●) were unchanged following coadministration with dCF. N = 7 to 10 mice for each dose.

Fig. III-2.


A
B
100
80
60
40
20
% MPE
0.001
0.01
0.1
1
10
Dose agonist (nmol)
0.001
0.01
0.1
1
10
Dose agonist (nmol)

antinociception following 5'-NH_2dAdo or dCF pretreatment. 5'-NH_2dAdo (1 nmol, i.t.) interacted differently with opioid receptor-selective agonists. 5'-NH_2dAdo pretreatment (10 min before opioid agonist administration) significantly increased antinociception induced by DAMGO and deltorphin II at various time points (Fig. III-3). Pretreatment with dCF (200 nmol, i.t. 10 min) had little effect on antinociception induced by opioid agonists, although the time course for morphine-induced antinociception was extended to 90 min (data not shown). dCF (200 nmol, i.t.) administered 1 h before opioid administration was without effect on opioid-induced antinociception (data not shown).

The calculated 10-60 min AUC values for each opioid agonist following 5'-NH_2dAdo or dCF pretreatment are shown in Fig. III-4. The time-dependency of an ED_{50} dose of DAMGO and deltorphin II, but not morphine, was significantly potentiated following pretreatment with 5'-NH_2dAdo (Fig. III-4A). No observable changes were seen for opioid agonist 10-60 min AUC values in mice pretreated with dCF (Fig. III-4B).

The effects of 5'-NH_2dAdo and dCF on U50,488-induced antinociception were also investigated. U50,488-induced antinociception was significantly potentiated by 5'-NH_2dAdo pretreatment and the duration of effect was extended (data not shown). Potentiation of U50,488-induced antinociception was also noted following dCF pretreatment (data not shown). Potentiation of U50,488-induced antinociception by either 5'-NH_2dAdo or dCF, however, was accompanied by profound motor incoordination, precluding any definitive interpretation of its significance.

Fig. III-3. Time-response curves following administration (i.t.) of an ED_{50} dose of opioid agonists following pretreatment with either normal saline or 5'-NH_2dAdo. 5'-NH_2dAdo (1 nmol) pretreatment (10 min) before saline administration (○) produced only slight antinociception in the tail flick test at times 10, 30 and 60 minutes. Antinociception following pretreatment with saline (□) 10 min before morphine (A), DAMGO (B) or deltorphin II (C) was compared to those effects seen following 5'-NH_2dAdo (1 nmol; ▵) pretreatment. * indicates antinociception significantly different than saline ($P < .05$).

Fig. III-3.

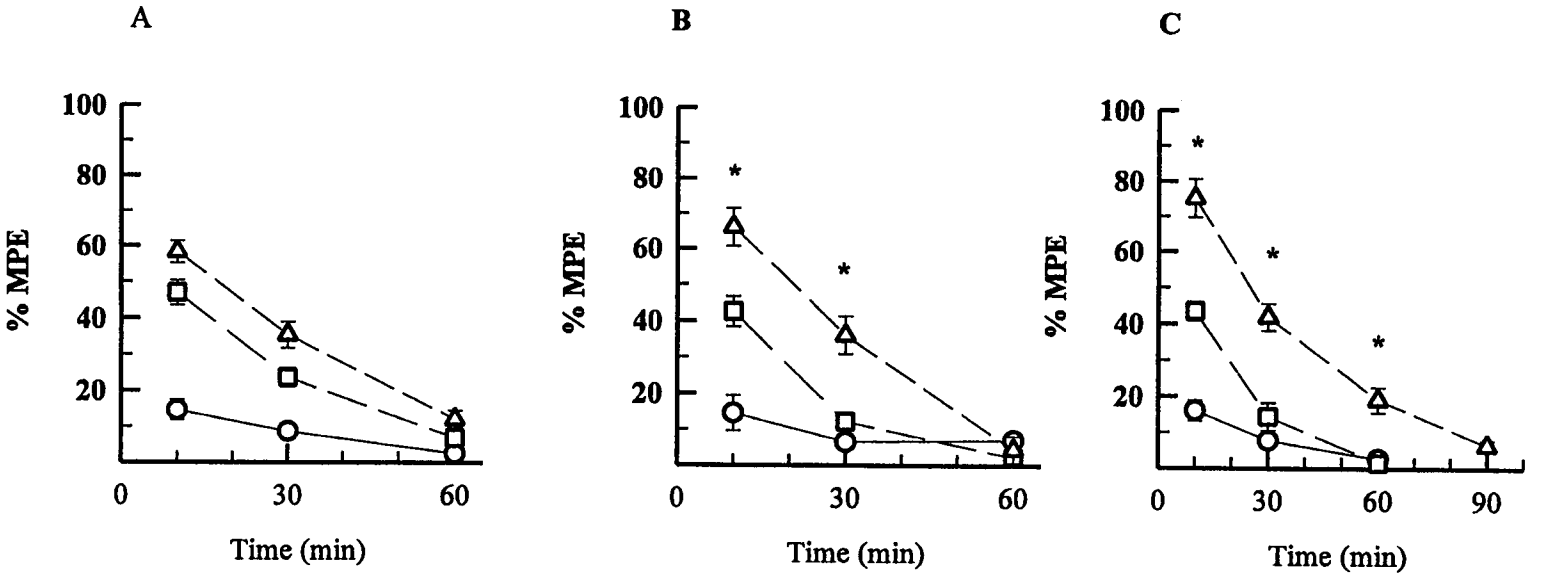
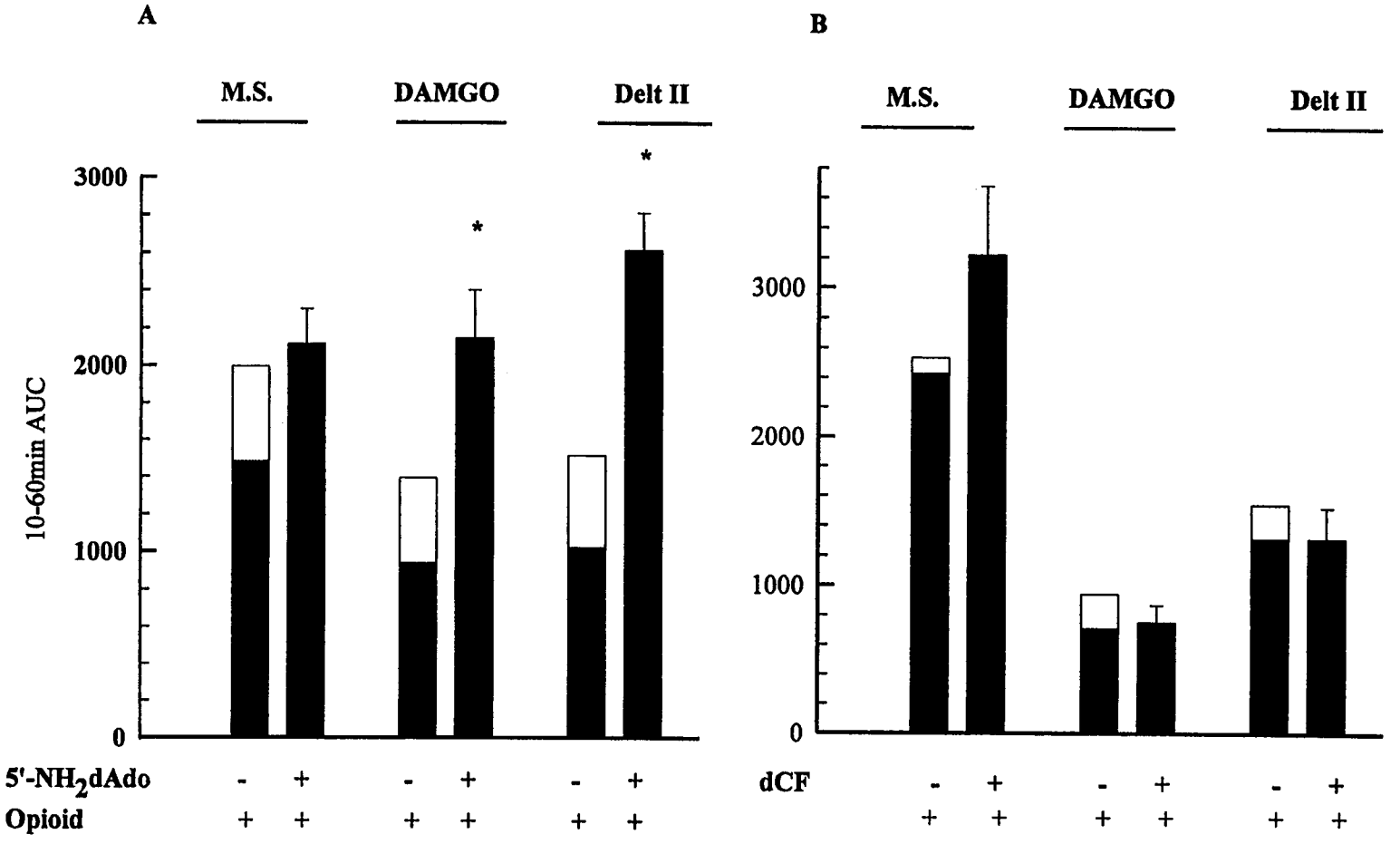


Fig. III-4. AUC values for opioid receptor agonists following pretreatment with saline or either 5'-NH_2dAdo or dCF. 10-60 min AUC values following i.t. administration of opioid receptor agonists were calculated according to Simpson's Rule. Mice (N = 7 to 10 for each group) were pretreated with either saline (left columns of each pair) or either (A) 5'-NH_2dAdo (1 nmol) or (B) dCF (200 nmol) (right columns of each pair) 10 min before opioid receptor agonist administration. The AUC contribution of 5'-NH_2dAdo or dCF in each experiment was determined by pretreating mice with 5'-NH_2dAdo or dCF 10 min before administration of saline (left open columns). Significant enhancement (*; $P < .05$) of opioid-induced antinociception was determined by comparing 5'-NH_2dAdo/opioid or dCF/opioid AUC values to the sum of 5'-NH_2dAdo or dCF and opioid AUC values (see Sec. 2.3.1). [Delt II = deltorphin II.]

Fig. III-4.


A
M.S.
DAMGO
Delt II
3000
2000
1000
0
10-60min AUC
*
*
5'-NH2dAdo
Opioid
- + - + - +
+ + + + + +
B
M.S.
DAMGO
Delt II
3000
2000
1000
0
dCF
- + - + - +
+ + + + + +

Isobolographic analyses of interactions between endogenous adenosine and opioid agonists

As heterogenous interactions between selective opioid receptor agonists and adenosine analogues are observed when analyzed isobolographically (Chapter II), interactions between 5'-NH_2dAdo and opioid receptor-selective agonists were also conducted. Isobolographic analyses could only be performed for interactions between 5'-NH_2dAdo and opioid receptor-selective agonists because dCF did not induce time- or dose-dependent antinociception.

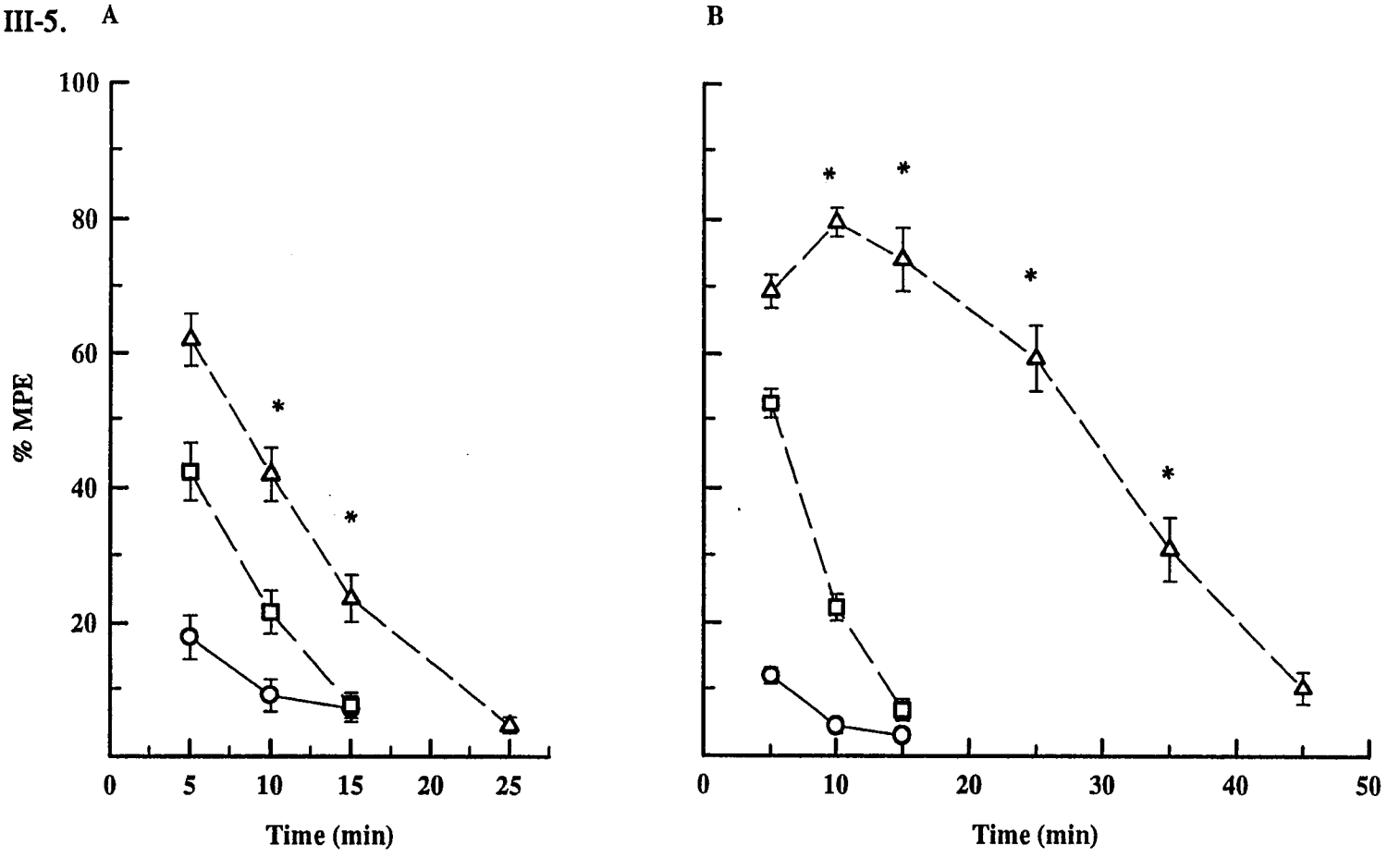
Additive interactions were observed when 5'-NH_2dAdo was coadministered with morphine or DAMGO in a 1:1 constant dose ratio (Table III-1), yielding total fraction values not significantly less than 1. Coadministration of 5'-NH_2dAdo with deltorphin II or DPDPE resulted in clear synergistic interactions (Table III-1). Isobolographic analysis of interactions between 5'-NH_2dAdo and U50,488 revealed additive interactions, with total fractions near unity. The relative degree of interaction between combinations of 5'-NH_2dAdo and opioid receptor-selective agonists, (demonstrated by the shift of the total fraction away from unity) was of the following rank order: deltorphin II > DPDPE > morphine $\geq$ DAMGO $\geq$ U50,488 $\geq$ 1.

Effect of adenosine kinase and adenosine deaminase inhibition on adenosine-induced antinociception

The effects of 5'-NH_2dAdo and dCF on exogenous adenosine-induced antinociception were investigated. Administration (i.t.) of each agent was timed so that maximal effect would occur simultaneously. 5'-NH_2dAdo had significant, but limited effects on adenosine-induced antinociception (Fig. III-5A). Antinociception induced by

Fig. III-5. Time course of exogenously administered adenosine following pretreatment with either 5'-NH_2dAdo or dCF. Time-response curves following administration (i.t.) of an ED_{50} dose of adenosine (110 nmol) following administration with either saline (□), 5'-NH_2dAdo (A; 1 nmol 5 min before) or dCF (B; 200 nmol coadministered) (△). Antinociception induced by 5'-NH_2dAdo (A) or dCF (B) are shown by the symbol (○). * indicates antinociception significantly different than saline ($P < .05$).

Fig. III-5.


A
B
100
80
60
40
20
% MPE
0
5
10
15
20
25
Time (min)
0
10
20
30
40
50
Time (min)
*

adenosine was greatly potentiated, however, by coadministration with dCF (Fig. III-5B). 5'-NH_2dAdo slightly, and dCF profoundly, prolonged adenosine-induced antinociception (Fig. III-5). Fig. III-6 shows the 5-15 min AUC values for exogenous adenosine-induced antinociception following 5'-NH_2dAdo or dCF treatment. Slight, but significant enhancement of exogenous adenosine-induced antinociception was seen following 5'-NH_2dAdo administration; an almost 3-fold enhancement, in contrast, was seen following dCF administration. Pretreatment with 200 nmol dCF (i.t.) 1 hr before exogenous adenosine administration produced results similar to those seen following adenosine/dCF coadministration (Fig. III-6).

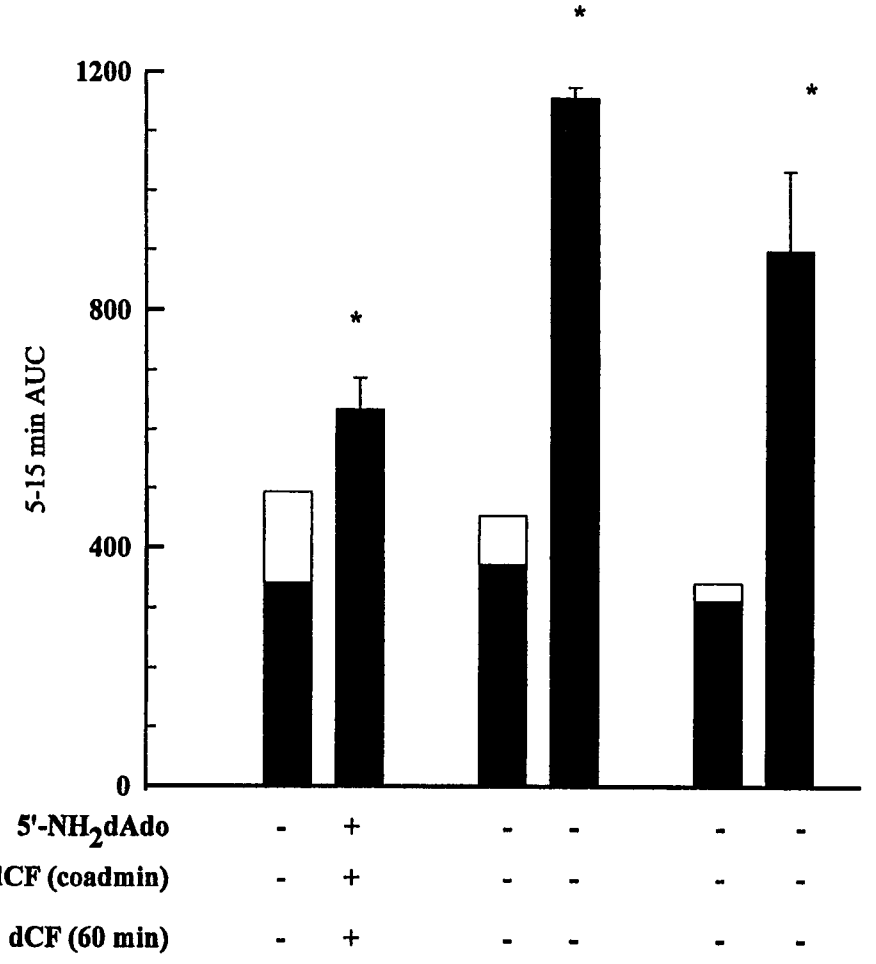
Discussion

Antinociception induced by i.t. administration of adenosine and adenosine analogs has been repeatedly demonstrated (reviewed Sawynok and Sweeney, 1989). Adenosine receptor agonists appear to mimic endogenous purinergic systems that may normally modulate nociceptive input from primary afferents. Adenosine, or nucleotides metabolized to adenosine, may be released from endogenous sites by opioids (DeLander and Hopkins, 1986; 1987b; Sweeney *et al.*, 1987a,b; 1989), serotonin (DeLander and Hopkins, 1987b; Sawynok and Reid, 1991) and following activation of non-nociceptive primary afferents (reviewed Salter *et al.*, 1993). In addition, manipulation of adenosine uptake or clearance alters nociceptive behaviors in mice and rats (DeLander and Hopkins 1987a; Sweeney *et al.*, 1989).

At least four distinct pathways are thought to regulate intra- and extra-cellular levels of endogenous adenosine: S-Adenosylhomocysteine hydrolase, the nucleoside

Fig. III-6. AUC values for exogenous adenosine-mediated antinociception following pretreatment with 5'-NH_2dAdo or dCF. AUC values (5-15 min) for adenosine-induced antinociception were calculated by Simpson's rule following pretreatment with 5'-NH_2dAdo (1 nmol 10 min before; 5'-NH2dAdo) coadministration with deoxycoformycin (200 nmol; dCF) or a 60 min pretreatment with dCF (200 nmol) (see legend Fig. III-3 for details). * indicates antinociception significantly different than saline control values ($P < .05$).

Fig. III-6.



transport system(s), adenosine kinase and adenosine deaminase. Although adenosine transport may be faster than subsequent metabolism (Young and Jarvis, 1983; Gu and Geiger, 1992), numerous studies have shown phosphorylation of adenosine by adenosine kinase to be a primary metabolic pathway regulating intracellular levels of adenosine (Winn *et al.*, 1980). The present studies demonstrated that, of the pathways responsible for adenosine metabolism, adenosine kinase plays a more significant role than adenosine deaminase in the regulation of endogenous adenosine modulating nociceptive input. 5'-NH_2dAdo, an inhibitor of adenosine kinase, induced time- and dose-dependent antinociception administered alone and enhanced antinociception when administered in combination with selected opioid analogs i.t. Investigations using dCF and preliminary investigations with EHNA, inhibitors of adenosine deaminase, revealed only limited antinociception or enhancement of opioid-induced antinociception, but profound enhancement of exogenously-administered adenosine. These results are comparable to the significance of adenosine kinase, relative to adenosine deaminase, in other models such as evoked seizures (Zhang *et al.*, 1993). In our investigations, spinal inhibitors of adenosine deaminase also appear to be less selective for inducing antinociception, causing significant motor impairment at doses that induced only minimal antinociception.

The apparent minimal involvement of adenosine deaminase in regulating endogenous adenosine levels modulating antinociception has been somewhat surprising. Adenosine deaminase generally has a lower affinity (K_D), but higher maximal enzymatic capacity (V_{max}) for adenosine, than adenosine kinase (Arch and Newsholme, 1978). Inhibitors of adenosine deaminase increase extracellular adenosine levels (Ballarin *et al.*, 1991) and induce behavioral and biochemical effects (reviewed Klohs and Kraker, 1992).

In addition, dCF potentiates certain actions of adenosine (Davies *et al.*, 1982). Although adenosine deaminase activity in the spinal cord is low compared to other brain regions, adenosine deaminase is concentrated in dorsal spinal cord lamina (Geiger and Nagy, 1986) and is present in small diameter primary afferent nerve terminals (Nagy and Daddona, 1985) suggesting potential involvement in regulation of nociceptive neurotransmission. dCF forms a very stable complex (irreversible) with cytosolic adenosine deaminase once dCF permeates the nucleoside transport system(s) (Roger-Brown *et al.*, 1978). dCF, however, has been demonstrated to be a poor permeant of the nucleoside transport system(s) (Newby, 1981). Our lack of effect following dCF administration, therefore, could have been due to the lack of adenosine deaminase inhibition by dCF because of poor transport across the cellular membrane. Inhibition of adenosine deaminase following i.t. administration has not been measured directly in our laboratory, but other investigators have shown maximal tissue deposition of dCF in central sites as early as 15 min after i.p. injection (McConnell *et al.*, 1978) and complete adenosine deaminase inhibition within 2 h (Geiger *et al.*, 1987). We have examined potential dCF (i.t.)-mediated actions for up to 360 min and no significant dCF-mediated effects were seen. Therefore, it seems unlikely that limited actions observed following dCF administration (i.t.) can be adequately explained by a failure of dCF to reach its site of action. Limited antinociception induced by dCF and the general absence of significant interactions between dCF and opioid receptor agonists argue against a role for adenosine deaminase in regulation of endogenous adenosine modulating nociceptive input.

It is important to note, however, that dCF dramatically enhanced adenosine-mediated actions when adenosine was administered i.t. dCF potentiation of exogenous

adenosine-mediated antinociception could be explained by dCF inhibition of adenosine uptake via the nucleoside transport system(s) (see above and Chapter IV). Although this is a possibility, our results suggest dCF-mediated effects are not due to nucleoside transport system(s) inhibition. Similar effects on adenosine-mediated antinociception were seen following coadministration or a 1 h pretreatment with dCF. In addition, opioid-induced release of adenosine is suggested to occur via the nucleoside transport system(s) (Sweeney *et al.*, 1993). Pretreatment with nucleoside transport system(s) inhibitors, therefore, might be expected to inhibit opioid-mediated antinociception. Other investigations in our laboratory have shown differing degrees of antagonism or potentiation of opioid-induced antinociception when nucleoside transport inhibitors are coadministered (see Chapter IV). Therefore, although dCF may temporarily "compete" for the nucleoside transport system(s) while gaining access to the intracellular compartment, the relative lack of effect of dCF on opioid-mediated antinociception suggests this action is of minor importance in the current studies. Overall, our observations with dCF suggest enzymatic control of adenosine levels by adenosine deaminase are of minor importance under physiologic conditions. Under conditions characterized by increased levels of adenosine, however, the significance of adenosine deaminase may be enhanced dramatically [see Zetterström *et al.* (1982)].

The authors are unaware of any reports detailing localization of adenosine kinase at specific spinal sites, but this enzyme is found throughout the central nervous system with heterogeneous distributions and activities in various brain regions (Phillips and Newsholme, 1979). In contrast to adenosine deaminase, adenosine kinase is a 'high affinity, low capacity' enzyme (Arch and Newsholme, 1978) that is substrate inhibited

at higher adenosine concentrations (Fox *et al.*, 1982). Inhibition of adenosine kinase, therefore, could induce significant changes in intracellular adenosine levels. As shown above, theophylline-reversible antinociception induced following adenosine kinase inhibition is likely a consequence of increased intracellular adenosine concentrations which could subsequently increase neuronal adenosine release [via the bi-directional nucleoside transport system(s)] and activation of adenosine receptors. Also, the increased potency or duration of opioid receptor agonist(i.t)-induced antinociception by 5'-NH_2dAdo, suggests increased intracellular adenosine concentrations also enhance opioid-induced antinociception. As suggested previously (Davies *et al.*, 1986; Zhang *et al.*, 1993), increased adenosine concentrations following inhibition of adenosine kinase may result in the increase of available pools of adenosine for release or may create an unfavorable concentration gradient for adenosine reuptake. Regardless of the precise mechanism(s), adenosine kinase inhibition appears to increase extracellular adenosine concentrations which leads to the activation of extracellular adenosine receptors and enhanced antinociception is the likely consequence.

Inhibition of adenosine kinase resulted in differing effects on antinociception induced by selective opioid receptor agonists. Synergistic interactions were seen for dose- and time-response curves and for isobolographic analysis of interactions between 5'-NH_2dAdo and deltorphin II, a *delta* opioid receptor-selective agonist. Synergism in inducing antinociception was also observed between 5'-NH_2dAdo and DPDPE, another *delta* opioid receptor-selective agonist. These data are consistent with synergism reported earlier (Chapter II) for interactions between adenosine agonists and *delta* opioid receptor-selective agonists administered intrathecally. As discussed above (Introduction and

Chapter II), synergism observed following activation of spinal purinergic and *delta* opioid systems suggests that these systems act independently to modulate nociceptive input. The noncompetitive inhibition by theophylline on *delta* opioid induced antinociception (DeLander *et al.*, 1992), however, indicates a basal adenosine "tone" may be necessary for full antinociceptive efficacy induced by *delta* opioid receptor agonists.

Coadministration of 5'-NH_2dAdo and morphine or DAMGO, a *mu* opioid receptor-selective agonist, displayed mixed results. Generally, antinociception resulting from these interactions was additive in nature and similar to results previously reported for antinociception induced by coadministration (i.t) of adenosine receptor agonists with morphine or DAMGO (Chapter II). Additive interactions are consistent with the hypothesis that opioid-stimulated release of adenosine is one mechanism for opioid induced antinociception. The present data revealing greater than additive antinociception for DAMGO AUC following pretreatment with 5'-NH_2dAdo allows one to hypothesize that appropriate selection of doses of 5'-NH_2dAdo may 'preload' neurons with adenosine leading to enhanced opioid actions.

These investigations demonstrate the significance of adenosine kinase in regulating physiologic concentrations of spinal adenosine that modulate nociceptive input. Inhibition of adenosine deaminase had little effect in this model, but may be of greater significance in conditions that result in higher local adenosine concentrations. The extent to which our investigations with 5'-NH_2dAdo parallel interactions of adenosine analogs with opioid receptor-selective agonists (Chapter II) provides additional support for the existence of an endogenous purinergic system modulating of nociceptive input and for significant interactions between spinal purinergic and opioid systems.

TABLE III-1. Antinociception induced by coadministration (i.t.) of 5'-NH_2dAdo and opioid receptor agonists in a 1:1 constant dose ratio[a] in mice.

	ED_{50} Agonist (i.t.) alone (95% CI)[b,c]	ED_{50} 5'-NH_2dAdo (95% CI)	ED_{50} Opioid Agonist (95% CI)	Total Fraction[d]
5'-NH_2dAdo	3.9 (2.6-5.8) n			
MS	102 (48-219) p	1.0 (0.6-1.7) n	32.2 (18.0-56.0) p	0.55
DAMGO	8.5 (7.0-10.4) p	1.1 (0.3-4.6) n	3.3 (0.8-13.6) p	0.66
Deltorphin II	2.6 (1.7-3.8) n	307 (99-955) p	359 (116-1114) p	0.22*
DPDPE	1.6 (1.2-2.3) n	708 (404-1242) p	354 (202-621) p	0.40*
U50,488	70 (44-110) n	1.4 (0.4-4.7) n	33 (10-109) n	0.84

[a]Doses of agonists coadministered proportional to ED_{50} values of individual agonists administered alone.
[b]From Pharm Basic.pcs, procedure 8; Tallarida and Murray, 1981.
[c]Doses for agonists: n = dose in nmol; p = dose in pmol.
[d]As described by Tallarida *et al.* (1989), see "Methods.".
*Indicates a mutiplicative interaction. Experimental ED_{50} values significantly different from theoretical additivity ($P < .05$).

CHAPTER IV

EFFECTS OF NUCLEOSIDE TRANSPORT INHIBITION: INDUCTION OF ANTINOCICEPTION AND EFFECTS ON ADENOSINE- AND OPIOID-INDUCED ANTINOCICEPTION

Abstract

Endogenous purinergic systems are important in spinal mechanisms of sensory afferent processing. Antinociception induced by spinal *mu* opioid receptor selective agonists appears to be mediated in part by opioid-stimulated adenosine release. Nucleoside transport system(s) have been implicated both in adenosine release and reuptake at spinal sites. The present investigations were designed to determine the significance of nucleoside transport system(s) inhibition *in vivo* in antinociception induced by opioids administered intrathecally (i.t.) in mice. Dilazep, but not dipyridamole or s-(4-nitrobenzyl)-6-thioinosine (NBMPR), nucleoside transport system(s) inhibitors, induced time- and dose-dependent antinociception in the tail flick test, putatively via spinal adenosine reuptake inhibition. Each nucleoside transport system(s) inhibitor, at doses without significant effects alone, enhanced adenosine-mediated antinociception when coadministered (i.t.). Concurrent treatment of mice with opioid receptor selective agonists and nucleoside transport system(s) inhibitors had varying effects on antinociception depending on the timing of the nucleoside transport inhibitor. In general, antinociception induced by *mu* opioid receptor selective agonists was inhibited by pretreatment, not effected following coadministration, and enhanced by posttreatment, with nucleoside transport system(s) inhibitors. In contrast, antinociception induced by *delta* opioid receptor selective agonists was enhanced by nucleoside transport system(s)

inhibitors in all treatment protocols. These results provide *in vivo* evidence that alterations in adenosine movements into or out of spinal neurons via the nucleoside transport systems can induce antinociception and effect opioid-mediated antinociception. These data support the hypothesis that adenosine has significant but independent roles in antinociceptive effects induced by *mu* and *delta* opioid receptor- selective agonists.

Introduction

Multiple spinal systems, including opioid and adenosine (purinergic) systems, modulate nociceptive neurotransmission in the dorsal horn (Yaksh and Malmberg, 1994). A role for spinal purinergic systems as modulators of nociceptive input is supported by observations that: 1) Intrathecal (i.t.) administration of adenosine or adenosine analogs induce methylxanthine-reversible antinociception in a wide variety of nociceptive tests (reviewed by Sawynok and Sweeney, 1989); 2) Adenosine receptor binding is high in dorsal horn laminae (known termini areas of primary afferent nociceptive neurons) (Choca *et al.*, 1987, 1988; Geiger *et al.*, 1984); 3) adenosine deaminase (ADA)-immunoreactivity is highest in superficial dorsal horn regions (Geiger and Nagy, 1986; Nagy and Daddona, 1985); and 4) binding of radiolabelled nucleoside transport system(s) inhibitors is also highest in dorsal horn laminae (Geiger and Nagy, 1984; 1985). In addition, spinal adenosine derived from other adenine nucleotides is thought to be involved in nociceptive modulation. For example, the analgesic responses following peripheral vibration are thought to be mediated via adenosine derived from low-threshold primary afferent nerves releasing ATP (Salter and Henry, 1987; Salter *et al.*, 1993).

Recently, our laboratory has demonstrated that manipulation of endogenous

adenosine levels induces antinociception in the mouse tail flick and hot plate assays (Chapters I and II). Additionally, i.t. administration of adenosine receptor antagonists induces thermal hyperalgesia (Jurna, 1984; Sawynok *et al.*, 1986) under certain experimental conditions. These results indicate an endogenous purinergic 'tone' may be active at spinal sites modulating nociceptive neurotransmission. Facilitation of this 'tone' would be expected to induce antinociception, while inhibition of the purinergic 'tone' would result in facilitated nociceptive neurotransmission.

Opioid agonists induce antinociception via direct interactions with opioid receptors at spinal sites (Yaksh and Noueihed, 1985). In addition to direct effects on spinal nociceptive neurotransmission, results from a number of laboratories support stimulated adenosine release as an additional mechanism of opioid-mediated antinociception. In behavioral studies, methylxanthines administered i.t. inhibit morphine (i.t.)-induced antinociception (DeLander and Hopkins, 1986; DeLander *et al.*, 1992; Sweeney *et al.*, 1987b) at doses comparable to those required to block adenosine-induced antinociception. In *in vivo* (Sweeney *et al.*, 1987a) and *in vitro* (Sweeney *et al.*, 1987b, 1989, 1993) studies, morphine has been demonstrated to release adenosine *per se* from primary afferent neurons. This release occurs from synaptosomes prepared from dorsal but not ventral spinal cord (Sweeney *et al.*, 1989) and occurs via a nucleoside transporter(s) with differential sensitivities to nucleoside transport inhibitors (Sweeney *et al.*, 1993). Opioid-evoked release of adenosine is proposed to differ from nucleotide-derived basal adenosine release (Sweeney *et al.*, 1987b, 1993); however, the precise source of opioid-evoked adenosine is not fully understood (Cahill *et al.*, 1993; Sweeney *et al.*, 1993).

Spinal adenosine release apparently is not uniformly involved in all opioid-

mediated actions. Our laboratory has shown mixed effects of adenosine receptor antagonists on opioid agonist dose-response curves (DeLander and Wahl, 1989; DeLander *et al.*, 1992). In addition, studies investigating interactions between spinal adenosine and opioid antinociceptive systems reveal either additivity or synergism (Chapters I and II) depending on the opioid agonist coadministered. We have also demonstrated modulation of endogenous adenosine concentrations at spinal sites have various effects on opioid-mediated antinociception depending on which opioid receptor is activated (Chapter II).

Recently, Sweeney *et al.* (1993) have shown nucleoside transport inhibition can significantly increase basal extracellular adenosine concentrations, theorized to be due to inhibition of nucleotide-derived adenosine reuptake. In addition, nucleoside transport inhibition decreased the uptake of exogenously administered adenosine. Interestingly, these inhibitory effects were seen with the nucleoside transport inhibitor dipyridamole, but not nitrobenzylthioinosine. Both compounds, however, decreased potassium- and morphine-stimulated adenosine release. Thus, nucleoside transport inhibition can increase or decrease extracellular adenosine levels, dependent upon inhibition of reuptake or efflux, respectfully. The differences in spinal adenosine release by selective opioid receptor agonists and the possibility of nucleoside transport inhibitors inducing antinociception or effecting opioid-mediated antinociception led us to investigate the effects of dipyridamole and nitrobenzylthioinosine, as well as the water-soluble nucleoside transport inhibitor, dilazep, administered alone (i.t.) and in combination with *mu* and *delta* opioid agonists. Our studies demonstrate the regulation of spinal adenosine efflux or reuptake via nucleoside transport systems effects antinociception induced by

adenosine and has various effects on opioid receptor agonist-induced antinociception depending on the administration timing of the nucleoside transport inhibitor and the type of opioid receptor agonist.

Materials and methods

Assay for antinociception

Antinociception was determined using the radiant heat tail flick assay of D'Amour and Smith (1941) as described (see General Methods).

Experimental protocol

Antinociceptive effects of nucleoside transport inhibitors alone or in combination with various opioid receptor agonists were evaluated in the mouse tail flick assay. Individual time- and dose-response curves for each drug were determined in groups of 7–10 mice. Individual ED_{50} values and 95% confidence intervals (95% C.I.) were determined at peak effect with the use of a computer (Tallarida and Murray, 1981; procedure #8).

Preliminary investigations demonstrated nucleoside transport inhibition had varying effects on opioid agonist-induced antinociception depending on the time of nucleoside transport inhibitor administration. Studies were conducted using three nucleoside transport administration protocols on opioid-mediated antinociception: 1) pretreatment - nucleoside transport inhibitor administration 10 min before opioid agonist; 2) coadministration - coinjection of nucleoside transport inhibitor with opioid receptor agonist; and 3) posttreatment - nucleoside transport inhibitor administration 5 min after opioid receptor agonist. Additionally, various doses of opioid agonists were used in each

nucleoside transport inhibitor administration protocol. When preliminary studies revealed potentiation following administration of a nucleoside transport inhibitor and an opioid, full investigations of the interaction were conducted using an ED_{50} dose of opioid receptor agonist. When preliminary studies revealed inhibitory interactions, full investigations were conducted using an ED_{80} dose of opioid receptor agonist. Selection of ED_{50} or ED_{80} opioid doses allowed us to maximize the ability to detect significant positive or negative drug interactions. Control injections of nucleoside transport inhibitor vehicles with opioid receptor agonists were conducted for all experiments.

Drugs and drug administration

All drugs were solubilized and injected intrathecally (i.t.) in a total volume of 5 μl using modifications of methods described by Hylden and Wilcox (1980). For pre- and post-treatment studies, multiple i.t. injections were necessary. Neither drug vehicle nor drug administration protocols had significant effects on control values. All opioid receptor agonists and dilazep were dissolved in normal saline. NBMPR was dissolved in normal saline containing 7.5% (w/v) 2-hydroxypropyl β-cyclodextrin. DPR was dissolved in normal saline slightly acidified with HCl (final [HCl] = 0.0015 N).

Statistics

Comparison between groups following drug treatments were performed by one way analysis of variance or Kruskal-Wallis analysis of variance on ranks followed by appropriate *post-hoc t*-tests. Critical values that achieved $P < .05$ were considered statistically significant.

Results

Antinociceptive effect of nucleoside transport inhibitors administered i.t.

At the doses tested, NBMPR (25 nmol) and DPR (5 nmol) did not induce statistically significant antinociception (Fig. IV-1A) in the mouse tail flick assay. The more water-soluble nucleoside transport inhibitor, dilazep, however, induced time- and dose-dependent antinociception (Fig. IV-1A and B). Maximal antinociception (approximately 60% M.P.E.) occurred at 5 min following i.t. administration. Caudally-directed biting and scratching was observed at later time points (approx. 10 to 15 min) following high doses (> 25 nmol) of i.t. dilazep suggesting dilazep may also effect basal adenosine release or have other nonspecific effects (see discussion). Theophylline, an adenosine receptor antagonist, was coadministered with dilazep in an attempt to demonstrate that antinociception induced by dilazep was the result of increased extracellular adenosine concentrations activating adenosine receptors.

Coadministration of theophylline (111 nmol) and dilazep (25 nmol) i.t., however, induced immediate vigorous biting and scratching behavior precluding any meaningful evaluation of dilazep-induced antinociception (data not shown).

Effect of nucleoside transport system(s) inhibition on exogenous adenosine-induced antinociception

Coadministration of the nucleoside transport inhibitors, NBMPR (12.5 nmol; Fig. IV-2A), DPR (5 nmol; Fig. IV-2B) or dilazep (12.5 nmol; Fig. IV-2C) significantly enhanced and prolonged exogenous adenosine-induced antinociception. The relative effectiveness of the nucleoside transport inhibitors potentiating adenosine-induced

Fig. IV-1. Dose (A)- or time (B)-dependent antinociception induced by i.t. administration of nucleoside transport inhibitors. (A) Antinociception for nucleoside transport inhibitors was determined using the mouse tail flick assay 5 min following graded doses of dilazep (●) or the highest doses of NBMPR (▲; 25 nmol) or DPR (■; 5 nmol). (B) Dilazep (25 nmol)-mediated antinociception was evaluated at various time points following i.t. administration. * $P < .05$ compared to tail flick latencies following vehicle administration. N = 7 to 10 mice for each dose (A) and N = 9 mice for the group (B).

Fig. IV-1.

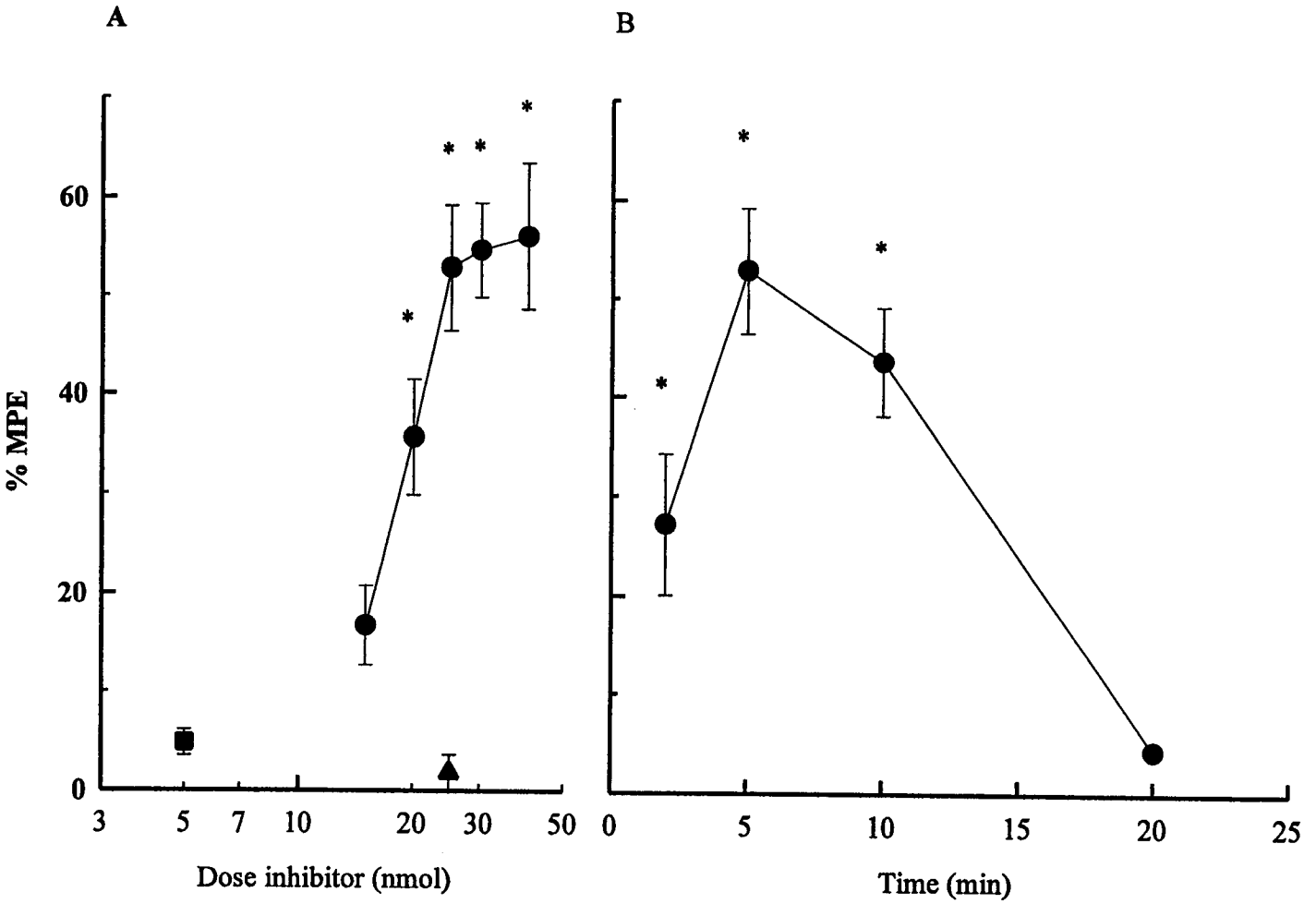
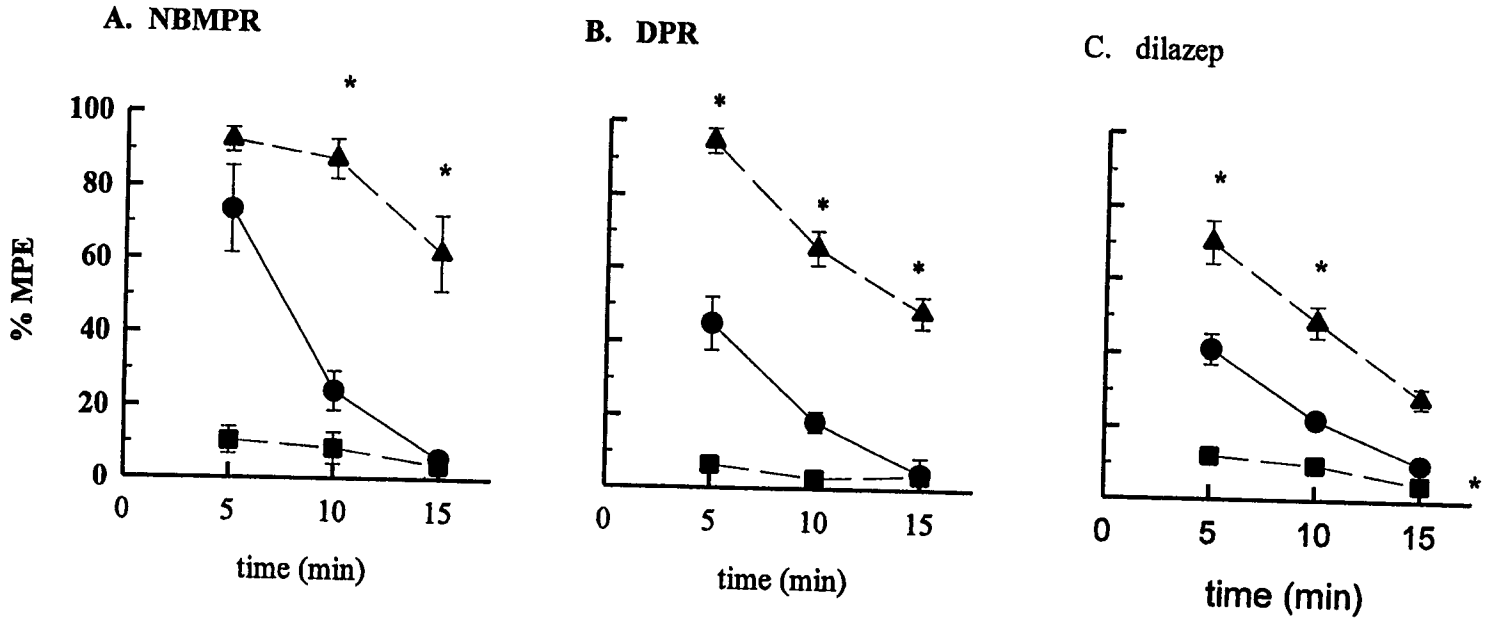

A
B
% MPE
60
40
20
0
3
5
7
10
20
30
50
Dose inhibitor (nmol)
0
5
10
15
20
25
Time (min)

Fig. IV-2. Effect of nucleoside transport inhibitor coadministration with exogenously administered (i.t.) adenosine hemisulfate. Adenosine hemisulfate (110 nmol) was administered alone (●) or with the nucleoside transport inhibitors (▲) NBMPR (12.5 nmol; A), DPR (5 nmol; B) or dilazep (12.5 nmol; C) and antinociception evaluated at the indicated time points. The effect of each nucleoside transport inhibitor (■) alone was not significantly different from control administration of vehicle solutions. * $P <$ .05 compared with the additive value of the nucleoside transport inhibitor alone and adenosine-induced antinociception in nucleoside transport inhibitor vehicle. N = 7 to 10 mice for each group.

Fig. IV-2.



antinociception was: NBMPR ≥ DPR > dilazep. Adenosine-mediated antinociception in the presence of NBMPR, DPR or dilazep returned to control values by approximately 40 min (data not shown).

Effect of nucleoside transport inhibition on opioid-induced antinociception

Previous studies have shown morphine releases adenosine *per se* (Sawynok *et al.*, 1989; Cahill *et al.*, 1993) via nucleoside transport systems (Sweeney *et al.*, 1993). Because inhibition of nucleoside transporters could inhibit both adenosine efflux and reuptake, various treatment protocols, therefore, were conducted to investigate if pre-, co- or post-treatment with nucleoside transport inhibitors effect opioid-mediated antinociception differently.

NBMPR, DPR and dilazep pretreatment significantly inhibited antinociception induced by the relatively *mu*-selective opioid receptor agonist, morphine (Fig. IV-3), and selective agonist, DAMGO (Fig. IV-4), in all experiments except for DPR administration with morphine. The effect of nucleoside transport inhibitor coadministration on *mu* opioid agonist-induced antinociception was less clear. NBMPR coadministration significantly inhibited morphine-induced antinociception (Fig. IV-3), but no other significant effects were observed. NTS(s) inhibitor posttreatment, however, generally potentiated morphine-(Fig. IV-3) and DAMGO-(Fig. IV-4) induced antinociception. Statistically significant increases were observed for all experiments except DPR posttreatment on DAMGO (Fig. IV-4) or dilazep posttreatment on morphine (Fig. IV-3).

Administration of deltorphin II with nucleoside transport inhibitors, regardless of the administration protocol, generally resulted in enhanced *delta* opioid agonist-induced

Fig. IV-3. Morphine(i.t.)-induced antinociception following various coadministration protocols with nucleoside transport inhibitors. Vehicles or graded doses of the nucleoside transport inhibitors, NBMPR, DPR or dilazep were administered (i.t.) 10 min before (○), coadministered with (▽) or 5 min following (□) i.t. administration of either an ED_{80} (400 pmol; pretreatment and coadministration experiments) or an ED_{50} (100 pmol; post-treatment experiments) dose of morphine sulfate. Effects of either 400 or 100 pmol morphine sulfate were normalized to 100 % to aid in displaying inhibitory (< 100 %) or enhancing (> 100 %) effects of nucleoside transport inhibition (see Materials and Methods for description). * $P < .05$ for antinociception induced by morphine following administration of nucleoside transport inhibitor vehicle. N = 7 to 10 mice for each point.

Fig. IV-3.

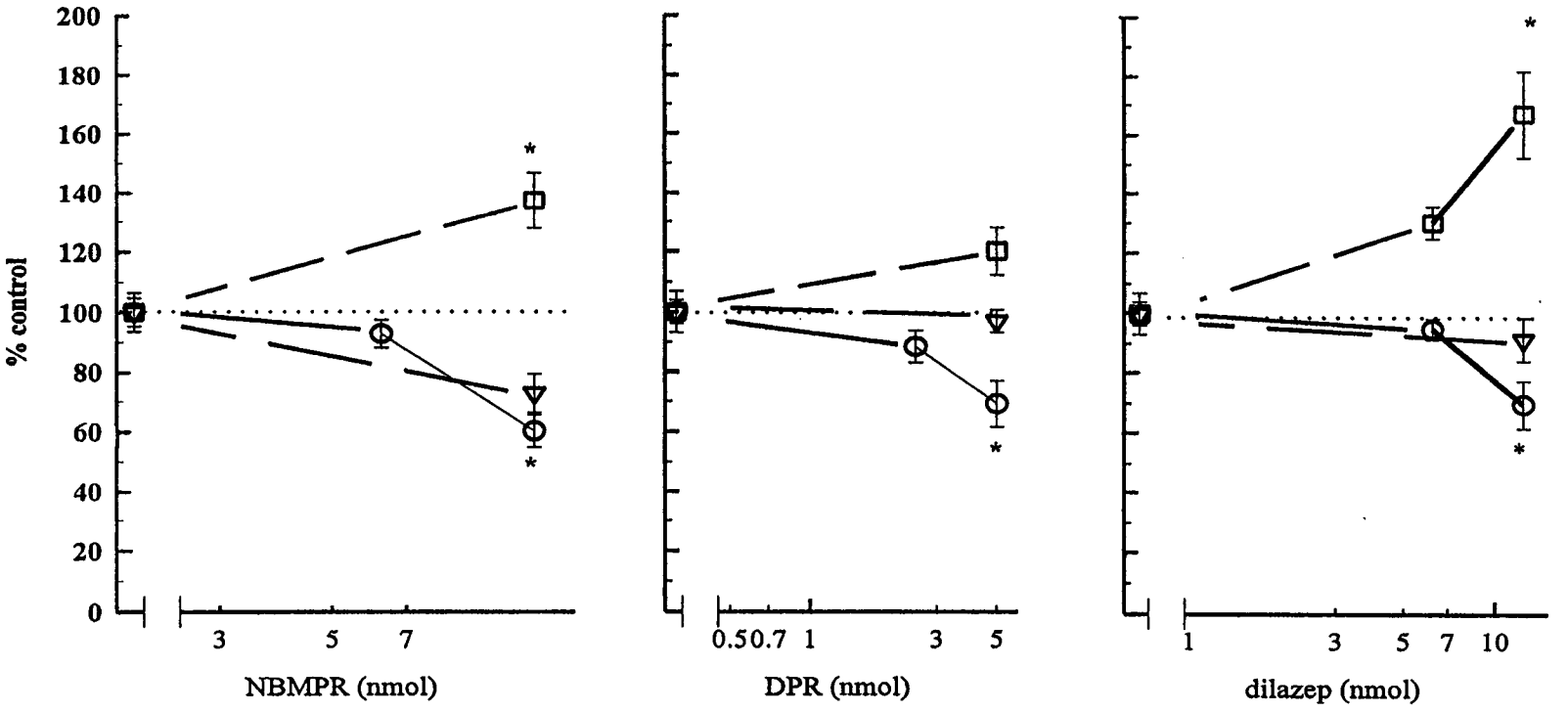
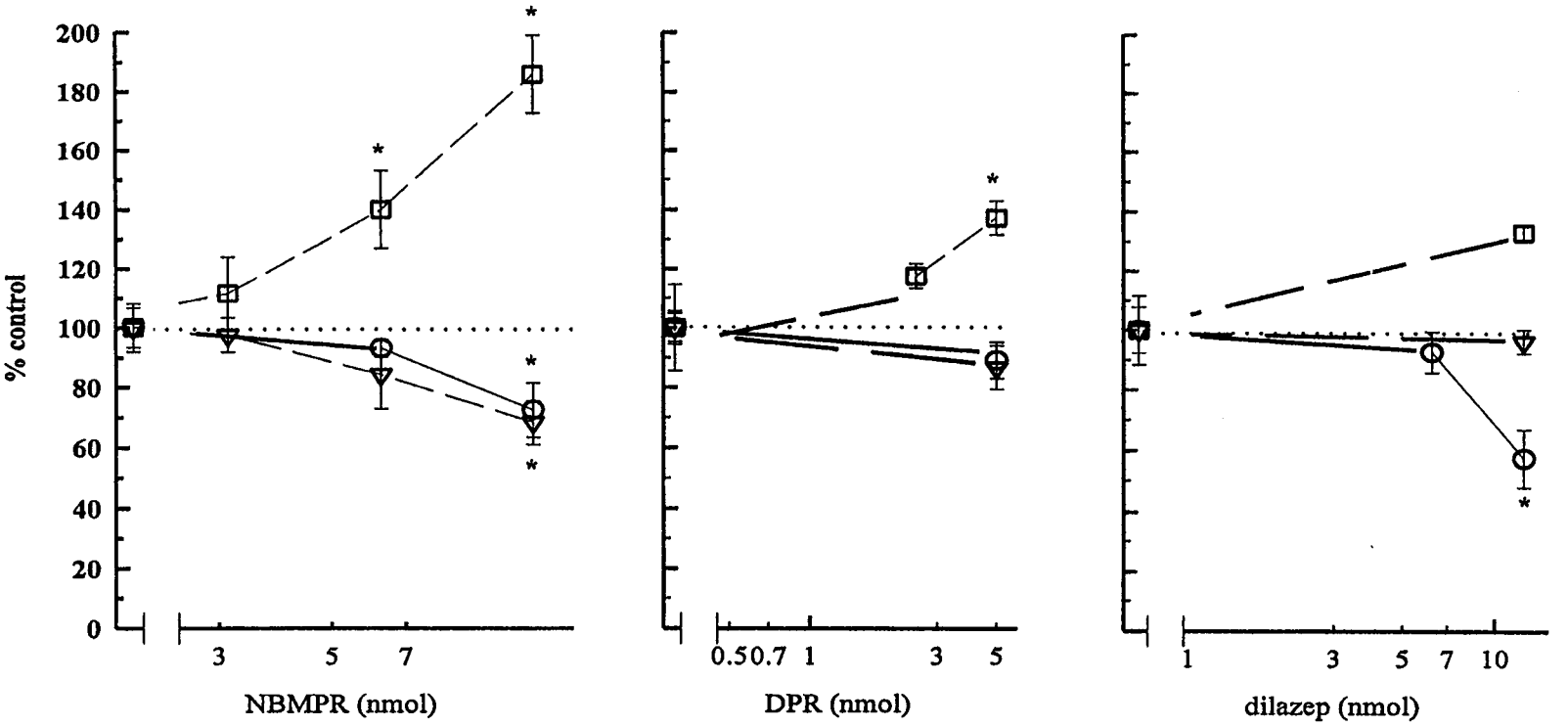

200
180
160
140
120
100
80
60
40
20
0
% control
*
*
3
5
7
NBMPR (nmol)
0.5
0.7
1
3
5
DPR (nmol)
*
*
1
3
5
7
10
dilazep (nmol)

Fig. IV-4. DAMGO(i.t.)-induced antinociception following various coadministration protocols with nucleoside transport inhibitors. Vehicles or graded doses of the nucleoside transport inhibitors, NBMPR, DPR or dilazep were administered (i.t.) 10 min before (○), concurrently with (▽) or 5 min following (□) the i.t. administration of either an ED_{80} (20 pmol) or an ED_{50} (5 pmol) dose of DAMGO (see legend fig. 3). * $P < .05$ for antinociception induced by DAMGO following administration of nucleoside transport inhibitor vehicle. N = 7 to 10 mice for each point.

Fig. IV-4.


% control
200
180
160
140
120
100
80
60
40
20
0
3
5
7
NBMPR (nmol)
0.5
0.7
1
3
5
DPR (nmol)
1
3
5
7
10
dilazep (nmol)

antinociception (Fig. IV-5). Significant potentiation of deltorphin II-induced antinociception was demonstrated following every treatment protocol except for pretreatment with dilazep (Fig. IV-5). Inhibitory or enhancing effects of nucleoside transport inhibitors on opioid-mediated antinociception are summarized in Table IV-1.

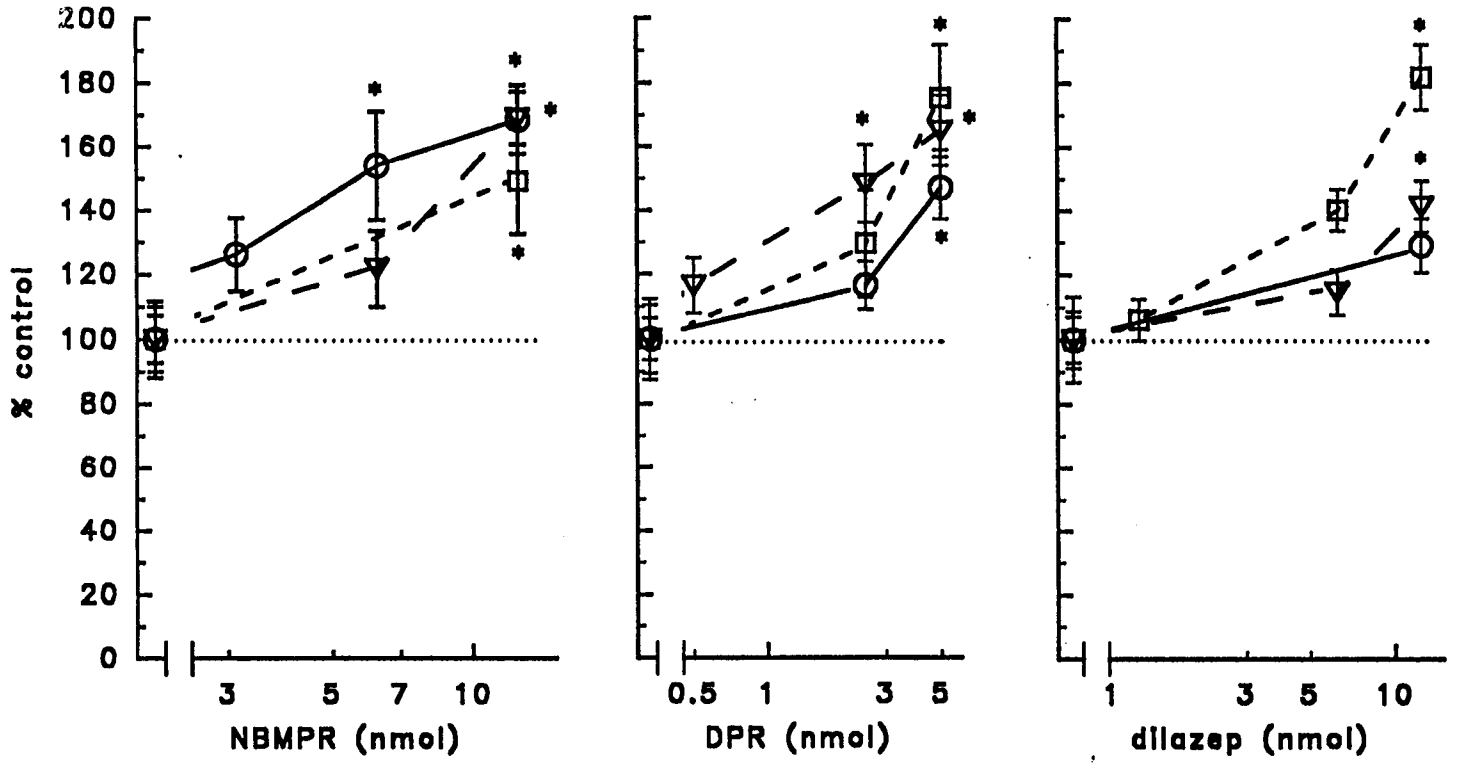
Discussion

Spinal purinergic systems are involved in synaptic transmission in the dorsal horns of the spinal cord (Salter *et al.*, 1993; Yaksh and Malmberg, 1994). Release of nucleotide(s) (i.e., ATP) are thought to be involved in non-noxious, low threshold primary afferent neurotransmission (Fyffe and Perl, 1984) and to be the primary source (up to 70%) of extracellular adenosine from spinal cord synaptosomes under basal conditions (Sweeney *et al.*, 1987b). Inhibition of the nucleoside transport systems might be hypothesized, therefore, to result in increased extracellular adenosine levels and to possibly induce antinociception.

Intrathecal administration of nucleoside transport inhibitors, in general, did not induce significant antinociception in the mouse tail flick assay. At the highest doses tested, DPR and NBMPR induced only slight, nonsignificant effects. The more water-soluble nucleoside transport inhibitor, dilazep, however, induced both time- and dose-dependent antinociception, indicating nucleoside transport systems may be involved in the reuptake of tonically-released nucleotide-derived adenosine. Inhibition of this uptake could allow greater synaptic adenosine levels and subsequently increased activation of extracellular adenosine receptors. It was not possible, however, to verify that dilazep-mediated antinociception was a result of activation of extracellular adenosine receptors.

Fig. IV-5. Deltorphin II(i.t.)-induced antinociception following various coadministration protocols with nucleoside transport inhibitors. Vehicles or graded doses of the nucleoside transport inhibitors, NBMPR, DPR or dilazep were administered (i.t.) 10 min before (○), concurrently with (▽) or 5 min following (□) the i.t. administration of an ED_{50} (2.5 nmol) dose of deltorphin II. The effect 2.5 nmol deltorphin II was normalized to 100 % to aid in expressing the enhancing (> 100 %) effects of nucleoside transport inhibition. * $P < .05$ for antinociception induced by deltorphin II following administration of nucleoside transport inhibitor vehicle. N = 7 to 10 mice for each point.

Fig. IV-5.


200
180
160
140
120
100
80
60
40
20
0
% control
3
5
7
10
NBMPR (nmol)
0.5
1
3
5
DPR (nmol)
1
3
5
10
dilazep (nmol)

Coadministration of dilazep with the adenosine receptor antagonist, theophylline, induced profound biting and scratching behavior. Delayed biting and scratching behavior induced by dilazep alone suggests dilazep may also influence an, as yet poorly characterized, basal release of adenosine via the nucleoside transport systems at later time points or have additional effects with delayed peak activity. Because biting and scratching behavior was not observed following NBMPR or DPR administration, nucleoside transport inhibitors may vary in their capacity to inhibit adenosine uptake and efflux.

Sweeney *et al.* (1993) demonstrated DPR, but not NBMPR, significantly enhanced basal adenosine levels in rat spinal cord synaptosome effluent and blocked uptake of exogenous adenosine. Both agents were effective at inhibiting morphine- and K^+-evoked adenosine release. Although others have shown DPR and NBMPR may not bind to identical sites on nucleoside transporters (Deckert *et al.*, 1988) and that NBMPR-insensitive transport systems exist (Shank and Baldy, 1990), it is unclear which transporter(s) dilazep inhibits. The absence of DPR- and NBMPR-induced anti-nociception in the present studies may be due to the inability of these compounds to inhibit multiple adenosine transporters in this heterogeneous family of transport systems. Support that inhibition of multiple transport systems must occur before significant extracellular adenosine levels accumulate include results from experiments by Ballarín *et al.* (1991) who demonstrated modest but significant increases in adenosine release from brain were seen following DPR administration, while profound increases were detected following coadministration of DPR, NBMPR and lidoflazine. Perhaps dilazep, but not NBMPR or DPR, inhibits multiple spinal nucleoside transport systems to significantly elevate basal extracellular adenosine levels required to induce antinociception. Because

dilazep was relatively weak at altering opioid-mediated antinociception compared to NBMPR or DPR (see discussion below), basal adenosine reuptake and opioid-evoked adenosine release may occur by different transport systems. Alternatively, dilazep has been shown to inhibit processes [i.e., Ca^{+2} channels (Nakagawa *et al.*, 1986)] other than nucleoside transporters. These additional actions may or may not explain dilazep-mediated antinociception and/or the delayed biting and scratching effects not seen with DPR or NBMPR.

Spinal administration of NBMPR, DPR and dilazep each had significant effects on spinal opioid- and adenosine-mediated antinociception. Pretreatment of nucleoside transport inhibitors significantly reduced morphine- and DAMGO-induced antinociception in the present studies. Morphine-stimulated adenosine release occurs via nucleoside transport system(s) (Sweeney *et al.*, 1993). Therefore, nucleoside transport inhibitor pretreatment would result in diminished *mu* opioid receptor-mediated antinociception. Post-treatment with nucleoside transport inhibitors, however, uniformly increased morphine- and DAMGO-induced antinociception. These latter results suggest inhibition of the reuptake of *mu* opioid-mediated adenosine release can prolong or enhance antinociception following *mu* opioid receptor activation. Our laboratory has previously suggested spinal NBMPR may also potentiate i.c.v. morphine-induced antinociception by inhibiting the reuptake spinal adenosine evoked by i.c.v. morphine (DeLander and Hopkins, 1987a). Decreased morphine- and DAMGO-induced antinociception by nucleoside transport inhibitor pretreatment and results from earlier studies in our laboratory showing adenosine receptor antagonism induces a parallel rightward shift of the dose-response curves for both morphine and DAMGO (DeLander *et al.*, 1992)

provide further support of the hypothesis that spinal adenosine release is a significant component of *mu* opioid receptor-mediated antinociception (see Sawynok *et al.*, 1989). Significantly enhanced effects with nucleoside transport inhibitor post-treatment suggest inhibition of the clearance of opioid-evoked adenosine release can result in significant potentiation of *mu* opioid receptor agonist-mediated antinociception.

In contrast to the time-dependent effects of nucleoside transport inhibitors on *mu* opioid receptor-mediated antinociception, nucleoside transport inhibition significantly increased *delta* opioid receptor-mediated antinociception, regardless of the time of nucleoside transport inhibitor administration. Our laboratory has previously demonstrated synergistic antinociceptive interactions between adenosine receptor and *delta* opioid receptor agonists (Chapter II). Increased endogenous adenosine levels following adenosine kinase inhibition also results in supra-additive interactions with *delta* opioid receptor agonists to induce antinociception (Chapter II). These results suggest adenosine and *delta* opioid receptor-mediated antinociception occur via distinct mechanisms and prior nucleoside transport inhibition would not likely inhibit *delta* opioid receptor-mediated antinociception. Based on observations that adenosine receptor antagonism shifts *delta* opioid agonist-induced antinociception in a non-parallel fashion (DeLander *et al.*, 1992), however, we have proposed a basal purinergic 'tone' may be important for full efficacy at *delta* opioid receptor agonists (Chapter II). Decreases in this purinergic 'tone' might decrease, while increases might enhance *delta* opioid receptor agonist-induced antinociception. Results from the present experiments suggest nucleoside transport inhibitors can increase this spinal purinergic 'tone' by inhibiting adenosine reuptake, resulting in enhanced antinociception.

Other proposed pharmacological actions of spinally administered nucleoside transport inhibitors do not adequately explain the results of the current investigations. In other brian areas, nucleoside transport inhibitor-induced vasodilation is well characterized (Zhang *et al.*, 1991). In the spinal cord, adenosine receptor agonists increase spinal cord blood flow (Karlsten *et al.*, 1992); this vasodilatory action of adenosine receptor agonists, however, is apparently unrelated to adenosine analog-induced antinociception. Vasodilation following nucleoside transport inhibition in the present study could explain the decreases of morphine- and DAMGO-mediated antinociception if the 'clearance' of these agents were accelerated via increases of spinal cord blood flow. Decreases of deltorphin II-mediated antinociception would be expected as well if changes in spinal cord blood flow significantly alter opioid agonist clearance. Pre- or post-treatment, as well as coadministration, of nucleoside transport inhibitors resulted only in enhanced *delta* opioid receptor-mediated antinociception, suggesting alterations of local blood flow are not significant in nucleoside transport inhibitor-mediated decreases in morphine- and DAMGO-induced antinociception.

In conclusion, these studies extend our understanding of the role of spinal adenosine in nociceptive processing. Treatment of animals with nucleoside transport inhibitors after administration of opioid receptor agonists generally enhanced opioid-mediated effects. These observations are consistent with the hypothesis that adenosine plays a role in opioid-induced analgesia and inhibition of adenosine reuptake can enhance opioid actions. Potential use of nucleoside transport inhibitors as adjuncts in opioid-induced analgesia, however, should be tempered by the recognition that interactions between all opioids and all nucleoside transport inhibitors are not identical. Depending

upon the specific opioid, nucleoside transport inhibitor and timing of drug administration, inhibition of opioid-induced antinociception may also be observed. Finally, differences observed for interactions between nucleoside transport inhibitors and specific opioid receptor agonists confirms our earlier observations concerning the relative involvement of adenosine in the mechanisms of action for antinociception induced by *mu* vs *delta* opioid receptor agonists.

TABLE IV-1. Summary of the effects of nucleoside transport inhibitor administration on adenosine- and opioid-induced antinociception[a].

Nucleoside Transport Inhibitor		MORPHINE	DAMGO	Delt II	Adenosine
NBMPR	pretreatment	↓	↓	↑	
	coadministration	↓	↔	↑	↑
	posttreatment	↑	↑	↑	
DPR	pretreatment	↔	↓	↑	
	coadministration	↔	↔	↑	↑
	posttreatment	↑	↔	↑	
dilazep	pretreatment	↓	↓	↔	
	coadministration	↔	↔	↑	↑
	posttreatment	↔	↑	↑	

[a] ↓ - significant inhibition; ↑ - significant potentiation; ↔ - no significant effect

CHAPTER V

ALTERATIONS IN SPINAL SENSORY AFFERENT PROCESSING: BEHAVIORAL EFFECTS FOLLOWING ADMINISTRATION OF ADENOSINE RECEPTOR ANTAGONISTS

Abstract

Previous studies in our laboratory demonstrate exogenous adenosine receptor agonists or inhibitors of endogenous adenosine-regulating pathways induce antinociception in a wide variety of antinociceptive assays. While characterizing spinal adenosine involvement in opioid-induced antinociception, we observed caudally-directed biting and scratching behavior following spinal administration of adenosine receptor antagonists. Importantly, this behavior occurs in the absence of additional sensory stimuli suggesting administration of adenosine receptor antagonists may block an endogenous inhibitory adenosine tone. Inhibition of this tone could lead to facilitated spinal nociceptive neurotransmission which manifests as biting and scratching behavior; similar to that observed following administration (i.t.) of excitatory amino acids or substance P. Inhibitors of adenosine-regulating systems, in the present investigations, inhibited biting and scratching behavior induced by spinally-administered excitatory amino acids or substance P. Inhibition of adenosine kinase, but not adenosine deaminase, significantly decreased biting and scratching induced by substance P and kainic acid. Adenosine kinase inhibition was less effective against AMPA-induced behavior, while no effects on NMDA-induced behavior was seen. Inhibition of biting and scratching following adenosine kinase inhibition was reversed by low doses of the adenosine receptor antagonist, theophylline. At higher doses, theophylline and 8-*p*(sulphophenyl)

theophylline (8-*p*SPTheo) induced dose- and time-dependent biting and scratching behavior similar to the excitatory amino acids or substance P. Theophylline- and 8-*p*SPTheo-induced behavior was inhibited by the A_1-selective adenosine receptor agonist, CPA, or the non-selective agonist, NECA, at various time points. NECA, however, failed to significantly reduce the overall behavior induced by 8-*p*SPTheo. Coadministration of excitatory amino acid antagonists or the tachykinin antagonist, spantide, had varying effects on theophylline-induced biting and scratching behavior. These studies further support the antinociceptive effects of endogenous adenosine and extend earlier results from our laboratory. Nociceptive behavior, involving both excitatory amino acid and tachykinin systems, is observed following spinal adenosine receptor antagonist administration. This behavior indicates a spinal adenosine "tone" may exist. Increases in this "tone" may inhibit, while decreases in spinal adenosine "tone" may disinhibit excitatory neurotransmission.

Introduction

Adenosine and adenosine analogs induce a wide variety of effects in the central and peripheral nervous systems. In general, adenosine receptor activation inhibits neuronal activity in many areas along the neuraxis (Kostopoulos and Phillis, 1977; Phillis and Wu, 1981; Stone, 1991; Snyder, 1985). Adenosine receptor agonists induce antinociception following systemic (Vapaatalo *et al.*, 1975; Holmgren *et al.*, 1983; Ahlijanian and Takemori, 1985), intracerebroventricular (Yarbrough and McGuffin-Clineschemidt, 1981; Mantegazza *et al.*, 1984; Herrick-Davis *et al.*, 1989) or intrathecal (i.t.) (Post, 1984; Holmgren *et al.*, 1986; Sawynok *et al.*, 1986; DeLander and Hopkins

1986, 1987a,b) administration. Radioligand binding studies suggesting the presence of both A_1 and A_2 adenosine receptors (Geiger *et al.*, 1984; Bruns *et al.*, 1986; Choca *et al.*, 1987), localization of adenosine deaminase (Geiger and Nagy, 1986), nucleoside transporters (Geiger and Nagy, 1985) and localization of adenosine-like immunoreactivity in the substantia gelatinosa (Braas *et al.*, 1986) further support a role for spinal adenosine in the modulation of sensory afferent input.

Administration of adenosine receptor agonists (i.t.) induces antinociception in several traditional behavioral assays for antinociception including tail flick and hot plate tests in mice (Post, 1984; DeLander and Hopkins, 1986, 1987a) and rats (Jurna, 1984; Holmgren *et al.*, 1986; Sawynok *et al.*, 1986), and inhibits biting and scratching behavior induced following i.t. administration of putative "pain" neurotransmitters (DeLander and Wahl, 1988). In addition, spinally administered adenosine analogs induce antinociception in several less "traditional" models including visceral nociception (Sosnowski *et al.*, 1989), phase 2 of the formalin test (Malmberg and Yaksh, 1993), thermal hyperalgesia following nerve compression (Yamamoto and Yaksh, 1991) and allodynia induced by i.t. strychnine (Sosnowski *et al.*, 1989) or prostaglandin $F_{2\alpha}$ (Minami *et al.*, 1992a). In human patients suffering from neuropathic pain, a recent study by Sollevi *et al.* (1995) demonstrated profound pain relief following intravenous administration of adenosine. Although this study did not investigate the site of adenosine-mediated analgesia, the spinal cord may be involved.

Facilitated nociceptive neurotransmission has been reported following adenosine receptor antagonist administration. Systemic administration of adenosine receptor antagonists facilitate a supraspinally integrated nociceptive threshold in awake rats

(Paalzow and Paalzow, 1973; Paalzow, 1994). Spinal administration of adenosine receptor antagonists induces thermal hyperalgesia under certain experimental conditions (Jurna, 1984; Sawynok *et al.*, 1986). Lastly, a recent report by Nagaoka *et al.* (1993) indicates spinal blockade of adenosine receptors may induce nociceptive behaviors.

To further examine the role of spinal adenosine in modulating sensory afferent input, experiments were conducted to evaluate if inhibitors of adenosine-regulating systems inhibit behavior induced by putative "pain" neurotransmitters. In addition, the potential for adenosine receptor antagonists administered spinally to induce aversive behavior was evaluated. Results from the present study support the hypothesis that an inhibitory spinal purinergic "tone" exists regulating sensory afferent input. Manipulations which increase this "tone" might block behavior induced by putative "pain" neurotransmitters. Manipulations which decrease this "tone", however, can lead to disinhibition of excitatory systems and facilitated nociceptive neurotransmission.

Materials and methods

Assays for nociception/antinociception

Caudally-directed biting and scratching behavior induced by i.t. injection of excitatory amino acids or substance P was quantitated as a measure of the degree of nociception induced by these putative pain neurotransmitters (see General Methods). The antinociceptive effect of inhibitors of adenosine-regulating systems to antagonize the behavior induced by the EAA agonists or substance P was determined following administration (i.t.) of the adenosine kinase inhibitor, 5'-amino 5'-deoxyadenosine (5'-NH_2-dAdo), or the adenosine deaminase inhibitor, 2'-deoxycoformycin (dCF).

Experimental protocol

Biting and scratching

Because previous studies (Paalzow and Paalzow, 1973; Paalzow, 1994; Jurna, 1984; Sawynok *et al.*, 1986; Nagaoka *et al.*, 1993; Chapter 2) suggest an inhibitory purinergic "tone" may exist, aversive behavioral effects of i.t. adenosine receptor antagonists were evaluated. Mice were injected with non-selective adenosine receptor antagonists, theophylline and 8-*p*-sulphophenyl theophylline (8-*p*SPTheo) intrathecally and caudally-directed biting and scratching was evaluated. Unlike behavior induced by the EAAs or substance P, adenosine antagonist-induced effects were persistent, lasting approximately for 10 min. Behavior induced by theophylline and 8-*p*SPTheo was quantitated over their entire duration of action.

Mice were coadministered theophylline with antagonists of EAA and NK-1 receptors to evaluate the possible involvement of EAA or NK-1 (tachykinin) receptor activation following spinal adenosine receptor antagonism. Caudally-directed biting and scratching behavior induced by theophylline was evaluated for 10min following administration alone or with D-2-amino-5-phosphonovaleric acid (APV), (±)-5-methyl-10,11-dihydro-5H-dibenzo(a,d) cycloheptene-5,10-imine maleate (MK-801), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 4-hydroxyquinoline-2-carboxylic acid (kynurenate), or (D-Arg^{1}, D-Trp^{7,9}, Leu^{11}) substance P (spantide).

Allodynia

Preliminary investigations were also conducted examining the potential allodynic effects of spinal adenosine receptor antagonists. Mice were administered adenosine

receptor antagonists (i.t.) or vehicle and immediately placed in a clear plexiglass observation chamber (12" X 12"). The magnitude of behaviors was carried out according to the method of Yaksh and Harty (1988). Briefly, the behavioral responses to light stroking of the hind quarters was assessed at various time points by light stroking of the flank of the mice with a smooth glass rod. The allodynic response [also known as touch-evoked agitation (TEA)] was ranked as follows: 0, no response; 1, mild squeaking with attempts to move away from the stroking probe; or 2, vigorous squeaking evoked by the stroking probe, biting at the probe and strong efforts to escape. Each mouse was tested every 5 min for 20 min, then at times 30, 45, 60 and 90 min. Control mice were tested for behaviors at each testing point. No behavior was observed in control animals; results for control groups, therefore, are not shown of Fig. V-8 for clarity purposes.

Drugs and drug administration

All drugs were solubilized and injected i.t. in a total volume of 5 μl. For coadministration studies, compounds were mixed together in solution. For studies involving multiple i.t. injections, drugs or the corresponding vehicles were administered at the times indicated. Administration protocols were such that the peak effect of each compound occurred simultaneously. Neither drug vehicle nor drug administration protocols had significant effects on control values. All compounds were dissolved in normal saline except 8pSPTheo (distilled water), CNQX (1.25% 2-Hydroxypropyl β-cyclodextrin) and kynurenate (0.1M NaOH).

Statistics

Comparisons between groups following drug treatments were performed by one way analysis of variance or Kruskal-Wallis analysis of variance on ranks followed by appropriate post-hoc *t*-tests. Critical values that achieved $P < .05$ were considered statistically significant.

Results

5'-NH_2dAdo (i.t.) pretreatment (10 min) dose-dependently inhibited caudally-directed biting and scratching behavior induced by kainic acid and substance P administered i.t. (Fig. V-1). 5'-NH_2dAdo (i.t.) partially inhibited biting and scratching induced by AMPA (maximal inhibition approx 50%) but was ineffective against behavior induced by NMDA at the highest dose of 5'-NH_2dAdo tested (1 nmol). Doses in this study and an earlier study (see Chapter III) of 5'-NH_2dAdo greater than 1 nmol induced slight hind limb flaccidity. Therefore, doses of 5'-NH_2dAdo were limited to 1 nmol or less to avoid possible motor effects. I.t. pretreatment (5 or 60 min) with an adenosine deaminase inhibitor, dCF (200 nmol), did not effect behavior induced by NMDA, AMPA, kainic acid or substance P (data not shown).

As shown in Fig. V-2, theophylline (6.25 - 55.5 pmol) dose-dependently reversed 5'-NH_2dAdo(1 nmol)-mediated inhibition of kainic acid- and substance P-induced biting and scratching. Importantly, theophylline reversed 5'-NH_2dAdo-mediated effects at doses which did not induce other behavioral effects alone (see below).

Higher doses of theophylline induced time- and dose-dependent biting and scratching behavior (Fig. V-3). Mice administered normal saline displayed typical

Fig. V-1. Inhibitory effects of the adenosine kinase inhibitor, 5'-NH_2dAdo, on nociceptive behavior induced by excitatory amino acids or substance P. Graded doses of 5'-NH_2dAdo were coadministered (i.t.) with doses of NMDA (△; 250 pmol), AMPA (□; 12.5 pmol), kainic acid (○; 60 pmol), or substance P (▽; 6 pmol) which induced approximately 80-100 behaviors (= 100% Control; C) over the first min of testing (mean ± s.e.m.; n = 7-10). * indicates significant reductions in excitatory amino acid- or substance P-induced effects ($P < .05$) compared to control values.

Fig. V-1.

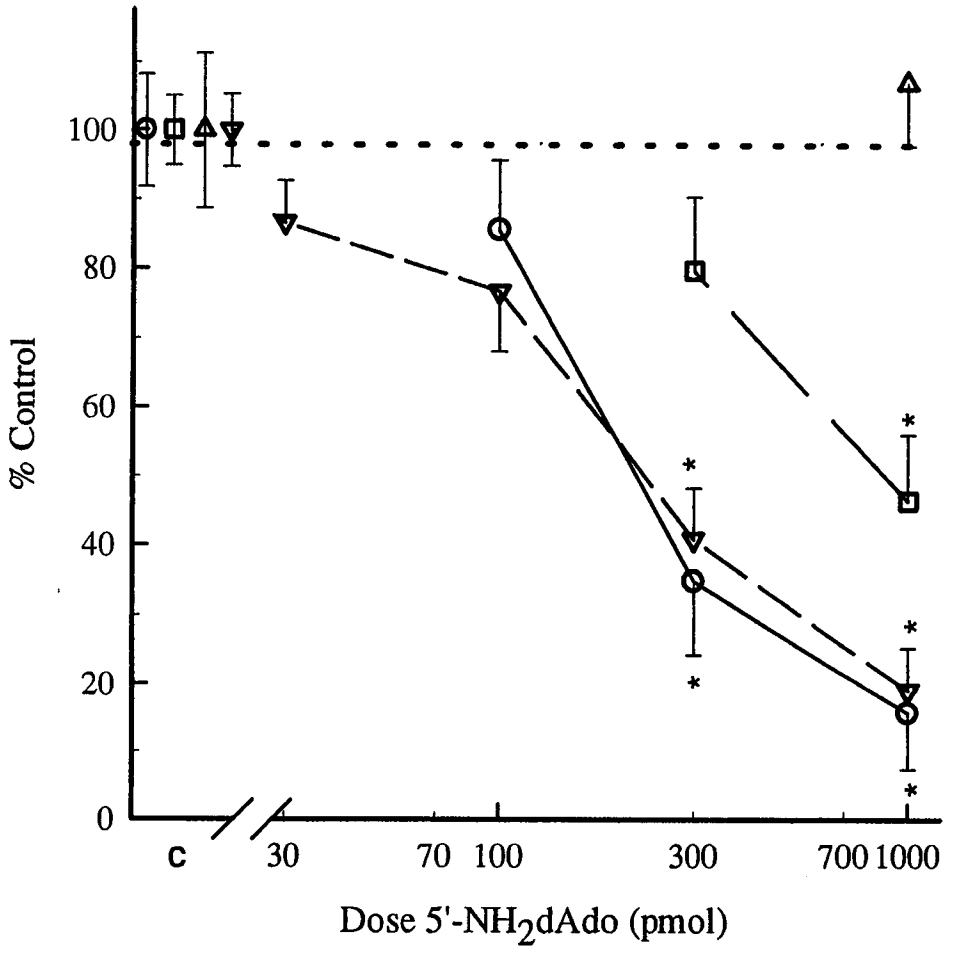

100
80
60
40
20
0
% Control
C
30
70
100
300
700
1000
Dose 5'-NH2dAdo (pmol)

Fig. V-2. Antagonism of 5'-NH_2dAdo-mediated inhibition of kainic acid- or substance P-induced biting and scratching by the adenosine receptor antagonist, theophylline. Saline (S) or graded doses of theophylline were coadministered (i.t.) with kainic acid (○; 60 pmol) or substance P (▽; 6 pmol) in the presence 5'-NH_2dAdo (1 nmol) and caudally-directed biting and scratching behavior (% Control) quantitated over the next minute (mean ± s.e.m.; n = 7-10). * indicates significantly increased biting and scratching behavior compared to behavior seen following coadministration of kainic acid or substance P and 5'-NH_2dAdo ($P < .05$).

Fig. V-2.

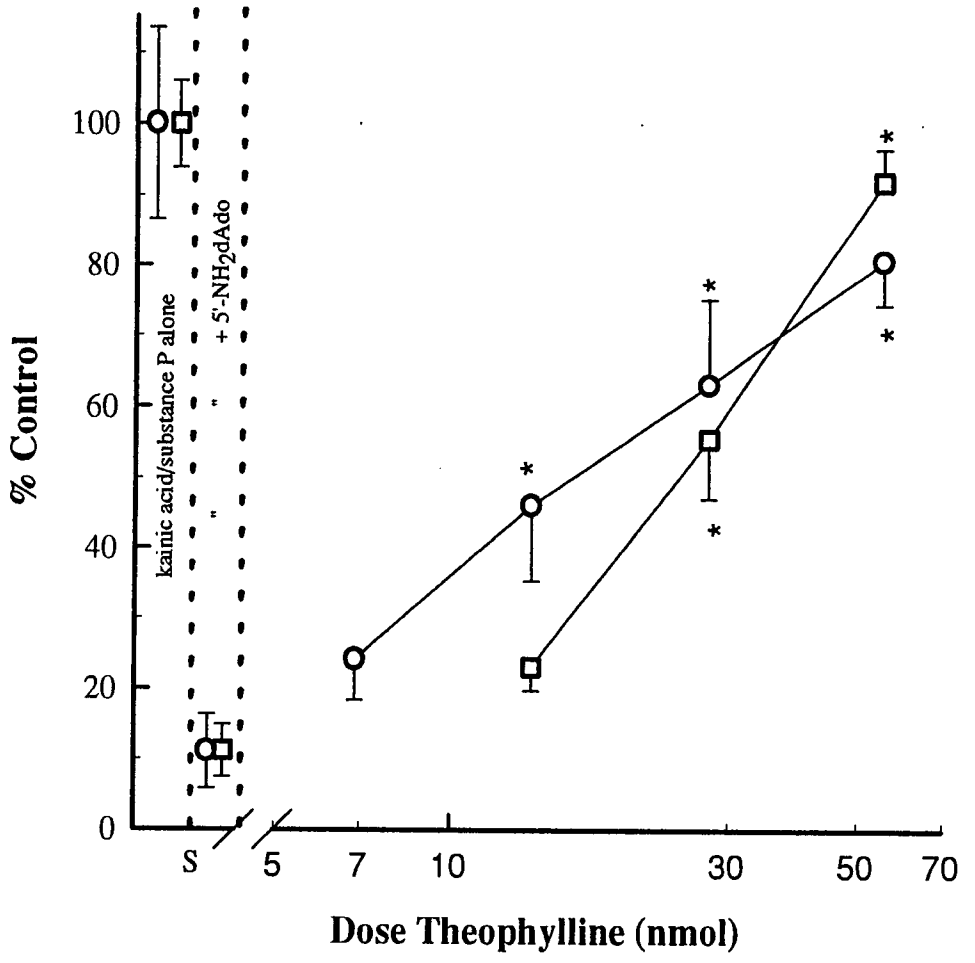
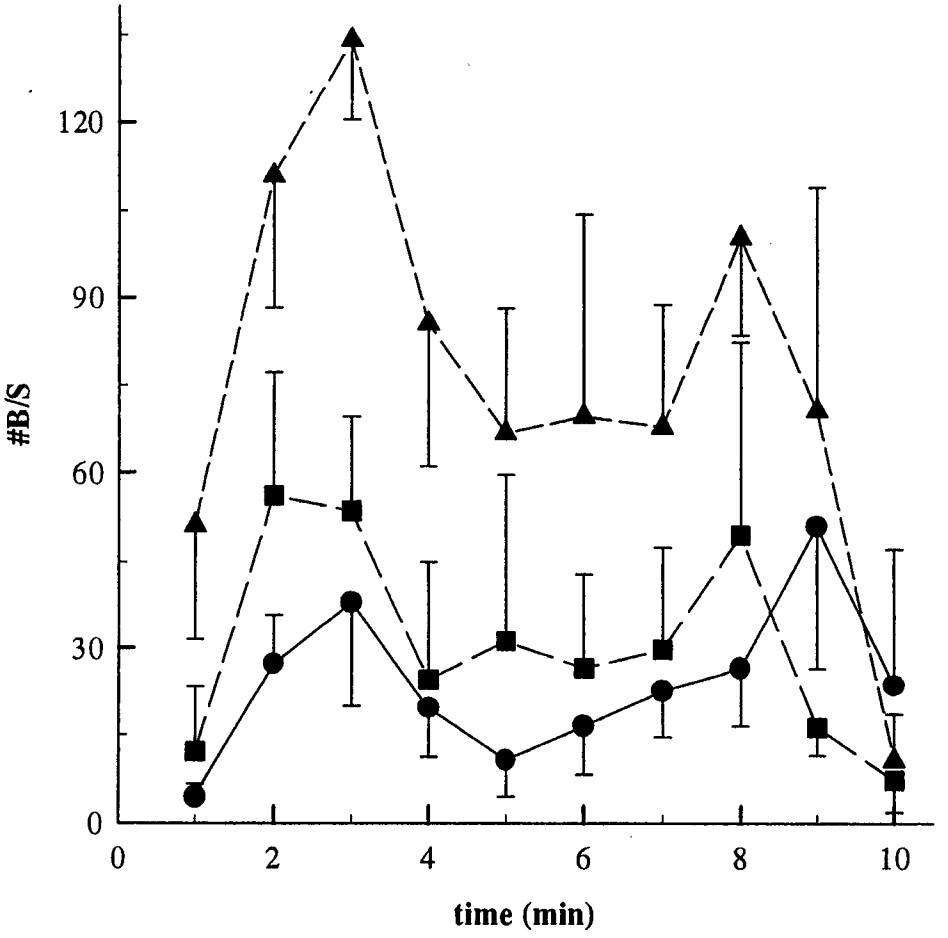

% Control
100
80
60
40
20
0
kainic acid/substance P alone
" + 5'-NH2dAdo
"
S
5
7
10
30
50
70
Dose Theophylline (nmol)

Fig. V-3. Caudally-directed biting and scratching behavior induced by the adenosine receptor antagonist, theophylline. Caudally-directed biting and scratching (#B/S) behavior (mean $\pm$ s.e.m.; n = 7-10) was quantitated each min following the administration (i.t.) of saline (●) or two doses of theophylline [111 nmol (■) or 222 nmol (▲)].

Fig. V-3.



"grooming" behavior commencing approximately 1 to 2 min following placement in the observation chamber. Such behavior was casual in nature, not forced, and vocalization was not observed at any timepoint. Grooming behavior which involved bites, licks or scratches toward the hind quarters or tail, however, was quantitated following normal saline administration in control animals. Increased biting and scratching behavior, and occasionally vocalization, was observed following i.t. adenosine receptor antagonist administration. Theophylline-induced biting and scratching was not significantly different than controls following administration of 111 nmol, but significantly increased behavior was evident approximately 3 min following injection of 222 nmol (Fig. V-3). Behaviors gradually returned to baseline values by approximately 10 min.

The more water-soluble antagonist, 8-*p*SPTheo, also induced caudally-directed biting and scratching behavior (Fig. V-4). Behavior induced by 100 nmol 8-*p*SPTheo was significantly increased during the first 5 min, but returned to control values by approximately 10 min. Average behavior (min^{-1}) induced by theophylline and 8-*p*SPTheo over the first 10 min are summarized in Table V-1. Preliminary investigations with the A_1-selective antagonist, DPCPX, resulted in slight biting and scratching behavior, but interpretation of results were complicated by significant vehicle effects (data not shown).

Pretreatment (5 min) with the A_1-selective adenosine receptor agonist, CPA (100 pmol), or the non-selective adenosine receptor agonist, NECA (25 pmol), in doses which significantly inhibit EAA- and SP-induced biting and scratching (DeLander & Wahl, 1988), had various effects on methylxanthine-induced behavior. Significant reductions of theophylline- (Fig. V-5) and 8-*p*SPTheo- (Fig. V-6)induced behavior at various time points were seen with adenosine receptor agonists. CPA significantly inhibited the biting

Fig. V-4. Caudally-directed biting and scratching behavior induced by the adenosine receptor antagonist, 8-*p*-sulphophenyl theophylline (8-*p*SPTheo). Caudally-directed biting and scratching (#B/S) behavior (mean ± s.e.m.; n = 7-10) was quantitated each min following the administration (i.t.) of distilled water (●) or increasing doses of 8-*p*SPTheo [25 nmol (■), 50 nmol (▲) or 100 nmol (▼)].

Fig. V-4.

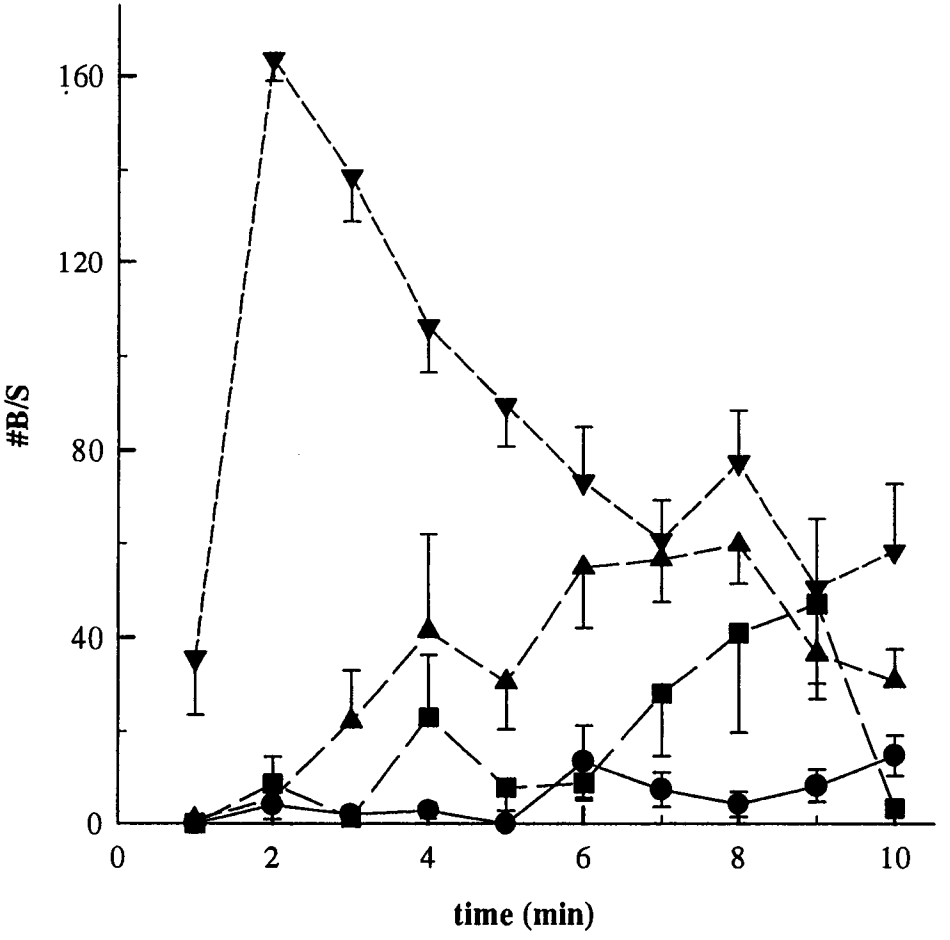


Fig. V-5. Inhibition of theophylline-induced nociceptive behavior by adenosine receptor agonists. Caudally-directed biting and scratching (#B/S) behavior (mean ± s.e.m.; n = 7-10) was quantitated each min following the administration (i.t.) of 222 nmol theophylline alone (●) or in the presence of (A) 100 pmol N^6-cyclopentyl adenosine (■) or (B) 25 pmol 5'-(N-ethyl)-carboxamido adenosine (■). * indicates significantly reduced biting and scratching behavior by the adenosine receptor agonists compared to behavior seen following theophylline administration alone ($P < .05$).

Fig. V-5.

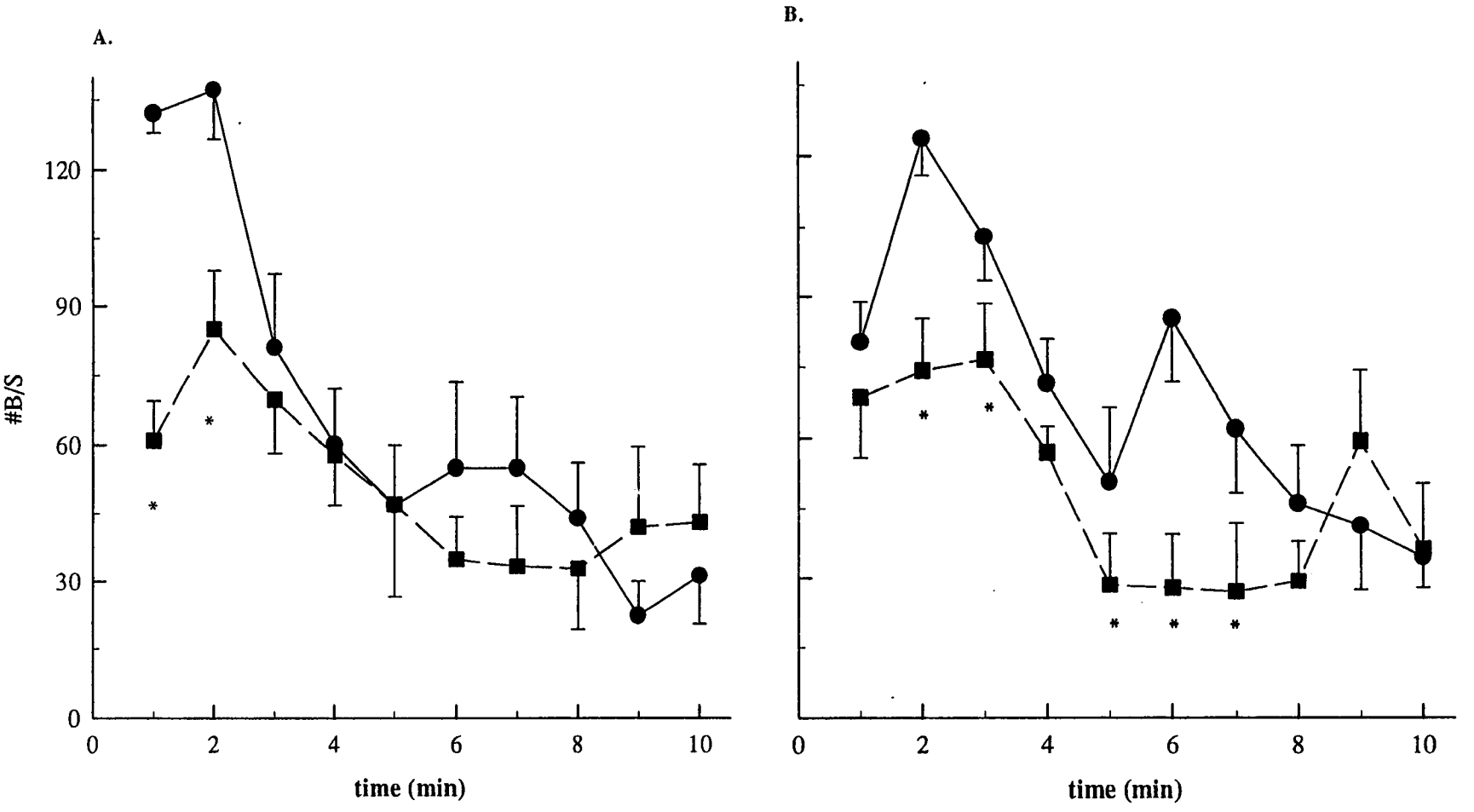
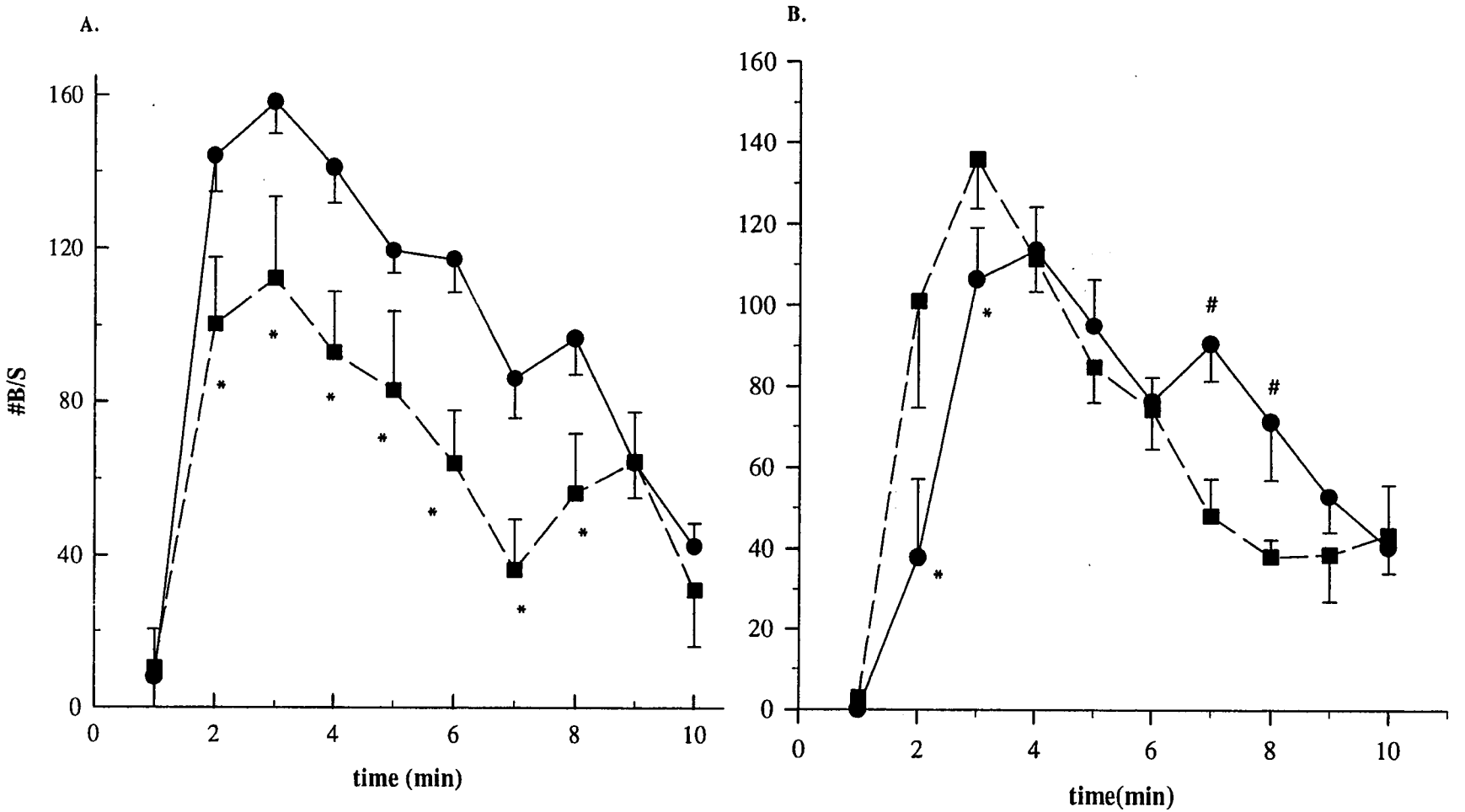


Fig. V-6. Inhibition of 8-*p*SPTheo-induced nociceptive behavior by adenosine receptor agonists. Caudally-directed biting and scratching (#B/S) behavior (mean ± s.e.m.; n = 7-10) was quantitated each min following the administration (i.t.) of 100 nmol 8-*p*SPTheo alone (●) or in the presence of (A) 100 pmol N^6-cyclopentyl adenosine (■) or (B) 25 pmol 5'-(N-ethyl)-carboxamido adenosine (■). * indicates significantly reduced biting and scratching behavior by the adenosine receptor agonists compared to behavior seen following 8-*p*SPTheo administration alone ($P < .05$).

Fig. V-6.


A.
B.
#B/S
time (min)
time(min)
0
40
80
120
160
0
20
40
60
80
100
120
140
160
0
2
4
6
8
10
*
#

and scratching behavior induced by theophylline and 8-*p*SPTheo and NECA significantly reduced theophylline-induced biting and scratching (Table V-1). NECA, however, failed to significantly reduce average behaviors induced by 8-*p*SPTheo (Table V-1), and appeared to decrease or increase behavior at selected time points (Fig. V-6B).

Coadministration of excitatory amino acid receptor antagonists or the neurokinin receptor antagonist, spantide, had varying effects on theophylline-induced biting and scratching behavior. Significant reductions were observed at selected time points following coadministration of theophylline with AP7, kynurenate, CNQX, and spantide (Fig. V-7 A, C and D), but the overall number of bites and scratches over 10 min was significantly reduced only following kynurenate and spantide administration (Table V-2). Coadministration of AP7 with spantide appeared to enhance the efficacy of spantide, significantly reducing bites and scratches induced by theophylline at most time points (Fig. V-7F) and decreasing average biting and scratching over the 10 min observation period (Table V-2).

In preliminary investigations, time- and dose-dependent tactile allodynia was observed following graded doses (i.t.) of adenosine receptor antagonists (Fig. V-8).

Discussion

Exogenous adenosine agonists regulate nociceptive processing at spinal levels (Sawynok and Sweeney, 1989; Salter *et al.*, 1993). Although multiple studies have been conducted characterizing the antinociceptive effects of exogenous adenosine receptor agonists, very few have investigated the involvement of endogenous purinergic systems.

Fig. V-7. Inhibition of caudally-directed biting and scratching induced by the adenosine receptor antagonist, theophylline, by antagonists at excitatory amino acid (EAA) or tachykinin receptors. Caudally-directed biting and scratching behavior (#B/S) was quantitated (mean ± s.e.m.; n = 7-10) every min following the administration (i.t.) of 222 nmol theophylline in the absence (○) or presence (□) of the EAA antagonists (A) *D*-2-amino-5-phosphonovaleric acid (AP7; 600 pmol), (B) (±)-5-methyl-10,11-dihydro-5H-dibenzo(a,d) cycloheptene-5,10-imine maleate (MK-801; 500 pmol), (C) 4-hydroxy-quinoline-2-carboxylic acid (kynurenate; 5 nmol), (D) 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 250 pmol), or (E) the tachykinin antagonist (*D*-Arg^{1}, *D*-Trp^{7,9}, Leu^{11}) substance P (spantide; 1 nmol), or (F) a combination of AP7 (600 pmol) plus spantide (1 nmol). * indicates significantly reduced, while # indicates significantly increased, biting and scratching behavior induced by theophylline in the presence of EAA or tachykinin antagonists ($P < .05$).

Fig. V-7.

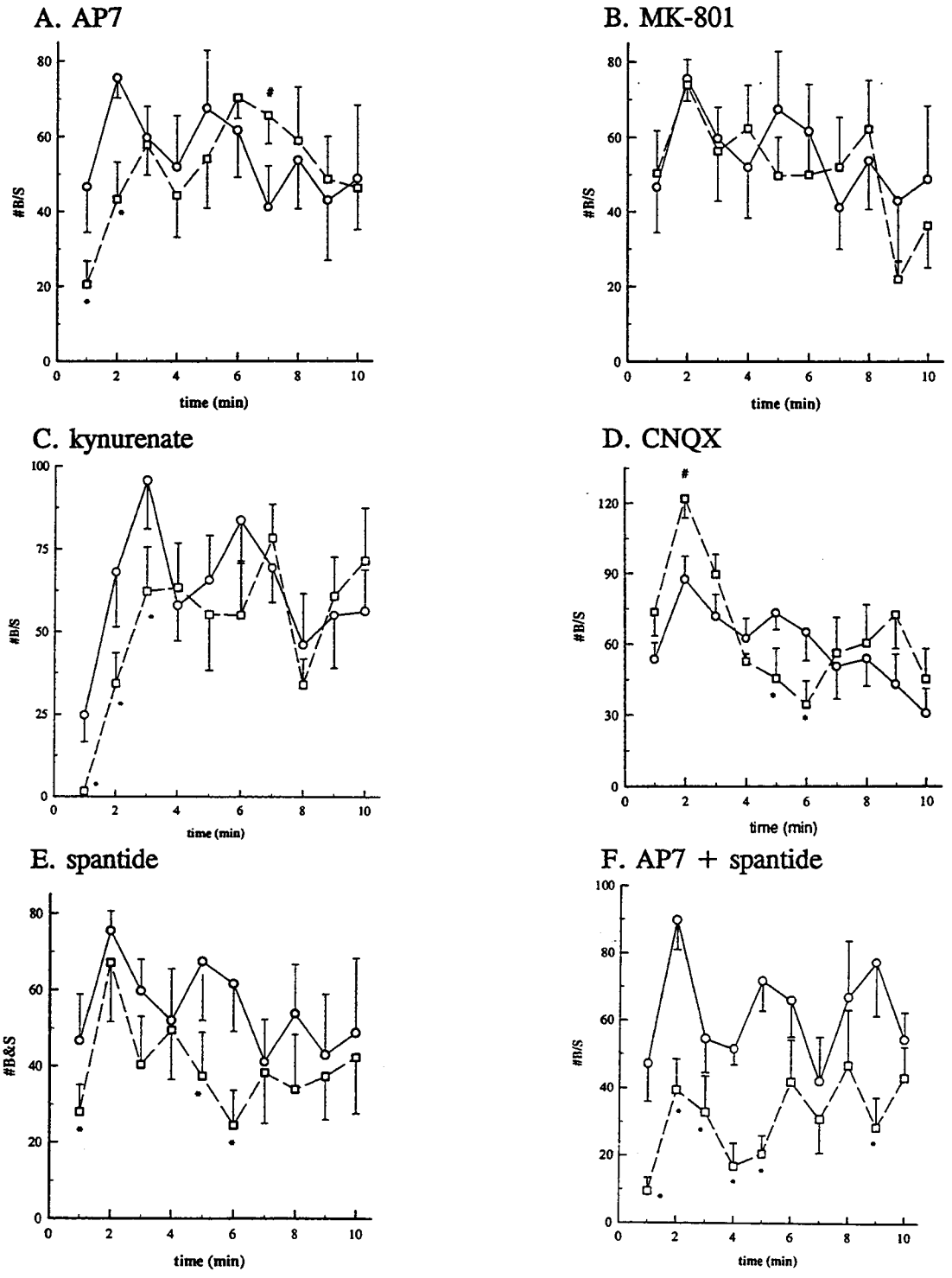
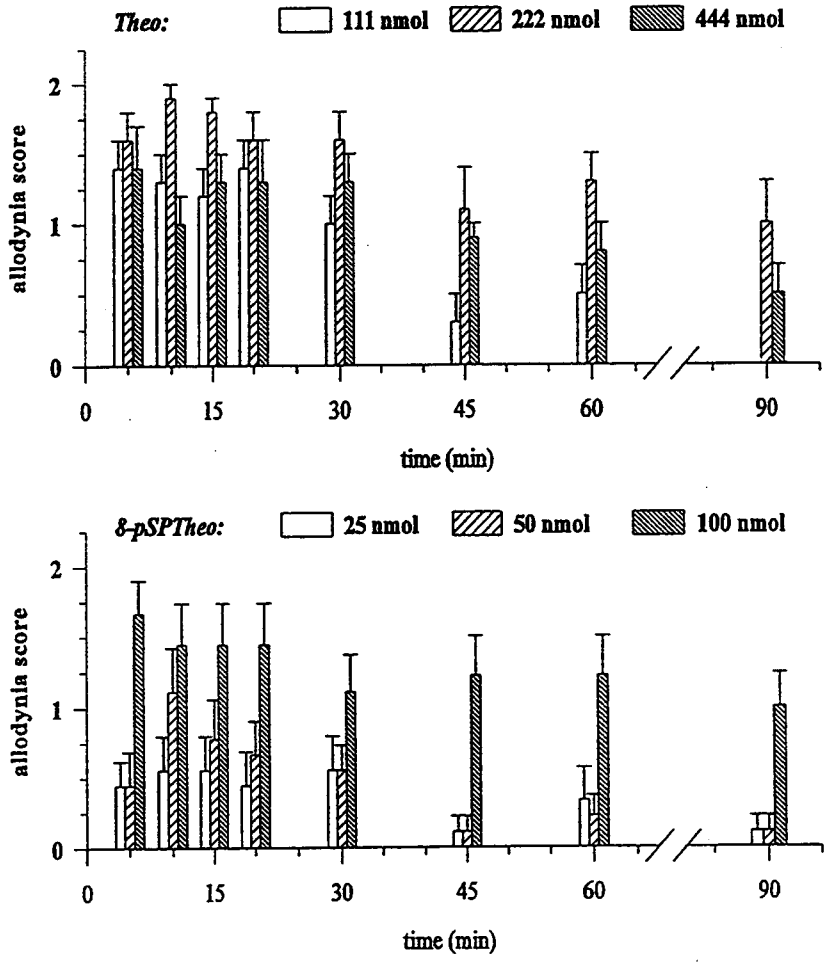

A. AP7
B. MK-801
C. kynurenate
D. CNQX
E. spantide
F. AP7 + spantide
#B/S
#B&S
time (min)

Fig. V-8. Methylxanthine-induced tactile allodynia following i.t. administration. Mice (n = 7-10) were administered vehicles (not shown) or graded doses of the adenosine receptor antagonists, theophylline of 8-*p*SPTheo, and tactile allodynia assessed at the time points indicated. Behaviors ($\pm$ s.e.m.) were evaluated on the 2 point system described by Yaksh and Harty (1988). No effect was seen by vehicles in any animal tested at any time point and are not shown for clarity. (see Materials and methods for details).

Fig. V-8.


Theo:
111 nmol
222 nmol
444 nmol
allodynia score
2
1
0
0
15
30
45
60
90
time (min)
δ-pSPTheo:
25 nmol
50 nmol
100 nmol
allodynia score
2
1
0
0
15
30
45
60
90
time (min)

Inhibition of spinal adenosine kinase activity by 5'-NH_2dAdo dose-dependently reduced behaviors induced by kainic acid and substance P, and partially inhibited AMPA-induced behavior. This theophylline-reversible effect suggests adenosine kinase inhibition might lead to decreased adenosine uptake, higher relative extracellular adenosine levels and subsequent adenosine receptor activation. 5'-NH_2dAdo inhibition of these behaviors is in agreement with earlier studies demonstrating adenosine receptor agonists inhibit behaviors induced by EAAs and substance P (DeLander and Wahl, 1988; 1989; 1991), although 5'-NH_2dAdo, in the present study, was ineffective against NMDA-induced biting and scratching. The lack of effect could reside in the fact that the dose of 5'-NH_2dAdo was limited to less than 1 nmol to avoid potential motor effects. The potential efficacy of higher doses of 5'-NH_2dAdo on NMDA-mediated effects can not be excluded. However, the lack of effect because of limited doses of 5'-NH_2dAdo seems unlikely since exogenous adenosine receptor agonists inhibit EAA and substance P-induced behavior at doses lower than those inducing motor incoordination (DeLander and Hopkins, 1987a; DeLander and Wahl, 1988; 1989; 1991). Increases of endogenous adenosine levels following adenosine kinase inhibition by 5'-NH_2dAdo may not be identical to receptor activation following exogenous agonist administration. The regional spinal distribution of adenosine kinase relative to adenosine receptors is unknown, and this possibility will require additional studies for validation.

In agreement with an earlier study in our laboratory (see Chapter III) and studies by other laboratories (Davies *et al.*, 1982; Zhang *et al.*, 1993), inhibition of adenosine deaminase induced much less dramatic effects. Inhibition of adenosine deaminase has been shown to induce behavioral effects (Radulovacki *et al.*, 1983), significantly enhance

extracellular adenosine levels (Ballarín *et al.*, 1991), and effect firing rates of central neurons (Phillis and Edstrom, 1976) indicating the importance of ADA activity may vary in different physiological processes. Most studies in which ADA inhibition failed to cause significant effects, however, demonstrated significant enhancement of adenosine-induced effects following inhibition of ADA (*c.f.* Chapter III; Davies *et al.*, 1982; Zhang *et al.*, 1993). Although not investigated in the present studies, inhibition of ADA in the presence of elevated adenosine levels might lead to potentiated effects.

In contrast to the antinociceptive actions following spinal adenosine receptor activation, facilitated nociceptive behavior is observed following the administration of methylxanthines, antagonists of adenosine receptors. Previous studies have shown administration of methylxanthines increase behavioral responses to nociceptive stimuli (Paalzow and Paalzow, 1973; Paalzow, 1994; Jurna, 1984; Sawynok *et al.*, 1986). These studies indicate adenosine may be tonically released (Jurna, 1984; Sawynok *et al.*, 1986) and this release may constantly regulate spinal sensory afferent input. Inhibition of this "tone", therefore, could lead to increased nociceptive transmission, observed as biting and scratching, or induce a "miscoding" of sensory afferent input, observed as tactile allodynia. Dose- and time-dependent biting and scratching induced by the adenosine receptor antagonists, in the present study, is consistent with the hypothesis that an endogenous adenosine tone exists at spinal sites.

Inhibition of methylxanthine-induced biting and scratching by CPA and NECA indicate this behavior is induced, at lease in part, via adenosine receptor blockade. Slightly better inhibition of biting and scratching was seen with the A_1-selective adenosine receptor agonist, CPA, and may indicate inhibition of A_1 adenosine receptors induce

methylxanthine-induced biting and scratching. Less inhibition by NECA may involve lower intrinsic activity at A_1 adenosine receptors than CPA, or may involve opposing effects following activation of both A_1 and A_2 receptors by this non-selective agent.

The possibility that theophylline- and 8-*p*SPTheo have other actions expressed behaviorally as biting and scratching can not be ruled out at the present time. Methylxanthines are reported to exert multiple effects in addition to adenosine receptor blockade including inhibition of phosphodiesterase and 5'-N, increases or decreases in intracellular Ca^{2+} concentrations, and modulation of GABAergic and noradrenergic neurotransmission (reviewed, Nehlig *et al.*, 1992 and Sawynok and Yaksh, 1993). Since the membrane permeable cAMP analog, 8-Br cAMP, or inhibition of PDE activity by 3-isobutyl-1-methylxanthine, potentiate the depolarizing responses of dorsal horn neurons to excitatory amino acids (Cerne *et al.*, 1992), such effects may, in part, mediate theophylline-induced biting and scratching. 8-*p*SPTheo, however, induced behaviors similar to theophylline. 8-*p*SPTheo is more hydrophilic and does not readily cross cellular membranes, indicating extracellular adenosine receptor blockade is the most likely mechanism behind the biting and scratching in the present studies.

Disruptions of an endogenous inhibitory purinergic tone by adenosine receptor antagonists might disinhibit the release or actions of excitatory neurotransmitters. Indeed, this type of interaction would be a predicted consequence of the gate control theory of pain (Melzack and Wall, 1965). Attempts were made to block methylxanthine-induced biting and scratching with excitatory neurotransmitter antagonists. Coadministration of excitatory neurotransmitter antagonists with methylxanthines resulted in decreased biting and scratching behavior at selected time points for all antagonists

except MK-801. Significant inhibition of overall behavior, however, could only be demonstrated by the non-selective excitatory amino acid antagonist, kynurenate, and the tachykinin antagonist, spantide. Since most excitatory neurotransmission (nociceptive and non-nociceptive) in the spinal cord is mediated via activation of non-NMDA receptors (reviewed, Wilcox, 1991 and Dickenson 1994), perhaps removal of the inhibitory tone of adenosine induces the recruitment of NMDA and tachykinin receptors to encode sensory afferent signals not normally coded by these receptors.

Multiple excitatory neurotransmitter systems are involved in the transmission of peripheral stimuli into central sites. In addition, these systems appear to work in conjunction in sensory afferent neurotransmission. Findings that 90 % of primary afferent neurons containing glutamate also contain substance P (Battaglia and Rustioni, 1988; Merighi *et al.*, 1991), that substance P enhances the basal release of excitatory amino acids in the spinal cord (Smullin *et al.*, 1990), and neurokinin and excitatory amino acid systems interact cooperatively to induce aversive behavior or physiological effects (reviewed, Urban *et al.*, 1994) all indicate multiple interactions between spinal excitatory systems. Inhibition of a single excitatory system, therefore, would not be expected to fully antagonize excitatory neurotransmission. Our findings that the non-selective EAA antagonist, kynurenate, and spantide, which can inhibit both tachykinin- and EAA-induced excitatory effects (Sakurada *et al.*, 1990), antagonize theophylline-induced biting and scratching may reflect cooperation between spinal excitatory systems. Disinhibition of either or both EAA or tachykinin systems following theophylline administration could lead to greatly enhanced excitatory effects since these compounds interact synergistically with each other (Mjellem-Joly *et al.*, 1991). Additionally, actions

of tachykinin agonists might be mediated in part by the subsequent release of excitatory amino acids (Brugger *et al.*, 1990; Okano *et al.*, 1993). Finally, adenosine receptor antagonists may not induce additional release of excitatory neurotransmitters, but may remove postsynaptic inhibitory effects of endogenous adenosine on neurons susceptible to excitatory neurotransmitters.

The present studies demonstrate manipulation of endogenous adenosine levels reduces aversive behavior induced by putative pain neurotransmitters. Increases of endogenous spinal adenosine levels, therefore, might represent a novel approach for inhibiting nociceptive neurotransmission. These studies also demonstrate disruption of spinal adenosine neurotransmission may result in disinhibited excitatory states manifesting as biting and scratching behavior or tactile allodynia.

TABLE V-1. Nociceptive behavior induced by adenosine receptor antagonists. Dose-dependency of adenosine receptor antagonists, theophylline and 8-*p*-sulphophenyl theophylline (8-*p*SPTheo), to induce caudally-directed biting and scratching behavior (left columns), and inhibition of this behavior by pretreatment (10 min) with the adenosine receptor agonists, N^6-cyclopentyl adenosine (CPA) or 5'-(N-ethyl)-carboxamido adenosine (NECA) (right columns).

I.t. administration[a]	ave #B/S[b]	I.t. administration	ave #B/S
saline	24.6 (5.7)	theophylline 222	66.4 (6.1)
theophylline 111	30.7 (10.2)	" + CPA[c]	50.7 (6.7)*
" 222	77.3 (8.3)*	theophylline 222	69.7 (2.9)
		" + NECA[d]	48.5 (5.3)*
d.w.[e]	5.7 (1.0)	8-*p*SPTheo 100	97.8 (3.6)
8-*p*SPTheo 25	16.9 (6.7)*	" + CPA	65.5 (9.1)*
" 50	34.1 (3.4)*	8-*p*SPTheo 100	68.4 (11.2)
" 100	85.5 (5.5)*	" + NECA	67.8 (12.9)

[a] Doses listed are in nmol.
[b] Caudally-directed biting and scratching (#B/S) was observed for 10 min following i.t. administration of drugs and the mean (±s.e.m.) behavior per min calculated.
[c] CPA dose = 100 pmol.
[d] NECA dose = 25 pmol.
[e] d.w. = distilled water.
* $p < .05$, paired Student's *t*-test.

TABLE V-2. Sensitivity of theophylline-induced biting and scratching behavior to the excitatory amino acid antagonists, AP7, MK-801, kynurenate, CNQX or the tachykinin antagonist, spantide (span).

	AP7[a]	MK-801	kynurenate	CNQX	span	AP7+span
Theo 222[b]	54.9 (5.4)[c]	54.9 (5.4)	62.3 (2.2)	58.8 (3.4)	54.9 (5.4)	62.2 (9.1)
" + antag	51.0 (5.3)	51.5 (4.0)	52.4 (5.9)*	64.7 (2.8)	39.4 (6.3)*	31.1 (3.6)*

[a] Doses of antagonists administered (pmol): AP7 - 600; MK-801 - 500; kynurenate - 250; CNQX - 5000; spantide (span) - 1000.

[b] Theophylline dose = nmol.

[c] Caudally-directed biting and scratching was observed for 10 min following i.t. administration of drugs and the mean (±s.e.m.) behavior per min calculated.

* $p < .05$, paired Student's *t*-test.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The significance of our investigations suggesting a tonic, endogenous purinergic system is present at spinal sites modulating sensory input, and the interactions of this system with opioid mechanisms of antinociception, is several fold. As suggested by early investigations in our laboratory and that of Sawynok and coworkers, stimulated release of adenosine appears to be one mechanism by which opioids induce antinociception (reviewed, Sawynok and Sweeney, 1989). These investigations were important in that they predicted additional investigations may reveal clinically significant interactions between opioids and adenosine, and they substantiated the possible existence of an endogenous spinal purinergic system modulating sensory input. Later investigations, carried out in conjunction with Dr. Frank Porreca (DeLander *et al.*, 1992), revealed that the significance of stimulated adenosine release, and thus the endogenous purinergic system, may vary depending upon the type of opioid receptor activated.

Methylxanthine-mediated antagonism of antinociception induced by *mu* opioid receptor-selective agonists appears to be competitive (DeLander *et al.*, 1992), which is consistent with stimulated release of endogenous adenosine as a consequence of *mu* opioid receptor activation. Investigations reported in this thesis provide additional support of this hypothesis. Coadministration (i.t.) of adenosine agonists with *mu* opioid receptor-selective agonists revealed simple additivity in their capacity to induce antinociception (Chapter II). Pharmacological theory would predict that redundant activation of a common pathway should result in an additive interaction. Findings from

the present studies, therefore, are consistent with, but do not prove, *mu* opioid receptor activation inducing the release of adenosine as a mechanism of action for antinociception. This mechanism of action, however, does not appear to be necessarily shared with other pharmacologic actions of opioids (DeLander *et al.*, 1992).

Very similar results were observed when *mu* opioid selective agonists were coadministered (i.t.) with an inhibitor of adenosine kinase (Chapter III). Inhibition of adenosine kinase caused antinociception and, as described by Zhang *et al.* (1993), adenosine kinase appeared to be more significant in the regulation of endogenous adenosine levels than adenosine deaminase. Inhibition of adenosine kinase in the presence of *mu* opioid selective agonists generally revealed an additive interaction for antinociception, again consistent with stimulated adenosine release as a mechanism of opioid-induced antinociception. One discrepancy in our findings was the observation that inhibition of adenosine kinase induced a greater than additive interaction with DAMGO, a *mu* selective opioid agonist, at selected time points. We believe this to be a consequence of the significance of adenosine kinase in regulating intracellular adenosine concentrations. In addition to the interaction described between adenosine kinase inhibitors and *mu* opioid receptor selective agonists, inhibition of adenosine kinase would be expected to elevate intracellular adenosine concentrations or create an unfavorable environment for the reuptake of adenosine from the synapse. Decreased rates of adenosine uptake would be expected to extend the duration of adenosine action and, consequently, the degree of DAMGO induced antinociception.

In *in vivo* and *in vitro* release studies, Sawynok and coworkers (see Introduction section) have observed adenosine release induced by opioid receptor agonists. These

investigators have also proposed that both release and reuptake of adenosine is mediated via NTS(s) (see Cahill *et al.*, 1993). Consistent with the hypothesis of Sawynok and coworkers and consistent with our hypothesis of stimulated adenosine release induced by *mu* opioid receptor activation, pretreatment of animals with NTS inhibitors decreased *mu* opioid receptor-selective induced antinociception. In contrast to pretreatment, but again consistent with these hypotheses, treatment of animals with NTS inhibitors after administration of *mu* opioid receptor-selective agonists enhanced antinociception apparently due to an increase in the duration of adenosine action in the synapse.

Stimulated adenosine release as a consequence of *mu* opioid receptor activation, therefore, is supported by a variety of different observations, including methylxanthine-mediated competitive antagonism of antinociception (Delander *et al.*, 1992), *in vivo* and *in vitro* evidence for opioid stimulated release of adenosine (Sawynok and Sweeney, 1989), additive interactions for antinociception following coadministration of a *mu* opioid receptor agonist and an adenosine receptor agonist (Chapter II) or adenosine kinase inhibitor (Chapter III) and inhibition of antinociception following pretreatment with an NTS inhibitor (Chapter IV). Confirmation of stimulated adenosine release following activation of *mu* opioid receptors highlights potential strategies for pain management. Clinicians should be aware that administration of NTS inhibitors may limit antinociception induced by *mu* opioid receptor selective agonists. Of equal importance is the recognition that coadministration of morphine (but not *mu* opioid receptor selective agonists) and either adenosine receptor agonists or agents which manipulate endogenous adenosine concentrations, may allow clinicians to use smaller doses of both agents to achieve analgesia, while avoiding potential side effects.

Stimulated adenosine release does not appear to be a consequence of *delta* opioid receptor activation. Coadministration of methylxanthines and *delta* opioid receptor agonists inhibited opioid-induced antinociception, but there appeared to be a non-parallel shift of the dose-response curve. The non-competitive nature of methylxanthine-mediated antagonism suggested a more complex interaction between adenosine and *delta* opioid receptor selective agonists than was observed for *mu* opioid receptor selective agonists. Examination of interactions between adenosine agonists and *delta* opioid receptor selective agonists revealed synergism (Chapter II). A supra-additive antinociceptive action was observed following coadministration of *delta* opioid receptor selective agonists with either adenosine receptor agonists (Chapter II) or an inhibitor of adenosine kinase (Chapter III). Synergism, observed following activation of two systems with a common pharmacologic effect, suggests that these systems utilize independent mechanisms of action. In preliminary *in vitro* investigations, Sawynok and coworkers (Dr. J. Sawynok - personal communication), in fact, have been unable to observe adenosine release following exposure of synaptosomes to *delta* opioid receptor selective agonists.

NTS inhibitors (Chapter IV) also interacted with *delta* opioid receptor selective agonists in a supra-additive fashion. Interestingly, enhancement of *delta* opioid receptor agonist-induced antinociception was independent of the time of treatment with inhibitors of the NTS(s). This observation suggests that adenosine is released tonically by mechanisms not effected by NTS inhibitors used in our investigations and that any manipulation to limit adenosine clearance may enhance antinociception induced by *delta* opioid receptor selective agonists. The capacity for methylxanthines to inhibit antinociception induced by *delta* opioid receptor selective agonists similarly suggests that,

although these systems have largely independent mechanisms of action, purinergic tone may be necessary for maximal antinociceptive efficacy following administration of *delta* opioid receptor selective agonists.

Non-competitive antagonism of *delta* opioid receptor selective agonist-induced antinociception by methylxanthines (DeLander *et al.*, 1992), the failure of *delta* selective agonists to induce adenosine release (Dr. J. Sawynok - personal communication), and supra-additive interactions for antinociception induced by coadministration of a *delta* opioid receptor selective agonists and adenosine receptor agonists, an adenosine kinase inhibitor or inhibitors of NTS, all support independent mechanisms of action for adenosine and *delta* opioid receptor selective agonists. The potential clinical implications of these investigations is significant. Coadministration of adenosine, or manipulations to increase endogenous adenosine, would be expected to enhance the analgesic potency of *delta* opioid receptor agonists, while using smaller doses of each agent. In addition, subsequent investigations seem likely to reveal an increased efficacy for *delta* opioid agonists in specific pain syndromes, when utilized in combination with strategies to increase activation of adenosine receptors.

Finally, our investigations clearly demonstrated the existence of an endogenous purinergic system that modulates nociceptive input and possibly helps to differentiate non-noxious and noxious stimuli. Pharmacologic manipulations to enhance endogenous adenosine concentrations suppressed behavior induced by putative pain neurotransmitters (Chapter V). In contrast, adenosine receptor antagonist administration induced of biting and scratching behavior, similar to that observed following administration of putative pain neurotransmitters (Chapter V). These observations suggest adenosine plays a role

in the tonic regulation of nociceptive input. Importantly, our ability to elicit actions through pharmacologic manipulation of an endogenous purinergic system provides a potential for 'use dependent' enhancement of processes modulating noxious transmission. This strategy may allow clinicians to avoid difficulties associated with exogenously administered adenosine receptor agonists.

Preliminary findings that adenosine receptor antagonists also induced allodynia allows us to extend our hypothesis to suggest that endogenous purinergic systems may prevent the 'miscoding' of non-noxious stimuli as noxious stimuli. The significance of endogenous adenosine in the modulation of non-noxious sensory input will require additional investigations, but our hypothesis is consistent with recent studies revealing unique efficacy for adenosine receptor agonists in opioid resistant pain syndromes (see Yaksh and Malmberg, 1994, for review).

The discovery of an endogenous purinergic system that modulates nociceptive processing is a significant contribution to our understanding of mechanisms of nociception and antinociception. We have revealed that adenosine receptor agonists and pharmacological manipulations that possibly increase endogenous adenosine concentrations can independently induce antinociception or interact with specific opioids to dramatically enhance opioid-induced antinociception. Involvement of spinal purinergic systems in antinociception, and perhaps sensory processing in general, provides important new strategies to explore in the management of pain syndromes sensitive and insensitive to traditional opioid therapy.

BIBLIOGRAPHY

AHCPR Guide: Acute Pain Management: Operative or Medical Procedures and Trauma. 1992. A. Jacox, D.B. Carr co-chairs; U.S. Dept of Health and Human Services, 145pgs.

AHCPR Guide: Management of Cancer Pain. 1994. A. Jacox, D.B. Carr, R. Payne co-chair; U.S. Dept of Health and Human Service, 257pgs.

Agarwal, R.P., T. Spector, and R.E. Parks, Jr. 1977. Tight-binding inhibitors--IV. inhibition of adenosine deaminases by various inhibitors. *Biochem Pharmacol* 26:359.

Ahlgren, S.C., and J.D. Levine. 1993. Mechanical hyperalgesia in streptozotocin-diabetic rats. *Neurosci* 52:1049.

Ahlijanian, M.K., and A.E. Takemori. 1985. Effects of (-)-N^6-(R-phenylisopropyl)-adenosine (PIA) and caffeine on nociception and morphine-induced analgesia, tolerance and dependence in mice. *European J Pharmacol* 112:171.

Alhaider, A.A., Lei, S.Z., and G.L. Wilcox. 1991. Spinal 5-HT_3 receptor-mediated antinociception: possible release of GABA. *J Neurosci* 11:1881.

Alhaider, A.A., and G.L. Wilcox. 1993. Nociceptive modulation by intrathecally administered 5-HT_{1A} and 5-HT_{1B} agonists in mice. *FASEB J* 4:A988

Aran, S., and D.L. Hammond. 1991. Antagonism of baclofen-induced antinociception by intrathecal administration of phaclofen or 2-hydroxy-saclofen, but not δ-aminovaleric acid in the rat. *J Pharmacol Exp Therap* 257:360.

Aran, S., and H.K. Proudfit. 1990. Antinociceptive interactions between intrathecally administered α-noradrenergic and 5'-N-ethylcarboxamide adenosine. *Brain Res* 519:287.

Arch, J.R.S., and E.A. Newsholme. 1978. Activities and some properties of 5'-nucleotidase, adenosine kinase and adenosine deaminase in tissues from vertebrates and invertebrates in relation to the control of the concentration and the physiological role of adenosine. *J Biochem* 174:965.

Balkowiec, A., K. Kukula, and P. Szulczyk. 1994. Influence of morphine on the activity of low-threshold visceral mechanoceptors in cats with acute pericarditis. *Pain* 59:251.

Ballarín, M, B.B. Fredholm, S. Ambrosio, and N. Mahy. 1991. Extracellular levels of adenosine and its metabolites in the striatum of awake rats: inhibition of uptake and metabolism. *Acta Physiol Scand* 142:97.

Barraco, R.A. 1991. Behavioral actions of adenosine and related substances. In: *Adenosine and adenine nucleotides as regulators of cellular function*, 1st edn, pp339-366. Ed. J.W. Phillis, CRC press: Boca Raton, FL.

Basbaum, A.I., and H.L. Fields. 1978. Endogenous pain control mechanisms: Review and hypothesis. *Ann Neurol* 4:451.

Basbaum, A.I., and H.L. Fields. 1984. Endogenous pain control systems: Brainstem spinal pathways and endorphin circuitry. *Ann Rev Neurosci* 7:309.

Battaglia, G., and A. Rustioni. 1988. Coexistence of glutamate and substance P in dorsal root ganglion neurons of the rat and monkey. *J Comp Neurol* 277:302.

Behbehani, M.M., and O. Dollberg-Stolik. 1994. Partial sciatic nerve ligation results in an enlargement of the receptive field and enhancement of the response of dorsal horn neurons to noxious stimulation by an adenosine agonist. *Pain* 58:421.

Besson, J.-M., and A. Chaouch. 1987. Peripheral and spinal mechanisms of nociception. *Physiol Rev* 67:67.

Boive, J. 1994. Central pain. In: *Textbook of Pain*, pp871-902. P.D. Wall and R. Melzack, eds. Churchill Livingstone Press, London.

Borst, M.M., A. Deussen, and J. Schrader. 1992. S-Adenosylhomocysteine hydrolase activity in human myocardium. *Cardiovasc Res* 26:143.

Braas, K.M., A.C. Newby, V.S. Wilson, and S.H. Snyder. 1986. Adenosine-containing neurons in the brain localized by immunocytochemistry. *J Neurosci* 6:1952.

Brosh, S., O. Sperling, Y. Bromberg, and Y. Sidi. 1990. Developmental changes in the activity of enzymes of purine metabolism in rat neuronal cells in culture and in whole brain. *J Neurochem* 54:1776.

Brugger, F., R.H. Evans, and N.S. Hawkins. 1990. Effects of *N*-methyl-D-aspartate antagonists and spantide on spinal reflexes and responses to substance P and capsaicin in isolated spinal cord preparations from mouse to rat. *Neurosci* 36:611.

Bruns, R.F. 1991. Role of adenosine in energy supply/demand balance. *Nucleosides Nucleotides* 10:931.

Bruns, R.F., G.H. Lu, and T.A. Pugsley. 1986. Characterization of the A_2 adenosine receptor labelled by [^{3}H]NECA in rat striatal membranes. *Molec Pharmacol* 29:331.

Burnstock, G. 1991. Distribution and roles of purinoceptor subtypes. *Nucleosides Nucleotides* 10:917.

Butcher, R.W., and E.W. Sutherland. 1962. Adenosine 3', 5'-phosphate in biological materials. I. Purification and properties of cyclic 3', 5'-nucleotide phosphodiesterase and use of this enzyme to characterize adenosine 3', 5'-phosphate in human urine. *J Biol Chem* 237:1244.

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^{++} channels from dorsal spinal cord synaptosomes. *Neurosci Abs* 18:1371.

Cahill, C.M., T.D. White, and J. Sawynok. 1993. Morphine activates ω-conotoxin-sensitive Ca^{2+} channels to release adenosine from spinal cord synaptosomes. *J Neurochem* 60:894.

Cerne, R., M. Jiang, and M. Randic. 1992. Cyclic adenosine 3'5'-monophosphate potentiates excitatory amino acid and synaptic responses of rat spinal dorsal horn neurons. *Brain Res* 596:111.

Chassin, M.M., R.H. Adamson, D.W. Zaharevitz, and D.G. Johns. 1979. Enzyme inhibition titration assay for 2'-deoxycoformycin and its application to the study of the relationship between drug concentration and tissue adenosine deaminase in dogs and rats. *Biochem Pharmacol* 28:1849.

Choca, J.I., R.D. Green, and H.K. Proudfit. 1988. Adenosine A_1 and A_2 receptors of the substantia gelatinosa are located predominantly on intrinsic neurons: An autoradiography study. *J Pharmacol Exp Therap* 247:757.

Choca, J.I., H.K. Proudfit, and R.D. Green. 1987. Identification of A_1 and A_2 adenosine receptors in the rat spinal cord. *J Pharmacol Exp Therap* 242:905.

Chou, W.S., A.K.S. Ho, and H.H. Lo. 1971. Effect of acute and chronic morphine and norepinephrine on brain adenyl cyclase activity. *Proc West Pharmacol Soc* 14:42.

Clements, J.R., K.R. Magnusson, J. Hautman, and A.J. Beitz. 1991. Rat tooth pulp projections to spinal trigeminal subnucleus caudalis are glutamate-like immunoreactive. *J Comp Neurol* 309:281.

Collier, H.O.J., G.W.L. James, and C. Schneider. 1966. Antagonism by aspirin an fenamates of bronchoconstriction and nociception by adenosine-5'-triphosphate. *Nature* 212:411.

Contreras, E., A. Germany, and M. Villar. 1990. Effects of some adenosine analogs on morphine-induced analgesia and tolerance. *Gen Pharmacol* 21:763.

Crain, S.M., and K. Shen. 1990. Opioids can evoke direct receptor-mediated excitatory effects on sensory neurons. *Trends in Pharmacol Sci* 11:77.

Crawley, J.N., J. Patel, and P.J. Marangos. 1981. Behavioural characterization of two long-lasting adenosine analogs: sedative properties and interaction with diazepam. *Life Sci* 29:2623.

Curtis, D.R., J.W. Phillips, and J.C. Watkins. 1959. Chemical excitation of spinal neurons. *Nature* 183:611.

Cushing, D.J., and S.J. Mustafa. 1991. Historical perspectives - Adenosine. In: *Adenosine and Adenine Nucleotides as Regulators of Cellular Functions*, J.W. Phillis, ed (CRC Press), pp. 3-13.

Dalsgaard, J., S. Felsby, P. Juelsgaard, and J. Froekjaer. 1994. Low-dose intra-articular morphine analgesia in day case knee arthroscopy: a randomized double-blinded prospective study. *Pain* 56:151.

D'Amour, F.E., and D.L. Smith. 1941. A method for determining loss of pain sensation. *J Pharmacol Exp Ther* 72:74.

Danzebrink, R.M., and G.F. Gebhart. 1991. Evidence that spinal 5-HT_1, 5-HT_2 and 5-HT_3 receptor subtypes modulate responses to noxious colorectal distension in the rat. *Brain Res* 538:64.

Davies, L.P. 1985. Pharmacological studies on adenosine analogues isolated from marine organisms. *Trends in Pharmacol Sci* 6:1.

Davies, L.P., J. Baird-Lambert, and D.D. Jamieson. 1982. Potentiation of pharmacological responses to adenosine, *in vitro* and *in vivo*. *Gen Pharmacol* 13:27.

Davies, L.P., J. Baird-Lambert, and J.F. Marwood. 1986. Studies on several pyrrolo[2,3-*d*] pyrimidine analogues of adenosine which lack significant agonist activity at A1 and A2 receptors but have potent pharmacological activity *in vivo*. *Biochem Pharmacol* 35:3021.

Davies, L.P., and J.W. Hambley. 1986. Regional distribution of adenosine uptake in guinea-pig brain slices and the effect of some inhibitors: Evidence for nitrobenzylthioinosine-sensitive and insensitive sites?. *Neurochem Int* 8:103.

Davies, L.P., J.W. Hambley, and G.A.R. Johnston. 1987. Reduced adenosine deaminase activity in the CNS of spontaneously-hypertensive rats. *Neurochem Int* 10:533.

Davies, L.P., D.D. Jamieson, J.A. Baird-Lambert, and R. Kazlauskas. 1984. Halogenated pyrrolopyrimidine analogues of adenosine from marine organisms: Pharmacological activities and potent inhibition of adenosine kinase. *Biochem Pharmacol* 33:347.

Deckert, J., P.F. Morgan, and P.J. Marangos. 1988. Adenosine uptake site heterogeneity in the mammalian CNS? Uptake inhibitors as probes and potential neuropharmaceuticals. *Life Sci* 42:1331.

DeClercq, E. 1987. s-Adenosylhomocysteine hydrolase inhibitors as broad-spectrum antiviral agents. *Biochem Pharmacol* 16:2567.

DeLander, G.E., and G.J. Hopkins. 1986. Spinal adenosine modulates descending antinociceptive pathways stimulated by morphine. *J Pharmacol Exp Therap* 239:88.

DeLander, G.E., and G.J. Hopkins. 1987a. Involvement of A_2 adenosine receptors in spinal mechanisms of antinociception. *European J Pharmacol* 139:215.

DeLander, G.E., and G.J. Hopkins. 1987b. Interdependence of spinal adenosinergic, serotonergic and noradrenergic systems mediating antinociception. *Neuropharmacol* 26:1791.

DeLander, G.E., H.I. Mosberg, and F. Porreca. 1992. Involvement of adenosine in antinociception produced by spinal or supraspinal receptor-selective opioid agonists: dissociation from gastrointestinal effects in mice. *J Pharmacol Exp Therap* 263: 1097.

DeLander, G.E., and J.J. Wahl. 1988. Behavior induced by putative nociceptive neurotransmitters is inhibited by adenosine or adenosine analogs coadministered intrathecally. *J Pharmacol Exp Therap* 246:565.

DeLander, G.E., and J.J. Wahl. 1989. Morphine (intracerebroventricular) activates spinal systems to inhibit behavior induced by putative pain neurotransmitters. *J Pharmacol Exp Therap* 251:1090.

DeLander, G.E., and J.J. Wahl. 1991. Descending systems activated by morphine (i.c.v.) inhibit kainic acid (i.t.)-induced behavior. *Pharmacol Biochem & Behav* 39:155.

Dellemijn, P.L., and H.L. Fields. 1994. Do benzodiazepines have a role in chronic pain management? *Pain* 57:137.

Deussen, A., M. Borst, and J. Schrader. 1988. Formation of *S*-Adenosylhomocysteine in the heart: An index of free intracellular adenosine. *Circulation Res* 63:240.

Dickenson, A.H. 1994. A cure for wind up: NMDA receptor antagonists as potential analgesics. *Trends Pharmacol Sci* 11:307.

Dickenson, A.H., and A.F. Sullivan. 1990. Differential effects of excitatory amino acid antagonists on dorsal horn nociceptive neurones in the rat. *Brain Res* 506:31.

Dourghty, P.M., and F.A. Lenz. 1994. Plasticity of the somatosensory system following neural injury. In: *Touch, temperature, and pain in health and disease: mechanisms and assessments*. J. Boive, P. Hansson and U. Lindblom, eds, pp 439-460. IASP Press, Seattle, WA.

Dray, A., L. Urban, and A. Dickenson. 1994. Pharmacology of chronic pain. *Trends Pharmacol Sci* 15:190.

Drury, A.N., and A. Szent-Györgyi. 1929a. The influence upon the heart of a substance present in heart muscle and other tissues. *J Physiol*, proceedings Jan. 19, 1929: (67):xiv.

Drury, A.N., and A. Szent-Györgyi. 1929b. The influence upon the heart of a substance related to nucleic acid. *J Physiol*, proceedings Apr. 27, 1929:xxxv.

Duggan, A.W., P.J. Hope, B. Jarrott, H.-G. Schaible, and S.M. Fleetwood-Walker. 1990. Release, spread and persistence of immunoreactive neurokinin A in the dorsal horn of the cat following noxious cutaneous stimulation. Studies with antibody microprobes. *Neurosci* 35:195.

Evans, R.H. 1989. The pharmacology of the segmental transmission in the spinal cord. *Progress Neurobiol* 33:255.

Fastbom, J., C. Post, and B.B. Fredholm. 1990. Antinociceptive effects and spinal distribution of two adenosine receptor agonists after intrathecal administration. *Pharmacol Toxicol* 66:69.

Fields, H.L., M.M. Heinricher, and P. Mason. 1991. Neurotransmitters in nociceptive modulatory circuits. *Ann Rev Neurosci* 14:219.

Fleetwood-Walker, S.M., P.J. Hope, R. Mitchell, N. El-Yassir, and V. Molony. 1988. The influence of opioid receptor subtypes on the processing of nociceptive inputs in the spinal dorsal horn of the cat. *Brain Res* 451:213.

Fox, I.H., T.D. Palella, D. Thompson, and C. Herring. 1982. Adenosine metabolism: modification by *S*-Adenosylhomocysteine and 5'-Methylthioadenosine. *Arch Biochem Biophys* 215:302.

Franco, R., E.I. Canela, and J. Bozai. 1986. Heterogeneous localization of some purine enzymes in subcellular fractions of rat brain and cerebellum. *Neurochem Res* 11:423.

Fredholm, B.B., M.P. Abbracchio, G. Burnstock, J.W. Daly, T.K. Harden, K.A. Jacobson, P. Leff, and M. Williams. 1994. VI. Nomenclature and classification of purinoceptors. *Pharmacol Rev* 46:143.

Fredholm, B.B., M. Ballarin, P. Gerwins, P. Hu, I. van der Ploeg, and F. Parkinson. 1991. Pharmacological implications of a multiplicity of adenosine actions in the CNS. *Nucleosides Nucleotides* 10:955.

Fyffe, R.E.W., and E.R. Perl. 1984. Is ATP a central synaptic mediator for certain primary afferent fibers from mammalian skin? *Proc Natl Acad Sci* 81:6890.

Galindo, A., K. Krnjevic, and S. Schwartz. 1967. Micro-iontophoretic studies on neurones in the cuneate nucleus. *J Physiol* 192:659.

Gamse, R., P. Holzer, and F. Lembeck. 1979. Indirect evidence for presynaptic location of opiate receptors on chemosensitive primary sensory neurones. *Archives of Pharmacol* 308:281.

Geiger, J.D., F.S. LaBella, and J.I. Nagy. 1984. Characterization and localization of adenosine receptors in rat spinal cord. *J Neurosci* 4:2303.

Geiger, J.D., J.L. Lewis, C.J. Macintyre, and J.I. Nagy. 1987. Pharmacokinetics of 2'-deoxycoformycin, an inhibitor of adenosine deaminase, in the rat. *Neuropharmacol* 26:1383.

Geiger, J.D., and J.I. Nagy. 1984. Heterogeneous distribution of adenosine transport sites labelled by [^{3}H]nitrobenzylthioinosine in rat brain: an autoradiographic and membrane binding study. *Brain Res Bull* 13:657.

Geiger, J.D., and J.I. Nagy. 1985. Localization of [^{3}H]nitrobenzylthioinosine binding sites in rat spinal cord and primary afferent neurons. *Brain Res* 347:321.

Geiger, J.D., and J.I. Nagy. 1986. Distribution of adenosine deaminase activity in rat brain and spinal cord. *J Neurosci* 6:2707.

Geiger, J.D., R.A. Padua, and J.I. Nagy. 1991. Adenosine deaminase regulation of purine actions. In: *Adenosine and Adenine Nucleotides as Regulators of Cellular Functions*, J.W. Phillis, ed (CRC Press), pp. 71-84.

Glaum, S.R., H.K. Proudfit, and E.G. Anderson. 1990. 5-HT_3 receptors modulate spinal nociceptive reflexes. *Brain Res* 510:12.

Go, V.L.W., and T.L. Yaksh. 1987. Release of substance P from the cat spinal cord. *J Physiol* 391:141.

Goodman, R.R., and S.H. Snyder. 1982. Autoradiographic localization of adenosine receptors in rat brain using (^{3}H)cyclohexyladenosine. *J Neurosci* 2:1230.

Gordh, T., I. Jansson, P. Hartvig, P.G. Gillberg, and C. Post. 1989. Interactions between noradrenergic and cholinergic mechanisms involved in spinal nociceptive processing. *Acta Anaesthesiologica Scandinavica* 33:39.

Gu, J.G., and J.D. Geiger. 1992. Transport and metabolism of D-[^{3}H] adenosine and L-[^{3}H] adenosine in rat cerebral cortical synaptoneurosomes. *J Neurochem* 58:1699.

Hammond, J.R. 1991. Comparative pharmacology of the nitrobenzylthioguanosine-sensitive and -resistant nucleoside transport mechanisms of Ehrlich ascites tumor cells. *J Pharmacol Exp Therap* 259:799.

Henderson, J.F., L. Brox, G. Zombor, D. Hunting, and C.A. Lomax. 1977. Specificity of adenosine deaminase inhibitors. *Biochem Pharmacol* 26:1967.

Herrick-Davis, K., S. Chippari, D. Luttinger, and S.J. Ward. 1989. Evaluation of adenosine agonists as potential analgesics. *European J Pharmacol* 162:365.

Hertz, L. 1991. Nucleoside transport in cells: kinetics and inhibitor effects. In: *Adenosine and adenine nucleotides as regulators of cellular function*, 1st edn, pp85-118. Ed. J.W. Phillis, CRC press: Boca Raton, FL.

Ho, D.H., J.K. Luce, and E. Frei III. 1968. Distribution of purine ribonucleoside kinase and selective toxicity of 6-methylthiopurine ribonucleoside. *Biochem Pharmacol* 17:1025.

Ho, I.K., H.H. Loh, and E.L. Way. 1972. Effect of cyclic AMP on morphine analgesia tolerance and physical dependence. *Nature* 238:397.

Ho, I.K., H.H. Loh, and E.L. Way. 1973. Cyclic adenosine monophosphate antagonism of morphine analgesia. *J Pharmacol Exp Therap* 185:336.

Holmgren, M., T. Hednar, G. Nordberg, and T. Mellstrand. 1983. Antinociceptive effects in the rat of an adenosine analogue, N^6-phenylisopropyladenosine. *J Pharm Pharmacol* 35:679.

Holmgren, M., T. Hedner, T. Mellstrand, G. Nordbert, and Th. Hedner. 1986. Characterization of the antinociceptive effects of some adenosine analogues in the rat. *Archives of Pharmacol* 334:290.

Holton, F.A., and P. Holton. 1954. The capillary dilator substances in dry powders of spinal roots; a possible role of adenosine triphosphate in chemical transmission from nerve endings. *J Physiol* 126:124.

Holton, P. 1959. The liberation of adenosine triphosphate on antidromic stimulation of sensory nerves. *J Physiol* 145:494.

Hong, Y., and F.V. Abbott. 1995. Peripheral opioid modulation of pain and inflammation in the formalin test. *European J Pharmacol* 277:21.

Hope, P.J., S.M. Fleetwood-Walker, and R. Mitchell. 1990. Distinct antinociceptive actions mediated by different opioid receptors in the region of lamina I and laminae III-V of the dorsal horn of the rat. *British J Pharmacol* 101:477.

Hunskaar, S., C. Post, O.B. Fasmer, and E. Arwestrom. 1986. Intrathecal injection of capsaicin can be used as a behavioural nociceptive test in mice. *Neuropharmacol* 25:1149.

Hwang, A.S., and G.L. Wilcox. 1989. Baclofen, γ-aminobutyric $acid_B$ receptors and substance P in the mouse spinal cord. *J Pharmacol Exp Therap* 248:1026.

Hylden, J.L.K., and G.L. Wilcox. 1980. Intrathecal morphine in mice, A new technique. *European J Pharmacol* 67:313.

Jiang, Q., A.E. Takemori, M. Sultana, P.S. Portoghese, W.D. Bowen, H.I. Mosberg, and F. Porreca. 1991. Differential antagonism of opioid *delta* antinociception by [D-Ala^2,Leu^5,Cys^6]enkephalin (DALCE) and naltrindole 5'-isothiocyanate (NTII): Evidence for *delta* receptor subtypes. *J Pharmacol Exp Therap* 257:1069.

Jurna, I. 1981. Aminophylline differentiates between the depressant effects of morphine on the spinal nociceptive reflex and on the spinal ascending activity evoked from afferent fibres. *European J Pharmacol* 71:393.

Jurna, I. 1984. Cyclic nucleotides and aminophylline produce different effects on nociceptive motor and sensory responses in the rat spinal cord. *Archives of Pharmacol* 327:23.

Kaneko, M., Y. Saito, Y. Kirihara, J.G. Collins, and Y. Kosaka. 1994. Synergistic antinociceptive interaction after epidural coadministration of morphine and licocaine in rats. *Anetshtsiology* 80:137.

Karlsten, R., T. Gordh, Jr., P. Hartvig, and C. Post. 1990. Effects of intrathecal injection of the adenosine receptor agonists R-phenylisopropyl-adenosine and *N*-ethylcarboxamide-adenosine on nociception and motor function in the rat. *Anesth Analg* 71:60.

Karlsten, R., T Gordh, and C. Post. 1992. Local antinociceptive and hyperalgesic effects in the formalin test after peripheral administration of adenosine analogues in mice. *Pharmacol Toxicol* 70:434.

Karlsten, R., T. Gordh, and B.A. Svensson. 1993. A neurotoxicologic evaluation of the spinal cord after chronic intrathecal injection of R-phenylisopropyl adenosine (R-PIA) in the rat. PhD thesis. ACTA Universitatis Upsaliensis, Uppsala, Sweden.

Kerr, F.W.L., and P.R. Wilson. 1978. Pain. *Ann Rev Neurosci* 1:83.

Khachaturian, H., M.E. Lewis, M.K.-H. Schäfer, and S.J. Watson. 1985. Anatomy of the CNS opioid systems. *Trends Neurol Sci* 8:110.

Klohs, W.D., and A.J. Kraker. 1992. Pentostatin: Future directions. *Pharmacol Rev* 44:459.

Kostopoulos, G.K., and J.W. Phillis. 1977. Purinergic depression of neurons in different areas of the rat brain. *Expl Neurol* 55:719.

Lamotte, C., C.B. Pert, and S.H. Snyder. 1976. Opiate receptor binding in primate spinal cord: Distribution and changes after dorsal root section. *Brain Res* 112:407.

Lee, K.S., and M. Reddington. 1986. Autoradiographic evidence for multiple CNS binding sites for adenosine derivatives. *Neurosci* 19:535.

Levine, J.D., and Y.O. Taiwo. 1989. Involvement of the mu-opiate receptor in peripheral analgesia. *Neurosci* 32:571.

Lin, B.B., M.C. Hurley, and I.H. Fox. 1988. Regulation of adenosine kinase by adenosine analogs. *Molec Pharmacol* 34:501.

Lord, J.A.H., A.A. Waterfield, J. Hughes, and H.W. Kosterlitz. 1977. Endogenous opioid peptides: Multiple agonists and receptors. *Nature* 267:495.

Lowe, S. 1953. The problem of synergism and antagonism of combined drugs. *Arzneim Forsch* 3:285.

Lowe, S. 1957. Antagonism and antagonists. *Pharmacol Rev* 9:237.

Macdonald, W.F., and T.D. White. 1985. Nature of extrasynaptosomal accumulation of endogenous adenosine evoked by K^+ and veratridine. *J Neurochem* 45:791.

Malcangio, M., P. Malmberg-Aiello, A. Giotti, C. Ghelardini, and A. Bartolini. 1992. *Neuropharmacol* 31:783.

Malmberg, A.B, and T.L. Yaksh. 1993. Pharmacology of the spinal action of ketorolac, morphine, ST-91, U50,488H, and L-PIA on the formalin test and an isobolographic analysis of the NSAID interaction. *Anesthesiology* 79:270.

Mansour, A., C.A. Fox, H. Akil, and S.J. Watson. 1995. Opioid-receptor mRNA expression in the rat CNS: Anatomical and functional implications. *Trends Neurosci* 18:22.

Mansour, A., H. Khachaturian, M.E. Lewis, H. Akil, and S.J. Watson. 1988. Anatomy of CNS opioid receptors. *Trends Neurosci* 11:308.

Mantegazza, P., R. Tammiso, F. Zambotti, L. Zecca, and N. Zonta. 1984. Purine involvement in morphine antinociception. *British J Pharmacol* 83:883.

Maxwell, D.J., W.M. Christie, A.D. Short, J. Storm-Mathison, and D. Ottersen. 1990. Central boutons of glomeruli in the spinal cord of the cat are enriched with L-glutamate-like immunoreactivity. *Neurosci* 36:83.

McConnell, W.R., W.J. Suling, L.S. Rice, W.M. Shannon, and D.L. Hill. 1978. Use of microbiologic and enzymatic assays in studies on the disposition of 2'-deoxy-coformycin in the mouse. *Cancer Treatment Rep* 62:1153.

McLachlan, E.M., W. Jang, M. Devor, and M. Michaelis. 1993. Peripheral nerve injury triggers noradrenerigic sprouting within dorsal root ganglia. *Nature* 363:543.

Melzack, R., and P.D. Wall. 1965. Pain mechanisms: A new theory. *Science* 150:971.

Mendell, L.M. 1966. Physiological properties of unmyelinated fibre projection to the spinal cord. *Exp Neurol* 16:316.

Merighi, A., J.M. Polak, and D.T. Theodosis. 1991. Ultrastructural visualisation of glutamate and aspartate immunoreactivities in the dorsal horn, with special reference to the co-localisation of glutamate, substance P and calcitonin-gene related peptide. *Neurosci* 40:67.

Merskey, H., and N. Bogduk. 1994. Classification of chronic pain. (IASP taskforce on taxonomy). IASP Press, Seattle, WA.

Miller, R.L., D.L. Adamczyk, W.H. Miller, G.W. Koszalka, J.L. Rideout, L.M. Beacham, E.Y. Chao, J.J. Haggerty, T.A. Krenitsky, and G.B. Elion. 1979. Adenosine kinase from rabbit liver: II. Substrate and inhibitor specificity. *J Biol Chem* 254:2346.

Minami, T., R. Uda, S. Horiguchi, S. Ito, M. Hyodo, and O. Hayaishi. 1992a. Effects of clonidine and baclofen on prostaglandin $F_{2\alpha}$-induced allodynia in conscious mice. *Pain Res* 7:129.

Minami, T., R. Uda, S. Horiguchi, S. Ito, M. Hyodo, and O. Hayaishi. 1992b. Allodynia evoked by intrathecal administration of prostaglandin $F_{2\alpha}$ to conscious mice. *Pain* 50:223.

Miyamoto, Y., N. Morita, Y. Kitabata, T. Yamanishi, S. Kishioka, M. Ozaki, and H. Yamamoto. 1991. Antinociceptive synergism between supraspinal and spinal sites after subcutaneous morphine evidenced by CNS morphine content. *Brain Res* 552:136.

Mjellem-Joly, N., A. Lund, O.-G. Berge, and K. Hole. 1991. Potentiation of a behavioural response in mice by spinal coadministration of substance P and excitatory amino acid agonists. *Neurosci Lett* 133:121.

Moldonado, R., O. Valverde, S. Turcaud, M.C. Fournie-Zaluski, and B.P. Roques. 1994. Antinociceptive response induced by mixed inhibitors of enkephalin catabolism in peripheral inflammation. *Pain* 58:77.

Monasky, M.S., A.R. Zinsmeister, C.W. Stevens, and T.L. Yaksh. 1990. Interaction of intrathecal morphine and ST-91 on antinociception in the rat: Dose-response analysis, antagonism and clearance. *J Pharmacol Exp Therap* 254:383.

Monterio, E.C., and J.A. Ribeiro. 1987. Ventilatory effects of adenosine mediated by carotid body chemoreceptors in the rat. *Naunyn Schmiedebergs Arch Pharmacol* 335:143.

Morris, B.J., and A. Herz. 1987. Distinct distribution of opioid receptor types in rat lumbar spinal cord. *Archives of Pharmacol* 336:240.

Murphy, R.M., and F.P. Zemlan. 1990. Selective 5-HT_{1A} and 5-HT_{1B} agonists differentially affect spinal nociceptive reflexes. *Neuropharmacol* 29:463.

Murray, T.F., and D.L. Cheney. 1982. Neuronal location of N^6-cyclohexyl-[^{3}H]adenosine binding sites in rat and guinea-pig brain. *Neuropharmacol* 21:575.

Nacimiento, W., and G.W. Kreutzberg. 1990. Cytochemistry of 5'-nucleotidase in the superior cervical ganglion of the rat: Effects of pre- and postganglionic axotomy. *Exp Neurol* 109:362.

Nagaoka, H., S. Sakurada, T. Sakurada, S. Takeda, Y. Nakagawa, K. Kisara, and Y. Arai. 1993. Theophylline-induced nociceptive behavioral response in mice: Possible indirect interaction with spinal *N*-methyl-D-aspartate receptors. *Neurochem Int* 22:69.

Nagy, J.I., and P.E. Daddona. 1985. Anatomical and cytochemical relationships of adenosine deaminase-containing primary afferent neurons in the rat. *Neurosci* 15:799.

Nakagawa, Y., M. Gudenzi, and S.J. Mustafa. 1986. Calcium entry blocking activity of dilazep in dog coronary artery. *Pharmacol* 33:148.

Nehlig, A., J.-L. Daval, and G. Debry. 1992. Caffeine and the central nervous system: mechanisms of action, biochemical, metabolic and psychostimulant effects. *Brain Res Rev* 17:139.

Newby, A.C. 1981. The interaction of inhibitors with adenosine metabolising enzymes in intact isolated cells. *Biochem Pharmacol* 30:2611.

Newby, A.C. 1984. Adenosine and the concept of 'retaliatory metabolites'. *Trends in Biol Sci* 2:42.

Newby, A.C. 1986. How does dipyridamole elevate extracellular adenosine concentration? *Biochem J* 237:845.

Nicholson, D., A. Reid, and J. Sawynok. 1991a. Effects of forskolin and phosphodiesterase inhibitors on spinal antinociception by morphine. *Pharmacol Biochem Behav* 38:753.

Nicholson, D., T.D. White, and J. Sawynok. 1991b. Forskolin and phosphodiesterase inhibitors release adenosine but inhibit morphine-evoked release of adenosine from spinal cord synaptosomes. *Can J Physiol Pharmacol* 69:877.

North, R.A. 1986. Opioid receptor types and membrane ion channels. *Trends in Neurosci* 9:114.

North, R.A. 1989. Membrane conductances and opioid receptor subtypes. *Trends in Pharmacol Sci* 14:81.

Ocaña, M., E. Del Posa, M. Barrios, L.I. Robles, and J.M. Baeyens. 1990. An ATP-dependent potassium channel blocker antagonizes morphine analgesia. *European J Pharmacol* 186:377.

Okano, K., Y. Kuraishi, and M. Satoh. 1993. Pharmacological evidence for involvement of excitatory amino acids in aversive responses induced by intrathecal substance P in rats. *Biol Pharm Bull* 16:861.

Ossipov, M.H., S. Harris, P. Lloyd, E. Messineo, S. Lin, and J. Bagley. 1990a. Antinociceptive interaction between opioids and medetomidine: Systemic additivity and spinal synergy. *Anesthesiology* 73:1227.

Ossipov, M.H., R. Lozito, E. Messineo, J. Green, S. Harris, S., and P. Lloyd. 1990b. Spinal antinociceptive synergy between clonidine and morphine, U69593, and DPDPE: Isobolographic analysis. *Life Sci Pharmacol Lett* 46:PL71.

Paalzow, G.H.M. 1994. Noradrenline but not dopamine involved in NMDA receptor-mediated hyperalgesia induced by theophylline in awake rats. *European J Pharmacol* 252:87.

Paalzow, G.H.M., and L. Paalzow. 1973. The effects of caffeine and theophylline on nociceptive stimulation in the rat. *Acta Pharmacol Toxicol* 32:22.

Pak, M.A., H.L. Haas, U.K.M. Decking, and J. Schrader. 1994. Inhibition of adenosine kinase increases endogenous adenosine and depresses neuronal activity in hippocampal slices. *Neuropharmacol* 33:1053.

Palella, T.D., C.M. Andres, and I.H. Fox. 1980. Human placental kinase. *J Biol Chem* 255:5264.

Paterson A.R.P., N. Kolassa, and C.E. Cass. 1981. Transport of nucleoside drugs in animal cells. *Pharmac Ther* 12:515.

Pertovaara, A. 1993. Antinociception induced by alpha-2-adrenoceptor agonists, with special emphasis on medetomidine studies. *Prog Neurobiol* 40:691.

Phillips, E., and E.A. Newsholme. 1979. Maximum activities, properties and distribution of 5' nucleotidase, adenosine kinase and adenosine deaminase in the rat and human brain. *J Neurochem* 33:553.

Phillis, J.W., and J.P. Edstrom. 1976. Effects of adenosine analogs on rat cerebral cortical neurons. *Life Sci* 19:1041.

Phillis, J.W., and J.R. Kirkpatrick. 1978. The actions of adenosine and various nucleosides and nucleotides on the isolated toad spinal cord. *Gen Pharmacol* 9:239.

Phillis, J.W., and P.H. Wu. 1981. The role of adenosine and its nucleotides in central synaptic transmission. *Prog Neurobiol* 16:187.

Plagemann, P.G.W., and R.M. Wohlhueter. 1980. Permeation of nucleosides, nucleic acid bases, and nucleotides in animal cells. *Curr Topics Membranes Transport* 14:225.

Planas, M.E., L. Rodriguez, S. Sanchez, O. Pol, and M.M. Puig. 1995. Pharmacological evidence for the involvement of the endogenous opioid system in the response to local inflammation in the rat paw. *Pain* 60:67.

Plummer, J.L., P.L. Cmielewski, G.K. Gourlay, H. Owen, and M.J. Cousins. 1992. Antinociceptive and motor effects of intrathecal morphine combined with intrathecal clonidine, noradrenaline, carbachol or midazolam in rats. *Pain* 49:145.

Pons, F., R.F. Bruns, and J.W. Daly. 1980. Depolarization-evoked accumulation of cyclic AMP in brain slices: the requisite intermediate adenosine is not derived from hydrolysis of released ATP. *J Neurochem* 24:1319.

Post, C. 1984. Antinociceptive effects in mice after intrathecal injection of 5'-N-ethylcarboxamide adenosine. *Neurosci Lett* 51:325.

Radulovacki, R., R.M. Virus, M. Djuricic-Nedelson, and R.D. Green. 1983. Hypnotic effects of deoxycoformycin in rats. *Brain Res* 271:392.

Reddy, S.V.R., J.L. Maderdrut, and T.L. Yaksh. 1980. Spinal cord pharmacology of adrenergic agonist-mediated antinociception. *J Pharmacol Exp Therap* 213:525.

Refsum, H., and P.M. Ueland. 1990. Clinical significance of pharmacological modulation of homocysteine metabolism. *Trends in Pharmacol Sci* 11:411.

Roerig, S.C., and J.M. Fujimoto. 1989. Multiplicative interaction between intrathecally and intracerebroventricularly administered *mu* opioid agonists but limited interactions between *delta* and *kappa* agonists for antinociception in mice. *J Pharmacol Exp Therap* 249:762.

Roerig, S.C., J.M. Fujimoto, and L.F. Tseng. 1988. Comparisons of descending pain inhibitory pathways activated by B-endorphin and morphine as characterized by supraspinal and spinal antinociceptive interactions in mice. *J Pharmacol Exp Therap* 247:1107.

Roerig, S.C., R.G. Hoffman, A.E. Takemori, G.L. Wilcox, and J.M Fujimoto. 1991. Isobolographic analysis of analgesic interactions between intrathecally and intracerebroventricularly administered fentanyl morphine and D-ala^2-D-leu^5-enkephalin in morphine-tolerant and nontolerant mice. *J Pharmacol Exp Therap* 257:1091.

Roerig, S.C., S.M. O'Brien, J.M. Fujimoto, and G.L. Wilcox. 1984. Tolerance to morphine analgesia: Decreased multiplicative interaction between spinal and supraspinal sites. *Brain Res* 308:360.

Rogler-Brown, T., R.P. Agarwal, and R.E. Parks, Jr. 1978. Tight binding inhibitors-VI: Interactions of deoxycoformycin and adenosine deaminase in intact human erythrocytes and Sarcoma 180 cells. *Biochem Pharmacol* 27:2289.

Rogler-Brown, T., and R.E. Parks, Jr. 1980. Tight binding inhibitors-VIII: Studies of the interactions of 2'-deoxycoformycin and transport inhibitors with the erythrocytic nucleoside transport system. *Biochem Pharmacol* 29:2491.

Runold, M., N.R. Prabhakar, J. Mitra, and N.S. Cherniack. 1987. Adenosine stimulates respiration by acting on vagal receptors. *Fed Proc* 46:825.

Sakurada, T., Y. Manome, K. Tan-No, S. Sakurada, and K. Kisara. 1990. The effects of substance P analogues on the scratching, biting and licking response induced by intrathecal injection of N-methyl-D-aspartate in mice. *British J Pharmacol* 101:307.

Salter, M.W., Y. DeKoninck, and J.L. Henry. 1992. ATP-Sensitive K^+ channels mediate an IPSP in dorsal horn neurones elicited by sensory stimulation. *Synapse* 11:214.

Salter, M.W., Y. DeKoninck, and J.L. Henry. 1993. Physiological roles for adenosine and ATP in synaptic transmission in the spinal dorsal horn. *Prog in Neurobiol* 42:125.

Salter, M.W., and J.L. Henry. 1987. Evidence that adenosine mediates the depression of spinal dorsal horn neurones induced by peripheral vibration in the cat. *Neurosci* 23:903.

Santos, J.N., K.W. Hempstead, L.E. Kopp, and R.P. Miech. 1968. Nucleotide metabolism in rat brain. *J Neurochem* 15:367.

Sawynok, J, M.J. Espey, and A. Reid. 1991a. 8-Phenyltheophylline reverses the antinociceptive action of morphine in the periaqueductal gray. *Neuropharmacol* 30:871.

Sawynok, J., and A. Reid. 1991. Noradrenergic and purinergic involvement in spinal antinociception by 5-hydroxytryptamine and 2-methyl-5-hydroxytryptamine. *European J Pharmacol* 204:301.

Sawynok, J., A. Reid, and D. Nance. 1991. Spinal antinociception by adenosine analogs and morphine after intrathecal administration of the neurotoxins capsaicin, 6-hydroxydopamine and 5,7-dihydroxytryptamine. *J Pharmacol Exp Therap* 258:370.

Sawynok, J., and M.I. Sweeney. 1989. The role of purines in nociception. *Neurosci* 32:557.

Sawynok, J., M.I. Sweeney, and T.D. White. 1986. Classification of adenosine receptors mediating antinociception in the rat spinal cord. *British J Pharmacol* 88:923.

Sawynok, J., and T.L. Yaksh. 1993. Caffeine as an analgesic adjuvant: A review of pharmacology and mechanisms of action. *Pharmacol Rev* 45:43.

Schatz, R.A., T.E. Wilens, and O.Z. Sellinger. 1981. Decreased transmethylation of biogenic amines after *in vivo* elevation of brain *S*-adenosyl-L-homocysteine. *J Neurochem* 36:1739.

Schrader, J. 1983. Metabolism of adenosine and sites of production in the heart. *In* Regulatory function of adenosine, ed. by R.M. Berne, T.W. Rall and R. Rubio (Martinus Nijhoff, Boston):133.

Schrader, J. 1991. Formation and metabolism of adenosine and adenine nucleotides in cardiac tissue. In: *Adenosine and adenine nucleotides as regulators of cellular function*, 1st edn, pp55-70. Ed. J.W. Phillis, CRC press: Boca Raton, FL.

Scott, T.G. 1967. The distribution of 5'-nucleotidase activity in the brain of the mouse. *J Comp Neurol* 129:97.

Seegmiller, J.E. 1985. Overview of possible relation of defects in purine metabolism to immune deficiency. *Annal N York Acad Sci* 451:9.

Shank, R.P., and W.J. Baldy. 1990. Adenosine transport by rat and guinea pig synaptosomes: basis for differential sensitivity to transport inhibitors. *J Neurochem* 55:541

Shimizu, H., S. Tanaka, and T. Kodama. 1972. Adenosine kinase of mammalian brain: Partial purification and its role for the uptake of adenosine. *J Neurochem* 19:687.

Smullin, D.H., S.R. Skilling, and A.A. Larson. 1990. Interactions between substance P, calcitonin gene-related peptide, taurine and excitatory amino acids in the spinal cord. *Pain* 42:93.

Snyder, S.H. 1985. Adenosine as a neuromodulator. *Ann Rev Neurosci* 8:103.

Sofuoglu, N., P.S. Portoghese, and A.E. Takemori. 1991. Cross-tolerance studies in the spinal cord of B-FNA-treated mice provides further evidence for *delta* opioid receptor subtypes. *Life Sci Pharmacol Lett* 49:PL153.

Sollevi, A. 1991. Effects of adenosine infusion on the conscious man. In: *Adenosine and Adenine Nucleotides as Regulators of Cellular Functions*, J.W. Phillis, ed (CRC Press), pp. 283-291.

Sollevi, A., M. Belfrage, T. Lundeberg, M. Segerdahl, and P. Hansson. 1995. Systemic adenosine infusion: a new treatment modality to alleviate neuropathic pain. *Pain* 61:155.

Solomon, R.E., and G.F. Gebhart. 1988. Mechanisms of effects of intrathecal serotonin on nociception and blood pressure in rats. *J Pharmacol Exp Therap* 245:905.

Solomon, R.E., and G.F. Gebhart. 1994. Synergistic antinociceptive interactions among drugs administered to the spinal cord. *Anesth Analg* 78:1164.

Sosnowski, M., C.W. Stevens, and T.L. Yaksh. 1989. Assessment of the role of A_1/A_2 adenosine receptors mediating the purine antinociception, motor, and autonomic function in the rat spinal cord. *J Pharmacol Exp Therap* 250:915.

Sosnowski, M., and T.L. Yaksh. 1989. Role of spinal adenosine receptors in modulating the hyperesthesia produced by spinal glycine receptor antagonism. *Anesth Analg* 69:587.

Stein, C., M.J. Millan, T.S. Shippenberg, K. Peter, and A. Herz. 1989. Peripheral opioid receptors mediating antinociception in inflammation. Evidence for involvement of *mu, delta,* and *kappa receptors*. *J Pharmacol Exp Therap* 248:1269.

Stone, T.W. 1991. Receptors for adenosine and adenine nucleotides. *Gen Pharmac* 22:25.

Sutherland, E.W., and T.W. Rall. 1958. Fractionation and characterization of a cyclic adenine ribonucleotide formed by tissue particles. *J Biol Chem* 232:1077.

Sweeney, M.I., T.D. White, K.H. Jhamandas, and J. Sawynok. 1987a. Morphine releases endogenous adenosine from the spinal cord *in vivo*. *Eur J Pharmacol* 141:169.

Sweeney, M.I., T.D. White, and J. Sawynok. 1987b. Involvement of adenosine in the spinal antinociceptive effects of morphine and noradrenalin. *J Pharmacol Exp Therap* 543:657.

Sweeney, M.I., T.D. White, and J. Sawynok. 1988. 5-Hydroxytryptamine releases adenosine from primary afferent nerve terminals in the spinal cord. *Brain Res* 462:346.

Sweeney, M.I., T.D. White, and J. Sawynok. 1989. Morphine, capsaicin and K^+ release purines from capsaicin-sensitive primary afferent nerve terminals in the spinal cord. *J Pharmacol Exp Therap* 248:447.

Sweeney, M.I., T.D. White, and J. Sawynok. 1990. 5-Hydroxytryptamine releases adenosine and cyclic AMP from primary afferent nerve terminals in the spinal cord *in vivo*. *Brain Res* 528:55.

Sweeney, M.I., T.D. White, and J. Sawynok. 1991. Intracerebroventricular morphine releases adenosine and adenosine 3'-5'-cyclic monophosphate from the spinal cord *via* a serotonergic mechanism. *J Pharmacol Exp Therap* 259:1013.

Sweeney, M.I., T.D. White, and J. Sawynok. 1993. Morphine-evoked release of adenosine from the spinal cord occurs via a nucleoside carrier with differential sensitivity to dipyridamole and nitrobenzylthioinosine. *Brain Res* 614:301.

Taiwo, Y.O., and J.D. Levine. 1990. Direct cutaneous hyperalgesia induced by adenosine. *Neurosci* 38:757.

Taiwo, Y.O., and J.D. Levine. 1991. Further confirmation of the role of adenyl cyclase and of cAMP-dependent protein kinase in primary afferent hyperalgesia. *Neurosci* 44:131.

Tallarida, R.J. 1992. Statistical analysis of drug combinations for synergism. *Pain* 49:93.

Tallarida, R.J., and R.B. Murray. 1981. Manual of pharmacologic calculation, Springer-Verlag, New York.

Tallarida, R.J., F. Porreca, and A. Cowan. 1989. Statistical analysis of drug- drug and site-site interactions with isobolograms. *Life Sci* 45:947.

Ueland, P.M. 1982. Pharmacological and biochemical aspects of S-adenosylhomocysteine and S-adenosylhomocysteine hydrolase. *Pharmacol Rev* 34:223.

Uhl, G.R., S. Childers, and G. Pasternak. 1994. An opiate-receptor gene family reunion. *Trends Neurosci* 17:89.

Urban, L., S.W.N. Thompson, and A. Dray. 1994. Modulation of spinal excitability: Co-operation between neurokinin and excitatory amino acid neurotransmitters. *Trends Neurosci* 17:432.

van Calker, D., M. Muller, and B. Hamprecht. 1979. Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J Neurochem* 33:999.

van der Weyden, M.B., and W.N. Kelley. 1976. Human adenosine deaminase: Distribution and properties. *J Biol Chem* 251:5448.

Vapaatolo, H. D. Onken, P.J. Neuvonen, and E. Westermann. 1975. Stereospecificity in some central and circulatory effects of phenylisopropyl-adenosine (PIA). *Arzneimittel-Forsch* 25:407.

Welch, S.P., and L.D. Dunlow. 1993. Antinociceptive activity of intrathecally administered potassium channel openers and opioid agonists: A common mechanism of action? *J Pharmacol Exp Therap* 267:390.

Westfall, D.P., H.H. Dalziel, and K.M. Forsyth. 1991. ATP as a neurotransmitter, cotransmitter and neuromodulator. In: *Adenosine and adenine nucleotides as regulators of cellular function*, 1st edn, pp295-307. Ed. J.W. Phillis, CRC press: Boca Raton, FL.

Wigdor, S., and G.L. Wilcox. 1987. Central and systemic morphine-induced antinociception in mice: Contribution of descending serotonergic and noradrenergic pathways. *J Pharmacol Exp Therap* 242:90.

Wilcox, G.L. 1988. Pharmacological studies of grooming and scratching behavior elicited by spinal substance P and excitatory amino acids. *Ann NY Acad Sci* 525:228.

Wilcox, G.L. 1991. Excitatory neurotransmitters and pain. In *Proceedings of the VIth World Congress on Pain*, pp 97.

Wild, K.D., T. Vanderah, H.I. Mosberg, and F. Porreca. 1991. Opioid δ receptor subtypes are associated with different potassium channels. *European J Pharmacol* 193:135.

Winn, H.R., T.S. Park, R.R. Curnish, R. Rubio, and R.M. Berne. 1980. Incorporation of adenosine and its metabolites into brain nucleotides. *Am J Physiol* 239:H212.

Woolf, C.J. 1994. The dorsal horn: State-dependent sensory processing and the generation of pain. In: *Textbook of Pain*, pp101-112. P.D. Wall and R. Melzack, eds. Churchill Livingstone Press, London.

Woolf, C.J., P. Shortland, and R.E. Coggeshall. 1992. Peripheral nerve injury triggers central sprouting of myelinated afferents. *Nature* 355:75.

Woolf, C.J., and Z. Wiesenfeld-Hallin. 1986. Substance P and calcitonin gene-related peptide synergistically modulate the gain of the nociceptive flexor withdrawal reflex in the rat. *Neurosci Lett* 66:226.

Woolfolk, D.R., and S.G. Holtzman. 1993. Restraint stress potentiates analgesia induced by 5'-N-ethylcarboxamidoadenosine: Comparison with morphine. *European J Pharmacol* 239:177.

Yaksh, T.L. 1981. The antinociceptive effects of intrathecally administered levonantradol and desacetyllevonantradol in the rat. *J Clin Pharmacol* 21:334S.

Yaksh, T.L. 1985. Pharmacology of spinal adrenergic systems which modulate spinal nociceptive processing. *Pharmacol Biochem Behav* 22:845.

Yaksh, T.L., R. Dirksen, and G.J. Harty. 1985. Antinociceptive effects of intrathecally injected cholinomimetic drugs in the rat and cat. *Anesthesiology* 117:81.

Yaksh, T.L., and G.J. Harty. 1988. Pharmacology of the allodynia in rats evoked by high dose intrathecal morphine. *J Pharmacol Exp Therap* 244:501.

Yaksh, T.L., and A.B. Malmberg. 1994. Central pharmacology of nociceptive transmission. In: *Textbook of pain*, 3rd ed., eds. P.D. Wall and R. Melzack, pp. 165–200, Churchill Livingstone, New York.

Yaksh, T.L., and R. Noueihed. 1985. The physiology and pharmacology of spinal opiates. *Ann Rev Pharmacol Toxicol* 25:433.

Yamada, Y., H. Goto, and N. Ogawawara. 1980. Purification and properties of adenosine kinase from rat brain. *Biochimica Biophysica Acta* 616:199.

Yamamoto, T., and T.L. Yaksh. 1991. Stereospecific effects of a nonpeptidic NK1 selective antagonist, CP-96,345: Antinociception in the absence of motor dysfunction. *Life Sci* 49:1955.

Yamashita, S., J.B. Park, P.D. Ryu, H. Inukai, M. Tanifuji, K. Murase. 1994. Possible presence of the ATP-sensitive K^+ channel in isolated spinal dorsal horn neurons of the rat. *Neurosci Lett* 170:208.

Yarbrough, G.G., and J.C. McGuffin-Clineschmidt. 1981. *In vivo* behavioral assessment of central nervous system purinergic receptors. *European J Pharmacol* 76:137.

Yeung, J.C., and T.A. Rudy. 1980. Multiplicative interactions between narcotic agonisms expressed at spinal and supraspinal sites of antinociceptive action as revealed by concurrent intrathecal and intracerebroventricular injections of morphine. *J Pharmacol Exp Therap* 215:633.

Young, J.D., and S.M. Jarvis. 1983. Nucleoside transport in animal cells. *Biosci Rep* 3:309.

Zetterström, T., L. Vernet, U. Ungerstedt, J. Tossman and B.B. Fredholm. 1982. Purine levels in the intact rat brain. Studies with an implanted perfused hollow fibre. *Neurosci Lett* 29:111.

Zhang, G., P.H. Franklin, and T.F. Murray. 1993. Manipulation of endogenous adenosine in the rat prepiriform cortex modulates seizure susceptibility. *J Pharmacol Exp Therap* 264:1415.

Zhang, G., and T.F. Murray. 1991. Manipulation of endogenous adenosine levels in rat prepiriform cortex modulates seizure susceptibility. *Soc Am Soc Pharmacol Exp Therap* 33:196.

Zhang, Y., J.D. Geiger, D.J. Legare, and W.W. Lautt. 1991. Dilazep-induced vasodilation is mediated through adenosine receptors. *Life Sci* 49:PL129.

Zieglgänsberger, W., and T.R. Tölle. 1993. The pharmacology of pain signalling. *Curr Opinion Neurobiol* 3:611.

Zimmerman, H. 1994. Signalling via ATP in the nervous system. *Trends Neurosci* 17:420.