INVESTIGATIONS OF INTERACTIONS BETWEEN NUCLEOSIDE TRANSPORTERS AND ADENOSINE RECEPTORS IN THREE CELL MODELS

by

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

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This thesis is dedicated to my family and friends whose unconditional support and encouragement is gratefully acknowledged

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ABSTRACT

Adenosine is a modulator that is involved in a large number of physiological processes in peripheral nerves, tissues and in the CNS. Adenosine levels are regulated by the intracellular and extracellular adenosine metabolizing enzymes as well as two main classes of nucleoside transporters, sodium-dependent and sodium-independent, which catalyze the movement of nucleosides across biological membranes. Once formed, adenosine can act on cell surface adenosine receptors. These studies were performed to (1) determine whether sodium-dependent transporters can function in the release of nucleosides during conditions that disturb metabolic or ionic homeostasis; (2) determine whether nucleoside release by sodium-independent (*es*) transporters can be differentially regulated by adenosine receptor stimulation; and (3) characterize the inhibitory effects of propentofylline on cAMP phosphodiesterase, adenosine transporters and three adenosine receptor types. Using mouse leukemia L1210/MA27.1 cells which possess only sodium-dependent nucleoside transporters, we found that disruption of the sodium-gradient with ouabain, a Na^+/K^+ATPase inhibitor or monensin, a sodium ionophore, caused release of the poorly metabolized nucleoside analogue [^{3}H]formycin B. These results suggest that adenosine may be released by sodium-dependent transporters, in addition to sodium-independent (*es*) transporters, during conditions such as ischemia that depress sodium gradients. To determine whether the release of nucleosides through *es* transporters and activation of adenosine receptors can affect further nucleoside release, we used DDT_1 MF-2 smooth muscle cells which possess adenosine A_1 and A_2 receptors as well as *es* transporters. We found that A_1 and A_2 stimulation by agonists cyclohexyladenosine

(CHA) and N-ethylcarboxamidoadenosine (NECA), respectively, did not modify [^{3}H]formycin B release from DDT$_1$ MF-2 smooth muscle cells. The effect of the nucleoside transport inhibitor, propentofylline, on adenosine receptor activation was examined in three Chinese hamster ovary (CHO) cell lines which had been transfected with A_1, A_{2a}, or A_{2b} receptors as well as a luciferase reporter gene under control of several copies of the cAMP response element. At high concentrations ($\geq$ 1 mM), propentofylline stimulated luciferase activity, likely by inhibiting cAMP phosphodiesterase activity. Propentofylline also caused significant inhibition of adenosine accumulation, with IC_{50} values of approximately 0.1 mM in all three cell types. Inhibition of receptors, stimulated with the agonist NECA, was evident for A_1 and A_{2a} receptors, and was of statistical significance for A_{2a} receptors. For receptors stimulated with adenosine this inhibition was reduced, indicating that the effects of propentofylline to inhibit adenosine uptake counteracts its effects to inhibit receptor activation. Whether these mechanisms contribute to the neuroprotective effects of propentofylline remain to be examined. These studies characterized several of the roles that specific transporters may play in regulating adenosine levels, and thus, the receptor-mediated effects of adenosine.

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ABBREVIATIONS

8-PT	8-phenyltheophylline
ADAC	adenosine amine congener
ADO	adenosine
ADP	adenosine 5'-diphosphate
APEC	2-[(2-aminoethylamino)-carbonylethylphenylethylamino]-5'-*N*-ethylcarboxamidoadenosine
APNEA	N^6-2-(4-aminophenyl)ethyladenosine
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
BW-A 522	3-(3-iodo-4-aminobenzyl)-8-(4-oxyacetate)-1-propylxanthine
Ca^{2+}	calcium ion
cAMP	cyclic adenosine 3',5'-monophosphate
cGMP	cyclic guanosine 3',5'-monophosphate
CGS 15843	9-chloro-2-(2-furanyl)-5,6-dihydro-[1,2,4]-triazolo[1,5]quinazolin-5-imine monomethanesulfonate
CGS 21680	2-[p-(2-carbonyl-ethyl)-phenylethylamino]-5'-N-ethylcarboxamido adenosine
CHA	cyclohexyladenosine
Ci	Curie
CNS	central nervous system
CPA	cyclopentyladenosine

CPT	cyclopentyltheophylline
CSC	8-(3-chlorostyryl)caffeine
CV 1808	2-phenylaminoadenosine
ei	equilibrative NBMPR-insensitive nucleoside transporter
es	equilibrative NBMPR-sensitive nucleoside transporter
EC_{50}	effective concentration at which half maximal effect occurs
FOR	forskolin
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPCPX	1,3-dipropyl-8-cyclopentylxanthine
Fig.	Figure
g	gravity force
g	grams
GTP	guanosine triphosphate
h	hours
I-ABOPX	1-propyl-3-(3-iodo-4-aminobenzyl)-8-(4-oxyacetate)phenylxanthine
IC_{50}	concentration of inhibitor at which maximum effect is inhibited by 50%
IMP	inosine monophophate
K^+	potassium ion
Km	Michaelis-Menten constant
Ki	inhibition constant

L-adenosine	9-β-L-ribofuranosyladenosine
mg	milligram
min	minute
ml	millilitres
mM	millimolar
mRNA	messenger ribonucleic acid
n	number of samples
N1/*cif*	Na^+ -dependent nucleoside transporter subtype 1
N2/*cit*	Na^+ -dependent nucleoside transporter subtype 2
N3/*cib*	Na^+ -dependent nucleoside transporter subtype 3
N4/*cit*	Na^+ -dependent nucleoside transporter subtype 4
N5/*cs*	Na^+ -dependent nucleoside transporter subtype 5
Na^+	sodium ion
NBMPR	nitrobenzylmercaptopurine riboside or nitrobenzylthioinosine
NECA	5'-*N*-ethylcarboxamidoadenosine
nM	nanomolar
NMDA	N-methyl-D-aspartate
pmol	picomoles
PPF	propentofylline
R-PIA	(-)-N^6-(R-phenylisopropyl)adenosine
RNA	ribonucleic acid
S-PIA	(+)-N^6-(S-phenylisopropyl)adenosine

SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
S.D.	standard deviation
sec	seconds
SEM	standard error of mean
μCi	microCurie
μl	microlitre
μM	micromolar
XAC	xanthine amine congener

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Chapter 1. Introduction

Adenosine, a ubiquitous nucleoside formed from the breakdown of adenosine triphosphate (ATP), is involved in a large number of physiological processes. These range from vasodilation (Morff and Granger, 1983; Runold et al., 1990; Sollevi 1986), immune responses (Maquart et al., 1994; Ramkumar et al., 1993) and lipolysis (Schwabe, 1983) to activity as a neuromodulator in the central nervous system (Latini et al., 1996; Fredholm, 1995). Adenosine was first shown to be a mediator of biological effects in 1929 when Drury and Szent-György demonstrated bradycardia and vasodilation after infusing adenosine and AMP to the mammalian heart (Drury and Szent-Györgyi, 1929). A wider interest in the role of adenosine followed from the demonstration that adenosine can be produced by the heart when deprived of oxygen (Berne, 1963; Gerlach et al., 1963). The observation that adenosine increased cAMP levels in rodent brain slices and that this accumulation was inhibited by methylxanthines such as theophylline and caffeine strongly suggested the presence of adenosine receptors (Sattin and Rall, 1970).

In addition to its peripheral modulatory role, adenosine has been found to have neuroprotective properties. In particular, it has been found to have a potent depressant effect on neurons (Dunwiddie, 1985; Fredholm and Dunwiddie, 1988) which results in a reduction of excitatory neurotransmission (Phillis et al., 1979; Okada and Ozawa, 1980). Consequently adenosine reduces neuronal injury associated with strokes and seizures.

Since adenosine appears to have significant cardio- and neuro-protective properties, therapeutic aims have been directed at developing agents which either mimic the effects of adenosine or enhance the activity of endogenous adenosine at its receptors.

1. Endogenous Adenosine

1.1 Adenosine Formation

Adenosine is formed primarily by metabolism of ATP, the end product of mitochondrial oxidative phosphorylation. Adenosine is also formed through hydrolysis of S-adenosylhomocysteine by S-adenosylhomocysteine hydrolase (Schrader et al., 1981). Once formed intracellularly, adenosine either is metabolized rapidly or released through cellular transport processes (Fig. 1).

Levels of adenosine are directly proportional to the relative rates of synthesis and hydrolysis of ATP. Under physiological conditions, plasma adenosine levels range between 0.1 and 1 μM (Onyd and Schrader, 1984). In unanaethetized, freely-moving rats, brain adenosine levels exist in nanomolar concentrations (Rudolphi et al., 1992). However, in conditions where there is an increase in neuronal activity or a decrease in oxygen and/or glucose availability, extracellular adenosine can increase to micromolar levels (Zetterström et al., 1982; Hagberg et al., 1987).

There is evidence that ATP can be released from cells (Burnstock, 1972; Burnstock, 1986) and subsequently hydrolyzed to ADP, AMP, and adenosine via ecto-nucleotidases (Zimmerman, 1992; Pearson et al., 1980) and possibly ecto-adenosine deaminase (see Geiger et al., 1991). The degree to which extracellular adenosine is formed from this pathway is variable between tissues due to differing activities of ecto-nucleotidase enzymes (Geiger et al., 1991).

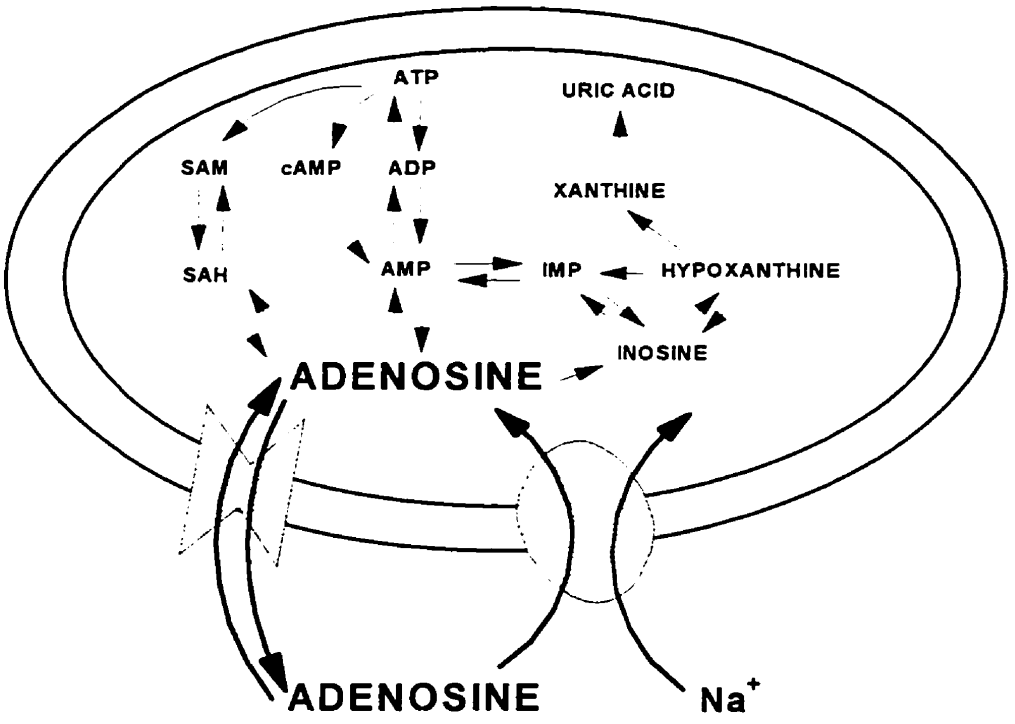


Fig. 1. Cellular metabolism, uptake and release of adenosine.

ABBREVIATIONS: AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; IMP, inosine monophosphate.

Taken from Parkinson and Anderson, 1995.

It is possible that cAMP may act as a source of adenosine following its metabolism to 5'AMP intracellularly or extracellularly (Cramer, 1977; Doore et al., 1975; Pull and McIlwain, 1977; Rosenberg and Li, 1995). Although this hypothesis has not been studied extensively, it is consistent with the small reduction in adenosine release from neural tissue observed during inhibition of phosphodiesterase, the catabolic enzyme for cAMP (Stone, et al., 1981; Rosenberg and Li., 1995).

De novo synthesis of adenosine involves the formation of 5'-inosine monophophate and subsequent conversion to 5'-AMP through the actions of adenylosuccinate synthetase and lyase (Schultz and Lowenstein, 1976). However, *de novo* synthesis of purines is expensive in terms of cellular energy and the concentration of enzymes responsible for this pathway are low in most tissues (Zimmer et al., 1973; Allsop and Watts, 1983). Therefore, this pathway represents a very minor contribution to the overall functional pool of adenosine.

1.2. Adenosine Metabolism

Once formed, adenosine is rapidly metabolized. Adenosine has an extremely short biological half-life in the range of 3-6 seconds (Rudolphi et al., 1992) which is due to metabolism by adenosine deaminase, adenosine kinase or S-adenosylhomocysteine hydrolase (Geiger et al., 1997). Adenosine deaminase is the enzyme that deaminates adenosine to form inosine. The activity of this enzyme is thought to be mainly localized to the cytosol, however, recent evidence suggests the presence of extracellular adenosine deaminase (Martin et al., 1995). Inosine has a low affinity for adenosine receptors (Bruns

et al., 1980) and therefore is not thought to be a relevant mediator of physiological functions through these receptors.

Adenosine kinase phosphorylates adenosine to AMP which is further phosphorylated to ADP and ATP. Adenosine kinase has an affinity (Km) in the low micromolar range and is saturated at near physiological concentrations of adenosine (Phillips and Newsholme, 1984; Mistry and Drummond, 1986). Substrate inhibition of adenosine kinase has been observed in rat heart when adenosine reaches supraphysiological levels (Fisher and Newsholme, 1984). Adenosine kinase activity appears to predominate at low adenosine concentrations whereas adenosine deaminase activity is greater at higher adenosine concentrations, and may predominate during hypoxia or ischemia (Kohn and Garfinkel, 1977).

S-adenosylhomocysteine hydrolase catalyzes another metabolic pathway for adenosine. This enzyme is located intracellularly and is responsible for the condensation of adenosine with homocysteine to form S-adenosylhomocysteine (Schrader et al., 1981; Lloyd and Schrader, 1987). Activity of this enzyme is limited due to the low availability of homocysteine (Snyder, 1985).

Intracellular concentrations of adenosine are maintained at low levels due to metabolism of adenosine. Extracellular concentrations are also maintained at low levels due to the presence of membrane-bound transport proteins which can mediate both uptake and release of adenosine (Fig 1.).

2. Adenosine Receptors

It was first observed by Sattin and Rall (1970) that adenosine receptor stimulation increased cAMP levels in the brain. It was later demonstrated that adenosine inhibited cAMP formation in fat cells (Fain et al., 1972). Therefore, the differential action of adenosine on cAMP formation provided an early biochemical means for identifying and classifying adenosine receptors. Pharmacologically, it is possible to distinguish each receptor type in terms of agonist and antagonist potencies in radioligand binding experiments and in functional biological assays (Williams and Jacobson, 1990; Van Galen et al., 1992; Abbrachio et al., 1993; Fredholm et al., 1992) . Burnstock classified purine-sensitive receptors into two subclasses; P1 and P2 receptors. P1 receptors exhibit an agonist preference of adenosine>AMP>ADP>ATP and are selectively blocked by methylxanthines. P2 receptors are selective for nucleotides ATP and ADP as well as diadenosine tetraphosphate and are insensitive to methylxanthines (Burnstock, 1978; 1980; Fredholm et al., 1994). Classification of purinergic P1 receptor into A_1 and A_2 adenosine receptors was proposed by Van Calker et al. in 1979 and is now generally accepted along with the A_{2a} and A_{2b} nomenclature. Current subclassification of A_1, A_{2a}, A_{2b} and A_3 receptors is based mainly on cloned sequences, agonist and antagonist potencies and biochemical characterization.

2.1. A_1 Receptors

Adenosine A_1 receptors couple to G_{i1}, G_{i2}, G_{i3} and G_o proteins (Freissmuth et al., 1991; Munshi et al., 1991) and are classically associated with the inhibition of adenylyl cyclase (Van Calker et al., 1978; Londos et al., 1980). These receptors have also been

demonstrated to inhibit Ca^{2+} conductance (Dolphin et al., 1986; Scholz and Miller, 1991; Mogul et al., 1993) stimulate K^+ conductance (Belardinelli and Isenberg, 1983; Trussell and Jackson, 1985) and affect phospholipase C activity (Gerwins and Fredholm, 1992; Gerwins, 1993).

High affinity agonists (Kd = 0.3-3 nM) for A_1 receptors in potency order include cyclopentyladenosine (CPA), cyclohexyladenosine (CHA), (–)-N^6-(R-phenyl-isopropyl)adenosine (R-PIA), and adenosine amine congener (ADAC). Agonists with moderate potency (3-30 nM) include 5'-*N*-ethylcarboxamidoadenosine (NECA), 2-chloroadenosine, and adenosine. An agonist which binds to the A_1 receptor with low affinity (30-350 nM) is (+)-N^6-phenylisopropyladenosine (S-PIA) (Fredholm et al., 1994).

A_1 receptors bind antagonists 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) and xanthine amine congener (XAC) with high affinity (0.5 - 2 nM). 8-Cyclopentyl-theophylline (CPT), 8-phenyltheophylline (8-PT) and CGS 15943 bind to A_1 receptors with intermediate potency (2-200 nM) and theophylline and 8-p-sulfophenyltheophylline bind with low potency (1 - 20 μM) (Fredholm et al., 1994).

Clones of the A_1 receptor from rat (Mahan et al., 1991; Reppert et al., 1991), bovine (Olah et al., 1992; Tucker et al., 1992), human (Liebert et al., 1992; Townsend-Nicholson and Shine, 1992; Ren and Stiles, 1994) and rabbit (Bhattacharya et al., 1993) encode proteins of 326 amino acids which have molecular weights of ~36,700 Daltons. There is approximately 87% overall amino acid identity of the species homologues of the A_1 receptor.

In situ hybridization and northern blotting techniques have demonstrated A_1 receptor mRNA to be highly expressed in the brain, particularly in the cortex, cerebellum, thalamus and hippocampus. A_1 receptor mRNA is also found in the spinal cord, fat cells, and testis (Olah and Stiles, 1995). Generally, the A_1 mRNA level correlates well with expression of the receptor protein.

2. 2. A_{2a} Receptors

A_{2a} receptors couple Gs proteins to stimulation of adenylyl cyclase and increased formation of cAMP (Van Calker et al., 1979). There is speculation on the existence of other G proteins which may be activated by stimulation of A_2 receptors such as G_{olf} (Fredholm, 1995). In addition, it has been shown that adenosine A_2 receptors potentiate P-type Ca^{2+} channels in hippocampal neurons through a mechanism involving cAMP-dependent protein kinases (Mogul et al., 1993).

High affinity A_{2a} agonists include CGS 21680, NECA, 2-[(2-aminoethylamino)-carbonylethylphenylethylamino]-5'-*N*-ethylcarboxamidoadenosine (APEC) and adenosine (1-20 nM). CGS 21680 appears to be selective for A_{2a} receptors. Other agonists which bind to A_{2a} receptors with lower affinity (>20 nM) include 2-chloroadenosine, CV 1808, R-PIA and ADAC. High affinity (20-100 nM) antagonists for A_{2a} receptors include XAC, 8-(3-chlorostyryl)caffeine (CSC), KF 17837, and CGS 15943. Agonists with intermediate (0.2-2 μM) affinity include CPT, DPCPX and 8-PT (Fredholm et al., 1994).

A_{2a} receptors have been cloned from canine (Maenhaut et al., 1990), rat (Fink et al., 1992) and human (Furlong et al., 1992) cDNA libraries. A_{2a} receptor mRNA encodes

a slightly larger protein than the other adenosine receptors; it has 410-412 amino acids and a molecular weight of ~45,000 Daltons (Olah and Stiles, 1995). The additional amino acids associated with the A_{2a} receptor as compared to the other receptors represents the elongated carboxy-terminus. Although the functional significance of the elongated tail is unknown, it is speculated that this region may represent potential phosphorylation sites for G protein-receptor kinases and could therefore be involved in desensitization processes (Olah and Stiles, 1995; Shimada et al., 1992).

A_{2a} receptors are located primarily in the brain with the highest abundance in the dopamine-rich regions including caudate putamen, nucleus accumbens, and tuberculum olfactorium (Fredholm, 1995). A_{2a} receptor mRNA has also been found in human heart, kidney and lung (Linden et al., 1993).

2.3. A_{2b} Receptors

Similar to A_{2a} receptors, A_{2b} receptors are Gs protein-linked stimulatory receptors. A_{2b} receptors have been cloned from rat (Stelhe et al., 1992; Rivkees and Reppert, 1992) and human (Pierce et al., 1992) brain cDNA libraries. The A_{2b} receptor consists of 332 amino acid residues and has a molecular weight of 36,000 Daltons (Fredholm, 1994). Simulation of adenylate cyclase activity and increased cAMP levels have been demonstrated with the cloned A_{2b} receptor (Rivkees and Reppert, 1992). Furthermore, when NECA was applied to *Xenopus* oocytes injected with rat A_{2b} receptor mRNA, an inward chloride current coupled to phospholipase C activation was demonstrated (Yakel et al., 1993). Marquardt et al. (1992) has demonstrated an A_{2b} receptor mediated

stimulation of calcium channel activity in oocytes injected with mouse A_{2b} receptor mRNA.

Whereas A_{2a} receptors are localized mainly in dopamine-rich areas in the brain, A_{2b} receptors are ubiquitous. High expression of rat A_{2b} mRNA was detected in the caecum, large intestine and urinary bladder. Brain, spinal cord and lung also expressed A_{2b} mRNA (Stehle et al., 1992).

The only reasonably high affinity (0.5 - 5 μM) agonist for A_{2b} receptors is NECA (Fredholm et al., 1994). At the 5-20 micromolar range 2-chloroadenosine, adenosine and R-PIA can stimulate A_{2b} receptors. In contrast to A_{2a} receptors, A_{2b} receptors do not bind CGS 21680. Interestingly, higher concentrations of adenosine are required to activate A_{2b} receptors versus A_{2a} receptors. This suggests that supraphysiological levels of adenosine are required to activate A_{2b} receptors whereas basal adenosine levels can stimulate A_{2a} receptors. There are no known selective antagonists for this receptor, but XAC, CPX, 8-PT and CGS 15943 are effective antagonists (Fredholm et al., 1994).

2. 4. A_3 Receptors

The exact signal transduction mechanism of the recently discovered A_3 receptor is not fully understood. Stimulation of A_3 receptors with NECA or R-PIA resulted in a pertussis toxin-sensitive inhibition of forskolin-stimulated adenylyl cyclase (Zhou et al., 1992). Inhibition of cAMP accumulation is consistent with activity of a G_i-type protein, however the exact G protein coupling for these receptors remains to be elucidated (Linden, 1994). A_3 receptors have been shown to be involved in regulation of inositol

triphosphate (IP_3)-induced increases in levels of intracellular Ca^{2+} in RBL-2H3 mast cells (Ramkumar et al., 1993) and G protein-dependent activation of phospholipase C in rat brain (Abbracchio et al., 1995).

Meyerhof et al. (1991) and Zhou et al. (1992) independently cloned A_3 receptors from rat testis and brain. The isolated cDNA encodes a 320 amino acid residue, 36,000 Dalton protein. Adenosine A_3 receptors from sheep (Linden et al., 1993) and human (Salvatore et al., 1993) have subsequently been cloned and display a 72% overall sequence identity to the rat A_3 receptor. In contrast to other cloned adenosine receptors, A_3 receptors exhibit a consensus site for N-linked glycosylation on both the amino terminus and the second extracellular loop.

The distribution of A_3 receptors varies depending on the species. In rat, A_3 receptor mRNA was primarily localized to testes (Zhou et al., 1992) whereas in sheep a wide distribution was evident (Linden et al., 1993). A tissue distribution profile of lung = liver >> brain = aorta > testis > heart was reported for humans (Salvatore et al., 1993). Demonstration of A_3 receptor mRNA in lung appears to be common in all species (Olah and Stiles, 1995).

High affinity agonists (<10 nM) for A_3 receptors include N^6-2-(4-aminophenyl)-ethyladenosine (APNEA) and N^6-benzyl-NECA. Agonists which bind with intermediate affinity (10-30 nM) include NECA and R-PIA. In contrast to other adenosine receptors, classical xanthines do not inhibit rat A_3 receptors (Fredholm, 1995). However, cloned sheep (Linden et al., 1993) and human (Salvatore et al., 1993) A_3 receptors do bind

certain xanthine antagonists (I-ABOPX) with micromolar affinity. A high affinity (1-20 nM) antagonist for A_3 receptors is BW-A 522 (Fredholm et al., 1994).

2.5. Adenosine Receptor-Mediated Effects and Therapeutic Use

2.5.1. Peripheral effects of adenosine

It has long been known that adenosine is a potent bradycardic and blood pressure lowering agent with marked vasoconstrictor effects in the kidney (Drury and Szent-Györgyi, 1929). Currently adenosine is used for treatments of cardiac arrhythmias such as paroxysmal supraventricular tachycardia. Adenosine acting on A_1 receptors in the heart, slows down conduction at the atrio-ventricular node by decreasing Ca^{2+} influx resulting in decreased depolarization (Collis, 1991; Mullane and Williams, 1991).

Adenosine infusion increases coronary blood flow due to vasodilatory actions (Berne, 1963). A_{2a} activation in vascular beds is also associated with a reduction in blood pressure (Olsson and Pearson, 1990). These effects are predominantly mediated by an increase in cAMP formation, however, it has been suggested that ATP-dependent K^+ channels may also mediate vasodilation (Daut et al., 1990). Although adenosine represents a novel hypotensive agent via vasodilatory activity, its ability to cause bradycardia through A_1 receptors represents a major drawback. However, use of selective A_2 receptor agonists to achieve only vasorelaxant effects may be possible (Mullane and Williams, 1991).

Antithrombotic effects of adenosine have also been demonstrated. Activation of the A_{2a} receptor on platelets elevates intracellular cAMP levels, resulting in the inhibition

of platelet aggregation produced by ADP and other agents (Olsson and Pearson, 1990). Furthermore this anti-platelet aggregation effect of adenosine and its analogues is blocked by methylxanthines (Mills and Smith, 1971).

Another potential therapeutic use for adenosine or its analogues is to provide protection to the heart during ischemia or infarction. It was found that adenosine is released in large quantities during ischemia and is important for stabilizing tissues and cells under stressful conditions (Downey et al., 1993). Ischemic preconditioning, whereby a sublethal period of ischemia leads to resistance of the myocardium to subsequent ischemia, was initially proposed by Ely et al. (1985). Subsequent studies have documented that stimulation of A_1 and possibly A_3 adenosine receptors may mediate these effects (Thornton et al., 1992).

Adenosine has also been implicated as an anti-inflammatory autacoid. Inhibition of neutrophil aggregation, reduction in free radical production and prevention of leukocyte accumulation in inflamed areas has been demonstrated (Schrier et al., 1990). These actions may be A_2 receptor-mediated (Lappin and Whaley, 1984; Mandler et al., 1982). A_{2b} receptor stimulation by adenosine may also be responsible for enhancing antigen-stimulated degranulation in mast cells (Yakel et al., 1993). Other observations strongly suggest A_3 receptor involvement over A_{2b} receptors (see Palmer and Stiles, 1995).

Adenosine has been shown to increase the release of histamine from human lung preparations (Ott et al., 1992) and thus has been implicated in asthmatic episodes. The adenosine receptors involved in the release of allergic mediators in the lung were originally characterized as "atypical" since their effects were weakly blocked by methylxanthines

(Hughes et al., 1984). However, recent evidence suggests that A_3 receptors may be involved in the etiology of asthma by functioning to increase release of allergic mediators from mast cells (Meade et al., 1996).

2.5.2. Central effects of adenosine

Adenosine is present in the brain under physiological conditions and is suggested to be a potent neuromodulator in that it regulates neurotransmitter release, neuronal firing rate, glial cell function and cerebral blood flow. Adenosine is involved in physiological and pathophysiological conditions of the CNS including alertness, nociception, tremor disorders, and ischemia (Fredholm, 1995).

Adenosine is a neuromodulator of the mesopontine cholinergic neurons involved in arousal. Whole-cell and extracellular recordings in brainstem slices show that mesopontine cholinergic neurons are under tonic inhibitory control by adenosine via the modulation of low-threshold calcium currents (Rainnie et al., 1994). Furthermore, extracellular adenosine levels decrease during sleep (Rainnie et al., 1994) and increase during prolonged wakefulness (Porkka-Heiskanen et al., 1997).

Adenosine receptors found in the spinal cord appear to play a modulatory role in sensory transmission. A_1 and A_2 receptors are present in Rolando's gelatinous substance, a region particularly implicated in the control of pain (Choca et al., 1988). Activation of these receptors by systemic, central or intrathecal administration of adenosine is accompanied with antinociceptive effects (Post, 1984; Sawynok et al., 1991).

Adenosine has been touted as an endogenous anticonvulsant (Dragunow et al., 1985) and has anti-epileptic properties (Ault and Wang, 1986; Jarvis et al., 1991). The anticonvulsant actions of adenosine and analogues are likely mediated via interaction with A_1 receptors. Stimulation of A_1 receptors leads to a presynaptic inhibition of release and postsynaptic inhibition of the actions of excitatory neurotransmitters such as glutamate or acetylcholine (Andiné et al., 1990; Phillis et al., 1991). A resultant reduction in neuroexcitability occurs. In contrast, acute treatment with adenosine receptor antagonists such as caffeine results in epileptogenesis. However, when these antagonists are given chronically in doses that resemble habitual coffee consumption, there is decreased incidence of seizures following N-methyl-D-aspartate (NMDA) receptor activation or GABA receptor blockade (Fredholm, 1995). These findings are consistent with upregulation of adenosine A_1 receptors in individuals who chronically ingest caffeinated products.

Interestingly, adenosine A_{2a} receptors are co-localized with dopamine D_2 receptors in the dopamine-rich receptor sites in the brain (Stehle et al., 1992; Fink et al., 1992). Activation of A_{2a} receptors by CGS 21680 leads to a decrease in binding of dopamine receptor agonists to the D_2 receptors (Ferré et al., 1992). It has also been shown that adenosine receptor antagonists increase signalling through D_2 receptors (Ferré et al., 1992). The possibility of selectively increasing or decreasing activation of post synaptic dopamine D_2 receptors by modulating adenosine A_{2a} receptors has been proposed (Fredholm, 1995) and could have implications for both Parkinson's disease and schizophrenia.

Adenosine also appears to have a neuroprotective role in cerebral ischemia (Rudolphi et al., 1992). Adenosine acts as an endogenous cerebroprotective agent in three principal ways. First, adenosine exhibits a direct A_{2a} receptor- mediated vasodilatory effect leading to an increase in cerebral blood flow. Cerebral vasodilation due to an indirect effect of adenosine on presynaptic inhibition of catecholamine release may also occur (Hedqvist and Fredholm, 1976). Second, adenosine or agonist binding to presynaptic A_1 receptors results in an inhibition of glutamate release (Andiné et al., 1990) whereas application of the antagonist 8-PT results in increased extracellular glutamate in ischemic brain tissue (Sciotti et al., 1992). Postsynaptically, adenosine helps to maintain Ca^{2+} homeostasis by inhibiting membrane depolarization, which prevents the opening of voltage dependent Ca^{2+} channels (Rudolphi et al., 1992). Stimulation of A_1 receptors causing decreased neuronal activity and stimulation of A_{2a} receptors causing increased cerebral blood flow aids the restoration of the balance between oxygen supply and demand as well as between ATP synthesis and use. Third, adenosine causes direct inhibition of the formation of free radicals through inhibition of the activation of neutrophils (Rudolphi et al., 1992). Adenosine may also indirectly inhibit free radical production by activating antioxidant enzymes, which limits the lesions induced by free radicals (Simon et al., 1984).

Potential therapeutic strategies related to adenosine include adenosine receptor agonists and inhibitors of adenosine metabolism. While adenosine receptor subtype selectivity is best achieved through receptor agonists, these compounds are limited, at least at present, by tissue selectivity. For example, adenosine A1 agonists have desirable neuroprotective properties but also produce cardiac depression, an effect that limits their

clinical development. In contrast, the effects of inhibitors of adenosine metabolism are dependent on adenosine levels, therefore, tissue selectivity is achieved by stimuli that induce localized adenosine production. As adenosine's effects occur through the interaction of extracellular adenosine with adenosine receptors while adenosine metabolism is, primarily, intracellular, nucleoside transport processes are considered the first step in adenosine metabolism.

3. Nucleoside Transport

Three basic research strategies have been used to characterize nucleoside transporters. First, measuring the transport of specific permeants enables the determination of affinity constants and maximum transport capacities. Second, radioligand binding assays with specific, high affinity inhibitors are used to determine abundance of transporters in various cells and relative selectivity of permeants for transporter binding sites. Third, molecular cloning provides a useful method to examine the structure of transporters.

As adenosine is rapidly metabolized within cells, it is important to differentiate between accumulation of adenosine *per se* and of adenosine metabolites. Therefore, *transport* is defined as the transfer of the unmetabolized, native permeant across the cell membrane, whereas *uptake* refers to the accumulation of permeant without regard to its possible metabolism (Geiger and Nagy, 1990). Thus, very short incubation times are required to accurately measure transport of adenosine through nucleoside transporters.

Adenosine is able to cross cellular membranes via passive diffusion, facilitated transport or secondary active transport. Seven functionally distinct nucleoside transporters have been characterized in peripheral tissues from several species (Cass, 1995). These have been subdivided into two broad classes; sodium-dependent and sodium-independent transporters.

3.1. Sodium-dependent Transport

Sodium-dependent transporters couple the unidirectional movements of nucleosides and sodium ions across the plasma membrane. Sodium-dependent transporters have been divided further into five subclasses based on permeant selectivity and sensitivity to blockade by the transport inhibitor nitrobenzylthioinosine (NBMPR) (Table 1.). N1/*cif* transporters are *c*oncentrative NBMPR-*i*nsensitive and generally selective for purines including the purine analogue *f*ormycin B (Vijayalakshmi and Belt, 1988; Crawford et al., 1990). N2/*cit* transporters are also *c*oncentrative and NBMPR-*i*nsensitive, however they are selective for pyrimidines such as *t*hymidine (Vijayalakshmi and Belt, 1988). Unlike N2/*cit* transporters, N4/*cit* accept guanosine as a permeant (Gutierrez et al., 1992; Gutierrez and Giacomini, 1993). *C*oncentrative, NBMPR-*i*nsensitive transporters termed N3/*cib* exhibit *b*road selectivity for both purines and pyrimidines (Wu et al., 1992; and Huang et al., 1993). They also transport two sodium ions per nucleoside unlike other sodium-dependent transporters which transport nucleosides and sodium ions in a 1:1 ratio. Finally, *cs* transporters which have been recently designated N5, are *c*oncentrative and *s*ensitive to low nanomolar concentrations

of NBMPR (Cass, 1995). Adenosine appears to be a permeant for all the sodium-dependent nucleoside transporters characterized to date, although the maximum velocity of transport varies widely (Yao et al., 1996).

There appears to be a heterogeneous distribution of sodium-dependent transporters among cells and tissues. The distribution of sodium-dependent transporters in dissociated and cultured cells is listed in Table 2.

Three different transport proteins with sodium-dependent nucleoside transport activity have been cloned and are termed SNST1, CNT1 and CNT2. SNST1 has no significant homology to CNT1 or CNT2, however it shows sequence homology to the Na^+/glucose cotransporter, SGLT1 (Pajor and Wright, 1992). Expression of SNST1 in *Xenopus* oocytes results in low sodium-dependent cotransport activity consistent with N3/*cib* nucleoside transport processes (Pajor, 1994). Northern analysis detected mRNA for SNST1 in rabbit kidney and heart, but not in liver or intestine.

Expression of CNT1 in *Xenopus* oocytes resulted in sodium-dependent uridine transport activity of 20,000 fold increase over basal (Huang et al., 1994). The observation that uridine, thymidine, cytidine and adenosine, but not inosine or guanosine, inhibited uridine and thymidine influx catalyzed by CNT1 was consistent with CNT1 being an N2 transporter (Griffith and Jarvis, 1996). Distribution of CNT1 mRNA was detected in rat intestine and kidney, but not heart, brain, spleen, lung, liver or skeletal muscle (Huang et al., 1994).

CNT2 was recently cloned from rat jejunum and expressed in *Xenopus* oocytes (Yao et al., 1996). The expressed mRNA appears to have sodium-dependent nucleoside

TABLE 1. Functional properties of nucleoside transporter subclasses

	Equilibrative		Concentrative				
Trivial	*es*	*ei*	*cif*	*cit*		*cib*	*cs*
Numerical			N1	N2	N4	N3	N5
Na^+-dependent	-	-	+	+	+	+	+
Na^+/nucleoside stoichiometry			1:1	1:1	1:1	2:1	nd
Inhibited by:							
NBMPR	+	-	-	-	-	-	+
dipyridamole	+	+	-	-	-	-	+
dilazep	+	+	-	-	-	-	nd
propentofylline	+	+	+	nd	nd	nd	nd
Permeants:							
adenosine	+	+	+	+	+	+	+
uridine	+	+	+	+	+	+	nd
guanosine	+	+	+	-	+	+	nd
inosine	+	+	+	-	-	+	nd
formycin B	+	+	+	-	-	+	+
tubercidin	+	+	-	-	nd	+	nd
thymidine	+	+	-	+	+	+	nd

Adapted from Cass, 1995.

Abbreviations: NBMPR, nitrobenzylthioinosine; nd, not determined

TABLE 2. Tissue distribution of dependent transporters

Tissue Preparation	Species	Tissue Type	Transporter Subtype
Dissociated Cells			
	rat	choroid plexus	N2, N3
		macrophages	N1
		jejunum	N2
		hepatocytes	N1
		renal epithelium	N1, N2, N3
	mouse	macrophages	N1, N2, N3
		peritoneal exudate cells	N3[5]
		splenocytes	N1
		thymocytes	nd[6]
		bone marrow granulocyte-macrophage progenitor cells	nd
		enterocytes	N1, N2
	rabbit	choroid plexus	N2, N3
		intestinal brush border vesicles	N1, N2, N3
		renal epithelium	N1, N2, N3
	hamster	peritoneal exudate cells	N3[4]
	guinea-pig	enterocytes	N1, N2, N3
	bovine	renal epithelium	N1, N2
	human	renal epithelium	N4
		leukemic	N5
Cultured Cell lines			
rat	intestinal epithelial	IEC-6 cells	N1
	carcinoma	Walker 256 cells	N1
human	colon carcinoma	Caco-2 cells	N3
	leukemic	HL 60 cells	N3
opossum	proximal tubule	OK cells	N1[3]
mouse	leukemic	L1210 cells	N1
	leukemic	P388 cells	N1[1]
	lymphoma	S49 cells	N1[1]
	fibroblast	L929 cells	N1[1]
	macrophage	RAW 309 Cr.1 cells	N1[1]
pig	proximal tubule	LLC-PK_1 cells	N1[2]

[1] Plagemann and Aran, 1990
[2] Griffith et al., 1992
[3] Doherty and Jarvis, 1993
[4] Baer and Moorji, 1991
[5] Baer and Moorji, 1990
[6] nd, not determined

All other information adapted from Griffith and Jarvis, 1996 and Cass, 1995.

transport consistent with N1/*cif* permeant selectivity. CNT2 shows considerable sequence similarity to CNT1 indicating that they come from a single gene family (Che et al., 1995). Nevertheless, there are divergent regions in the N- and C- terminal regions and CNT2 exhibits a unique ATP/GTP binding motif and additional putative protein kinase A and C phosphorylation sites suggestive of differential regulation of the two carriers (Che et al., 1995; Griffith and Jarvis, 1996).

3.2. Sodium-independent Transport

Sodium-independent transporters are equilibrative transport processes that move nucleosides bidirectionally across plasma membranes. These have been further subdivided into *e*quilibrative-*s*ensitive (*es*) and *e*quilibrative-*i*nsensitive (*ei*) on the basis of their sensitivity to inhibition by NBMPR (Jarvis and Young, 1987; Plagemann et al., 1988) and have been identified to be the products of separate genes (Belt and Noel, 1988).

Equilibrative nucleoside transporters in mammalian cells accept a variety of nucleosides as substrates. However, the affinities of zero-*trans* influx processes for the different nucleosides also exhibits a wide range from approximately 20 μM to 5 mM (Griffith and Jarvis, 1996). (Zero-*trans* influx refers to the transport of a substrate from one side of the membrane where its concentration is varied, to the other side where its concentration is initially zero (Plagemann and Wohlhueter, 1980)). Adenosine has been shown to exhibit large differences in its affinity for the *es* transporter. For example, in many cells the Km for adenosine influx at room temperature is 20 - 50 μM (Griffith and Jarvis, 1996). This differs substantially from cultured bovine chromaffin cells and plasma

vesicles from chromaffin tissue. In these tissues, the Km was estimated to be 1-2 μM (Deligado et al., 1991; Sen et al., 1993). This variation in Km values may be affected by the metabolic lability of adenosine as Km values for adenosine kinase are typically about 1 μM.

3.2.1. *es* nucleoside transporters

Equilibrative-sensitive nucleoside transport is the best characterized nucleoside transport process in mammalian cells. This system is sensitive to inhibition by low nanomolar concentrations of NBMPR and exhibits directional symmetry such that the maximum velocities of influx and efflux are similar (Cass et al., 1974; Belt, 1983; Vijayalakshmi and Belt, 1988; Griffith and Jarvis, 1996).

Although *es* transporters in different cell types all exhibit broad permeant selectivity, substantial differences exist between kinetic constants, turnover numbers, carrier mobility, and size of *es* transporters in various species and tissues. This may indicate that multiple isoforms of this transporter exist.

Polyclonal antibodies to the *es* transporter of human erythrocytes were species cross-reactive among rat, rabbit and pig *es* transporters (Kwong et al., 1992). However, in another study, these anti-erythrocyte *es* antibodies detected only human synctiotrophoblast brush-border nucleoside transporter and not the kinetically similar *es* nucleoside transporter in basolateral membranes of the human placenta (Barros et al., 1995) indicating that at least two isoforms of *es* are present in the human placenta. Recently, a human placental cDNA with functional characteristics of *es* transport

processes was cloned and termed hENT1 (Griffith et al., 1997). hENT1 encoded a 456 amino acid residue glycoprotein which showed no significant sequence similarity to other known transporters.

3.2.2. *ei* nucleoside transport

Equilibrative-insensitive nucleoside transport is resistant to inhibition by nanomolar concentrations of NBMPR (Belt et al., 1993). Much less is known about the *ei* transporter in comparison to the *es* transporter as there are no selective inhibitors, antibodies or molecular probes for this carrier protein. As of yet, this transporter has not been cloned.

Similar to *es* transporters, *ei* carriers show broad substrate specificity for nucleoside permeants. However, there is evidence that within the same cell population, *ei* transporters have a lower affinity for nucleosides than do *es* transporters (Griffith and Jarvis, 1996).

3.2.3. Distribution of transporters

Many cells, including mouse leukemic L1210 cells, possess both *es* and *ei* transporters. However, relative proportions of *es* and *ei* transporters may vary (Hammond and Johnstone, 1989). Some cells, such as human erythrocytes (Plagemann and Woffendin, 1988), S49 mouse lymphoma cells (Plagemann and Wohlhuenter, 1984), cultured chromaffin cells (Deligado et al., 1990; Sen et al., 1993) and DDT_1 MF-2 cells (Parkinson et al., 1996) possess only *es* transporters. Conversely, other cells including the

Novikoff N1S1-67 rat hepatoma cell line possess *ei* but not *es* transporters (Plagemann and Wohlhueter, 1984).

3.3. Transport Inhibitors

In addition to the use of nucleoside transport inhibitors for characterizing transport processes in experimental models, therapeutic uses have been investigated. By inhibiting removal of extracellular adenosine, transport inhibitors can enhance receptor mediated effects of endogenous adenosine (Van Belle, 1988; Geiger and Fyda, 1991; Jacobson et al., 1991). The use of nucleoside transport inhibitors provides an attractive therapeutic strategy as ideally these compounds have minimal effects until adenosine production is elevated. As a result, the beneficial effect is localized to tissues that are producing adenosine and adverse drug reactions are minimized. Nucleoside transport inhibitors can be categorized based on their chemical structures into purine ribosides such as NBMPR, pyrimidopyrimidine derivatives such as dipyridamole, substituted piperazines including lidoflazine and analogues, tertiary amine diazepine compounds such as dilazep, and xanthines such as propentofylline.

3.3.1. Nitrobenzylthioinosine

As mentioned previously, NBMPR has been used to characterize *es* transporters. Because binding of [^{3}H]NBMPR corresponds stoichiometrically to inhibition of nucleoside transport (Cass et al., 1974), it is a useful tool for determining the presence and number of transporters in cells and tissue preparations. NBMPR has not been used clinically due to

its poor solubility and metabolic instability. However, nanomolar concentrations of NBMPR have been shown to cross the blood-brain barrier in rats (Anderson et al., 1996). Therefore, it may provide a model for testing the neuronal effects of nucleoside transport inhibitors *in vivo* (Anderson et al., 1996).

3.3.2. Dipyridamole

Dipyridamole is a well known coronary vasodilator and antithrombotic drug (Persantine®). Its actions are likely mediated through inhibition of adenosine transport and thus enhancing the half-life of adenosine in the plasma (Woffendin and Plageman, 1987). Dipyridamole is also an inhibitor of cGMP phosphodiesterase activity and reactive oxygen species formation (Rhodes et al., 1985; Iuliano et al., 1989; Bult et al., 1991). Similar to [^{3}H]NBMPR binding, [^{3}H]dipyridamole has been used for the pharmacological characterization of adenosine transport sites as it can bind to both *es* and *ei* transporter sites in guinea pig (Marangos and Deckert, 1987). Interestingly, dipyridamole exhibits only a low affinity for inhibition of *es* transporters in rat cells and tissues as well as several other species (Griffith and Jarvis, 1996).

3.3.3. Lidoflazine and analogues

Lidoflazine and its analogues mioflazine, R75231 and soluflazine are inhibitors of *es* transporters and have also been shown to inhibit *ei* mediated nucleoside transport (Hammond, 1991). Mioflazine is unable to distinguish between *es* and *ei* transport, however R75231 exhibits selectivity for the *es* carrier. Soluflazine differs as well in that it

is a more effective inhibitor of *ei* transport than of *es* (Lee and Jarvis, 1988; Hammond, 1991; Griffith et al., 1990). These drugs have been used to produce vasodilation, decrease tissue damage post-myocardial ischemia, cause sedation, increase "quality of sleep", and are able to cross the blood brain barrier (Wauquier et al., 1987).

3.3.4. Dilazep

Dilazep inhibits nucleoside transport via both equilibrative transporters as well as the N5/*cs* sodium-dependent transporter (Paterson et al., 1993). Dilazep has been used clinically as a vasodilator for decreasing coronary and total vascular resistance, and increasing coronary blood flow in anginal patients (Marzilli et al., 1984). Dilazep is water soluble and is a potent inhibitor of *es* transporters with K_i values between 1-10 nM (Griffith and Jarvis, 1996). This compound is metabolized by endogenous esterases (Geiger and Fyda, 1991) and also inhibits Na^+ and Ca^{2+} channel activity (Hoque et al., 1995).

3.3.5. Propentofylline

Propentofylline is a neuroprotective compound (Parkinson et al., 1994) that increases adenosine levels (Andiné et al,. 1990). Propentofylline inhibits nucleoside transport processes at concentrations that are associated with neuroprotection *in vivo* (Parkinson et al., 1993). At low micromolar concentrations, propentofylline inhibits *es* transporters whereas at higher concentrations, propentofylline will also inhibit *ei* and N1/*cif* transporters (Parkinson et al., 1993). This xanthine derivative has been found to

antagonize A_1, A_{2a} and A_{2b} adenosine receptors (Fredholm et al., 1992) which paradoxically, is inconsistent with its neuroprotective properties. This contradiction has not yet been resolved but is addressed in Chapter 4.

3.4. Nucleoside Release

Adenosine can be released from cells under basal conditions and by a variety of stimuli. Depolarization of cells, glutamate receptor agonists, electrical stimulation, metabolic stress, or hypertonic sodium solutions have been used to stimulate efflux of adenosine in various cell and tissue preparations (White and Hoehn, 1991; Manzoni et al., 1994; Baudourin-Legros et al., 1995). To date, direct evidence for vesicular release of adenosine, analogous to neurotransmitter efflux, has not been shown (Cahill et al., 1993). Bi-directional nucleoside transport carriers are most commonly implicated in release of adenosine.

Because adenosine is rapidly metabolized by intracellular and extracellular enzymes, it is difficult to achieve a stable releasable pool of intracellular adenosine. [^{3}H]Adenosine can be loaded in cells in the presence of inhibitors of metabolic enzymes. Unfortunately, several of these inhibitors, including the adenosine kinase inhibitor iodotubercidin (Parkinson and Geiger, 1996) and the adenosine deaminase inhibitor deoxycoformycin (Chen et al., 1984; Wiley et al., 1991), have been shown to block nucleoside transport processes. Therefore, due to the difficulty in characterizing adenosine release *per se*, metabolically stable analogues provide useful tools to study this system.

Formycin B is a metabolically stable inosine analogue which is a permeant for *es*, *ei*, N1/*cif*, N3/*cib* and N5/*cs* transporters (Plagemann et al., 1990 Plagemann and Woffendin 1989; Dagnino and Paterson, 1990; Wu et al., 1993; Paterson et al., 1993). L-Adenosine, the stereoisomer of physiological D-adenosine has also been used to examine transport activity. [^{3}H]L-Adenosine is unaffected by adenosine kinase or adenosine deaminase and is therefore useful in nucleoside accumulation and release studies (Gu et al., 1991; Foga et al., 1996). After cells are loaded with the metabolically stable radiolabeled permeant, extracellular media is removed and cells are resuspended in permeant-free buffer. Subsequent release of the radiolabeled permeant can be measured. Nucleoside release through *es* transporters has been demonstrated in several cell types (Jarvis, 1986; Phillis et al., 1989; Plagemann and Woffendin, 1989; White and MacDonald, 1990; Cunha and Sebastiao, 1993; Foga et al., 1996).

3.4.1. Transport inhibitors and nucleoside release

The nucleoside transport inhibitors dilazep, dipyridamole, and NBMPR have been employed to study adenosine efflux as well as uptake in various cell and tissue preparations (Clark and Dar, 1989; Green, 1980; Caciagli et al., 1988; Meghji et al., 1989). In peripheral cells, low concentrations of dipyridamole and NBMPR can block efflux through *es* transporters (Jarvis and Young, 1986; Plagemann et al., 1990a,b). However, much higher concentrations of these transport inhibitors are required to inhibit efflux through the *ei* transporter (Jarvis and Young, 1986). In rat hippocampal and hypothalamic slices, inhibitors decreased the evoked released of adenosine (Fredholm and

Jonzon, 1981; Jonzon and Fredholm, 1985) suggesting equilibrative transporters may play a large role in adenosine efflux in the central nervous system.

Nucleoside transport inhibitors have been used to prolong the presence of extracellular adenosine by blocking uptake. To potentiate adenosine receptor mediated effects, nucleoside transport inhibitors must block adenosine uptake to a greater degree than release. However, efflux through the *es* transporter can be blocked with the nucleoside transport inhibitors NBMPR and dipyridamole (Jarvis and Young, 1986; Plagemann et al., 1990). In animal brain preparations, the transport inhibitors dipyridamole, lidoflazine, propentofylline and NBMPR indeed increased basal levels of endogenous adenosine (Cahill et al., 1993; Ballarin et al., 1991; Pazzagli et al., 1993; Park and Gidday, 1990; Wallman-Johansson and Fredholm, 1994). These findings suggest that extracellular adenosine is primarily derived from the hydrolysis of released ATP (White and Hoehn, 1991).

During conditions that cause enhanced utilization of cellular ATP, such as glutamate excitotoxicity, much of the resultant adenosine production appears to occur intracellularly. If transport inhibitors block uptake and release with equal potency and efficacy, these compounds could decrease extracellular adenosine and, potentially, enhance excitotoxicity. Thus it is important to determine whether transport inhibitors block uptake and release similarly during both basal and stimulated conditions. It is also important to determine whether the same transporter subtypes mediate both adenosine uptake and release.

3.4.2. Release of nucleosides via sodium-dependent transporters

Sodium-dependent nucleoside transporters normally function in cellular uptake. However, recent evidence indicates that sodium-dependent glutamate transporters which also normally mediate inward transport, can be reversed after depolarization, ATP depletion or glycolytic inhibition (Madl and Burgesser, 1993; Gemba et al., 1994). In conditions of abnormal metabolism such as hypoxia, it is proposed that reversal of the sodium-dependent glutamate transporter provides an important source of extracellular glutamate (Szatkowski and Attwell, 1994). Similarly, intracellular adenosine greatly increases in hypoxic conditions and can be released down its concentration gradient. However, some studies have shown that cellular release of adenosine can be resistant to *es* and *ei* transport inhibitors in various CNS tissue preparations (Hollins and Stone, 1980; Fredholm and Jonzon, 1981; Caciagli et al., 1988; Cahill et al., 1993; Wallman-Johanson and Fredholm et al., 1994). Thus, in Chapter 2, we report our investigations on nucleoside release via sodium-dependent nucleoside transporters.

3.5. Regulation of Nucleoside Transport Processes

Recent evidence indicates that nucleoside transport can be regulated by a variety of factors including hormones, secretagogues, direct activation of intracellular signaling pathways, and adenine nucleotides. When protein kinase A or C are directly activated in cultured bovine chromaffin cells, inhibition of adenosine transport occurs via *es* transporters (Delicado et al., 1991; Sen et al., 1993). Furthermore, studies with bovine chromaffin cells or pig kidney cells indicate that nucleoside uptake may be regulated by

cAMP-dependent phosphorylation (Sen et al., 1993; Sayos et al., 1994). Forskolin or cAMP analogues decreased both adenosine uptake and [^{3}H]NBMPR binding in these cell preparations. Both direct molecular modification of the *es* transporter and a decrease in cell surface transporter number have been proposed as mechanisms to account for the inhibition of adenosine transport (Delicado et al., 1994; 1991).

The effects of signal transduction pathways on release of adenosine have not been studied as extensively as their effects on uptake due to the difficulty of achieving releasable pools of intracellular adenosine. However, direct alteration of G protein function influenced adenosine release via equilibrative transporters in cultured cerebellar granule neurons (Sweeney, 1996). Thus, it appears that signal transduction mechanisms may alter release of nucleosides through *es* transporters in some cell systems. Since adenosine can be released under conditions of metabolic stress and can then activate adenosine receptors, it is of interest to know whether there is a subsequent feedback regulation on adenosine transport processes. In Chapter 3, we examine whether release of the nucleoside formycin B was affected by adenosine receptor agonists.

Summary

Knowledge of adenosine receptors has progressed steadily. Receptor binding studies have provided preliminary evidence for new receptor subtypes in the brain. Development of novel ligands have permitted a more in-depth evaluation of the distribution and function of the main receptor subclasses in both central and periperal tissues. Furthermore, molecular cloning of adenosine receptors has provided important

information regarding potential new receptor subtypes, species- and tissue- selectivity as well as structure-activity relationships.

However, our understanding of the role of adenosine, and the potential for adenosine based therapeutic strategies in physiological and pathophysiological conditions is still limited. We need to increase our knowledge about both intracellular and extracellular adenosine formation during basal conditions or stimuli of various kinds. The influence of multiple transporter subtypes on the delivery of adenosine to its receptors and removal of adenosine from its receptors also requires further study. In addition, the potential for regulation of transporter function in response to stressors that elevate adenosine levels needs to be evaluated.

[1]Chapter 2. Uptake and Release of [^{3}H]Formycin B via Sodium-Dependent Nucleoside Transporters in Mouse Leukemic L1210/MA27.1 Cells

ABSTRACT

At least seven functionally-distinct nucleoside transport processes exist; however, mouse leukemic L1210/MA27.1 cells possess only one subtype, a Na^+-dependent transporter termed N1/*cif*. The capacity of this transporter subtype to release nucleosides from L1210/MA27.1 cells was investigated using the poorly metabolized inosine analog [^{3}H]formycin B. Uptake of [^{3}H]formycin B into these cells was inhibited by replacement of Na^+ in the buffer with choline, or by blocking Na^+/K^+ATPase with 2 mM ouabain, inhibiting glycolysis with 5 mM iodoacetic acid, or inhibiting nucleoside transport with 1 mM phloridzin. Sodium stimulated uptake with an EC_{50} value of 12 mM. To measure release of [^{3}H]formycin B, cells were loaded with [^{3}H]formycin B (10 μM) then washed and resuspended in buffer. Replacement of Na^+ in the buffer with choline enhanced [^{3}H]formycin B release by 20 - 47%, and significant stimulation of release was observed with Na^+ concentrations of 30 mM or less. Resuspending loaded cells into Na^+-buffer containing 2 mM ouabain or 10 μM monensin, a Na^+ ionophore, significantly enhanced [^{3}H]formycin B release during 20 min by 39% or 29%, respectively. Release of [^{3}H]formycin B into choline buffer was inhibited 26.5% by 10 mM phloridzin and 39.6% by 10 mM propentofylline, compounds known to inhibit various transporters including Na^+-dependent nucleoside transporters. Release was also inhibited significantly by 100

[1] Borgland SL and Parkinson FE, 1997, Uptake and release of [^{3}H]formycin B via sodium-dependent nucleoside transporters in mouse leukemic L1210/MA27.1 cells. J Pharmacol Exp Ther. 281: 347-353.

μM concentrations of dilazep, dipyridamole and NBMPR, inhibitors with selectivity for Na^+-independent nucleoside transporters. In the absence of Na^+, the permeants adenosine and uridine enhanced [^{3}H]formycin B release by up to 40.9% and 21.4%, respectively. These data indicate that in the absence of an inwardly directed Na^+-gradient, Na^+-dependent nucleoside transporters can function in the release of nucleosides.

INTRODUCTION

Nucleoside transport processes are membrane-bound carrier proteins that mediate the transfer of nucleosides across plasma membranes. Seven transporters have been characterized according to function (Cass, 1995) and are divided into two broad classes: Na^+-independent and Na^+-dependent processes. Na^+-independent transporters are carrier-mediated processes that catalyze cellular influx or efflux of nucleosides with the direction of movement determined by the nucleoside concentration gradient. Two equilibrative transporters are distinguished by their sensitivity to the transport inhibitor nitrobenzylthio-inosine (NBMPR) and are termed equilibrative sensitive (*es*) and equilibrative insensitive (*ei*), respectively (Vijayalakshmi and Belt, 1988). Na^+-dependent transporters couple the influx of Na^+ to the influx of nucleosides; thus, in the presence of a transmembrane Na^+-gradient nucleosides can be concentrated within cells to levels in excess of those in the extracellular environment. Five Na^+-dependent nucleoside transporters have been described and are termed N1 - N5. N1, also called *cif*, accepts purines and uridine as permeants, while N2, also called *cit*, and N4 are pyrimidine-selective. N3 and N5, also called *cib* and *cs*, respectively, have broad permeant selectivity and accept both purines

and pyrimidines. N5 (*cs*) is unique among the currently identified Na^+-dependent transporters for its sensitivity to inhibition by low nanomolar concentrations of NBMPR. Dipyridamole and dilazep inhibit both *es* and *ei* but are poor inhibitors of Na^+-dependent transporters (Cass, 1995).

Nucleoside transport processes are an important component of nucleoside salvage pathways and provide cells with nucleosides that are required for cellular metabolism. In addition, adenosine is an endogenous nucleoside that has autocrine and paracrine regulatory effects. In brain, adenosine is an inhibitory neuromodulator and extracellular adenosine levels are regulated by nucleoside transport processes. Because adenosine levels also increase during stroke and cellular release of adenosine can be resistent to inhibitors of *es* and *ei* transporters (Geiger and Fyda, 1991), we investigated whether Na^+-dependent nucleoside transporters can mediate nucleoside release during conditions that perturb transmembrane Na^+-gradients. Recent evidence indicates that glutamate transporters, which are dependent on Na^+ and normally function in cellular uptake, can mediate glutamate release following depolarization, ATP depletion or glycolytic inhibition (Madl and Burgesser, 1993; Gemba *et al.*, 1994). It has been proposed that this is an important source of extracellular glutamate during conditions of abnormal metabolism, such as stroke (Szatkowski and Attwell, 1994).

Murine leukemia L1210 cells possess both Na^+-independent (*es* and *ei*) and Na^+-dependent (N1/*cif*) nucleoside transporter activities (Crawford *et al.*, 1990b). Mutation strategies led to the isolation of L1210/MA27.1 cells which retain only an N1/*cif* nucleoside transporter (Crawford *et al.*, 1990a); thus, these cells provide a model system

to examine the function of Na^+-dependent nucleoside transporters. We investigated cellular release of [^{3}H]formycin B, a poorly metabolized inosine analogue (Plagemann *et al.*, 1990; Dagnino and Paterson, 1990; Wu *et al.*, 1993) that is a permeant of N1/*cif* transporters present in L1210/MA27.1 cells (Crawford *et al.*, 1990a), and found evidence for Na^+-dependent transporter-mediated release of [^{3}H]formycin B.

MATERIALS AND METHODS

Materials: Mouse leukemic L1210/MA27.1 cells were provided by Dr. J.A. Belt. [^{3}H]Formycin B was purchased from Moravek Biochemicals (Brea, CA). [^{3}H]Adenosine, ^3H_2O and [^{3}H]polyethylene glycol were from DuPont NEN (Boston, MA). NBMPR was obtained from Research Biochemicals International (Natick, MO). RPMI 1640 and heat-inactivated horse serum were purchased from Gibco BRL (Burlington, Ontario). Dilazep was provided by F. Hoffmann-LaRoche Ltd (Basel, Switzerland). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture: Mouse leukemic L1210/MA27.1 cells were maintained in logarithmic phase growth in RPMI 1640 culture medium with 10% heat-inactivated horse serum. Cells were harvested by centrifugation at 100 x *g* for 10 min, washed twice with Na^+ buffer (in mM: NaCl, 118; KCl, 4.9; $MgCl_2$, 1.2; KH_2PO_4, 1.4; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 25; glucose, 11; $CaCl_2$, 1; pH 7.4, 300 ± 10 mOsm) then resuspended in Na^+ buffer to 10^6 cells/ml. For some experiments, cells were washed and resuspended in buffer in which NaCl was replaced with equimolar choline chloride (choline buffer). For experiments with iodoacetic acid, glucose was omitted from

the buffer. Osmolarity of buffers was adjusted, as necessary, to 300 ± 10 mOsm with NaCl or choline chloride.

Measurements of [^{3}H]Formycin B Uptake: [^{3}H]Formycin B (10 μM; 6 μCi/ml) uptake into L1210/MA27.1 cells was measured using an oil-stop centrifugation method as previously described (Parkinson *et al.*, 1993).

The effect of ouabain, an inhibitor of Na^+/K^+ ATPase, iodoacetic acid, an inhibitor of glycolysis, or phloridzin, an inhibitor of Na^+-dependent nucleoside transport (Lee *et al.*, 1990), on [^{3}H]formycin B uptake was assessed. Cells were preincubated with 2 mM ouabain for 40 min at 37°C (Dagnino *et al.*, 1991), 5 mM iodoacetic acid for 20 min at 37 °C (Plagemann and Aran, 1990), or 1 mM phloridzin for 15 min at 22°C (Huang *et al.*, 1993) and [^{3}H]formycin B uptake (22°C) was determined. The effect of nucleoside transport inhibitors on [^{3}H]formycin B uptake was determined with cells preincubated for 15 min (22°C) with 100 μM concentrations of NBMPR, dilazep or dipyridamole.

The effect of graded Na^+ concentrations on [^{3}H]formycin B uptake was determined by preparing and incubating (15 min, 22°C) cells in buffers containing 0, 6, 12, 30, 59 or 118 mM NaCl. Aliquots of cells were added to reaction mixtures containing [^{3}H]formycin B in identical Na^+ concentrations. After uptake intervals of 180 seconds, reactions were terminated and cell-associated radioactivity was determined.

Measurements of [^{3}H]Formycin B Release: Cells were washed and resuspended at 5 x 10^6 cells/ml in Na^+ buffer and loaded with 10 μM (1 μCi/ml) [^{3}H]formycin B for 30 or 70 min at 37°C. To determine total cellular loading of [^{3}H]formycin B, aliquots of cells (100 μl) were centrifuged (13,000 x *g*) through oil and associated radioactivity was

determined. To assay cellular release of [^{3}H]formycin B, 100 µl aliquots of cells were transferred to 1.5 ml microcentrifuge tubes, centrifuged (13,000 x *g*) for 5 seconds and loading buffer was aspirated. Cell pellets were cooled on ice then resuspended in either Na^+ or choline buffer (22°C; 500 µl) and 400 µl aliquots were transferred to 1.5 ml microcentrifuge tubes containing 200 µl oil. Following release intervals of 1 - 20 min, cells were centrifuged through oil and both supernatants (350 µl) and cell pellets were analyzed for radioactivity. Cells resuspended into buffer at 4°C were used to estimate release at 0 min. Cell viability following resuspension was determined by trypan blue exclusion assays and was routinely greater than 95%.

The effect of extracellular Na^+ concentrations on [^{3}H]formycin B release was determined by resuspending [^{3}H]formycin B-loaded cells in 4°C or 37°C buffer containing 0, 30, 59, or 118 mM NaCl. Values of release at 0 min were subtracted from 10 and 20 min release values for each buffer.

To determine the effects of ouabain, iodoacetic acid or the Na^+-ionophore monensin on [^{3}H]formycin B release, cells loaded for 30 min with [^{3}H]formycin B were resuspended in Na^+ buffer (4°C or 37°C) alone or in Na^+ buffer containing 2 mM ouabain, 10 µM monensin or 5 mM iodoacetic acid. Release of [^{3}H]formycin B during time intervals of 0, 10 or 20 min was measured as described above. To test whether these treatments affected cell viability, trypan blue dye exclusion or intracellular water volume was measured. To determine intracellular volume, cells were incubated in Na^+ buffer for 30 min at 37°C, centrifuged, and resuspended in buffer as described above. After 20 min at 37°C, ^3H_2O (0.7 µCi/ml) or [^{3}H]polythylene glycol (0.7 µCi/ml) was added and cells

were incubated for a further 3 min. Cells were then centrifuged through oil and cell pellets were assayed for tritium content.

The effects of inhibitors or permeants of nucleoside transport processes on release of [^{3}H]formycin B were evaluated. Cells were loaded with [^{3}H]formycin B in Na^+ buffer for 30 min at 37°C. Cell aliquots (100 µl) were centrifuged (13,000 x *g*) for 5 seconds, supernatants were removed, and pellets were resuspended in 500 µl choline buffer in the absence or presence of the nucleoside transport inhibitor phloridzin, dilazep, dipyridamole, NBMPR, or propentofylline or in the absence or presence of the N1/*cif* transporter permeant adenosine or uridine. Cells were incubated for 10 or 20 min at 37°C and then centrifuged through oil.

Measurements of [^{3}H]Adenosine Release: The effect of iodoacetic acid on [^{3}H]adenosine release was determined as described above, using cells loaded for 30 min (37°C) with [^{3}H]adenosine (10 µM; 1 µCi/ml).

RESULTS

Cellular Accumulation of [^{3}H]Formycin B in L1210/MA27.1 Cells: Cellular uptake of [^{3}H]formycin B was greater with cells in Na^+ buffer than with cells in choline buffer; the rates of uptake were (mean ± SEM) 7.6 ± 0.3 pmol/10^6 cells/min and 0.2 ± 0.4 pmol/10^6 cells/min, respectively. For cells in Na^+ buffer, uptake of [^{3}H]formycin B was reduced by treatment of the cells with 2 mM ouabain, 5 mM iodoacetic acid or 1 mM phloridzin; the rates of uptake were (mean ± SEM) 1.5 ± 0.2, 1.8 ± 0.4, and 0.6 ± 0.3 pmol/10^6 cells/min, respectively (Fig. 1). Uptake of [^{3}H]formycin B was inhibited 23.6%

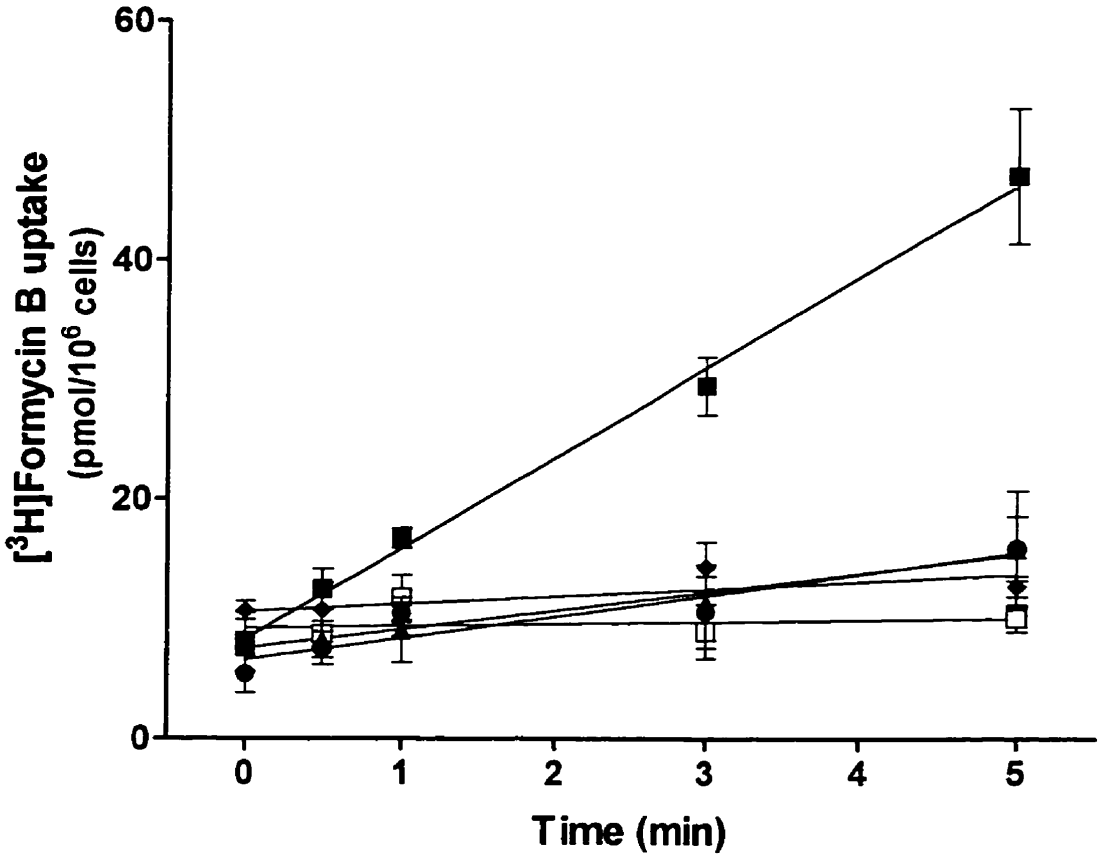


Fig. 1. Cellular accumulation of [^{3}H]formycin B in L1210/MA27.1 cells. Cells were harvested then washed and resuspended in Na^+ (closed symbols) or choline (open squares) buffer. Cells were incubated with [^{3}H]formycin B (10 μM) in buffer alone (squares) or in Na^+ buffer containing 2 mM ouabain (closed triangles), 5 mM iodoacetic acid (closed circles), or 1 mM phloridzin (closed diamonds). Symbols represent means and bars represent S.E.M. for 3 separate experiments, each performed in quadruplicate.

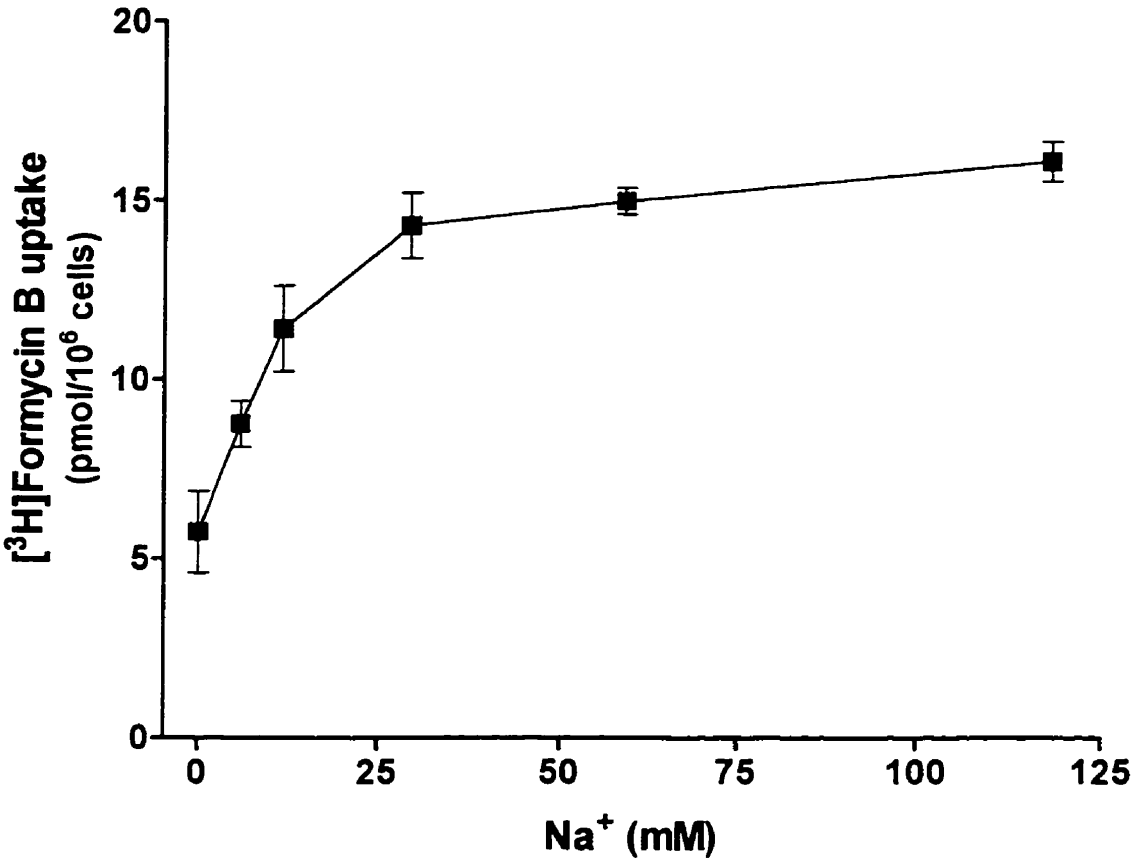


Fig. 2. Effect of extracellular Na^+ concentration on [^{3}H]formycin B uptake by L1210/MA27.1 cells. Cells were harvested and washed then resuspended in buffer containing 0, 6, 12, 30, 59 or 118 mM NaCl. Choline chloride was added to maintain constant osmolarity. Uptake of [^{3}H]formycin B during 180 second intervals was measured. Symbols represent means and bars represent S.E.M. of 3 experiments performed in quadruplicate.

by 100 μM NBMPR, 59.2% by 100 μM dilazep and 56.6% by 100 μM dipyridamole (data not shown). Sensitivity of [^{3}H]formycin B uptake to Na^+ was determined by measuring cellular accumulation in the presence of graded concentrations of NaCl. The EC_{50} value obtained by non-linear regression analysis was 12 mM Na^+ (Fig. 2).

Release of [^{3}H]Formycin B from L1210/MA27.1 Cells: Total [^{3}H]formycin B loaded in 70 min was 99000 ± 12000 dpm/10^6 cells (mean ± S.D.; n = 2). Release was stimulated by resuspending cells in Na^+ or choline buffer at 22°C. During 10 min intervals, the percent of total loaded [^{3}H]formycin B that was released into Na^+- or choline-buffer was 31 ± 4% (mean ± S.D.) or 53 ± 7%, respectively (Fig. 3). The rate of release of [^{3}H]formycin B at 22°C was (mean ± S.D.) 3.2 ± 0.3 pmol/5 X 10^6 cells/min in choline buffer and 1.1 ± 0.2 pmol/5x10^6 cells/min in Na^+ buffer (Fig.3).

Total [^{3}H]formycin B loaded in 30 min was 90000 ± 3000 dpm/10^6 cells (mean ± S.E.M.; n = 26). Release of [^{3}H]formycin B was examined at 37°C in the presence of several concentrations of Na^+. No effect of Na^+ concentration on release at 0 min was apparent (data not shown), however release at 10 or 20 min in buffer containing 0 or 30 mM Na^+ was significantly ($p < 0.05$, ANOVA with Tukey's HSD post-tests) greater than release in buffers containing 118 mM Na^+ (Fig. 4). The percent of total loaded [^{3}H]formycin B that was released into Na^+-buffer (118 mM NaCl) during 0, 10 or 20 min was (mean ± S.E.M) 16 ± 1%, 54 ± 1%, and 65 ± 1%, respectively. Release during 10 or 20 min (37°C) was 47% or 20% greater in buffer containing 118 mM choline chloride than in buffer containing 118 mM NaCl (Fig.4).

Release of [^{3}H]formycin B was enhanced by treatment of loaded cells with 2 mM ouabain or 10 μM monensin for 10 or 20 min (Fig.5). Following 20 min treatment with ouabain or monensin, release of [^{3}H]formycin B was significantly ($p<0.05$, paired t-test) increased by 39% or 29%, respectively. In contrast, release was inhibited by treatment with 5 mM iodoacetic acid (Fig. 5). Release was significantly ($p<0.05$, paired t-test) inhibited by 35% relative to control, following 20 min exposure to 5 mM iodoacetic acid (Fig. 5). Because the glycolytic inhibitor iodoacetic acid may elevate endogenous adenosine levels, which could then competitively inhibit release of [^{3}H]formycin B, we tested the effect of iodoacetic acid treatment on tritium release following loading of cells with [^{3}H]adenosine (Fig. 6). Following 10 or 20 min treatments with iodoacetic acid, tritium release was significantly increased by 303% or 364%, respectively. Ouabain, monensin or iodoacetic acid treatment had no significant effect on intracellular volume or on cell viability (data not shown).

Inhibitors of nucleoside transport processes were examined for effects on [^{3}H]formycin B release from L1210/MA27.1 cells (Table 1) and significant inhibition of [^{3}H]formycin B release was observed with each of the transport inhibitors used. Phloridzin, which inhibits nucleoside uptake by Na^+-dependent but not by Na^+-independent nucleoside transporters, produced significant inhibition of [^{3}H]formycin B release only at 10 mM, the highest concentration used. Propentofylline, which can inhibit adenosine uptake by both Na^+-dependent and Na^+-independent nucleoside transporters, significantly inhibited [^{3}H]formycin B release at both 1 and 10 mM. The classical inhibitors of Na^+-independent nucleoside transport, dipyridamole, NBMPR, and dilazep, also produced

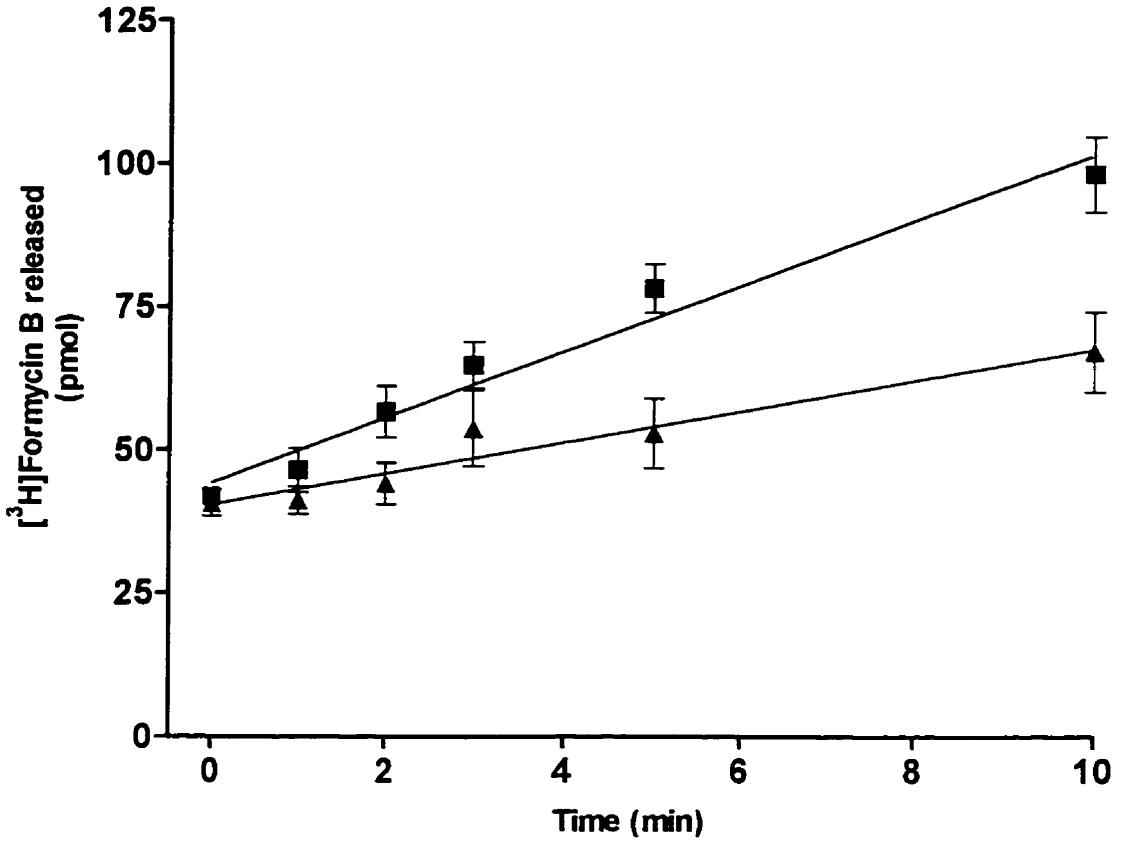


Fig. 3. Effect of extracellular Na^+ on release of [^{3}H]formycin B from L1210/MA27.1 cells. Cells were loaded with [^{3}H]formycin B in Na^+ buffer for 70 min (37°C). Cells were centrifuged briefly (5 seconds, 13,000 x *g*) and extracellular [^{3}H]formycin B was removed. Cells were resuspended in Na^+ (filled triangles) or choline (filled squares) buffer at 22°C. Release was terminated by centrifuging cells through oil. Symbols represent means and bars represent S.D. of 2 separate experiments, performed in quadruplicate.

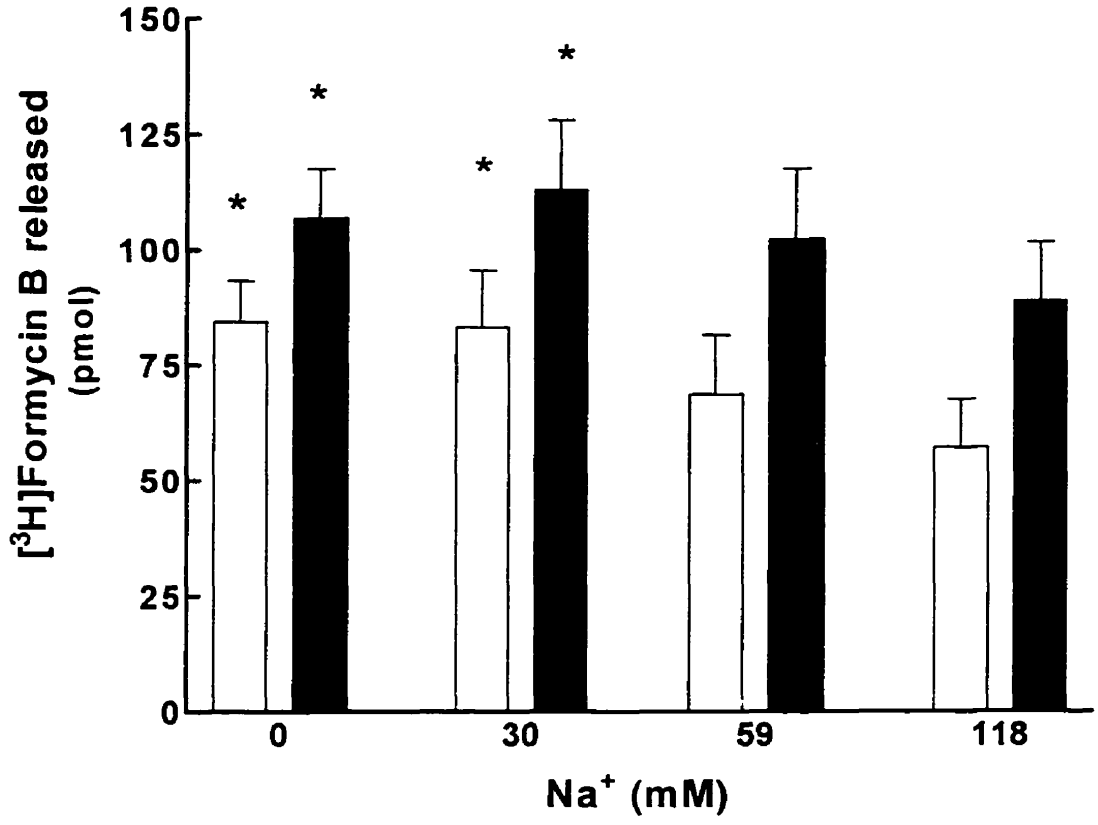
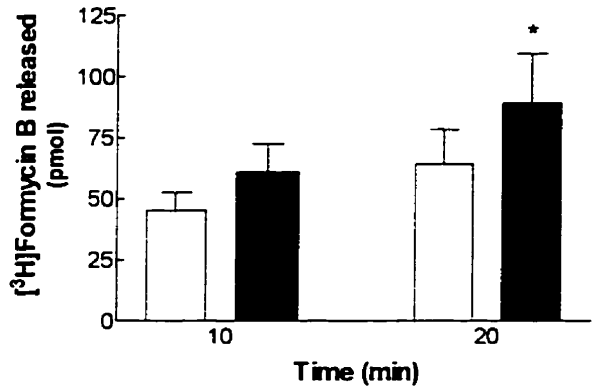


Fig. 4. Effect of Na^+ concentration on release of [^{3}H]formycin B from L1210/MA27.1 cells. Cells were incubated with [^{3}H]formycin B for 30 min (37°C) in the presence of Na^+. Cells were pelleted (5 seconds; 13,000 x *g*) and extracellular [^{3}H]formycin B was removed. Cells were resuspended in buffers containing 0, 30, 59 or 118 mM NaCl at 37°C and incubated for 10 (open bars) or 20 min (closed bars) before pelleting through oil (30 seconds; 13,000 x *g*). Release of [^{3}H]formycin B at 0 min was estimated with cells resuspended into buffers at 4°C and was 31.6 - 34.7 pmol. Values for 0 min were subtracted from values for 10 and 20 min release intervals. Bars represent mean ± S.E.M. of 3 separate experiments performed in quadruplicate. (*$p<0.05$ ANOVA with Tukey's HSD post-test comparing [^{3}H]formycin B released in the presence of 0, 30 or 59 mM NaCl to that released at 118 mM NaCl).


[3H]Formycin B released (pmol)
125
100
75
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25
0
10
20
Time (min)
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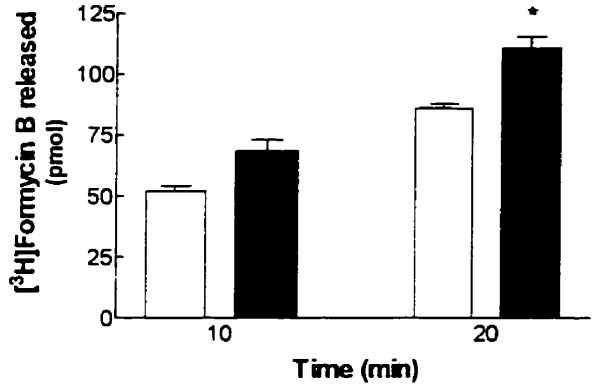
[3H]Formycin B released (pmol)
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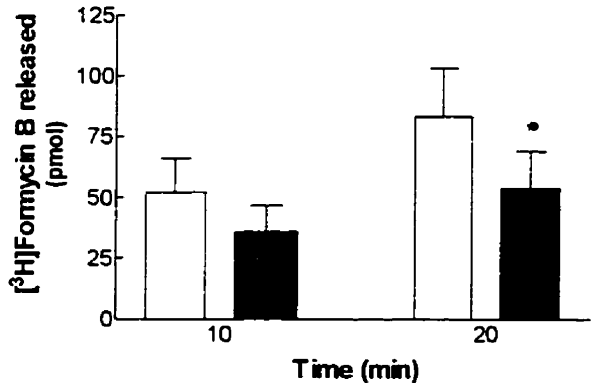

[3H]Formycin B released (pmol)
125
100
75
50
25
0
10
20
Time (min)
*

Fig. 5. Effect of ouabain, monensin or iodoacetic acid on release of [^{3}H]formycin B from L1210/MA27.1 cells. Cells were loaded with [^{3}H]formycin B (10 μM) in Na^+ buffer for 30 min (37°C). Cells were centrifuged briefly (5 seconds, 13,000 x *g*) and extracellular [^{3}H]formycin B was removed. Cells were resuspended into Na^+ buffer (37°C) in the absence (open bars) or presence (filled bars) of 2 mM ouabain (top panel), 10 μM monensin (center panel) or 5 mM iodoacetic acid (lower panel). Tritium content of supernatants was measured 10 or 20 min following resuspension. [^{3}H]Formycin B content of supernatants at 0 min, determined by resuspending cells into Na^+ buffer (4°C) in the absence or presence of ouabain, monensin or iodoacetic acid, was subtracted from 10 and 20 min values. Bars represent mean ± S.E.M. for 3 separate experiments performed in quadruplicate (*$p<0.05$, paired t-test comparing [^{3}H]formycin B release in the presence and absence of inhibitor).

significant inhibition of [^{3}H]formycin B release; at 100 μM concentrations release was inhibited by approximately 10% with dilazep and 25% with NBMPR or dipyridamole. At concentrations of 10 μM, dipyridamole and NBMPR inhibited release by 0 - 10%. Of the inhibitors tested and at the concentrations used, propentofylline produced the greatest inhibition of release (38%).

The effect of the nucleoside transporter permeants, adenosine and uridine, on release of [^{3}H]formycin B was tested (Table 2). In contrast to the inhibitory effects of nucleoside transport inhibitors, release of [^{3}H]formycin B during 10 or 20 min exposure to adenosine or uridine at concentrations of 100 μM - 10 mM was significantly greater than release in choline buffer alone. At 10 μM, the lowest concentration tested, release was significantly greater than control following 20 min, but not 10 min, exposure to adenosine or uridine. At concentrations of 100 μM - 10 mM, adenosine produced greater elevation of [^{3}H]formycin B release than did uridine.

DISCUSSION

The main finding of this study was that release of [^{3}H]formycin B from L1210/MA27.1 cells was Na^+-dependent; removal of extracellular Na^+ or disruption of transmembrane Na^+-gradients enhanced [^{3}H]formycin B release.

As shown previously (Parkinson *et al.*, 1993; Crawford *et al.*, 1990a), uptake of nucleosides by mouse leukemic L1210/MA27.1 cells, was inhibited by removal of extracellular Na^+. In the presence of physiological levels of Na^+, the uptake of [^{3}H]formycin B during a 5 min interval was 5-fold greater than in the absence of Na^+. An EC_{50} value of 12 mM Na^+ was obtained, which agrees with the value (13 mM) for

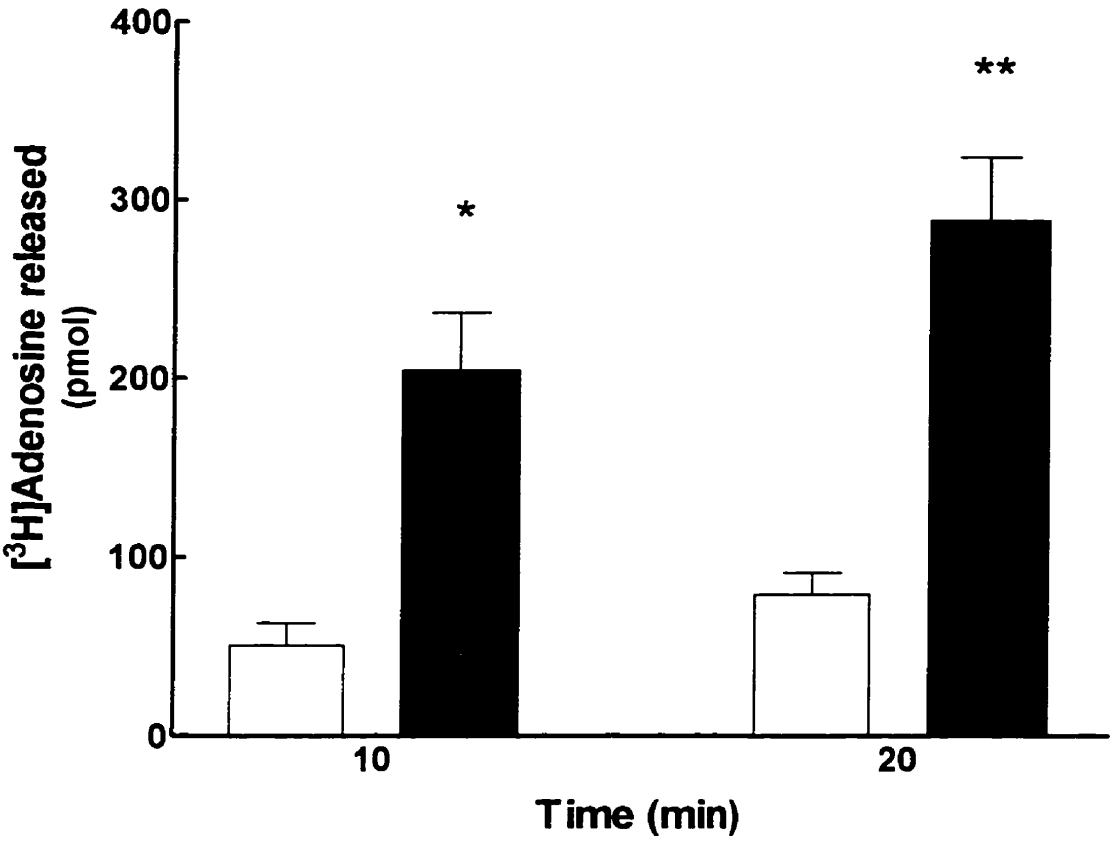


Fig. 6. Effect of iodoacetic acid on release of [^{3}H]adenosine from L1210/MA27.1 cells. Cells were loaded with 10 μM [^{3}H]adenosine in Na^+ buffer for 30 min (37°C). Cells were centrifuged briefly (5 seconds, 13,000 x *g*) and extracellular tritium was removed. Cells were resuspended into Na^+ buffer (37°C) in the absence (open bars) or presence (filled bars) of 5 mM iodoacetic acid. Tritium content of supernatants was measured 10 or 20 min following resuspension. Tritium content of supernatants at 0 min was determined by resuspending cells into Na^+ buffer (4°C) in the absence or presence of iodoacetic acid and was subtracted from values for 10 and 20 min release intervals. Bars represent mean ± S.E.M. for 3 separate experiments performed in quadruplicate. (*$p<0.05$, **$p<0.01$; paired t-test comparing tritium release in the presence and absence of iodoacetic acid).

TABLE 1. Effect of nucleoside transport inhibitors on release of [^{3}H]formycin B.

Cells were loaded with [^{3}H]formycin B, extracellular tritium was removed and cells were resuspended in choline buffer (37°C) in the absence or presence of test compounds. Following 10 or 20 min release intervals, tritium content of supernatants was determined. [^{3}H]Formycin B released in the presence of inhibitors is expressed as a percent of control release (mean ± SEM), determined in the absence of added compounds. Experiments consisted of controls and two drug concentrations and were performed in quadruplicate and repeated at least three times.

Inhibitor	10 min	20 min
Phloridzin		
10 mM	76.5 ± 1.5*	73.5 ± 3.0*
1 mM	93.6 ± 2.2	93.7 ± 4.8
Propentofylline		
10 mM	61.9 ± 2.5*	60.4 ± 1.9*
1 mM	81.5 ± 1.4*	78.8 ± 2.8*
Dilazep		
100 μM	91.9 ± 3.2	88.6 ± 2.9*
Dipyridamole		
100 μM ◊	76.6 ± 4.8*	78.2 ± 2.8*
10 μM Φ	91.6 ± 0.8	91.3 ± 5.1
NBMPR		
100 μM ◊	74.9 ± 3.6*	80.2 ± 1.8*
10 μM Φ	89.3 ± 0.9*	103.1 ± 5.4

*p<0.05 ANOVA with Tukey's HSD post-test; relative to control values

[◇] dissolved in 1% DMSO; [^{3}H]formycin B released in 1% DMSO was 99.1 ± 1.7 and 100.8 ± 2.0 percent of control at 10 min and 20 min, respectively.

[Φ] dissolved 0.1% DMSO; [^{3}H]formycin B released in 0.1% DMSO was 97.8 ± 1.9 and 99.8 ± 1.1 percent of control at 10 min and 20 min, respectively.

TABLE 2. Effect of the nucleosides adenosine and uridine on release of [^{3}H]formycin B. Cells were loaded with [^{3}H]formycin B, extracellular tritium was removed and cells were resuspended in choline buffer in the absence or presence of test compounds. Following 10 or 20 min release intervals, tritium content of supernatants was determined. [^{3}H]Formycin B released in the presence of nucleoside is expressed as a percent of control release (mean ± SEM), determined in the absence of nucleoside. Experiments, consisting of controls and two drug concentrations, were performed in quadruplicate and repeated at least three times.

Nucleoside	10 min	20 min
Adenosine		
10 mM	140.5 ± 0.6*	135.9 ± 4.3*
1 mM	131.0 ± 4.7*	136.8 ± 5.1*
100 μM	140.9 ± 2.0*	128.9 ± 1.7*
10 μM	103.3 ± 2.06	110.4 ± 2.1*
Uridine		
10 mM	119.7 ± 2.1*	121.4 ± 1.7*
1 mM	110.8 ± 2.4*	111.9 ± 0.1*
100 μM	119.2 ± 2.1*	118.2 ± 0.7*
10 μM	102.5 ± 1.1	108.3 ± 1.7*

*$p<0.05$ ANOVA with Tukey's HSD post-test; relative to control values

nucleoside transporter-mediated uptake of 6-mercaptopurine in rat intestinal brush-border membrane vesicles (Iseki *et al.*, 1996). Phloridzin, an inhibitor of Na^+-dependent transporters for glucose as well as those for nucleosides (Lee *et al.*, 1988; Lee *et al.*, 1990), inhibited [^{3}H]formycin B uptake by 73% over 5 min. Disruption of transmembrane Na^+-gradients by blocking Na^+/K^+ ATPase activity with ouabain or by depressing cellular ATP stores with the glycolytic inhibitor iodoacetic acid decreased [^{3}H]formycin B uptake to 30-35% of control.

Following loading of cells with [^{3}H]formycin B, release was enhanced by removal of extracellular Na^+, or by treating cells with phloridzin, ouabain or monensin indicating that nucleoside release from these cells is stimulated by conditions that perturb transmembrane Na^+-gradients.

In contrast to the stimulatory effects of ouabain, monensin and Na^+ replacement, the glycolytic inhibitor iodoacetic acid decreased [^{3}H]formycin B release. By depressing intracellular ATP levels, iodoacetic acid can depress Na^+/K^+ ATPase activity and cause intracellular Na^+ overload (Gemba *et al.*, 1994), and thus, would be expected to have similar effects on nucleoside release as ouabain and monensin. We hypothesized that, by depressing ATP levels, iodoacetic acid elevated levels of intracellular adenosine which then competitively inhibited release of [^{3}H]formycin B. Consistent with this hypothesis, we found that iodoacetic acid stimulated tritium release in cells loaded with [^{3}H]adenosine. The difference in release of these two compounds indicates that [^{3}H]adenosine is the better permeant for outward transport. Previously, it has been shown that Na^+-dependent influx of 1 μM adenosine (190 pmol/10^9 cells/s) was approximately 8-fold faster than that

of 1 μM formycin B (24 pmol/10^9 cells/s) in L1210 cells (Crawford *et al.*, 1990b) and that adenosine has greater affinity than formycin B for N1/*cif* transporters (Vijayalakshmi and Belt 1988).

An interesting finding of these studies was that treatment of cells with phloridzin, ouabain or Na^+-replacement buffer was more effective in inhibiting [^{3}H]formycin B uptake than in stimulating [^{3}H]formycin B release. At least three factors may contribute to this difference. First, each of these treatments may elevate intracellular adenosine levels. In this case, total nucleoside release may be underestimated by measuring [^{3}H]formycin B release, since simultaneous release of nonradioactive adenosine may competitively inhibit [^{3}H]formycin B release. Second, uptake studies were performed with cells pretreated with the desired buffers and drugs; however, since pretreatment was not possible for release studies, release was measured from the beginning of exposure of cells to the various treatment conditions. Because the drugs were not at equilibration with their respective target sites prior to initiation of release, this could lead to underestimation of the effects of the cell treatments on [^{3}H]formycin B release. Third, the finite intracellular volume of the cells meant that intracellular [^{3}H]formycin B concentrations were not constant for the duration of the release time intervals. Each of these three factors would have the effect of lowering the measured [^{3}H]formycin B release.

Differences were also observed in the Na^+ concentration-dependence of [^{3}H]formycin B uptake and release; for example, uptake was unaffected but release was stimulated by reducing the buffer Na^+ concentration from physiological to 30 mM. This may indicate that intracellular levels of Na^+ are higher in cells used for release assays than

in cells used for uptake assays. It is possible that intracellular Na^+ levels are elevated prior to initiation of release intervals, since cells are loaded with [^{3}H]formycin B in the presence of Na^+ buffer.

Release of [^{3}H]formycin B was depressed by millimolar concentrations of low affinity inhibitors of Na^+-dependent nucleoside transporters, such as propentofylline (Parkinson *et al.*, 1993) and phloridzin (Lee *et al.*, 1988; Lee *et al.*, 1990). Release was also decreased by 10 - 100 μM concentrations of NBMPR, dipyridamole and dilazep, inhibitors that at nanomolar concentrations are selective for Na^+-independent nucleoside transporters (Cass, 1995). Several studies have measured adenosine release in the presence or absence of NBMPR or dipyridamole at concentrations of 10 - 100 μM (Hoehn and White, 1990; Craig and White, 1993; Green, 1980; Cunha *et al.*, 1996). Inhibition of release has been interpreted as evidence of release mediated by equilibrative transporters. However, the present study indicates that NBMPR, dipyridamole and dilazep can inhibit nucleoside uptake and release mediated by Na^+-dependent transporters. Thus, high (> 10 μM) concentrations of these compounds should be used with caution in investigations of cellular release mechanisms for nucleosides.

Stimulation of release by adenosine and uridine may indicate trans-acceleration in the absence of a Na^+-gradient. This phenomenon, commonly observed with Na^+-independent nucleoside transporters (Jarvis, 1986), can occur when transporter permeants are simultaneously present on both sides of the membrane. In the presence of a Na^+-gradient, Na^+-dependent transporters function as symporters and translocate nucleosides in an inward direction. As long as the Na^+-gradient is maintained, the intracellular

accumulation of permeants does not appear to affect permeant uptake. Our data suggest, however, that disruption of transmembrane Na^+-gradients may uncouple nucleoside transport from Na^+ translocation, and in this situation transport of nucleosides in one direction may accelerate the transfer in the opposite direction.

Carrier-mediated release of neurotransmitters, including glutamate, GABA and dopamine, has been demonstrated by elevating intracellular Na^+ levels, replacing extracellular Na^+, blocking Na^+/K^+ ATPase activity, or inhibiting glycolysis (Gemba *et al.*, 1994; Eshleman *et al.*, 1994; Levi and Raiteri, 1993; Belhage *et al.*, 1993). Furthermore, it has been suggested that carrier-mediated release of glutamate is a significant source of excitotoxic extracellular glutamate in cerebral ischemia (Szatkowski and Attwell, 1994). Adenosine released via reversal of Na^+-dependent nucleoside transporters may contribute to the micromolar levels of extracellular adenosine that arise during cerebral ischemia. Molecular evidence indicates that mRNA for N1/*cif* and N2/*cit* transporters is widely distributed in brain (Anderson *et al.*, 1996). Other sources that may contribute to elevated extracellular adenosine levels include release via Na^+-independent transporters and release of ATP followed by enzymatic dephosphorylation to adenosine.

In summary, we have demonstrated that by disrupting transmembrane Na^+-gradients, reversal of Na^+-dependent nucleoside transporters can mediate cellular release of nucleosides. The evidence that this release is transporter-mediated includes inhibition by transport inhibitors and stimulation by transporter permeants. Adenosine, a nucleoside with diverse receptor-mediated effects, may be released from cells by this process during

conditions, such as ischemia, that depress cellular transmembrane Na^+-gradients by compromising intracellular ATP levels and/or Na^+/K^+ ATPase function.

[2]Chapter 3. Effect of Adenosine Receptor Agonists on Release of the Nucleoside Analogue [^{3}H]Formycin B from Cultured Smooth Muscle DDT_1 MF-2 Cells

ABSTRACT

Four receptor subtypes for adenosine have been characterized. We examined whether the stimulation of adenosine receptors has a regulatory effect on transporter-mediated nucleoside release. We used DDT_1 MF-2 smooth muscle cells which possess only nitrobenzylthioinosine (NBMPR)-sensitive transporters as well as both A_1 and A_{2a} receptors. Cells were loaded with the metabolically stable nucleoside analogue [^{3}H]formycin B and then resuspended in buffer. Release of [^{3}H]formycin B from cells was inhibited by the transport inhibitor NBMPR indicating that release was mediated by a nucleoside transport process. Neither N-ethylcarboxamidoadenosine (NECA) nor CGS 21680 affected release. Cyclohexyladenosine (CHA) produced a concentration-dependent inhibition of [^{3}H]formycin B release with an IC_{50} value of 3 - 6 μM. Inhibition of release by CHA was not blocked by dipropylcyclopentylxanthine indicating that the effect of CHA was not due to stimulation of A_1 receptors. We performed competition binding studies with [^{3}H]NBMPR and graded concentrations of CHA and found that CHA inhibited [^{3}H]NBMPR binding to the *es* transporters with a K_i value of 2.9 μM. Thus, CHA inhibited [^{3}H]formycin B release by direct interactions with transporters. We conclude that release of the nucleoside formycin B from DDT_1 MF-2 cells is not regulated by adenosine A_1 or A_{2a} receptor activation.

[2] Borgland SL and Parkinson FE, 1997, Effect of adenosine receptor agonists on release of the nucleoside analogue [^{3}H]formycin B from cultured smooth muscle DDT_1 MF-2 cells. Eur J Pharmacol. *Submitted.*

INTRODUCTION

Adenosine, an endogenous nucleoside with autocrine and paracrine regulatory actions, is formed from the dephosphorylation of ATP. The concentration of this nucleoside is tightly regulated by purine enzymes as well as by transport processes. Intracellular adenosine concentrations are kept at nanomolar concentrations due to the activity of the enzymes adenosine kinase, which phosphorylates adenosine to adenosine monophosphate, and adenosine deaminase, which deaminates adenosine to inosine (Geiger and Fyda, 1991). Nucleoside transporters catalyze the movement of nucleosides across biological membranes. There are two broad classes of transport proteins (Griffith and Jarvis, 1996) which facilitate the movement of adenosine across cellular membranes. Under normal conditions, sodium/nucleoside co-transporters move adenosine unidirectionally into cells by utilizing the sodium gradient (Cass, 1995). Equilibrative (sodium-independent) transporters can move adenosine bidirectionally across plasma membranes by facilitated diffusion. Equilibrative transporters can be further subdivided into two classes, equilibrative-sensitive (*es*) and equilibrative-insensitive (*ei*), based on their sensitivity to inhibition to nanomolar concentrations of nitrobenzylthioinosine (NBMPR), (Vijayalakshmi and Belt, 1988).

Nucleoside transport processes are important components of nucleoside salvage pathways and provide cells with nucleosides essential for maintaining cellular metabolism. In addition, nucleoside transporters regulate interstitial levels of adenosine. Inhibitors of *es* equilibrative transporters, such as NBMPR, inhibit the removal of adenosine from the interstitium. As a result, adenosine persists in the extracellular environment and is

available to interact with adenosine receptors. Thus, the receptor-mediated effects of adenosine can be potentiated by nucleoside transport inhibitors (Geiger and Fyda, 1991).

Four adenosine receptor subtypes have been characterized and cloned and are termed A_1, A_{2a}, A_{2b}, or A_3 (Fredholm et al., 1994). Activation of A_1 and A_3 receptors is generally inhibitory as adenylyl cyclase activity is inhibited. A_1 receptors can enhance K^+ conductance (Belardinellini and Isenberg, 1983) and inhibit Ca^{2+} conductance (Dolphin et al., 1986). A_{2a} and A_{2b} receptors are G_s protein linked stimulatory receptors that enhance cAMP formation.

Cellular release of nucleosides via equilibrative transporters has been previously demonstrated with human erythrocytes (Plagemann and Woffendin, 1989) and hamster DDT_1 MF-2 cells (Foga et al., 1996). Studies with bovine chromaffin cells or pig kidney cells indicate that nucleoside uptake may be regulated by cAMP-dependent phosphorylation (Sen et al., 1993; Sayos et al., 1994). Forskolin or cAMP analogues decreased both adenosine uptake and [^{3}H]NBMPR binding in these cell preparations. Furthermore, activation of protein kinase C has been shown to inhibit adenosine uptake in chromaffin cells (Delicado et al., 1991). The effects of signal transduction pathways on release of adenosine have not been studied as extensively as their effects on uptake because intracellular concentrations of adenosine are maintained at low levels. Nevertheless, Sweeney (1996) demonstrated that alteration of G protein function influenced adenosine release via equilibrative transporters in cultured cerebellar granule neurons. Thus, it appears that signal transduction mechanisms can affect the function of *es* transporters, at least in some cell systems.

Since adenosine that is released from cells can activate its cell surface receptors, this study was designed to test whether adenosine receptor activation affects transporter-mediated release of nucleosides. For this study we chose DDT_1 MF-2 smooth muscle cells, which appear to possess only nucleoside transporters of the *es* subtype (Parkinson et al., 1996) as well as A_1 and A_{2a} adenosine receptors (Ramkumar et al., 1989). We investigated cellular release of [^{3}H]formycin B, a poorly metabolized nucleoside analogue (Plagemann and Woffendin, 1989; Dagnino and Paterson, 1990; Wu et al., 1993) which can permeate *es* transporters in DDT_1 MF-2 cells (Parkinson et al., 1996).

MATERIALS AND METHODS

Materials: [^{3}H]Formycin B was purchased from Moravek Biochemicals (Brea, CA) and [^{3}H]nitrobenzylthioinosine was obtained from DuPont Canada (Mississauga, Ontario). Cyclohexyladenosine (CHA), nitrobenzylthioinosine (NBMPR), dipropylcyclopentylxanthine (DPCPX), CGS 21680, and N-ethylcarboxamidoadenosine (NECA) were purchased from Research Biochemicals International (Natick, MA). Formycin B, Triton X-100, trypan blue and HEPES were obtained from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium and fetal bovine serum were obtained from Gibco BRL (Burlington, Ontario). Dilazep was provided by F. Hoffmann-LaRoche Ltd. (Basel, Switzerland).

Cell Culture: DDT_1 MF-2 smooth muscle cells, originally isolated from steroid-induced leiomyosarcoma of Syrian hamster vas deferens (Norris et al., 1974), were obtained from American Type Culture Collection. Cells were grown in suspension and

maintained as exponentially proliferating cultures in Dulbecco's modified Eagle's medium supplemented with 4.5 g/l glucose, 5% qualified fetal bovine serum and 2 mM L-glutamine as previously described (Parkinson et al., 1996).

[^{3}H]Formycin B Efflux Measurements: Cells were harvested by centrifugation (100 x *g* for 10 min), washed twice (100 x *g* for 5 min) and resuspended (3 x 10^6 cells/ml) in physiological buffer containing NaCl, 120 mM; $MgCl_2$, 1 mM; K_2HPO_4, 3 mM; $CaCl_2$, 1.2 mM; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 20 mM; and glucose, 10 mM. Osmolarity of the buffer was adjusted as necessary to 300 ± 10 mOsmol and pH was adjusted to 7.4 with NaOH.

Cells were loaded 1 h at 22°C with 10 μM [^{3}H]formycin B, a metabolically stable nucleoside analogue (Wu et al., 1993) that is a permeant for nucleoside transporters in DDT_1 MF-2 cells (Parkinson and Geiger, 1996). Cells were pelleted (5 sec, 11,000 x *g*), extracellular [^{3}H]formycin B was removed and pellets were placed on ice. Release was stimulated by resuspending cells in 500 μl buffer alone or buffer containing 10 μM NBMPR, an inhibitor of *es* nucleoside transporters; 30 μM or 300 nM CHA, a selective A_1 receptor agonist; 30 μM NECA, an A_1/A_2 mixed agonist; 10 μM DPCPX, an A_1 receptor antagonist; or 10 μM CGS 21680, a selective A_{2a} receptor agonist. Cells were incubated for 90 seconds at either 22°C or 37°C and then a 400 μl aliquot was centrifuged (30 sec; 16,000 x *g*) over oil (85 parts silicon oil: 15 parts paraffin oil; 200 μl) to separate cells from the extracellular medium. Samples of the supernatant were taken for radioactive determination by liquid scintillation spectroscopy. The microcentrifuge tubes

were washed three times with dH_20, the oil was removed, and the pellets were dissolved in 10% Triton X-100 for determination of radioactivity.

Cell viability was assayed by trypan blue exclusion at the end of each experiment and was routinely greater than 95%.

[^{3}H]Nitrobenzylthioinosine Binding: Cells were harvested, washed twice and resuspended in buffer as above. Cells, 25,000 per ml assay volume, were incubated (22°C) with 0.5 nM [^{3}H]NBMPR and CHA (10 nM-100 μM) for 1 hour and reactions were terminated by filtration through Whatman GF/B filters using a Brandel cell harvester. Total binding was measured in the presence of [^{3}H]NBMPR alone and nonspecific binding was measured in the presence of 100 μM dilazep. [^{3}H]NBMPR concentrations were corrected for ligand depletion. The K_i value for CHA was determined with the Cheng and Prusoff equation (Cheng and Prusoff, 1973) using a K_D value for NBMPR of 0.26 nM (Parkinson et al., 1996).

Data Analysis: [^{3}H]Formycin B release measurements were in triplicate and [^{3}H]NBMPR binding measurements were in duplicate. Each experiment was performed at least three times and all values are reported as mean ± S.E.M. Nonlinear regression was performed using the software package GraphPad PRISM version 2. To test for significant differences between two means, t-tests were used. To test for significant differences between three or more means, data were analyzed using a one way ANOVA with a Bonferroni post-hoc test. A significance level of $p \leq 0.05$ was chosen *a priori*.

RESULTS

Release of [^{3}H]formycin B in the presence of the transport inhibitor, NBMPR (10 μM), was significantly inhibited by 43% at 22°C and 37% at 37°C (Fig. 1). The A_1 receptor agonist CHA (30 μM) significantly inhibited [^{3}H]formycin B release by 41% at 22°C. Release at 37°C in the presence of CHA was inhibited by 20%, however this was not statistically significant. [^{3}H]Formycin B release was not significantly increased in the presence of the A_1/A_2 mixed receptor agonist, NECA (30 μM) (Fig. 1). The selective A_{2a} receptor agonist, CGS 21680 (10 μm), did not alter [^{3}H]formycin B release (Fig. 2).

To examine further the effect of CHA on release of [^{3}H]formycin B, we examined release in the presence of 0.1 μM to 30 μM CHA. Concentration-dependent inhibition of [^{3}H]formycin B release by CHA was observed with a half maximal inhibition constant (IC_{50}) of 2.7 ± 1.5 μM at 22°C and 6.4 ± 1.5 μM at 37°C (Fig. 3). These values were not significantly different.

To test whether this inhibition of [^{3}H]formycin B was due to stimulation of A_1 receptors, the effect of the selective A_1 receptor antagonist, DPCPX (10 μM), was investigated (Fig. 4). DPCPX had no effect on release and did not reverse the inhibitory effect of CHA indicating that the effect of CHA was not due to stimulation of A_1 receptors.

To evaluate whether CHA was affecting [^{3}H]formycin B release directly by blocking the *es* transporter, we performed competition binding assays with [^{3}H]NBMPR

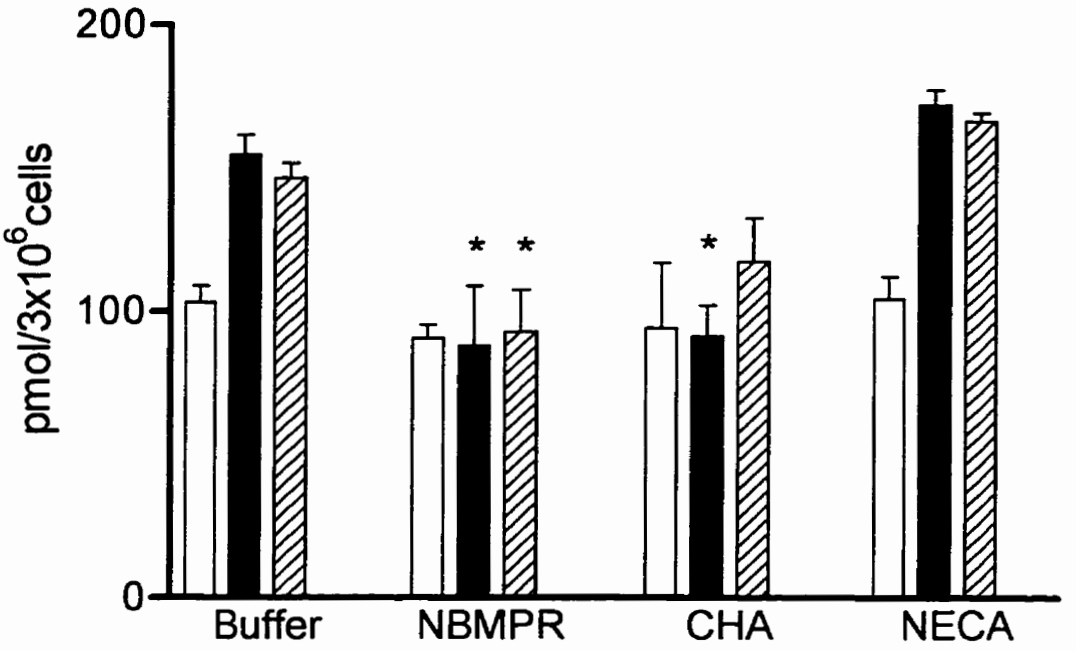


Fig.1. Release of [^{3}H]Formycin B (10 μM) from DDT$_1$-MF-2 smooth muscle cells in the presence of 10 μM NBMPR, 30 μM CHA or 30 μM NECA at 4°C (open bars), 22°C (filled bars) or 37°C (hatched bars). Bars represent mean ± SEM from at least 3 experiments (*$p<0.05$; ANOVA with Bonferroni post-hoc test).

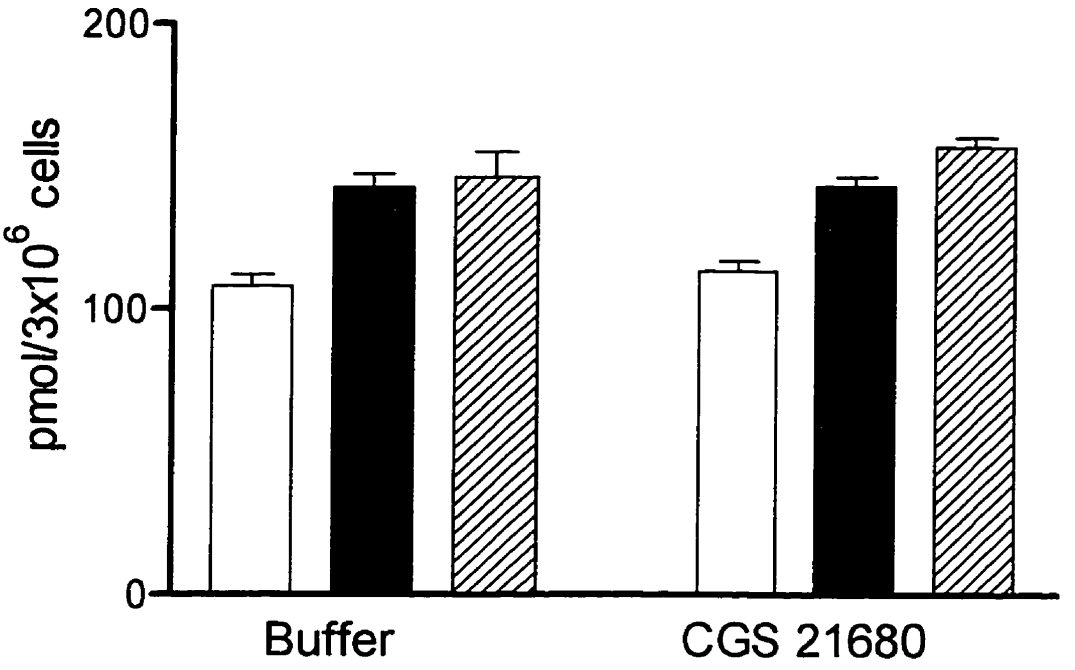


Fig. 2. Release of [^{3}H]Formycin B from DDT$_1$ MF-2 smooth muscle cells in buffer or buffer with 10 μM CGS 21680 at 0°C (open bars), 22°C (filled bars) or 37°C (hatched bars). Bars represent mean ± SEM from three experiments.

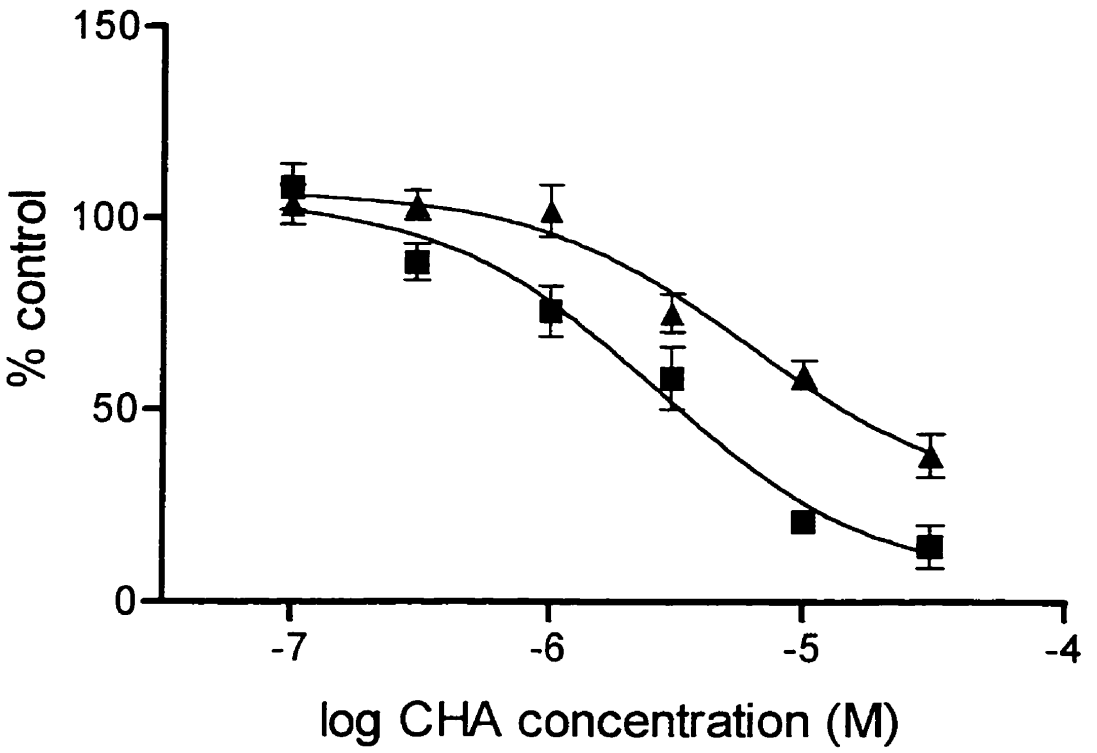


Fig. 3. Concentration-dependent inhibition of [^{3}H]formycin B release by CHA at 22°C (triangles) or 37°C (squares). Release was stimulated by resuspending cells in either 22°C or 37°C buffer containing 0.1 μM to 1 mM CHA. Symbols represent mean ± SEM of at least 3 experiments. Control represents release in the absence of CHA.

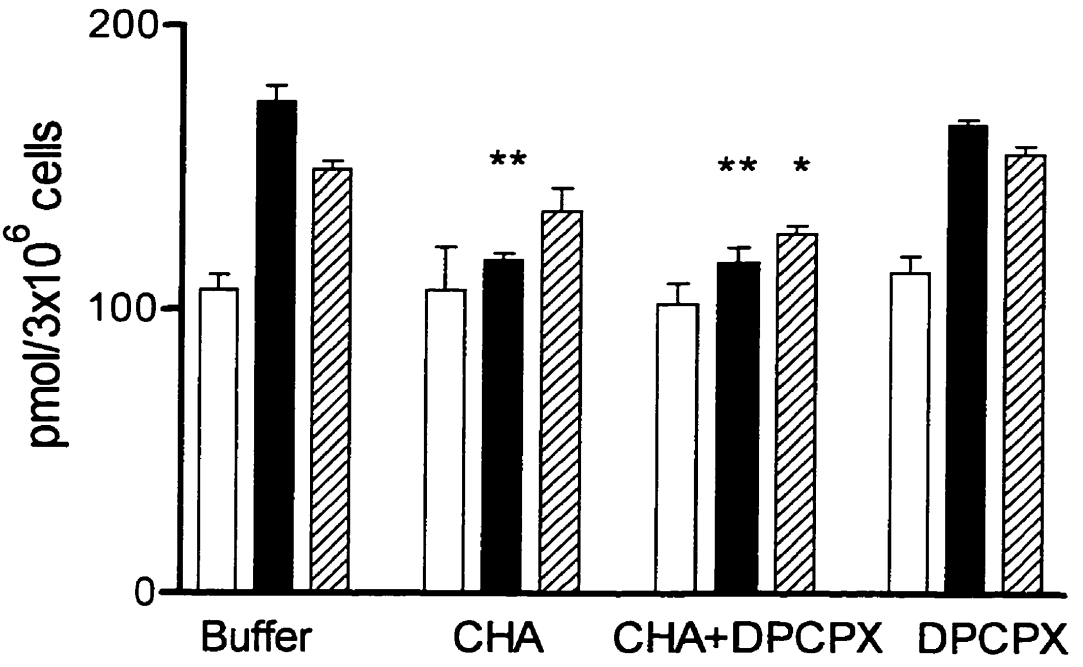


Fig. 4. Release of [^{3}H]formycin B from DDT$_1$ MF-2 smooth muscle cells in the presence of buffer, or buffer containing 30 μM CHA, 10 μM DPCPX, or 10 μM DPCPX and 30 μM CHA. [^{3}H]Formycin B release at 4°C (open bars), 22°C (filled bars), or 37°C (hatched bars) was measured. Bars represent mean ± SEM of at least 3 experiments. (*$p<0.05$, **$p<0.01$; ANOVA with Bonferroni post-hoc test).

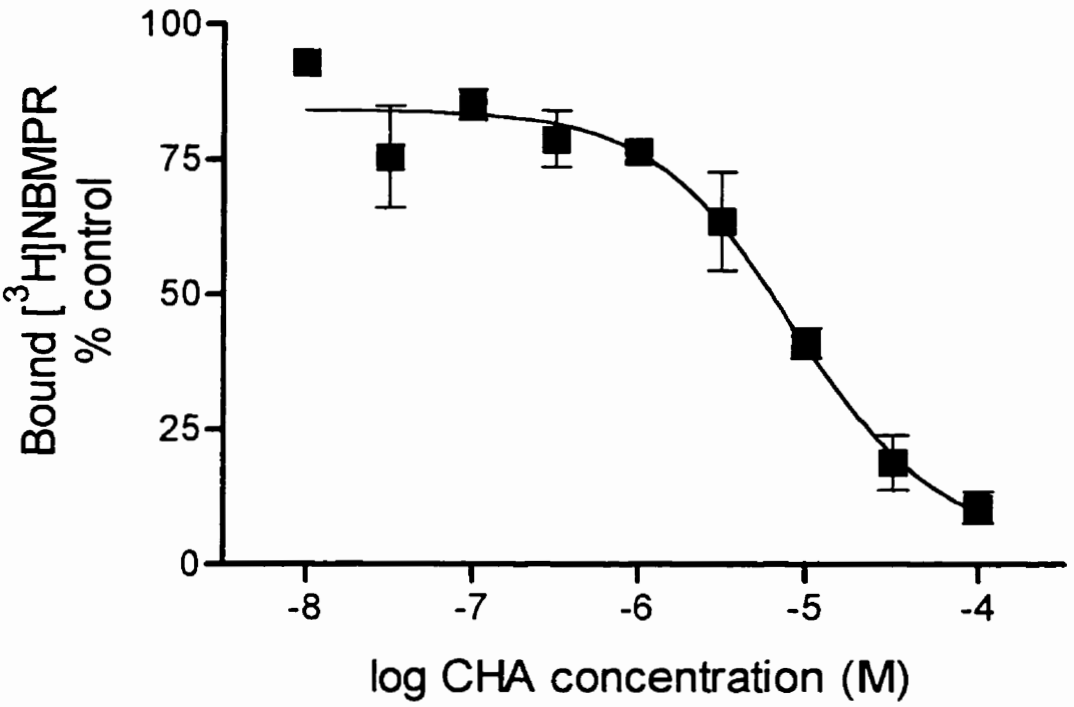


Fig. 5. Concentration-dependent inhibition of site-specific [^{3}H]NBMPR (0.5 nM) binding by CHA (10 nM to 100 μM). Cells (25,000/ml assay volume) were incubated for 1 h with radioligand and graded concentrations of CHA. Site-specific binding (control) of [^{3}H]NBMPR was the difference between binding in the absence and presence of dilazep (100 μM) or unlabeled NBMPR (1 μM). Symbols represent mean ± SEM of three experiments performed in duplicate.

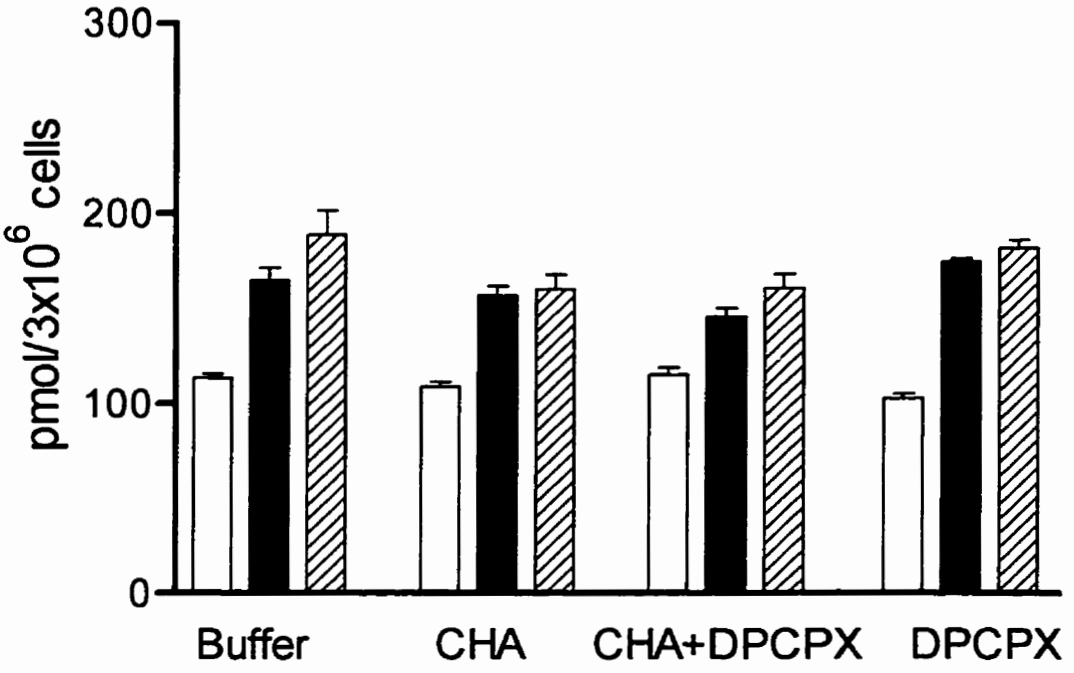


Fig. 6. [^{3}H]Formycin B release in the presence of buffer alone, 300 nM CHA, 300 nM CHA and 10 μM DPCPX, or 10 μM DPCPX alone. Release was stimulated at 4°C (open bars), 22°C (filled bars) or 37°C (hatched bars). Bars represent mean ± SEM of three experiments.

(Fig. 5). CHA produced a concentration-dependent inhibition of [^{3}H]NBMPR binding to DDT_1 MF-2 cells and an K_i value of 2.9 ± 0.5 μM was obtained.

A concentration of CHA (300 nM) that did not inhibit [^{3}H]NBMPR binding but can cause significant activation of A_1 receptors in these cells (data not shown), was examined for inhibition of [^{3}H]formycin B release (Fig. 6). At this concentration, CHA did not inhibit release of [^{3}H]formycin B from DDT_1 MF-2 cells.

DISCUSSION

The main finding of this study was that [^{3}H]formycin B release from DDT_1 MF-2 cells was not modulated by A_1 or A_{2a} receptor stimulation but was inhibited by compounds that directly blocked nucleoside transport processes.

We used a poorly metabolized inosine analogue, formycin B, for this study because adenosine is rapidly metabolized by intracellular and extracellular enzymes. Thus, it is difficult to achieve a stable releasable pool of intracellular adenosine. While it is possible to load cells with [^{3}H]adenosine in the presence of inhibitors of adenosine metabolism, several inhibitors of adenosine metabolism, such as the adenosine kinase inhibitor iodotubercidin, can block nucleoside transport processes and interfere with uptake or release assays (Parkinson and Geiger, 1996). Formycin B is a poorly metabolized nucleoside that is a permeant for *es* nucleoside transporters and equilibrates across cell membranes (Cass, 1995). These properties make formycin B a useful probe for assaying cellular release of nucleosides.

Cells were loaded with [^{3}H]formycin B by imposing an inwardly directed concentration gradient. Release was initiated by removing extracellular [^{3}H]formycin B and resuspending cells in buffer, a procedure that reversed the concentration gradient. Release was inhibited by the transport inhibitor NBMPR, indicating that release occurred through *es* transporters. [^{3}H]Formycin B release through *es* transporters in human erythrocytes has been observed previously (Plagemann and Woffendin, 1989).

To determine if there was a temperature-dependence of transporter function, we measured release at either 22°C or 37°C. Release of [^{3}H]formycin B from cells resuspended in buffer at 22°C was equivalent to release at 37°C during the 90 sec release intervals. Extracellular [^{3}H]formycin B was present following resuspension of cells in buffer at 4°C which is likely due to a small volume of residual loading buffer. However, this amount was consistent between experiments; furthermore, release at 4°C was not inhibited by NBMPR and therefore was not *es* transporter mediated. Extracellular tritiated nucleoside associated with cells resuspended into buffer at 4°C has also been observed in other studies investigating nucleoside release processes (Borgland and Parkinson, 1997; Foga et al., 1996).

For these experiments, maximally effective concentrations of A_1 and A_{2a} receptor agonists were chosen so as to cause significant receptor activation with a minimum release interval. Significant inhibition of [^{3}H]formycin B release by 30 μM CHA was observed with IC_{50} values of 3 - 6 μM. Previously, CHA has been shown to inhibit [^{3}H]adenosine accumulation in rat brain cells with a K_i of 14.5 μM (Geiger et al., 1988). However, the A_1 receptor antagonist DPCPX did not reverse the inhibition of [^{3}H]formycin B release by

CHA. This indicates that inhibition of release by CHA was not mediated by A_1 receptor activation. We tested whether CHA could interact directly with *es* transporters and found that CHA inhibited [^{3}H]NBMPR binding with a K_i value similar to the IC_{50} value for inhibition of [^{3}H]formycin B release. Similarly, in rat brain preparations, an IC_{50} value of 8.8 μM was obtained for the displacement of [^{3}H]NBMPR binding by CHA (Geiger et al., 1985). A concentration of CHA that did not block [^{3}H]NBMPR binding but was effective in adenosine A_1 receptor assays had no effect on [^{3}H]formycin B release. This indicates that inhibition of [^{3}H]formycin B release from DDT_1 MF-2 cells by CHA was due to direct inhibition of *es* transporters.

No effect on [^{3}H]formycin B release was observed with NECA, a mixed A_1/A_2 receptor agonist, or CGS 21680, a selective A_2 receptor agonist. Similarly, NECA had little effect on adenosine accumulation in rat dissociate brain cells (Geiger et al., 1988). Thus, nucleoside release from DDT_1 MF-2 cells was not regulated by adenosine A_2 receptor activation. In contrast to our results, NECA enhanced adenosine transport in cultured bovine chromaffin cells (Deligado et al., 1990). The mechanism of this effect is not clear, however, since the effects of NECA were not mimicked by forskolin, which may indicate that adenosine receptors stimulatory for adenylyl cyclase were not involved.

Previous studies investigating regulation of nucleoside uptake have focused primarily on direct stimulation of components of second messenger pathways. Delicado et al. (1991) found that protein kinase C activators inhibited nucleoside uptake in cultured chromaffin cells. Inhibition of adenosine uptake by cAMP analogues or direct stimulation of adenylyl cyclase with forskolin has also been demonstrated (Sen et al., 1990). In

cerebellar granule cells, Sweeney (1996) observed that pertussis toxin decreased adenosine release while cholera toxin potentiated adenosine release, indicating that the activity of equilibrative transporters in cerebellar granular cells was modulated by activation of G proteins. Thus, depending on the cell type, activators of cAMP formation have been shown to increase or decrease the activity of nucleoside transporters. In the present study with DDT_1 MF-2 cells, we found no effect on nucleoside release processes by concentrations of NECA or CGS 21680 that activate adenosine A_{2a} receptors and increase cAMP levels.

In summary, A_1 and A_2 adenosine receptor stimulation does not modify [^{3}H]formycin B release from DDT_1 MF-2 smooth muscle cells. The inhibition of [^{3}H]formycin B release observed with 30 μM CHA was due to direct interactions with the transport process and not to receptor stimulation.

Chapter 4. Modulation of Adenosine Receptor Activity by Propentofylline in Chinese Hamster Ovary Cell Lines Transfected with Human A_1, A_{2a} or A_{2b} Receptors

ABSTRACT

Propentofylline is a xanthine derivative shown to be neuroprotective *in vivo*. Propentofylline has been shown to block adenosine transport processes which would be expected to potentiate adenosine receptor stimulation. However, propentofylline has also been shown to antagonize adenosine receptors directly. Furthermore, propentofylline is a weak cAMP phosphodiesterase inhibitor, an effect which would mimic some of the receptor-mediated effects of adenosine. We investigated the concentration dependent inhibitory effects of propentofylline on cAMP phosphodiesterase, adenosine transport processes, and adenosine A_1, A_{2a} and A_{2b} receptors. We used CHO cells which were transfected with human adenosine A_1, A_{2a} or A_{2b} receptors and a luciferase reporter gene under control of a promoter sequence containing several copies of the cAMP response element. Cells were treated with the drugs of interest for 30 minutes at 37°C, washed and incubated for 3.5 hours at 37°C, and assayed for luciferase activity. At concentrations of 1 and 10 mM, propentofylline increased luciferase activity probably due to inhibition of cAMP phosphodiesterase. Uptake of [^{3}H]adenosine (30 min) was inhibited by propentofylline with IC_{50} values of 0.17 mM, 0.14 mM and 0.18 mM for A_1, A_{2a} and A_{2b} receptor-transfected cells, respectively. The effect of propentofylline on agonist-mediated changes in luciferase was examined using the mixed A_1/A_2 agonist NECA. Propentofylline did not inhibit NECA-stimulated A_{2b} receptors but a trend towards inhibition of A_1

receptors and significant inhibition of A_{2a} receptors was observed. The effect of propentofylline on adenosine-mediated changes in luciferase was examined. Propentofylline did not modify adenosine-mediated changes of luciferase in cells expressing A_{2b} receptors or in forskolin-stimulated cells expressing A_1 receptors. However, propentofylline (10 μM) inhibited adenosine-stimulated A_{2a} receptors. In this experimental system, propentofylline did not potentiate the effects of adenosine. Whether propentofylline can potentiate the effects of endogenously produced adenosine, for example, to enhance the neuroprotective effects of adenosine, remains to be determined.

INTRODUCTION

Adenosine, formed from the breakdown of 5'-adenosine triphosphate, is an endogenous neuroprotective agent that is released from cells under conditions of metabolic stress (Rudolphi et al., 1992a) and acts on one of four cloned cell surface receptors termed A_1, A_{2a}, A_{2b}, or A_3 (Fredholm et al., 1994).

A_1 receptors are abundant in brain and stimulation of these receptors can decrease cAMP formation, increase K^+ conductance and decrease Ca^{2+} conductance (Rudolphi et al., 1992b). Stimulation of A_1 receptors leads to presynaptic inhibition of release and postsynaptic inhibition of action of excitatory neurotransmitters, particularly glutamate (Andiné et al, 1990; Dunwiddie, 1985). Adenosine A_{2a} and A_{2b} receptors are coupled to activation of adenylyl cyclase via stimulatory G-proteins. Adenosine A_{2a} receptors, are mainly localized in the dopamine rich regions of the brain (Ferré et al., 1992) whereas A_{2b} receptors appear to have a ubiquitous distribution. The role of adenosine A_3 receptors in the brain is poorly characterized.

Once formed intracellularly, adenosine can be released via nucleoside transporters. Although both sodium-dependent and sodium-independent transporters exist, the latter are generally implicated in the release of nucleosides under conditions of metabolic stress (Griffith and Jarvis, 1996). These bi-directional transporters can be further subdivided in to equilibrative-sensitive (*es*) and equilibrative-insensitive (*ei*) based on their sensitivity to the nucleoside transport inhibitor nitrobenzylthioinosine (Cass, 1995). Since inhibitors of nucleoside transport can increase extracellular concentrations and thereby enhance receptor-mediated effects of adenosine (Dresse et al., 1982), novel pharmaceuticals using this strategy have been proposed.

Propentofylline is a novel xanthine derivative in late-phase clinical trials that is known to be neuroprotective *in vivo* (Mrsulja et al., 1985; DeLeo 1987; Dux et al., 1990). In animal studies, it has been shown to reduce neuronal damage due to Ca^{2-} accumulation in cerebral ischemic gerbils (DeLeo et al., 1987) and rats (Hagberg et al., 1990). In humans, a significant clinical improvement in cognitive function has been observed in treatment of dementias due to cerebrovascular disease and Alzheimer's disease (Moller et al., 1994).

Although the exact mechanism of action of propentofylline has not been fully elucidated, it has several effects at the cellular level which may be responsible for its neuroprotection. Neuroprotective effects of propentofylline are consistent with elevation of adenosine levels in ischemic brain (Andiné et al., 1990). Propentofylline can block three adenosine transporter subtypes; *es*, *ei*, and the sodium-dependent N1/*cif* (Parkinson et al., 1993). Thus, neuroprotective effects of this drug may result from inhibition of adenosine

transport resulting in an elevation of extracellular adenosine levels and thereby an enhancement of adenosine A_1 and/or A_2 receptor stimulation (Parkinson et al., 1994). Propentofylline has also been shown to inhibit neutrophil activation (Banati et al., 1994) and stimulate nerve growth factor (Nabeshima et al., 1993), although the molecular mechanisms that mediate these effects have not been identified.

However, similar to other xanthine derivatives, propentofylline is able to antagonize A_1, A_{2a} and A_{2b} receptors (Fredholm et al., 1992) as well as inhibit cyclic nucleotide phosphodiesterase isoforms (Meskini et al., 1994). Although this compound can block adenosine receptors, we hypothesize that there are concentrations at which propentofylline can potentiate rather than inhibit adenosine-mediated effects.

In this study, we investigated the concentration dependence of propentofylline for enhancing the receptor-mediated effects of adenosine using three Chinese Hamster Ovary (CHO) cell lines which have been stably transfected with different human adenosine receptors; A_1, A_{2a}, or A_{2b} (Castañón and Spevak, 1994). These cells have integrated in their genome a luciferase reporter gene under control of a promotor sequence containing several copies of the cAMP response element (Himmler et al., 1993). The activity of the reporter gene is dependent on cellular levels of cAMP and can be monitored by bioluminescence.

MATERIALS AND METHODS

Materials: Adenosine, forskolin, Tris and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). N-Ethylcarboxamidoadenosine (NECA)and CGS

21680 were obtained from Research Biochemicals International (Natick, MA) and [^{3}H]adenosine was from DuPont NEN (Boston, MA). G-418 (geneticin), MEM α-medium and dialyzed fetal bovine serum were purchased from Gibco BRL (Baltimore, MD). Luciferase Substrate was obtained from Promega (Madison, WI). Propentofylline was generously donated by Dr. K. Rudolphi (Hoechst AG; Frankfurt, Germany).

Cell Culture: Three Chinese Hamster Ovary (CHO) cell lines, transfected with human adenosine A_1, A_{2a} or A_{2b} receptors and a luciferase reporter gene under control of a promotor sequence containing several copies of the cAMP response element, (Castañón and Spevak, 1994) were obtained from Dr. Castañón (Ernst Boehringer Institute; Vienna, Austria). The cell lines were maintained as exponentially proliferating cultures in nucleotide free MEM α-medium supplemented with 10 % dialyzed fetal calf serum and the neomycin analogue G-418 (700 μg/ml). For assays, cells were seeded on 6-well plates and used when confluent.

Luciferase Assays: CHO cells in 6 well plates were washed with serum-free media. For all experiments drugs were prepared in serum free media and applied to cells in a volume of 2 ml for 30 min at 37°C. After 30 min, cells were washed with serum free media and incubated for 3.5 h at 37°C also in serum-free media. After 3.5 h, cells were washed with citrate-saline buffer (134 mM KCl, 15 mM sodium-citrate; pH adjusted to 7.0-7.4) and scraped from cell culture plates. Cells were centrifuged (2 min; 13,000 x *g*) and buffer was removed. Cell pellets were resuspended in 100 μl Tris-Triton solution (100 mM Tris, pH 7.8; 0.1% Triton X-100) and incubated at 4°C for 15 min. Aliquots were centrifuged (15 min; 4°C; 13,000 x *g*) to pellet nuclei. Supernatants were transferred

to fresh microcentrifuge tubes. Aliquots (20 µl) were used for luciferase assays and 50 µl aliquots were used for protein determinations. Bioluminescence due to luciferase activity was measured on a luminometer (Biocan Scientific; Tropics) after addition of Luciferase Substrate (Promega; Madison WI).

Cells were treated with propentofylline (1 µM - 10 mM) to test for direct effects of propentofylline on luciferase. The effect of propentofylline (10 µM - 10 mM) on agonist-mediated changes in receptor activity was assayed in each cell line using the A_1/A_2 mixed agonist NECA (1 µM for A_1 receptors; 100 nM for A_{2a} receptors; 10 µM for A_{2b} receptors). Concentrations of NECA were chosen according to EC_{50} values obtained by Castañón and Spevak (1994). CHO cells transfected with A_1 receptors were stimulated with 2 µM forskolin (Castañón and Spevak, 1994). Concentration dependence of adenosine (10 nM - 1 mM) on A_1 receptors in forskolin-stimulated cells was examined. For cells expressing A_{2a} or A_{2b} receptors, adenosine concentrations of 100 nM to 1 mM were used. To determine the effect of propentofylline on adenosine-mediated A_1 receptor stimulation, adenosine (1 µM) and propentofylline (1 µM - 10 mM) were applied to forskolin-stimulated cells. For cells expressing A_{2a} and A_{2b} receptors, adenosine (100 nM for A_{2a} receptors; 1 µM for A_{2b} receptors) and propentofylline (10 µM- 10 mM) were applied to cells. To determine the effect of an A_{2a} selective agonist, CGS 21680, on luciferase activity in cells transfected with A_{2a} or A_{2b} receptors, graded concentrations (0.01 - 100 µM) of this drug were applied to cells.

Adenosine Accumulation: CHO cells in 6 well plates were washed twice with physiological buffer (in mM: NaCl, 118; KCl, 4.9; $MgCl_2$, 1.2; KH_2PO_4, 1.4; 4-(2-

hydroxyethyl)-1-piperazineethanesulfonic acid, 25; glucose, 11; $CaCl_2$, 1; pH 7.4, 300 ± 10 mOsm). [^{3}H]Adenosine (100 μM) in the absence or presence of propentofylline (1 μM-10 mM) was applied for either 20 sec or 30 min at 22°C. Uptake of [^{3}H]adenosine (100 μM) at 0 sec was determined in the presence of 30 μM dipyridamole. Solutions were aspirated and cells were washed three times with ice cold buffer. Cells were dissolved in 1 M NaOH (60 min; 37°C) and then taken for radioactive determination by liquid scintillation counting.

Protein Determination: Samples (1:10 dilution in water) were assayed for protein concentration using the method of Lowry et al. (1951). Standards were prepared using bovine serum albumin in water with 10% Tris-Triton. Since samples from adenosine accumulation studies were dissolved in 1 M NaOH, standards for these experiments were prepared using 0.1 M NaOH.

Data Analysis: Experiments were performed four times in triplicate unless otherwise indicated. Half maximal inhibition constants (IC_{50}) and effective concentrations (EC_{50}) were obtained by non-linear regression using the software package GraphPad PRISM version 2. To test for significant differences between groups, data were analyzed using a one way ANOVA with a Bonferroni post-hoc test. A significance level of $p<0.05$ was chosen *a priori*.

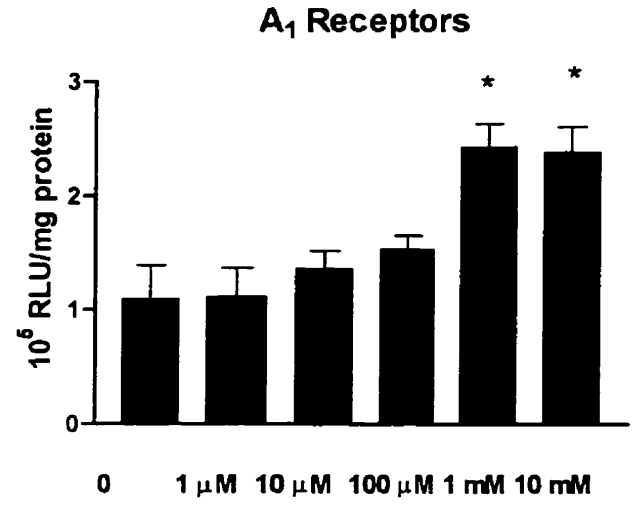
RESULTS

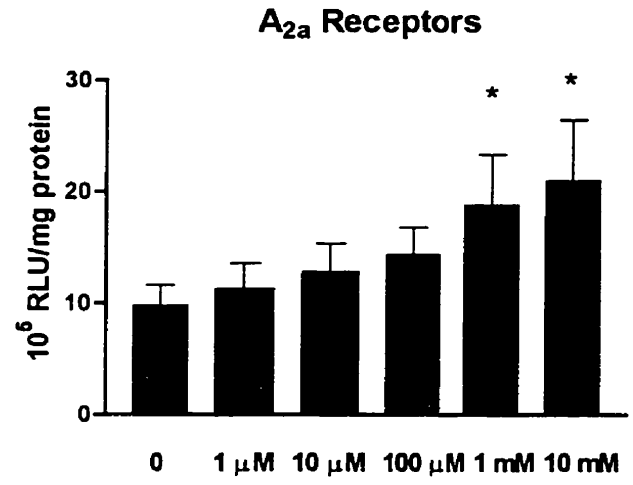
Propentofylline Concentration Response. Propentofylline can inhibit cAMP phosphodiesterase (Meskini et al, 1994) so to determine the concentration at which

propentofylline directly activates luciferase we treated the cells with propentofylline at concentrations of 1 μM to 10 mM. In all three cell lines, significant increases in luciferase activity were evident at 1 and 10 mM propentofylline (Fig. 1). EC_{50} values of 0.17 ± 1 mM, 0.27 ± 1.7 mM and 0.56 ± 1.3 mM were obtained for A_1, A_{2a}, and A_{2b} receptor-transfected cell lines, respectively.

Effects of Propentofylline on Agonist-Mediated Changes in Luciferase Levels. The A_1/A_2 receptor agonist NECA was used to assay for A_1, A_{2a} or A_{2b} receptor function. In the A_1 receptor-transfected cell line (Fig. 2a), 2 μM forskolin was used to stimulate cAMP production. Forskolin increased luciferase activity, which was inhibited by NECA. Propentofylline produced a concentration-dependent increase in luciferase activity which reached statistical significance at 1 and 10 mM. NECA significantly stimulated luciferase activity in A_{2a} and A_{2b} receptor-transfected cells (Fig. 2b, 2c). Propentofylline (10 μM and 100 μM) caused a significant 26% and 32% reduction in NECA-stimulated cAMP in A_{2a} receptor-transfected cells, respectively (Fig. 2b). There was no significant reduction in NECA-stimulated cAMP in A_{2b} receptor transfected cells. However, there was a significant increase in cAMP production in the presence of 10 mM propentofylline (Fig. 2c).

Adenosine Concentration-Response. Each cell line was assayed for receptor function using graded concentrations (10 nM - 1 mM) of adenosine. In cells expressing A_1 receptors, 2 μM forskolin was used to stimulate cAMP production. Although 10 nM adenosine inhibited luciferase activity, significance was achieved at concentrations of 100 nM adenosine and greater. An EC_{50} value of 72 ± 3.9 nM was obtained (Fig. 3a).


A1 Receptors
10^6 RLU/mg protein
3
2
1
0
*
*
0
1 μM
10 μM
100 μM
1 mM
10 mM


A2a Receptors
10^6 RLU/mg protein
30
20
10
0
*
*
0
1 μM
10 μM
100 μM
1 mM
10 mM

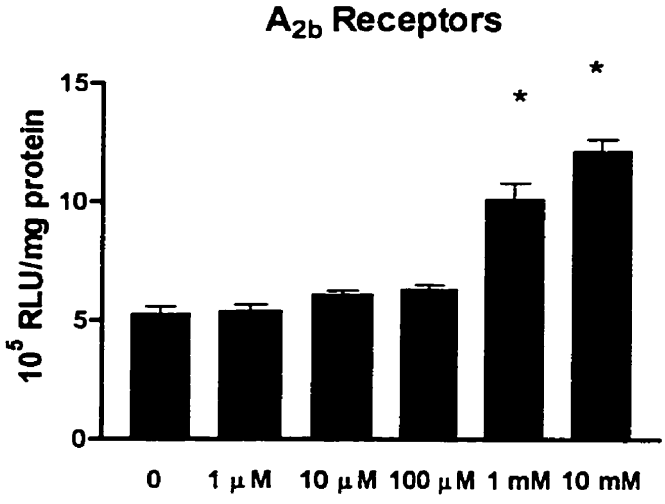
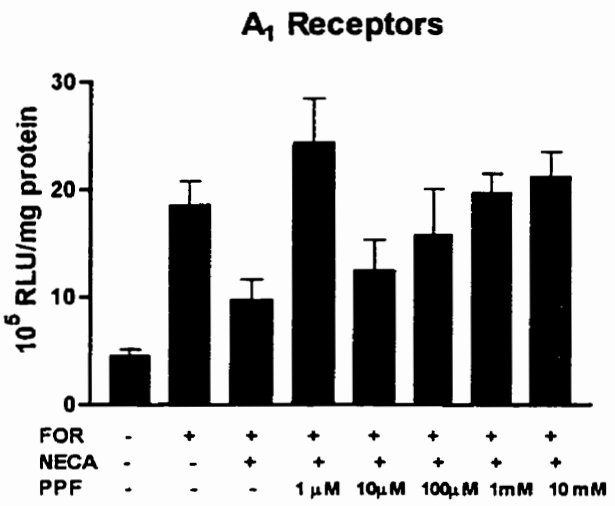
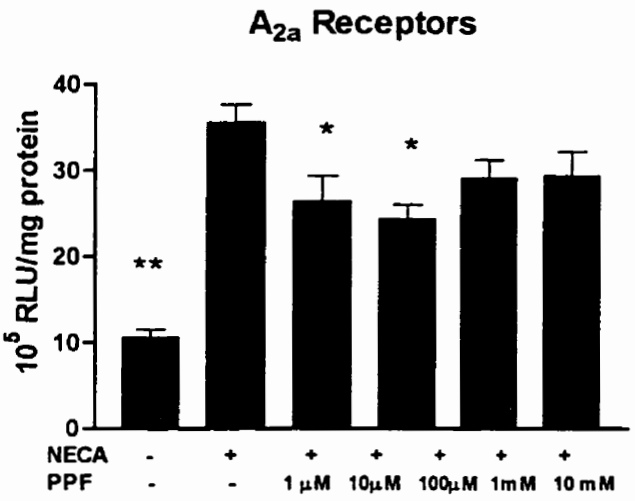

A2b Receptors
10^5 RLU/mg protein
15
10
5
0
*
*
0
1 μM
10 μM
100 μM
1 mM
10 mM

Fig. 1. Concentration-dependent stimulation of luciferase activity by propentofylline in CHO cells transfected with A_1 receptors (A), A_{2a} receptors (B), or A_{2b} receptors (C). Concentrations of 1 μM to 10 mM propentofylline were applied to cells for 30 min at 37°C. Luciferase activity in cells was determined 3.5 h after removal of propentofylline. Data are represented as Relative Light Units (RLU) per mg protein. Bars represent means ± S.E.M. of four experiments (*$p<0.05$; ANOVA with Bonferroni post-hoc test comparing data for each concentration of propentofylline to data in the absence of propentofylline).


A1 Receptors
30
20
10
0
10^5 RLU/mg protein
FOR - + + + + + + +
NECA - - + + + + + +
PPF - - - 1 μM 10μM 100μM 1mM 10 mM


A2a Receptors
40
30
20
10
0
10^5 RLU/mg protein
**
*
*
NECA - + + + + + +
PPF - - 1 μM 10μM 100μM 1mM 10 mM

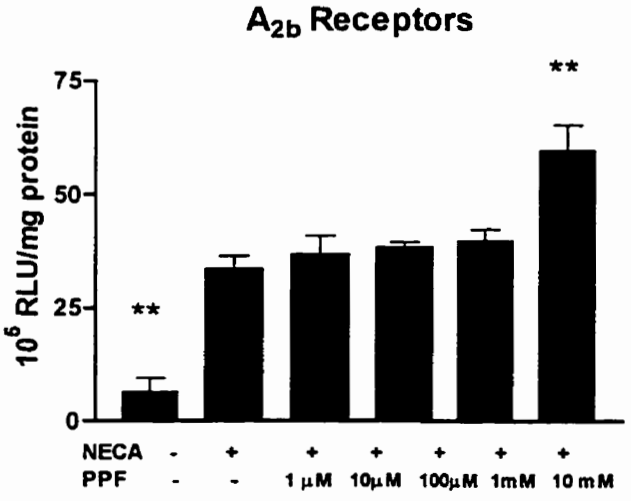
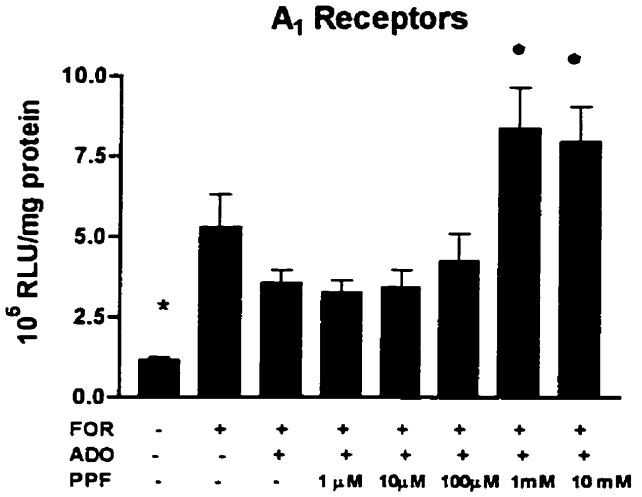
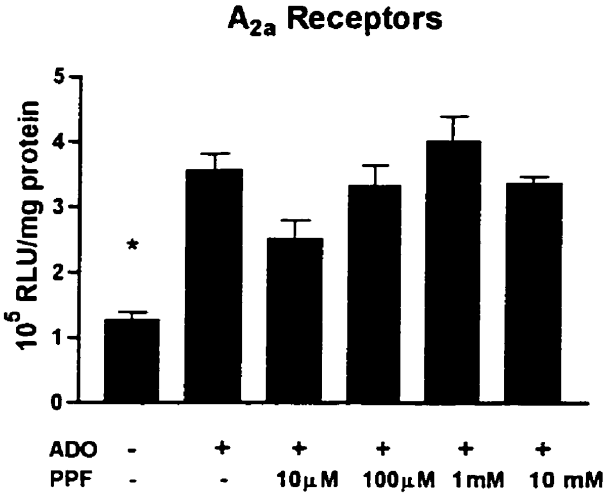

A2b Receptors
75
50
25
0
10^5 RLU/mg protein
**
**
NECA - + + + + + +
PPF - - 1 μM 10μM 100μM 1mM 10 mM

Fig. 2. Effect of propentofylline on agonist-mediated changes in luciferase activity for cells expressing A_1 receptors (A), A_{2a} receptors (B), or A_{2b} receptors (C). The A_1/A_2 mixed agonist, NECA, was applied to cells (1 μM for A_1; 100 nM for A_{2a}; 10 μM for A_{2b}) with or without 10 μM to 10 mM propentofylline (PPF) for 30 min at 37°C. In cells expressing A_1 receptors, cAMP production was stimulated with forskolin (FOR; 2 μM). Data are expressed as Relative Light Units (RLU) per mg protein. Bars represent mean ± SEM of four experiments. Statistical differences in luciferase activity, relative to cells treated with forskolin and NECA (A) or NECA alone (B,C) are indicated. (*$p<0.05$, **$p<0.01$; ANOVA with Bonferroni post-hoc test).


A1 Receptors
10.0
7.5
5.0
2.5
0.0
10^6 RLU/mg protein
*
FOR - + + + + + + +
ADO - - + + + + + +
PPF - - - 1 μM 10μM 100μM 1mM 10 mM


A2a Receptors
5
4
3
2
1
0
10^6 RLU/mg protein
*
ADO - + + + + +
PPF - - 10μM 100μM 1mM 10 mM

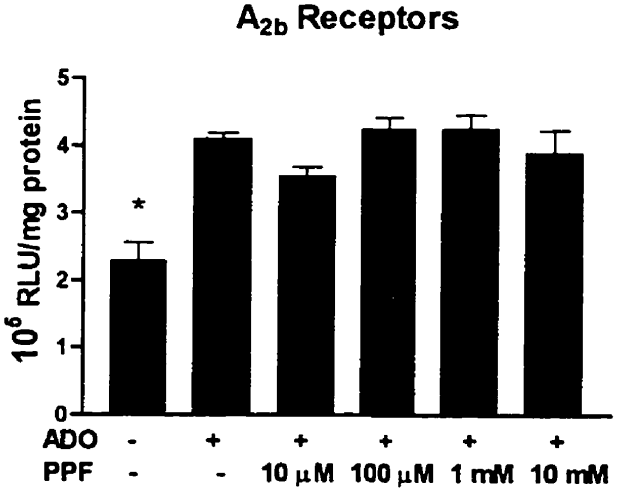
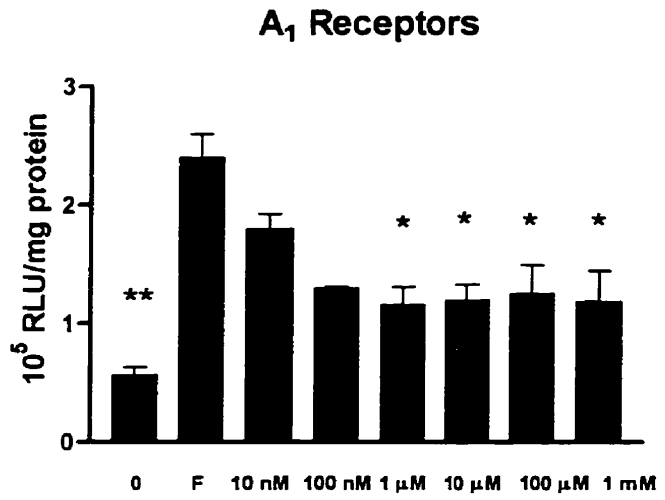
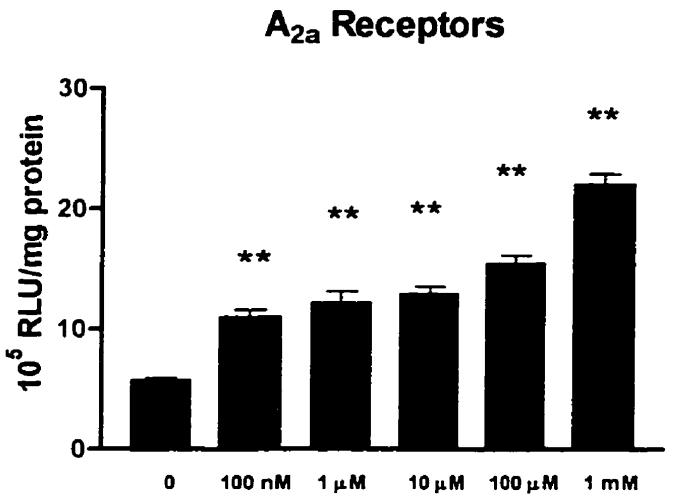

A2b Receptors
5
4
3
2
1
0
10^6 RLU/mg protein
*
ADO - + + + + +
PPF - - 10 μM 100 μM 1 mM 10 mM

Fig. 3. Concentration-dependent effects of adenosine on luciferase activity in cells expressing A_1 receptors (A), A_{2a} receptors (B), or A_{2b} receptors (C). Forskolin (2 μM) was used to stimulate cAMP production in A_1 receptor-transfected cells. Adenosine (10 nM - 1 mM for A_1 receptors; 100 nM - 1mM for A_{2a} or A_{2b} receptors) was applied to cells for 30 min at 37°C. Data are expressed as Relative Light Units (RLU) per mg protein. Bars represent means ± SEM of four experiments (*$p<0.05$, **$p<0.01$; ANOVA with Bonferroni post-hoc test comparing data for each adenosine concentration to data obtained in the absence of adenosine).


A1 Receptors
10^5 RLU/mg protein
3
2
1
0
**
*
*
*
*
0
F
10 nM
100 nM
1 μM
10 μM
100 μM
1 mM


A2a Receptors
10^5 RLU/mg protein
30
20
10
0
**
**
**
**
**
0
100 nM
1 μM
10 μM
100 μM
1 mM

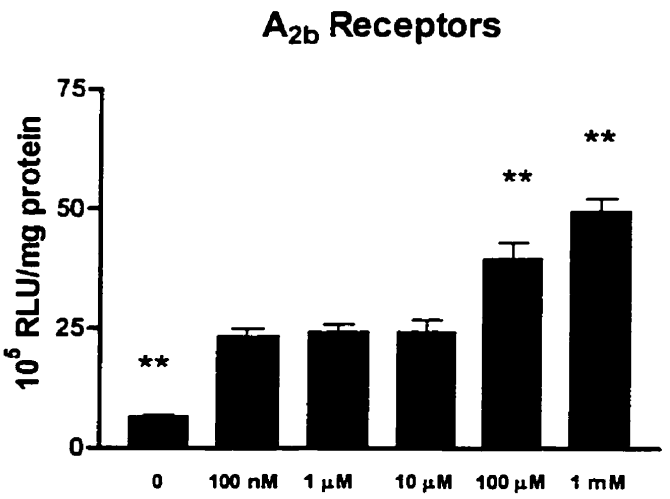

A2b Receptors
10^6 RLU/mg protein
75
50
25
0
**
**
**
0
100 nM
1 μM
10 μM
100 μM
1 mM

Fig. 4. Effect of propentofylline on adenosine-mediated changes in luciferase activity following activation of A_1 (A), A_{2a} (B), or A_{2b} (C) receptors by adenosine. Adenosine (1 μM for A_1; 100 nM for A_{2a}; 1 μM for A_{2b} receptors) was applied to cells with or without propentofylline (PPF; 1 μM to 10 mM) for 30 min at 37°C. In cells expressing A_1 receptors, luciferase activity was stimulated by addition of 2 μM forskolin (FOR). Data are expressed as Relative Light Units (RLU) per mg protein. Bars represent means ± S.E.M. of four experiments (*$p<0.05$, **$p<0.01$; ANOVA with Bonferroni post-hoc test comparing data obtained in the presence of adenosine and forskolin (A) or adenosine only (B,C) to data obtained in the added presence of propentofylline or in the absence of adenosine).

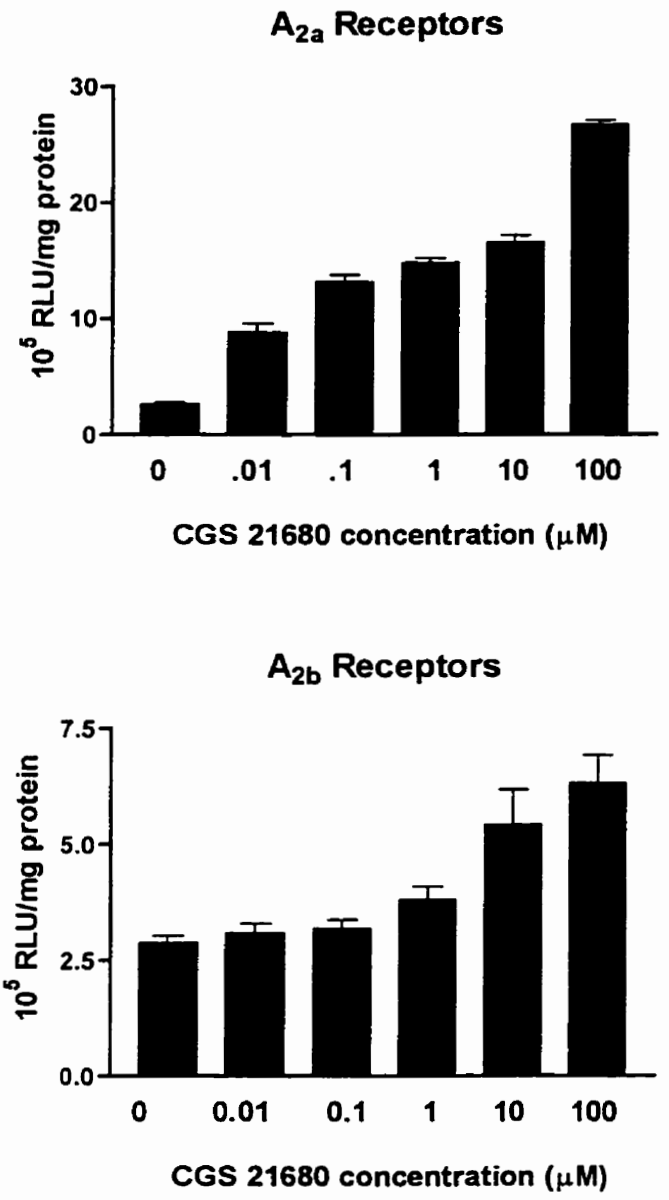
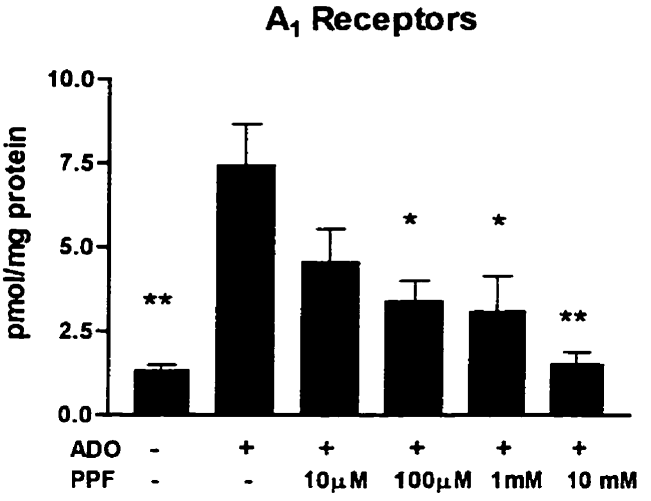
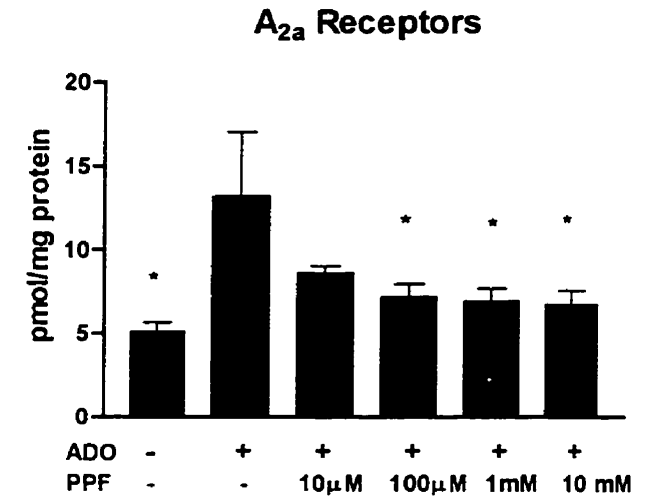


Fig. 5. Effect of the A_{2a} selective agonist CGS 21680 on luciferase activity in cells expressing A_{2a} (A) and A_{2b} (B) receptors. CGS 21680 (0.01 μM to 100 μM) was applied to cells for 30 min at 37°C. Data are expressed as Relative Light Units (RLU) per mg protein. Bars represent means ± SEM of four experiments. (*$p<0.05$, **$p<0.01$; ANOVA with Bonferroni post-hoc test comparing data obtained in the presence of CGS 21680 to all data obtained in the absence of CGS 21680).


A1 Receptors
10.0
7.5
5.0
2.5
0.0
pmol/mg protein
**
*
*
**
ADO - + + + + +
PPF - - 10μM 100μM 1mM 10 mM


A2a Receptors
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10
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0
pmol/mg protein
*
*
*
*
ADO - + + + + +
PPF - - 10μM 100μM 1mM 10 mM

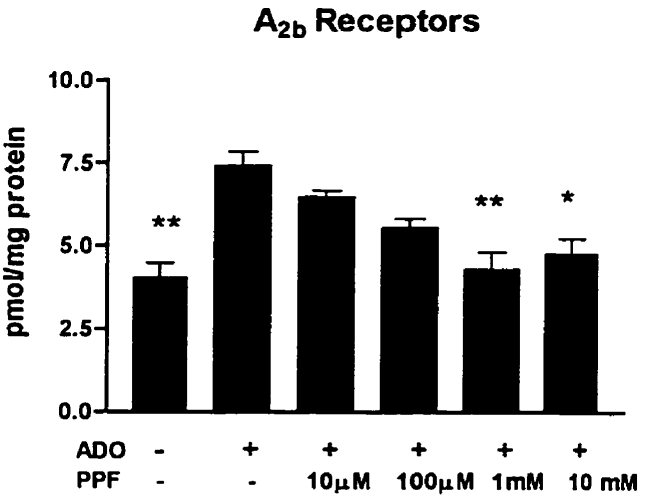
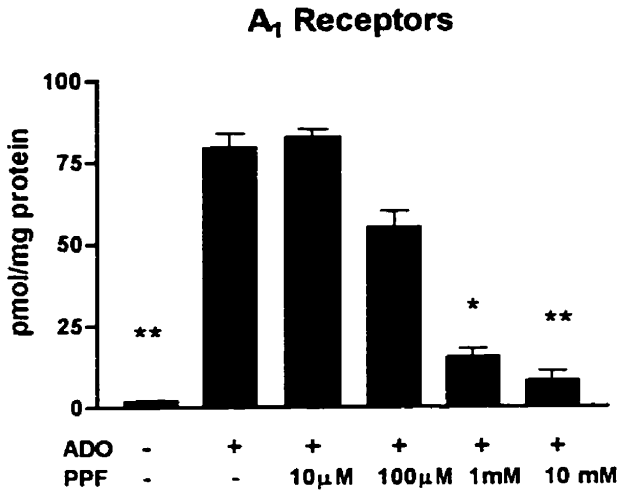
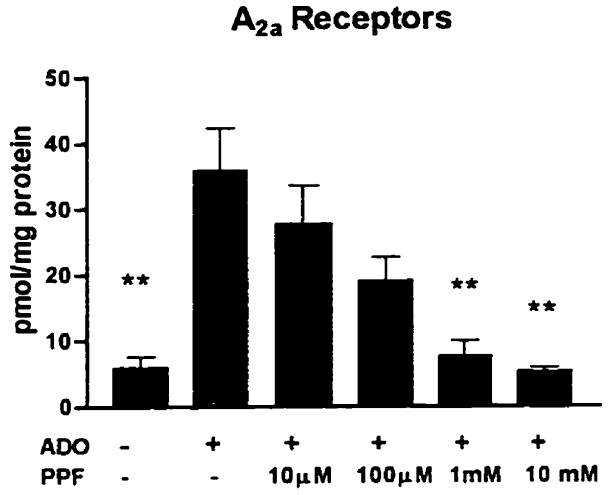

A2b Receptors
10.0
7.5
5.0
2.5
0.0
pmol/mg protein
**
**
*
ADO - + + + + +
PPF - - 10μM 100μM 1mM 10 mM

Fig. 6. Effect of propentofylline on [^{3}H]adenosine transport, during 20 sec, in cells transfected with A_1 (A), A_{2a} (B), or A_{2b} (C) receptors. Cells were incubated with [^{3}H]adenosine (100 μM) alone or in the presence of propentofylline (PPF; 10 μM - 10 mM) for 20 sec. Maximal inhibition was achieved in the presence of 30 μM dipyridamole. Bars represent means ± SEM of four experiments. (*$p<0.05$, **$p<0.01$; ANOVA with Bonferroni post-hoc test comparing data obtained from cells treated with propentofylline or dipyridamole to data obtained from cells with [^{3}H]adenosine alone).


A1 Receptors
pmol/mg protein
100
75
50
25
0
**
*
**
ADO - + + + + +
PPF - - 10μM 100μM 1mM 10 mM


A2a Receptors
pmol/mg protein
50
40
30
20
10
0
**
**
**
ADO - + + + + +
PPF - - 10μM 100μM 1mM 10 mM

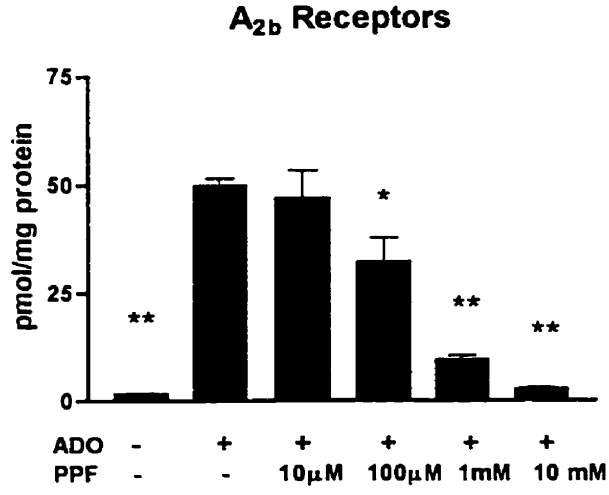

A2b Receptors
pmol/mg protein
75
50
25
0
**
*
**
**
ADO - + + + + +
PPF - - 10μM 100μM 1mM 10 mM

Fig. 7. Effect of propentofylline on [^{3}H]adenosine accumulation, during 30 min, in cells transfected with A_1 (A), A_{2a} (B), or A_{2b} (C) receptors. Cells were incubated with [^{3}H]adenosine (100 μM) alone or in the presence of propentofylline (10 μM - 10 mM) for 30 min. Maximal inhibition of [^{3}H]adenosine accumulation occurred in the presence of 30 μM dipyridamole. Bars represent means ± SEM of three experiments for A_1 and four experiments for A_{2a} or A_{2b} receptor-transfected cells. (*$p<0.05$, **$p<0.01$; ANOVA with Bonferroni post-hoc test comparing data obtained from cells treated with propentofylline or dipyridamole to data obtained from cells incubated with [^{3}H]adenosine alone).

Luciferase activity was increased by adenosine in a concentration-dependent manner in A_{2a} and A_{2b} receptor- transfected cells; EC_{50} values of 0.11 ± 3.5 mM and 0.79 ± 3.2 mM, respectively were obtained (Fig. 3b, 3c).

Effect of Propentofylline on Adenosine-Mediated Changes in Luciferase Levels. Concentrations of adenosine that have significant effects on luciferase production were used in combination with graded concentrations of propentofylline to determine whether propentofylline inhibits or potentiates adenosine-mediated receptor activation. In cells expressing A_1 receptors, adenosine (1 μM) inhibited forskolin-stimulated luciferase activity (Fig. 4a). Propentofylline at concentrations less than 100 μM, did not alter inhibition of luciferase activity mediated by adenosine. However, in the presence of 1 mM and 10 mM propentofylline, luciferase activity was greater than forskolin-stimulated luciferase activity alone. In cells expressing A_{2a} receptors, 100 nM adenosine increased cAMP levels over control. Propentofylline (10 μM) inhibited adenosine-stimulated luciferase activity (Fig. 4b). In contrast, propentofylline did not significantly inhibit adenosine (1 μM)-stimulated luciferase activity in A_{2b} receptor-transfected cells (Fig. 4c).

Effect of CGS 21680 on Cells Expressing A_{2a} or A_{2b} Receptors. To ensure there was no contamination of A_{2a} receptor-transfected cells with A_{2b} receptor-transfected cells or vice versa, graded concentrations of the selective A_{2a} agonist CGS 21680 was applied. A significant increase in luciferase activity occurred at 10 nM CGS 21680 in cells expressing A_{2a} receptors (Fig. 5a). In A_{2b} receptor-transfected cells, a significant increase in luciferase activity occurred at a 1000 fold greater concentration (Fig. 5b).

Effect of Propentofylline on [^{3}H]Adenosine Accumulation. Figure 6 illustrates [^{3}H]adenosine accumulation during 20 seconds by cells transfected with A_1, A_{2a} and A_{2b} receptors. Propentofylline inhibited [^{3}H]adenosine uptake in all three cell lines with IC_{50} values of 160 ± 5 μM, 17 ± 2 μM, and 75 ± 3 μM for cells expressing A_1, A_{2a} or A_{2b} receptors, respectively. [^{3}H]Adenosine accumulation in 30 min was also determined for each receptor type (Fig. 7). Propentofylline inhibited accumulation similarly in the three cell lines with IC_{50} values of 0.14 ± 0.6 mM, 0.14 ± 0.2 mM, and 0.18 ± 0.3 mM for A_1, A_{2a}, and A_{2b} receptor-transfected cell lines, respectively.

DISCUSSION

It has been suggested that propentofylline indirectly enhances activity at adenosine receptors by blocking removal of adenosine from the vicinity of its receptors (Fredholm and Lindstrom, 1986). However, propentofylline has also been shown to antagonize adenosine receptors (Fredholm et al., 1992). The effect of propentofylline on cAMP production was studied in three CHO cell lines which were transfected with A_1, A_{2a} or A_{2b} receptors as well as a luciferase reporter gene under control of a promoter sequence containing several copies of the cAMP response element (Castañón and Spevak, 1994).

Previous studies have reported that propentofylline is able to inhibit cyclic nucleotide phosphodiesterase isoforms (Meskini et al., 1994). Similarly, our study demonstrated that propentofylline (≥1 mM) increased luciferase levels, probably by inhibiting cAMP phosphodiesterase. This phenomenon was observed for each cell line. The effects of propentofylline on cAMP phosphodiesterase were distinguished from its

effects at adenosine receptors, as lower concentrations of propentofylline alone did not significantly increase luciferase levels.

Propentofylline is a known inhibitor of *es* and *ei* nucleoside transporters as well as a sodium-dependent subtype of nucleoside transporter (Parkinson et al., 1993). We determined concentrations of propentofylline which inhibited cellular accumulation of adenosine in CHO cells expressing A_1, A_{2a} or A_{2b} receptors. In all three cell lines, propentofylline inhibited uptake of [^{3}H]adenosine at 20 sec, a time point likely to represent a greater accumulation of adenosine *per se* than its metabolites. Uptake of [^{3}H]adenosine during 30 min was similarly inhibited by propentofylline. CHO cells possess 30 to 40 % *ei* transporters (Plagemann and Wohlhueter, 1984) with the remaining transport mediated by *es* transporters. Previously (Parkinson et al., 1993) it was shown that propentofylline inhibits both *es* and *ei* transporters with IC_{50} values of 9 μM and 170 μM respectively; thus, the IC_{50} values reported in the present study (Fig. 7) are similar to those reported previously for *ei* transporters.

The effects of propentofylline on agonist- and adenosine-stimulated A_{2b} receptors were examined. Concentrations of the A_1/A_2 mixed receptor agonists NECA (10 μM) and adenosine (1 μM) that had significant effects on luciferase production were chosen. Propentofylline (≤ 1 mM) did not inhibit agonist or adenosine-stimulated A_{2b} receptors. However, luciferase levels significantly increased with 10 mM propentofylline, an effect likely due to cAMP phosphodiesterase inhibition.

A_{2a} receptors were stimulated with lower concentrations of NECA (1 μM) or adenosine (100 nM) as these receptors are more sensitive to stimulation by these

compounds. Propentofylline, at 10 μM or 100 μM, significantly inhibited NECA-mediated stimulation of A_{2a} receptors, while only the 10 μM concentration inhibited adenosine-stimulated A_{2a} receptors. This is likely due to a direct inhibitory effect of propentofylline at these receptors which is consistent with previous observations (Fredholm et al., 1992; Zhang et al., 1996).

A_{2a} receptor-transfected cells were not contaminated with A_{2b} receptor-transfected cells because, similar to previously reported results, CGS 21680 was significantly more potent for transfected A_{2a} than A_{2b} receptors expressed in CHO cells (Castañón and Spevak, 1994). Furthermore, agonist and adenosine-stimulated A_{2a} receptors were inhibited by propentofylline unlike A_{2b} receptors.

Castañón and Spevak (1994) reported an IC_{50} value of approximately 1 μM for NECA applied to forskolin-stimulated CHO cells transfected with A_1 receptors. Therefore, we chose this concentration to demonstrate propentofylline's effect on agonist-induced changes in receptor activation. As expected, NECA inhibited forskolin stimulated cAMP in the A_1 receptor-transfected cells. A dose-dependent increase in luciferase was observed in the presence propentofylline (1 - 100 μM), which was not observed for cells stimulated with adenosine. The effects of adenosine or NECA were not observed in the presence of propentofylline at concentrations of 1 or 10 mM. However, at these concentrations, the effects of propentofylline on cAMP phosphodiesterase would mask any effects at A_1 receptors.

An autoradiographic study with rat striatum determined that propentofylline was approximately10 times more potent for inhibiting ligand binding to A_1 receptors than to A_2

receptors (Parkinson and Fredholm, 1991). This preference for A_1 receptors has also been demonstrated with functional assays in rat hippocampal slices (Fredholm et al., 1992). The reason why this drug had a significant inhibitory effect on NECA stimulation at A_{2a} but not A_1 receptors is not clear and may indicate reduced sensitivity to propentofylline of human, compared to rat, A_1 receptors. This may also explain why the effects of adenosine at A_{2a} but not A_1 receptors were inhibited by propentofylline. The effect of adenosine at A_1 receptors was not significantly inhibited or potentiated by propentofylline even though propentofylline did inhibit adenosine uptake and might, therefore, be expected to prevent depletion of extracellular adenosine due to cellular uptake. It is possible that, under conditions of these experiments, cellular uptake did not affect the adenosine concentration sufficiently to alter receptor stimulation.

In these studies, we observed that trends for each receptor appeared to be similar between experiments, however, the measured luciferase levels differed. Whether the variability in luciferase levels is due to use of cells in later generations or use of different batches of luciferase substrate, is not known. Although we did not use cells beyond 20 generations, we have not determined the effect of passage number on reporter gene function.

Propentofylline did not enhance adenosine mediated receptor effects in our experiments even though we demonstrated clear inhibition of cellular uptake by propentofylline. It is possible that this drug has a greater effect on adenosine levels *in vivo* than *in vitro* due, for example, to the relative sizes of the extracellular compartments. Propentofylline's ability to enhance extracellular adenosine concentration has been shown

during ischemic conditions (Andiné et al., 1990). Therefore, neuroprotective effects of propentofylline resulting from indirect increases in adenosine levels due to transport inhibition cannot be ruled out. Since propentofylline can cross the blood-brain barrier, it is an attractive therapeutic agent for treatment of cerebrovascular disorders.

In conclusion, propentofylline inhibited cAMP phosphodiesterase and [^{3}H]adenosine accumulation in CHO cells expressing human A_1, A_{2a} or A_{2b} receptors. Propentofylline significantly inhibited NECA- and adenosine-stimulated receptors only in cells expressing adenosine A_{2a} receptors. Propentofylline was not shown to potentiate the effects of adenosine in any of these cell lines. However, propentofylline may be able to potentiate endogenously produced adenosine, and thereby play a role in enhancing neuroprotection by adenosine.

Chapter 5. General Discussion

Adenosine is involved in a large number of physiological processes mediated through specific adenosine receptors and activation of signal transduction mechanisms. The concentration of adenosine is tightly regulated by purine enzymes as well as by nucleoside transporters that carry adenosine across cell membranes. The primary source of adenosine is derived from the hydrolysis of ATP. During conditions of metabolic stress, there is an increase in adenosine production and this adenosine can be released down its concentration gradient. Alternately, extracellular adenosine may be derived from released ATP, which is dephosphorylated extracellularly. Regardless of the source, actions of adenosine are mediated by A_1, A_{2a}, A_{2b} or A_3 cell surface adenosine receptors.

Two classes of nucleoside transporters have been described; these consist of sodium- independent and sodium-dependent transporters (Cass, 1995). One of the functions of nucleoside transport processes is to facilitate the movement of adenosine across cellular membranes. Since adenosine receptors are located on plasma membranes, it is possible that transporters are important to initiate, as well as to terminate, adenosine receptor stimulation. Both classes of transporters have been studied extensively for their ability to mediate uptake of adenosine and other nucleosides. The role of nucleoside transporters in release of adenosine has received much less attention. Therefore, the studies reported in Chapter 2 and Chapter 3 were performed to clarify further the mechanisms and regulation of release processes.

Release of adenosine is thought to occur primarily through bidirectional *es* transporters (Griffith and Jarvis, 1996). Since, ischemic conditions can depress the sodium

gradient and there is evidence for reversal of sodium-dependent neurotransmitter transporters (Madl and Burgesser, 1993; Gemba et al., 1994), we examined release of a poorly metabolized nucleoside analogue, formycin B, following disruption of transmembrane sodium gradients in a cell line which possess only sodium-dependent nucleoside transport processes (Belt and Noel, 1988).

Before examining release, we confirmed that uptake of [^{3}H]formycin B into L1210/MA27.1 cells was sodium-dependent. Uptake of [^{3}H]formycin B was completely inhibited with a Na^+/K^+ ATPase inhibitor, ouabain and the glycolysis inhibitor iodoacetic acid. Release of [^{3}H]formycin B was inhibited by the presence of an inwardly directed sodium gradient. Perturbation of the sodium gradient by ouabain or the sodium ionophore, monensin, enhanced [^{3}H]formycin B release. Release was inhibited by the nucleoside transport inhibitors, phloridzin and propentofylline. Furthermore, compounds selective for sodium-independent nucleoside transporters, nitrobenzylthioinosine, dipyridamole, and dilazep inhibited release only at high concentrations. Finally, in the absence of sodium, [^{3}H]formycin B release was enhanced by the permeants adenosine and uridine indicating transacceleration. Thus, disruption of sodium gradients may uncouple nucleoside transport from sodium translocation.

From this study, it appears that by disrupting transmembrane sodium-gradients, reversal of sodium-dependent transporters can mediate release of nucleosides. During ischemic conditions where the sodium gradient is perturbed due to depression of ATP levels and/or compromising Na^+/K^+ ATPase function, extracellular adenosine may be derived from efflux through sodium-dependent transporters. Although sodium-dependent

transporters are present in brain, the degree to which adenosine released from these transporters mediates receptor activation requires further study.

Regulation of nucleoside transport also provides further insight into the modulatory effects of adenosine, especially during cerebral ischemia. Since adenosine can be released under conditions of metabolic stress and act on cell surface adenosine receptors, it is possible that receptor activation can modify subsequent adenosine release. Previous studies, which have primarily concentrated on regulation of uptake of adenosine, have indicated that nucleoside transport function may be under complex regulation (Delicado et al., 1990; 1991; Sen et al., 1990; Sayos et al., 1994; Sweeney, 1996). Our study presented in Chapter 3 discusses whether adenosine may act as a feedback modulator to alter its own release. To do this, we used cultured smooth muscle DDT_1 MF-2 cells which possess A_1 and A_2 receptors as well as predominantly *es* transporters. We measured release of the permeant [^{3}H]formycin B after stimulating adenosine receptors with the selective A_1 agonist CHA, the selective A_2 agonist CGS 21680 or the A_1/A_2 mixed agonist, NECA. Stimulation of receptors with NECA or CGS21680 had no effect on release of [^{3}H]formycin B. However, at a concentration of 30 μM, CHA inhibited release . This inhibition of release caused by CHA was not reversed using the selective A_1 antagonist, DPCPX, indicating that it was not A_1 receptor activation causing release. We, therefore, examined whether release was inhibited due to direct interactions of CHA with the nucleoside transporter. CHA inhibited binding of [^{3}H]nitrobenzylthioinosine, a transport inhibitor which binds with high affinity to *es* transporters, at concentrations similar to those that inhibited [^{3}H]formycin B release. To determine if CHA could alter

transport through receptor signaling, we applied to DDT_1 MF-2 cells at a concentration of CHA which does not inhibit nucleoside transporters. Release of [^{3}H]formycin B was not inhibited or potentiated with this concentration of CHA. Therefore, we concluded that CHA inhibited [^{3}H]formycin B release via direct interaction with the transport process and not by a receptor-mediated interaction. These studies conclude that adenosine receptor stimulation does not modify [^{3}H]formycin B release from DDT_1 MF-2 smooth muscle cells. These findings are in contrast to previous reports that have demonstrated alterations in adenosine transport by direct stimulation of signal transduction mechanisms (Delicado et al, 1991; Sweeney, 1996).

Adenosine is considered to be an important inhibitory neuromodulator in the CNS. Nucleoside transport inhibitors have been proposed as therapeutic agents for treatment of various pathological conditions in the CNS. Inhibition of the transport of adenosine into cells can enhance receptor-mediated effects of adenosine and produce therapeutic effects. In particular, the xanthine derivative propentofylline, is in late stage clinical trials for treatment of dementias related to Alzheimer's disease and cerebrovascular disorders. The neuroprotective effects of propentofylline are consistent with its inhibitory action at nucleoside transporters resulting in potentiation of adenosine at its receptors (Parkinson et al., 1993). However, propentofylline can also antagonize adenosine receptors (Fredholm et al., 1992) and inhibit cyclic nucleotide phosphodiesterase isoforms (Meskini et al., 1994). The importance of these various effects of propentofylline for its neuroprotective properties is unclear. One possible mechanism of propentofylline's action that has been suggested previously involves raised cAMP levels due to phosphodiesterase inhibition.

This results in increased cAMP-dependent protein kinase activity with consequent inhibition of voltage-gated Na^+ channels and reduced excitotoxicity (Lloyd and Bagley, 1997).

In the study presented in Chapter 4, we characterized the inhibitory effects of propentofylline on cyclic nucleotide phosphodiesterase, adenosine transporters and three adenosine receptor subtypes. For these experiments, we used CHO cells that have been transfected with human adenosine A_1, A_{2a} or A_{2b} receptors as well as a reporter gene construct containing a luciferase gene with a cAMP response element-rich promoter. Propentofylline inhibited both cAMP phosphodiesterase and adenosine accumulation in all receptor-transfected CHO cells. Propentofylline inhibited the receptor mediated effects of the mixed agonist NECA, but did not inhibit the receptor mediated effects of adenosine. At a concentration of 10 μM, propentofylline inhibited only A_{2a} receptor stimulation. As stimulation of A_{2a} receptors promote excitatory amino acid release, a neuroprotective effect of propentofylline may result from inhibition of A_{2a} receptor-mediated excitotoxicity. Propentofylline clearly inhibited adenosine uptake into each of the three cell lines, however, there was no evidence of potentiation of the receptor- mediated effects of adenosine in our experimental protocol.

Adenosine levels greatly increase during hypoxic or ischemic conditions and stimulation of adenosine receptors mediates neuroprotective effects. The *es* transport inhibitor propentofylline is neuroprotective, at least in part, due to inhibition of adenosine uptake and potentiation of adenosine's receptor mediated effects. However, further development of nucleoside transport inhibitors for potential therapeutic use has been

limited by a lack of knowledge on two key points. First, the transport inhibitors that are available are selective only for equilibrative transporters. Currently, there is evidence for sodium-dependent transport in the brain. Sodium-dependent transporters normally function in the unidirectional uptake of adenosine. Thus, these transporters could still mediate transport when equilibrative transport is blocked and they may participate in adenosine release during ischemia. Second, inhibitors of equilibrative transport are able to inhibit both nucleoside uptake and release in some cell types. Consequently, it has not been clearly demonstrated that these inhibitors can allow release yet block uptake of adenosine during ischemia. The studies herein allow us to better understand the role of specific transporters in regulating adenosine levels, and, thus, adenosine's receptor-mediated effects, and provide a foundation for exploring these issues.

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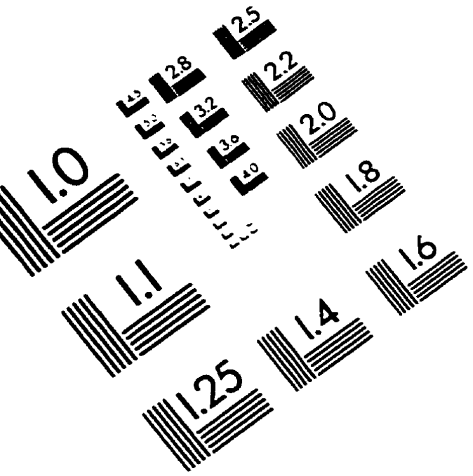
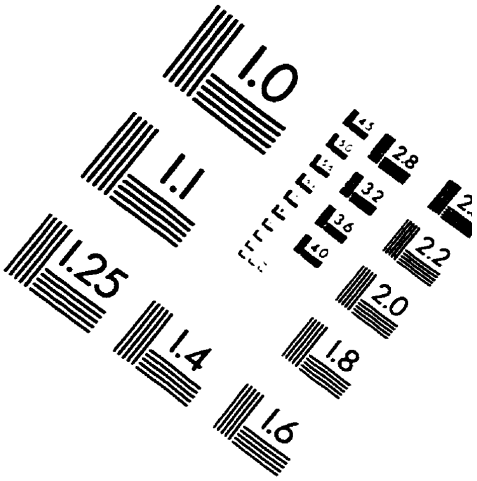
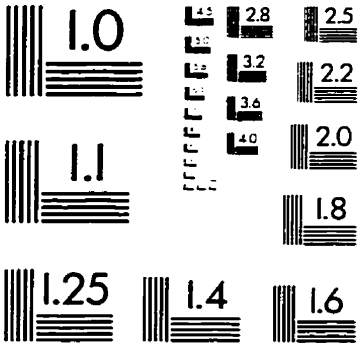
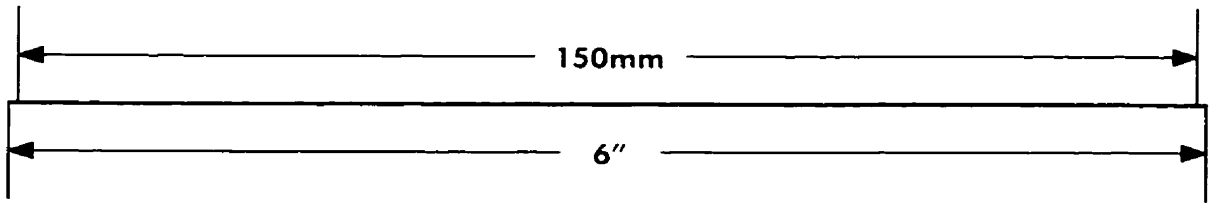
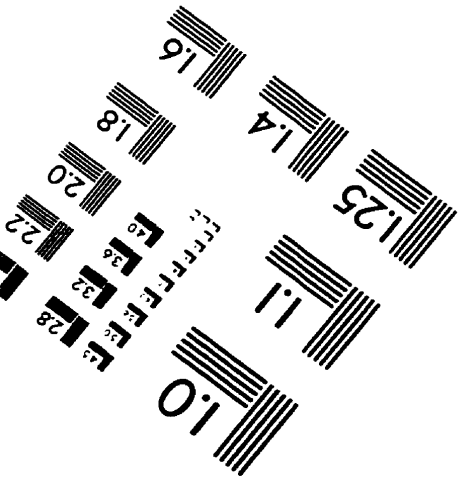
IMAGE EVALUATION TEST TARGET (QA-3)

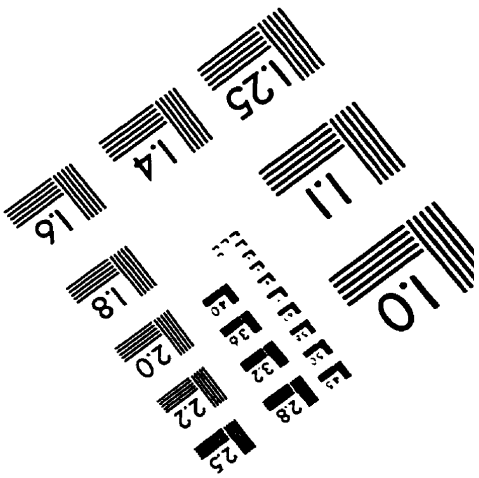












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