CHARACTERIZATION OF BIDIRECTIONAL ADENOSINE TRANSPORTERS IN RAT BRAIN SYNAPTOSOMES AND CLONAL DDT$_1$ MF-2 SMOOTH MUSCLE CELLS USING [^{3}H]L-ADENOSINE.

by

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A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree of

MASTER OF SCIENCE

Department of Pharmacology and Therapeutics
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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES
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Irene Oksana Foga   1997 (c)

This thesis is dedicated to
my family for their outstanding
patience and understanding.

ABSTRACT

Membrane-bound nucleoside transport processes and their inhibitors regulate intra- and extra-cellular levels of endogenous D-adenosine and the actions of adenosine are mediated through adenosine receptors. Our research involved (1) developing a method to measure adenosine transport and release in animal cells and CNS tissue, (2) characterizing the transport processes involved, and (3) determining the effects of transport inhibitors on these processes. Using rat brain synaptosomal preparations we showed that $[^3H]L$-adenosine, the metabolically stable enantiomer of $[^3H]D$-adenosine, was released in a concentration- and time-dependent, and temperature-sensitive manner that was linear with synaptosomal protein content. Release of $[^3H]L$-adenosine was inhibited by the nucleoside transport inhibitors dipyridamole, nitrobenzylthioinosine (NBI) and dilazep, as well as unlabelled L-adenosine, D-adenosine and uridine, suggesting that release occurred at least in part by NBI-sensitive equilibrative nucleoside transporters. To determine further which subtype of equilibrative nucleoside transporter was involved, we conducted similar studies using cells, DDT_1 MF-2 cells, that contain only NBI-sensitive (es) nucleoside transporters. In DDT_1 MF-2 cells, accumulation of $[^3H]D$-adenosine was more rapid than that of $[^3H]L$-adenosine. $[^3H]D$-Adenosine uptake was inhibited by unlabelled L-adenosine to a greater degree than $[^3H]L$-adenosine uptake was inhibited by unlabelled D-adenosine. Nitrobenzylthioinosine inhibited uptake of $[^3H]D$- and $[^3H]L$-adenosine. $[^3H]L$-Adenosine release was temperature- and time-dependent, and was inhibited by NBI. Our studies support the use of $[^3H]L$-adenosine as a useful probe for the characterization adenosine transport and release, and show that release occurs in part by nucleoside transporters that

are sensitive to inhibition by nanomolar concentrations of NBI. Such studies may help

identify therapeutically useful adenosine transport inhibitors.

Among the other students in the department, I wish to send a special thank you to Alvaro Bras, for his vast knowledge of pharmaceutics and reasoning on too many topics to mention.

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A dear friend once acknowledged with 'The Island of Research', that in our world, truly the only rule that has meaning is : 'Do not block the path of inquiry'.

To my family, I am in great debt for their time, patience and understanding. I was never the easiest individual to live with during this degree, somehow we all survived, thank you.

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LIST OF ABBREVIATIONS

AR	adenosine receptor
ADO	adenosine
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
C	control
Ca^{2+}	calcium
Ci	Curie
CNS	central nervous system
DDT	clonal smooth muscle DDT_1 MF-2 cells
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DPR	dipyridamole
DZP	dilazep
ei	NBI-insensitive (resistant) nucleoside transporters
es	NBI-sensitive nucleoside transporters
g	gravity force
g	grams
h	hours
IC_{50}	concentration at which 50% of maximal effect is inhibited
IMP	inosine 5'-monophosphate

K_d	dissociation constant
K_m	Michaelis-Menten constant
K_T	affinity constant of transport
L-adenosine	9-β-L-ribofuranosyladenosine
mg	milligram
min	minute
ml	milliliters
mM	millimolar
n	number of samples
NBI	nitrobenzylthioinosine
nm	nanometer
nM	nanomolar
pM	picomolar
pmol	picomoles
r	correlation coefficient
sec	seconds
S.E.M.	standard error of mean
μCi	microCurie
μl	microliter
μM	micromolar
v	volume
wt	weight

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REVIEW OF THE ADENOSINE LITERATURE

1. Introduction

Endogenous D-adenosine, hence forth simply refered to as adenosine, is a purine nucleoside whose physiological actions were first demonstrated about 70 years ago (Drury and Szent-Gyorgi, 1929); important early discoveries include findings that adenosine could be used to treat cardiovascular diseases (Honey *et al.*, 1930; Drury, 1936) and that the actions of adenosine in the CNS were mediated by cell-surface receptors (Sattin and Rall, 1970). Subsequently, adenosine was shown to inhibit neurotransmitter release (Fredholm and Dunwiddie, 1988), to affect adenylate cyclase activity following adenosine receptor activation, and to affect receptor-signal transduction systems in addition to adenylate cyclase (Williams, 1989). Furthermore, adenosine has been shown to act as a neuroprotective agent (Dragunow and Faull, 1988), an endogenous anticonvusant (During and Spencer, 1992; O'Brien, 1988), a prototypic neuromodulator (Williams, 1989), an anti-ischemic agent (Phillis, 1989; Rudolphi *et al.*, 1992b), as well as an anxiolytic and hypnotic (Jain *et al.*, 1995). Both cardio- and neuro-protective actions of adenosine have been shown to be mediated through specific cell surface adenosine receptors.

2. Adenosine receptors

Cell surface adenosine receptors are G protein-coupled receptors. Adenosine receptors have seven hydrophobic α-helices, each composed of ~22-26 amino acids, that transverse the cell membrane. The regions connecting the membrane-spanning 2-3, 4-5,

and 6-7 domains, as well as the amino terminus of the receptor, are orientated into the extracellular space. Regions connecting transmembrane domains 1-2, 3-4, and 5-6, as well as the carboxy-terminal tail of the receptor, are located cytoplasmically. Common features shared among adenosine receptors and many G protein-coupled receptors include an aspartate-arginine-tyrosine sequence in the second intracellular loop, cysteines in extracellular regions that may be involved in intra-receptor disulfide bond formation, and a conserved cysteine residue acting as a potential site for receptor palmitoylation in the carboxy-terminal tail (except in the A2A receptor) (Liebert *et al.*, 1989; Linden *et al.*, 1991; see Olah and Stiles, 1995). However, they differ in that adenosine receptors are smaller in size and contain a short amino terminus.

2.1. Adenosine receptor classification

In general, purinergic receptors, or purinoceptors, have been classified as either P1 (adenosine) or P2 (nucleotide) receptors. Most P1 purinoceptors have a rank order of agonist potencies of adenosine > AMP > ADP > ATP and are selectively blocked by methylxanthines. These receptors were initially subclassified according to their ability to modulate adenylate cyclase activity; A1 receptors inhibited whereas A2 receptors stimulated adenylate cyclase activity. However, changes in cAMP levels due to (in)activation of adenylate cyclase did not always correlate with events modulated by adenosine which suggested involvement of other second-messenger systems (Morgan, 1991). Current subclassification of A1, A2A, A2B, and A3 receptors is based mainly on cloned sequences, agonist and antagonist potencies, and physiological and biochemical

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actions. In contrast, P2 purinoceptors, with a general rank order of agonist potencies of ATP > ADP > AMP > adenosine, are insensitive to antagonism by methylxanthines and functionally affect a variety of second messenger systems including G-protein-coupled cascades and ligand-gated ion fluxes (Fredholm *et al.*, 1994). At least six P2 receptors have been identified including P2X, P2Y, P2U, P2T, P2Z, and P2D (Fredholm, 1995) based on molecular structures, agonist and antagonist potencies, and biochemical and pharmacological actions.

2.1.1. A1 Adenosine receptor (A1R) characterization

A1 adenosine receptor clones (Maenhaut *et al.*, 1990; Libert *et al.*, 1991; Mahan *et al.*, 1991; Tucker *et al.*, 1992; and Fink *et al.*, 1992), are made up of 326-328 amino acids, have a molecular mass of ~36,700 Daltons, and have 87% amino acid identity with the species homologues (Olah and Stiles, 1995). A1R's bind antagonist radioligands 1,3-dipropyl-8-cyclopentyl-xanthine (DPCPX) and xanthine amine congener (XAC) with high affinity (Kd ~ 1 nM) and agonists with the potency order of N^6-(R-phenylisopropyl)-adenosine (R-PIA) greater than 5'-N-ethyl-carboxamidoadenosine (NECA) (Olah and Stiles, 1995; see Table 1). A1Rs in brain are found at high densities in cortex, cerebellum, hippocampus and thalamus (Stehle *et al.*, 1992).

2.1.2. A2 Adenosine receptor (A2AR and A2BR) characterization

Two distinct A2 adenosine receptors that stimulated adenylate cyclase activity were proposed because high-affinity sites (A2AR) were found only in striatal membranes (Daly *et al.*, 1983), and low-affinity sites (A2BR) were observed in intact cells from all brain regions. Human A2ARs were cloned by Furlong *et al.* (1992) and Meng *et al.* (1994), have an amino acid sequence that is 82% identical with the canine subtype, and species homologues are 410-412 amino acids corresponding to ~45,000 Daltons. Selective A2A agonists include CV1808, CGS21680 and NECA, and antagonists include XAC, CSC and KF 17837 (Gurden *et al.*, 1993; see Table 1). A2ARs are predominately found in the basal ganglia (Schiffman *et al.*, 1991; Fink *et al.*, 1992), and to a lesser degree in the hippocampus and cortex (Cuhna *et al.*, 1994). Human A2BRs were cloned by Stehle *et al.* (1992), have an amino acid sequence that is 73% identical to rat, and species homologues are ~330 amino acids corresponding to <45,000 Daltons. A2BRs bind with low-affinity the agonist radioligand [^{3}H]NECA, but do not bind [^{3}H]CGS21680 or [^{125}I]-PAPA-APEC (see Table 1). A2BRs are diffusely distributed throughout the brain and have been found on glial as well as neurons (Stehle *et al.*, 1992).

2.1.3. A3 Adenosine receptor (A3R) characterization

Pharmacologically and molecularly, A3 adenosine receptors are distinct from A1 and A2 adenosine receptors (Zhou *et al.*, 1992). Rat A3Rs were cloned by Meyerhof *et al.* (1991) and Zhou *et al.* (1992), have an amino acid sequence that is 42-47% identical

Table 1. Classification of adenosine receptors (P1 purinoceptors)

Name	A1R	A2AR	A2BR	A3R
G-protein coupling	G_i	G_s	G_s	Yes, type not determined.
Effectors	$\downarrow$cAMP, $\uparrow$IP$_3$, $\uparrow$K$^+$, $\downarrow$Ca^{2+}	$\uparrow$cAMP	$\uparrow$cAMP	$\downarrow$cAMP
Agonists	CCPA>CPA> NECA> CGS21680 CHA, R-PIA, ADAC	PAPA-APEC, CGS21680> NECA> CV1808> R-PIA> CPA Ado	NECA	APNEA> R-PIA=NECA > CGS21680
Antagonists	DPCPX, XAC, 8-cyclopentyl-theophylline	XAC, CSC KF 17837 CGS15943 CP66713	XAC, DPCPX 8-PT CGS15943	BW-A 522
Distribution in CNS	cortex, hippocampus, cerebellum	caudate/ putamen, nucleus accumbens, olfactory tubercle	ubiquitous but low abundance	Wide

* Abbreviations: BW-A 522, 3-(3-iodo-4-aminobenzyl)-8-(4-oxyacetate)-1-propylxanthine; CPA, N^6-cyclopentyladenosine; CHA, N^6-cyclohexyladenosine; R-PIA, N^6-(R-phenylisopropyl)-adenosine; ADAC, adenosine amine congener; NECA, 5'-N-ethyl-carboxamidoadenosine; CGS21680, 2-[p-(2-carbonyl-ethyl)-phenylethylamino]-5'-N-ethylcarboxamidoadenosine; CGS22988, [1R-(1α,2α,3β,5β)]-3-(2,6-diamino-N^2-(3-carbethoxyphenyl)-9H-purin-9-yl)-5'-(N-ethylcarbamoyl)-1,2-cyclopentanediol; PAPA-APEC, 2-[4-[2-[2-[phenylmethylcarbonylamino]ethylaminocarnyl]ethyl]phenyl]ethylamino-5'-N-ethylcarboxamidoadenosine; DCPX, 1,3-dipropyl-8-cyclopentyl-xanthine; XAC, xanthine amine congener; 8-PT, 8-phenyltheophylline; CV1808, 2-phenylaminoadenosine; CGS15943, 9-chloro-2-(2-furanyl)-5,6-dihydro-[1,2,4]-triazolo[1,5]quinazolin-5-imine monomethane-sulfonate; KF 1787, 1,3-dipropyl-8-(3,4-dimethoxystyryl)-7-methyl-xanthine; APNEA, N^6-2-(4-aminophenyl)ethyladenosine; CCPA, 2-chloro-cyclopentyl-adenosine; CP66713, 4-amino-8-chloro-1-phenyl[1,2,4]triazolo[4,3-a]- quinozaline.

Adapted from Fredholm (1995; 1994), Luthin and Linden (1995), Olah and Stiles (1995).

to that of canine A1 and A2 adenosine receptors, and species homologues are made up of 320 amino acids. A3Rs bind with high-affinity the agonist radioligand N^6-2-(4-amino-3-$[^{125}I]$iodophenyl)-ethyladenosine ($[^{125}I]$-APNEA) and, with less potency, N^6-(R-phenylisopropyl)-adenosine (R-PIA) and NECA (see Table 1). A3Rs in brain are expressed mainly in hippocampus, cortex, cerebellum, and striatum (Salvatore *et al.*, 1993).

3. Tissue levels of adenosine

The levels of adenosine available for stimulation of adenosine receptors are controlled by processes related to its production, release, re-uptake (transport), and metabolism. Regardless of whether adenosine originates endogenously or exogenously, its effects appear to be inexorably linked to its levels at receptor sites.

3.1. Measurement of endogenous adenosine levels

For precise and accurate measurements of endogenous adenosine levels, adenosine release, production/metabolism, and re-uptake must be inhibited completely and virtually instantaneously. Importantly, tissue levels of adenosine do correlate with adenosine actions. Experimentally, it has been well documented that sampling methods can have profound effects on measured levels of purines. For example, tissue levels of adenosine change rapidly and markedly due to post-mortem breakdown of adenine nucleotides. Therefore, accurate determination of basal levels of purines can only be obtained when post-mortem-induced increases in adenosine and decreases in cellular energy charge (1/2

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ADP + ATP): (AMP + ADP + ATP) or ATP/ADP ratios are prevented by rapid inactivation of enzymes responsible for adenine nucleotide breakdown and adenosine production. For brain, post-mortem-induced changes in tissue levels of purines were minimized when animals were killed by blow-freezing (Winn *et al.*, 1979) and focused microwave irradiation (Delaney and Geiger, 1995,1996; Phillis *et al.*, 1995). It appears that large differences are observed in adenosine levels among brain regions only in the absence of extensive post-mortem metabolism. In other tissues, great care must be taken to ensure that adenosine is neither being formed nor lost during the sampling period. In attempting to determine levels of adenosine near cell surface adenosine receptors, extracellular levels of adenosine in tissue transudates have been measured. Nanomolar concentrations of adenosine were measured after the large amounts of adenosine produced by tissues that were damaged with insertion of the microdialysis probe were allowed to dissipate and stabilize (Ballarin *et al.*, 1991), using the closed cranial window technique (Meno *et al.*, 1991), and using the cortical cup technique (Geiger *et al.*, 1991). To measure levels of endogenous adenosine accurately, it is necessary to decide first whether to measure total tissue levels or extracellular concentrations, then to sample near the relevant site(s) of action, and to take proper steps to limit pre- and post-sampling production/metabolism of adenosine (Geiger *et al.*, 1997).

4. Enzymatic regulation of endogenous adenosine levels

Purine metabolism is involved in the maintenance of intra- and extra-cellular levels of adenosine that under physiological conditions range between 0.1 and 1 μM (Onyd and

Schrader, 1984). A prominent pathway for adenosine formation is the sequential dephosphorylation of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and adenosine monophosphate (AMP). The degradation of AMP to adenosine is catalyzed by 5'-nucleotidase (Meghji *et al.*, 1989). A minor synthetic pathway for adenosine, at least in the brain, arises from the hydrolysis of S-adenosylhomocysteine by S-adenosylhomocysteine hydrolase (Schrader *et al.*, 1981). Once formed, adenosine is metabolised rapidly; adenosine's half-life in humans is only a few seconds. Adenosine may be metabolised by adenosine deaminase (ADA), adenosine kinase (AK), or S-adenosylhomocysteine hydrolase (see Fig. 1). ADA, located predominantly in cell cytosol, mediates the deamination of adenosine to inosine with a K_m for adenosine of 20-100 μM (Geiger and Nagy, 1990). Some evidence of ecto-ADA activity has been shown in a variety of tissues (see Geiger *et al.*, 1991, Martin *et al.*, 1995). AK is a cytosolic enzyme that phosphorylates adenosine to AMP with a K_m for adenosine of 0.2-2.0 μM (Geiger and Nagy, 1990). AK activity is inhibited by levels of endogenous adenosine only 5-times higher than basal levels (Phillis and Newsholme, 1979; Schrader *et al.*, 1983; Mistry and Drummond, 1986). AK has been isolated from yeast (Leibach *et al.*, 1971), a variety of mammalian sources (Palella *et al.*, 1980; Yamada *et al.*, 1980; Rottland and Miras-Portugal, 1985), certain microorganisms (Lobelle-Rich and Reeves, 1983; Datta *et al.*, 1987), and CNS tissues (for review see Geiger *et al.*, 1997). SAHH is an easily reversible intracellular enzyme, with a K_m of 1-5 μM for adenosine (Geiger and Nagy, 1990), that is involved in the metabolism of S-adenosylhomocysteine (SAH) to adenosine, and vice versa; SAH formation is favoured because homocysteine levels are low and tightly

Figure: 1. Intracellular and extracellular adenosine production and metabolism.
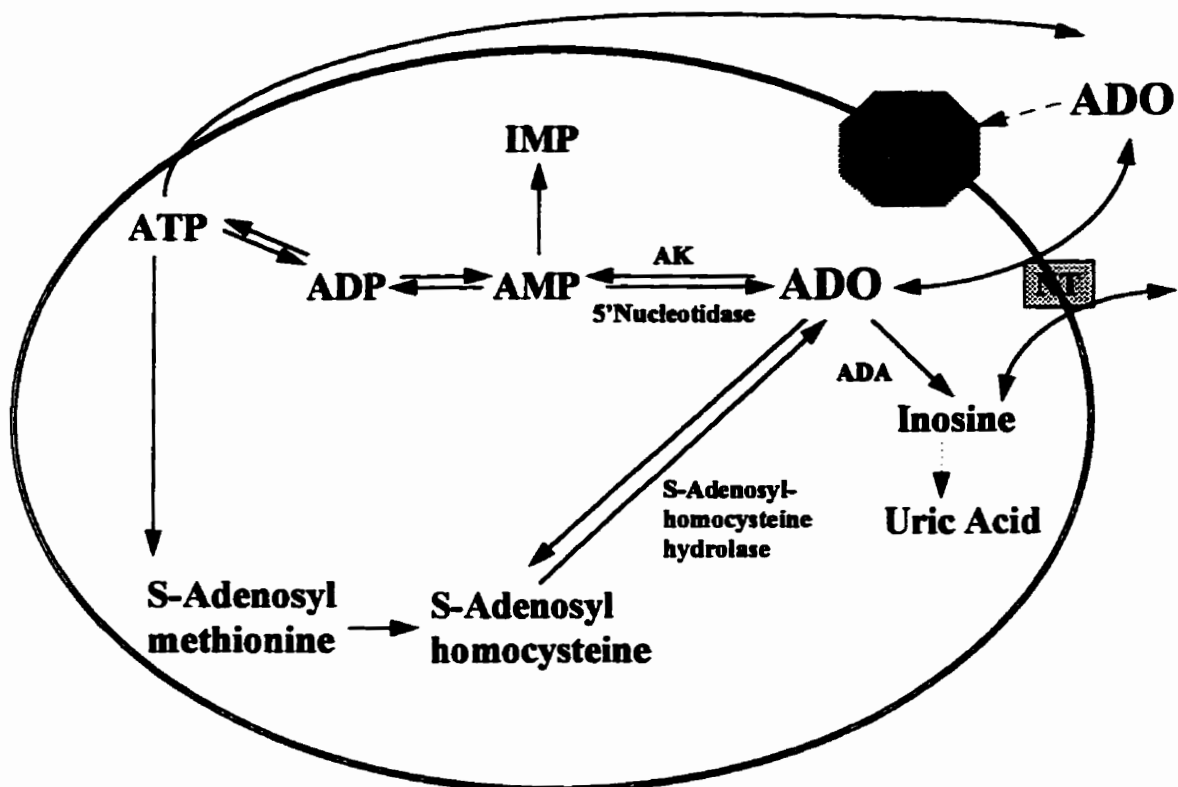
Abbreviations: ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate; AMP, adenosine 5'-monophosphate; IMP, inosine 5'-monophosphate; ADO, adenosine; AK, adenosine kinase; ADA, adenosine deaminase.

controlled (Geiger and Nagy, 1990).

5. Transport

Because transport processes control the extracellular levels and actions of adenosine (Wu and Phillis, 1984), characterization of these processes in CNS tissues is vital to our understanding of the neuroregulatory actions of adenosine. Transport is defined as the transfer of native permeant from one side of a membrane to another and is different from uptake which refers to an accumulation of permeant substrate without regard to its ultimate metabolic fate. Adenosine transport across plasma membranes in the CNS was first described by Santos *et al.* in 1968. Seven distinct nucleoside transport processes have been described based on their functional and pharmacological characteristics as well as, in some cases, their molecular structures. Currently, nucleoside transporters are classified according to their (1) ability to translocate nucleosides across cell membranes by facilitated diffusion equilibrative processes or sodium-dependent transport systems, (2) sensitivity or resistance to the blocking effects of nitrobenzylthioinosine (NBI) and other nucleoside transport inhibitors, and (3) preference for purine or pyrimidine nucleosides as permeants.

Equilibrative nucleoside transport processes typically are driven by nucleoside concentration gradients across cell membranes, appear to be bi-directional, are widely distributed among mammalian cells and tissues, exhibit broad permeant selectivities, transport all endogenous nucleosides, are inhibited by dipyridamole and dilazep, and are

subclassified according to their sensitivity to inhibition by NBI (equilibrative NBI-sensitive (*es*) transport and equilibrative NBI-insensitive (*ei*) transport).

Concentrative nucleoside transport processes are secondary-active systems that are inwardly driven by transmembrane Na^+ gradients, appear to be limited to specialised cells, and are subclassified into concentrative NBI-insensitive purine-selective [f = formycin] (*cif*) transport, two distinct concentrative NBI-insensitive pyrimidine-selective [t = thymidine] (*cit*) transporters, concentrative NBI-insensitive broadly selective (*cib*) transport, and concentrative NBI-sensitive (*cs*) transport (Table 2; for review see Geiger *et al.*, 1997). An alternate numerical classification of the concentrative nucleoside transporters; *cif* (N1), *cit* (N2 and N4), *cib* (N3), and *cs* (N5) represents the sequence in which the transporters were discovered (Crawford *et al.*, 1990a, b, c; Dagnino *et al.*, 1991; Williams and Jarvis, 1991; Huang *et al.*, 1993; Wu *et al.*, 1992).

5.1. Molecular structure of nucleoside transporters

Of the equilibrative nucleoside transporters much more is known about *es* than *ei*. The *es* transporter is located in, for example, erythrocytes and lymphocytes at densities of 10^2-10^4 transporters/cell (Jarvis *et al.*, 1982; Smith *et al.*, 1989) while in cultured human choriocarcinoma (BeWo) cells *es* transporter density is $>10^7$ transporters/cell (Boumah *et al.*, 1992). The *es* transporter is similar to glucose transporter GLUT1 (for review see Cass, 1995) in that it contains 11 potential transmembrane-spanning domains (Griffiths *et al.*, 1997), but *es* is not recognized by an antibody specific for GLUT1 (see Cass, 1995). When purified, the *es* transporter protein migrates at ~55 kDa on sodium dodecyl sulfate

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TABLE 2. Classification of nucleoside transporters

Class	Subclass	Cloned	Inhibitors	Permeants
Equilibrative (Na$^+$ independent)				
	es	Y	NBI, DPR, DZP	ALL
	ei	N	DPR, DZP	ALL
Concentrative (Na$^+$ dependent)				
	cif (N1)	Y		A, U, G, I, FB
	cit (N2)	Y		A, U, Th
	(N4)	N		A, U, G, Th
	cib (N3)	Y		ALL
	cs (N5)	N	NBI, DPR, DZP	A, FB

Abbreviations: A, adenosine; U, uridine; G, guanosine; I, inosine; FB, formycin B; Tu, tubercidin; Th, thymidine. ALL includes: A, U, G, I, FB, Tu, Th.

Adapted from Cass (1995).

polyacrylamide electrophoretograms and binds NBI with a stoichiometry of 1:1 with a K_d ranging from 0.1-1.0 nM (Cass *et al.*, 1974; Cass and Paterson, 1976, Jarvis and Young, 1980; Jarvis *et al.*, 1982; Paterson *et al.*, 1985; Agbanyo *et al.*, 1988). cDNA fragments (2.0-2.03 kb) encoding polypeptides, recognized by polyclonal antibodies specific for the human erythrocyte *es* transporter, have been isolated from a cDNA library prepared in a bacteriophage expression system from cultured human (BeWo) choriocarcinoma cells (Boumah *et al.*, 1994). Recently, a human placental cDNA with functional characteristics of an *es* transporter has been cloned by Griffiths *et al.* (1997). The cloned hENT1 encodes a 456 residue glycoprotein with a molecular mass of 50,249 Daltons that shows no significant sequence similarity to other known transporters. However, the predicted amino acid sequence showed some homology to ZK809.4 and F16H11.3 in the *Caenorhabditis elegans* genome, to the 517-residue hypothetical protein FUN26 of *Saccharomyces cerevisiae* (Oullettee *et al.*, 1993), and to the 36 kD mouse and human HNP36 proteins.

Recently, cDNAs of three different proteins with sodium-dependent nucleoside transport activity were cloned and expressed (Pajor and Wright, 1992; Huang *et al.*, 1994; Yao *et al.*, 1996) and termed SNST1, CNT1 and CNT2. The SNST1 sequence is homologous to the sodium/glucose cotransporter termed GLUT1 (Pajor and Wright, 1992). Recombinant SNST1 expressed in *Xenopus* oocytes is characteristic of N3/*cib* sodium/nucleoside cotransport activity, and the predicted sequence suggests a protein of 672 amino acids, 3 potential N-linked glycosylation sites, 12 transmembrane domains, and 2 sodium-binding sites. SNST1 mRNA was found in kidney and heart, but not liver and

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intestine. The sequence for rCNT1 is 27% homologous with a bacterial H^+/nucleoside cotransporter (Huang *et al.,* 1994). Recombinant CNT1 expression in *Xenopus* oocytes exhibits N2/*cit* characteristics, including sodium/nucleoside cotransport and transport of anti-HIV drugs including azidothymidne and dideoxycytidine (Huang *et al.,* 1994). The predicted cDNA sequence of rCNT1 suggests a protein of 648 amino acids, 14 transmembrane domains, 3.1% cysteine content, 3 N-linked and 4 O-linked glycosylation sites, and 4 protein kinase C-dependent phosphorylation sites. CNT1 mRNA was found in rat intestine and kidney, but not heart, brain, spleen, lung, liver or skeletal muscle (Che *et al.,* 1995). Sodium-dependent transport was observed in dissociated brain cells from rat and guinea pig (Johnston and Geiger, 1982; Geiger and Fyda, 1991), as well as primary cultures of rat neurons and astrocytes (Hosli and Hosli, 1988; Bender *et al.,* 1994), and dorsal brain stem synaptosomes (Lawrence *et al.,* 1994). Recently, mRNA for rCNT1 was located in rat brain choroid plexus, posterior hypothalamus, superior colliculus, brain stem, striatum, hippocampus, cerebellum, and cortex (Anderson *et al.,* 1996). cDNA encoding a second mammalian nucleoside transporter (SPNT) belonging to the rCNT1 family was isolated from rat liver (Che *et al.,* 1995). Subsequently, rCNT2 (nomenclature of Yao *et al.,* 1996), was isolated from rat jejunum expressed in *Xenopus laevis Oocytes* and exhibits N1/*cif* characteristics. Comparative analysis of rCNT2 and SPNT showed that both contain a similar open reading frame of cDNA, and both have a similar predicted amino acid sequence except for conservative substitutions of glycine for alanine at residue 419 and valine for isoleucine at residue 522 in rCNT2. The predicted cDNA sequence of rCNT2 suggests a protein of 662 amino acid residues, 64% of which are identical to that

of rCNT1. Recently, mRNA for rCNT2 was located in rat brain choroid plexus, posterior hypothalamus, superior colliculus, brain stem, striatum, hippocampus, cerebellum, and cortex (Anderson *et al.*, 1996).

5.2. Criteria for nucleoside transport studies

While transport of adenosine concerns only permeation of nucleoside across the plasma membrane by one or more mediated mechanisms, uptake is a combination of transport and metabolism. Adenosine influx through nucleoside transporters can be more rapid than its subsequent metabolism. Therefore, measurement of adenosine transport across plasma membranes in cells capable of metabolizing this substrate can only be measured accurately following very brief reaction intervals (initial zero-trans transport). Otherwise, phosphorylation of transported adenosine by AK and entrapment of its phosphorylated derivatives intracellularly may lead to an over-estimation of the levels of adenosine accumulated as well as K_m values that reflect AK activity more so than adenosine transport. Alternatively, deamination of adenosine by ADA may affect transport measurements because intracellularly accumulated adenosine or inosine may act as competitive substrates for the nucleoside transporters thus affecting both influx and efflux of adenosine down its concentration gradient. Metabolism of transported adenosine may be overcome by the addition of inhibitors of AK and ADA such as 5'-iodotuberocidin and 2'-deoxycoformycin, respectively. However, it is important to recognise that problems may arise because these inhibitors can serve as substrates for nucleoside transporters. Thus, criteria developed for an accurate measurement of adenosine transport

kinetics include the use of (1) very short incubation periods, (2) non-metabolised substrates for the transporter, or (3) tissues deficient in the enzymes thought to be responsible for metabolising the transported substrate (Paterson *et al.*, 1985).

The complex interactions between transport and metabolism has lead to the use of alternative substrates for nucleoside transporters including uridine and formycin B (Jones and Hammond, 1992; Lee and Jarvis 1988b; Plagemann and Wolhueter, 1983; Plagemann and Woffendin, 1989). Uridine, a pyrimidine nucleoside is, at least in some cells, not metabolised extensively under short incubation conditions. However, because nucleoside transporters have a relatively low affinity for uridine (K_m 300 μM) (Stefanovich, 1983; Lee and Jarvis, 1988a) its usefulness is limited (Geiger *et al.*, 1988). Formycin B, a poorly metabolized inosine analog, has been used to characterize sodium-dependent and equilibrative transporters in a variety of tissues including guinea-pig ventricular myocytes (Conant and Jarvis, 1994), S49 mouse lymphoma cells (Plagemann, 1991a; Plagemann, 1991b; Plagemann and Woffendin, 1989), and mouse leukemic L1210/MA27.1 cells (Borgland and Parkinson, 1997).

Recently L-adenosine has been proposed for the analysis of nucleoside transporters. L-Adenosine, the stereoisomer of D-adenosine (see Fig. 2), and other L-ribose-substituted nucleosides were first synthesized over 30 years ago, in hopes of developing more metabolically stable analogs for facilitation of nucleic acid studies and investigation of the therapeutic usefulness of nucleosides (Acton *et al.*, 1964). The L-isomer of adenosine was not metabolized by ADA in *Aspergillus oryzae* nor was the L-isomer of 5'-AMP dephosphorylated by 5'-nucleotidase in *Trimeresurus flavoviridis* (Asai

et al., 1967). Pharmacologically relevant concentrations of L-enantiomers of adenosine, AMP, ADP, ATP, and 5'-(*N*-ethylcarboxamido)adenosine were found to be inactive in tests of human platelet aggregation (Cusack *et al.*, 1981), and relaxation of guinea pig taenia coli (Cusack and Planker, 1979). [^{3}H]L-Adenosine was first used as a substrate for passive and facilitative diffusion systems in mouse erythrocytes and L-1210/AM cells (Gati *et al.*, 1989); transport rates of D-adenosine were higher than L-adenosine. In contrast, Gu *et al.* (1991) showed that rat cerebral cortex synaptoneurosomes lacked stereoselectivity for the ribose moiety of adenosine. [^{3}H]L-Adenosine was affected by neither by ADA nor by AK, thereby suggesting that it is more metabolically stable than [^{3}H]D-adenosine, and accumulation of L-adenosine and D- adenosine occurred by similar processes in rat brain synaptoneurosomes (Gu *et al.*, 1991). This was later supported by similar findings in chromaffin cells (Casillas *et al.*, 1993). Subsequently, [^{3}H]L-adenosine transport was shown to be significantly faster than its subsequent metabolism in rat cortical synaptoneurosomes (Gu and Geiger, 1992). We proposed to use [^{3}H]L-adenosine as a probe to characterize nucleoside transporter(s) involved in adenosine uptake and release in heterogeneous rat brain synaptosomal preparations. We hypothesized that such studies would help further identify adenosine transport inhibitors which could act as enhancers of extracellular levels of endogenous adenosine especially if (1) the inhibitors block influx to a greater degree than efflux (release) of intracellular adenosine or (2) the inhibitors block equally well the influx and efflux of adenosine, but significant amounts of adenosine are formed as a result of dephosphorylation of released adenine nucleotides.

Figure: 2. Chemical structure for D- and L-adenosine.
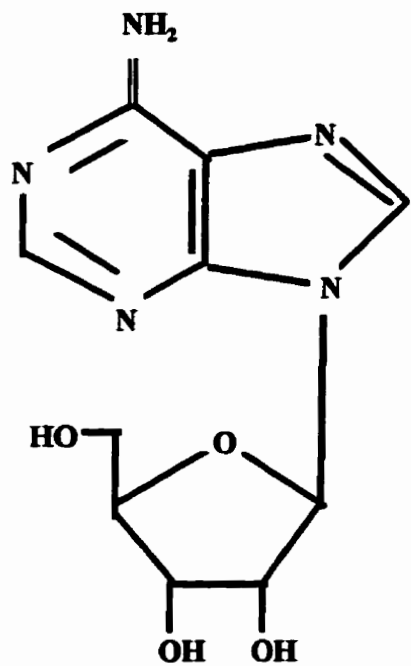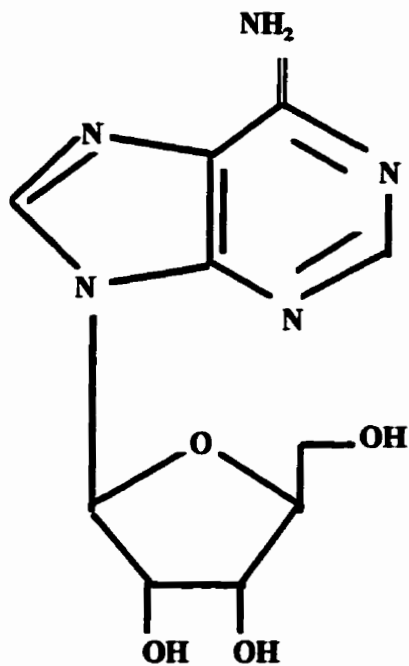
D-ADENOSINE   L-ADENOSINE

5.3. Release

Adenosine release may be induced by depolarization, veratridine, glutamate receptor agonists, electrical stimulation, glucose and oxygen deprivation, ischemia, and hypertonic sodium chloride (White and Hoehn, 1991: Manzoni *et al.*, 1994; Baudourin-Legros *et al.*, 1995). A portion of adenosine release from nerve preparations has been shown to be calcium-dependent, and to date adenosine has not been shown to be contained in or released from synaptic vesicles (Cahill *et al.*, 1993). The most common mechanism implicated in adenosine release is efflux through bidirectional nucleoside transporters. Characterization of adenosine release is difficult to do with radiolabeled adenosine because of its metabolic instability.

Direct involvement of nucleoside transporters in adenosine release may be studied by measuring zero-trans efflux of metabolically stable substrates for nucleoside transporters. Methodologically, cells are pre-loaded with [^{3}H]-labelled substrate, separated from their loading medium followed by resuspension in buffer not containing any substrate (zero-trans), wherein the subsequent release of [^{3}H]-labelled substrate is measured. Using this type of experimental approach, nucleoside release mediated through *es* transporters has been characterized in a variety of cells and tissues (Jarvis, 1986; Phillis *et al.*, 1989; Plagemann and Woffendin, 1989; Plagemann *et al.*, 1991b; White and MacDonald, 1990; Cunha and Sebastiao, 1993).

Evidence for adenosine efflux through its transporter has been obtained indirectly from findings that nucleoside transport inhibitors including DZP, DPR and NBI inhibit the basal release of adenosine from rat cerebellar synaptosomes (Clark and Dar, 1989), murine

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neuroblastoma cells deficient in AK (Green, 1980), primary cultures of rat glial cells (Caciagli *et al.*, 1988), and primary cultures of chick neurones and glia (Meghji *et al.*, 1989). These inhibitors also decreased the evoked release of adenosine from purified cholinergic nerve terminals of rat caudate nucleus (Richardson and Brown, 1987), cultured sympathetic neurons (Tolkovsky and Suidan, 1987), rat hippocampal and hypothalamic slices (Fredholm and Jonzon, 1981; Jonzon and Fredholm, 1985), and rabbit retina (Perez *et al.*, 1986).

6. Inhibitors of nucleoside transporters

There are five categories of nucleoside transport inhibitors based on their chemical structures; purine ribosides (NBI), pyrimidopyrimidine derivatives (DPR), substituted piperazines (mioflazine, soluflazine), tertiary amine diazepine compounds (dilazep), and xanthines (propentofylline) (see Fig. 3). NBI, although not clinically used partly due to its metabolic instability (Van Reempts *et al.*, 1988), is a valuable probe for nucleoside transport systems at the whole animal, *in vitro* and molecular levels (see Geiger and Fyda, 1991) mainly because it is a very potent and selective inhibitor of *es*. DPR is used clinically and experimentally as a dilator of coronary and cerebral arterioles, a platelet anti-aggregator, an enhancer of the pharmacological effects of modified nucleosides including 3'-azido-3'-deoxythymidine and 2',3'-dideoxycytidine, and an inhibitor of cGMP phosphodiesterase activity and reactive oxygen species formation (Dawicki *et al.*, 1985; Rhodes *et al.*, 1985, Iuliano *et al.*, 1989; Bult *et al.*, 1991). Both [^{3}H]NBI and [^{3}H]DPR have been used to study the localization of adenosine transporters in the CNS by

autoradiographic and membrane binding methods (Geiger and Nagy, 1984, 1985; Geiger, 1986; Bisserbe *et al.*, 1992). Mioflazine and soluflazine are prototype drugs that inhibit adenosine transport, produce vasodilation, decrease tissue damage secondary to myocardial ischemia, cause sedation, increase the quality of sleep, and appear to be the only adenosine transport inhibitors capable of entering the brain following parenteral administration (Wauquier *et al.*, 1987). DZP is clinically useful as a vasodilator for decreasing coronary and total vascular resistance, and increasing coronary blood flow in patients diagnosed with angina (Marzilli *et al.*, 1984). Propentofylline functions at low concentrations to inhibit *es* transporters while at higher concentrations it blocks *ei* and N1/*cif* transport (Parkinson *et al.*, 1993). In addition, propentofylline can antagonize A1, A2A and A2B adenosine receptors, inhibit microglial activation and stimulate nerve growth factor.

Transport inhibitors, by blocking adenosine influx, might be expected to enhance the extracellular levels of adenosine if the extracellular adenosine originated as released adenine nucleotide, or if the inhibitor was selective in decreasing the uptake but not the release of adenosine through bidirectional transporters (Pazzagli *et al.*, 1993; Ballarin *et al.*, 1991; Park and Gidday, 1990; Phillis *et al.*, 1989). Transport inhibitors might, alternatively, decrease extracellular levels of adenosine if adenosine is formed intracellularly and released through bidirectional transporters, and if the inhibitor blocked release to a greater degree than uptake. Thus, it is important to characterize the degree to which CNS nucleoside transporters mediate adenosine release, identify those adenosine transporters primarily involved in adenosine release and determine the physiological and

Figure: 3. Chemical structures of adenosine transport inhibitors.

NBI
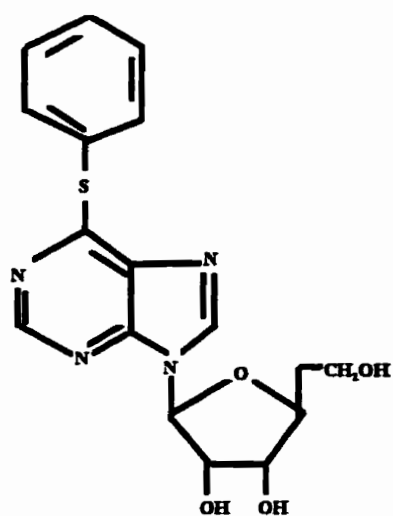
DPR
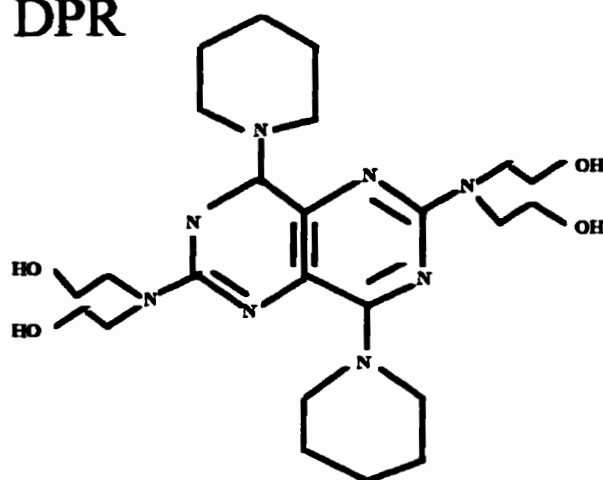
DZP
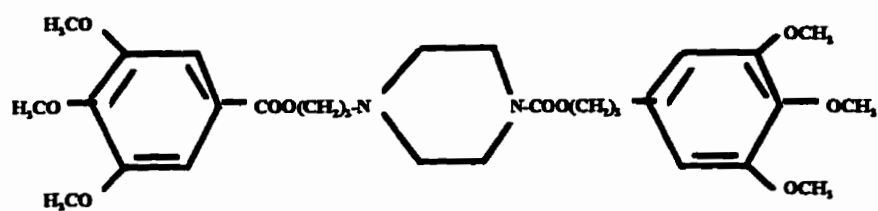
pathological significance of these processes so that adenosine transport/release blockers

may be exploited for therapeutic benefit. Towards this end the following studies were

performed.

[1]Chapter 1. Bi-directional Nucleoside Transporters Function in the Release of [³H]L-Adenosine from Rat Brain Synaptosomal Preparations.

ABSTRACT: Adenosine transport inhibitors as enhancers of extracellular levels of endogenous adenosine would presumably, only be effective if, for example, (1) the inhibitors block influx to a greater degree the efflux (release) of intracellular adenosine or (2) the inhibitors block equally well the influx and efflux of adenosine, but significant amounts of adenosine are formed as a result of dephosphorylation of released adenine nucleotides. Limited information is available regarding the directional symmetry of adenosine transporters in neural cells. Using rat brain crude P2 synaptosomal preparations preloaded with [³H]L-adenosine, an enantiomer of the physiological D-adenosine, our objectives were to determine (1) if [³H]L-adenosine, a substrate for adenosine transporters that is more metabolically stable than physiological D-adenosine, was being released from synaptosomal preparations, (2) the optimal conditions necessary to observe release, and (3) the degree to which this release was mediated by efflux through bidirectional nucleoside transporters. [³H]L-Adenosine release was found to be concentration and time dependent, temperature sensitive, and linear with synaptosomal protein. [³H]L-Adenosine was inhibited dose-dependantly by dipyridamole, nitrobenzylthioinosine, and dilazep; at concentrations of 100 μM inhibition was at least 40% for dipyridamole, 52% for nitrobenzylthioinosine, and 49% for dilazep. After loading with [³H]L-adenosine alone or [³H]L-adenosine plus unlabeled L-adenosine, D-adenosine, or uridine, [³H]L-adenosine

[1] Gu, J.G. Foga, I.O. Parkinson, F.E. and Geiger, J.D.: Involvement of bidirectional adenosine transporters in the release of L-[³H]adenosine from rat brain synaptosomal preparations. *Journal of Neurochemistry* 64:2105-2110, 1995.

release was inhibited 42% by L-adenosine, 69% by uridine, and 81% by D-adenosine. The inhibition of $[^3H]$L-adenosine release from the synaptosomal preparations by substrates for or inhibitors of nucleoside transporters suggests that a portion of the release was mediated by nucleoside transporters. This experimental system may prove useful for evaluating the effects of pharmacological agents on bidirectional transport of adenosine.

INTRODUCTION

The uptake of adenosine into neural cells by nucleoside transporters is an important mechanism by which extracellular levels of adenosine are controlled (Wu and Phillis, 1984; Geiger and Fyda, 1991). It follows, therefore, that inhibition of nucleoside transport may increase the extracellular levels of endogenous adenosine. In so doing, adenosine's actions, through binding to and activation of cell surface receptors, could be enhanced. Such an approach to the development of pharmacological agents could presumably yield agents that may selectively enhance the actions of adenosine when and where adenosine is being produced. If this is successful, adenosine transport inhibitors may be identified that could enhance some of the neuroprotective properties of adenosine (Williams, 1989; Geiger and Fyda, 1991; Rudolphi *et al.*, 1992a).

Less than 10 years ago, adenosine transport into a variety of cell types including those derived from central and peripheral tissues was thought to be handled by a single class of nucleoside transporters that were equilibrative and sensitive to the inhibitory actions of a wide range of nucleoside transport blockers. Subsequent to this, at least six classes of transporters have been identified and classified on the basis of inhibitor sensitivity, substrate specificity, and sodium ion dependence (Vijayalakshmi and Belt,

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1988; Geiger and Fyda, 1991; William and Jarvis, 1991; Jones and Hammond, 1992; Parkinson *et al.*, 1993). At least some of these nucleoside transporters have been shown to be bidirectional such that either influx or efflux of substrates can occur (Jarvis and Martin, 1986; Plagemann and Woffendin, 1989).

To our knowledge, it is presently unknown whether the best characterised adenosine transporter in the CNS, the equilibrative/inhibitor-sensitive system, or for that matter any of the CNS transporters identified to date, function as bidirectional transporters. Further, even for the equilibrative-sensitive adenosine transporter, it is unknown whether, or the degree to which, adenosine transport inhibitors can selectively block influx or efflux. Moreover, depending on tissue preparation and stimuli used, it has been shown that extracellular adenosine can result from either its release *per se* or from metabolism subsequent to the release of adenine nucleotides (White and Hoehn, 1991; Craig and White, 1993). On the basis of these findings, it appears difficult to predict *a priori* whether an inhibitor of adenosine transport would increase or decrease extracellular levels of adenosine. Thus, the development of adenosine transport inhibitors as therapeutic agents appears to be progressing in the absence of experimental systems with which to test for bidirectional function and of data necessary to predict whether a particular agent may allow adenosine to exit cells yet prevent its uptake.

One of the major problems in testing for directional symmetry and in determining effects of pharmacological agents on bidirectional transport of adenosine is that adenosine is metabolically unstable; this prevents "loading" cells with adenosine and subsequently determining release characteristics. We recently showed that L-adenosine, a more

metabolically stable stereoenantiomer of the physiological D-adenosine, was transported into synaptosomal preparations, at least in part, via nucleoside transporters (Gu *et al.*, 1991; Gu and Geiger, 1992). This suggests to us that L-adenosine may be useful in studying nucleoside transporter-mediated release processes. Here, we report some characteristics of [^{3}H]L-adenosine release from rat brain synaptosomal preparations and show that this release appears to be due, at least in part, to efflux through adenosine transporters. This system may be useful in characterizing the bidirectional function of CNS adenosine transporters and in identifying drugs capable of selectively influencing adenosine influx or efflux.

MATERIALS AND METHODS

Materials: [^{3}H]L-Adenosine (13 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA), ^{3}H$_2$O (1.0 mCi/g) was from Amersham Canada Ltd. (Oakville, Ontario). [^{3}H]D-Adenosine (61.5 Ci/mmol) was from DuPont Canada (Mississuaga, Ontario). Nitrobenzylthioinosine, dipyridamole, and D-adenosine were from Sigma Chemical Co. (St. Louis, MO). Generous gifts of L-adenosine and dilazep were supplied by Dr. K. Flora of the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute and F. Hoffmann La Roche, Basel, Switzerland, respectively. Statistical analyses were carried out by one way ANOVA with Bonferroni correction, Student's *t* tests, and nonlinear regressions using Instat and GraphPad PRISM (Biosoft). All data were expressed as mean values and S.E.M. Statistical significance was considered to be at the $p < 0.05$ level.

Synaptosomal Preparation: Male Sprague-Dawley rats weighing 300 ± 25 g obtained from the University of Manitoba Central Animal Care breeding facility were killed by decapitation. Brains were removed and placed on an ice-cold plate and cerebral cortices were dissected out and homogenized (25 strokes) in a Teflon-glass homogenizer containing 10 volumes (wt/v) of 0.32 M sucrose. Homogenates were centrifuged at 1,800 g for 5 min, and supernatants were centrifuged at 13,000 g for 20 min. Pellets (crude P2 synaptosomal fraction) were resuspended in HEPES buffer (in mM: NaCl, 110; glucose, 25; sucrose, 68.3; KCl, 5.3; $CaCl_2$, 1.8; $MgSO_4$, 1.0; HEPES, 20; pH 7.4) and centrifuged at 13,000 g for 20 min. All synaptosomal procedures were conducted at 4°C unless otherwise indicated.

Synaptosomal Release Assay: Synaptosomal pellets (~ 0.8 mg of protein /100 µl) were resuspended in HEPES buffer containing, unless otherwise indicated, 10 µM [^{3}H]L-adenosine (0.5 mCi/ µmol). Synaptosomes were incubated at 37°C for 30 min and 100 µl aliquots were transferred to microcentrifuge tubes and were centrifuged at 13,000 g for 2 min. Supernatants were aspirated and tubes containing pellets were kept on ice until taken for assay. All assays were completed within 1.5 h of tissue preparation. Pellets were washed with 1 ml of ice-cold HEPES buffer, followed by immediate aspiration. Release was initiated by vortex mixing after addition of 1 ml of prewarmed (37°C) HEPES buffer in the absence or presence of either nucleoside substrates or nucleoside transport inhibitors. Unless otherwise indicated, reactions were performed at 37°C for 30 sec,

conditions where release increased linearly with increasing incubation times. [3H]L-Adenosine release was terminated by centrifugation at 13,000 g for 1 min. Supernatants were removed and 0.5 ml aliquots were taken for measurement of radioactivity by scintillation spectroscopy. Pellets were digested in 1 ml of 0.1 M NaOH overnight and analysed for protein determination using the method of Lowry *et al.* [1951].

Release as a function of synaptosomal protein concentration was determined using 0.2-2 mg of synaptosomal protein loaded with 100 μM [3H]L-adenosine. [3H]L-Adenosine release as a function of the length of incubation period was determined at two temperatures, 37°C and 4°C at reaction time intervals that ranged from 0 to 600 sec. Synaptosomes (P2) loaded with [3H]L-adenosine at concentrations of 1, 10, and 100 μM were used to determine the release profile as a function of loading concentrations. The effects of various agents including KCl (50 mM), the nucleoside transport substrates (10 mM) L-adenosine, D-adenosine, and uridine, and the nucleoside transport inhibitors (100 μM) dipyridamole (DPR), nitrobenzylthioinosine (NBI), and dilazep (DZP) on [3H]L-adenosine release from synaptosomes (P2) loaded with 10 μM [3H]L-adenosine were determined by adding these agents simultaneously with buffer during resuspension of the final pellet. One percent dimethyl sulfoxide (DMSO), used for dissolving DPR and NBI, had no significant effect on [3H]L-adenosine release. Dose-dependent inhibition profiles for DPR, NBI, and DZP were determined using concentrations of these inhibitors ranging from 0.01-100 μM. To determine whether nucleoside transport substrates on the *cis* side of synaptosomal membranes could affect [3H]L-adenosine release, synaptosomes (P2)

were loaded with 10 µM [³H]L-adenosine plus 2.5 mM L-adenosine, D-adenosine, or uridine.

Adenosine metabolism was determined by a previously described TLC method (Gu and Geiger, 1992).

RESULTS

[³H]L-Adenosine was accumulated by crude P2 preparations of rat brain synaptosomes with typical tissue/medium ratios of ~1-2:10. [³H]L-Adenosine release experiments with purified fractions of microsomes and mitochondria, both of which are contained in the crude P2 fraction, were not conducted because of previous findings that adenosine was not released from these subcellular fractions (Hoehn and White, 1990). Synaptosomes (P2) preloaded with [³H]L-adenosine released more [³H]L-adenosine at 37°C than at 4°C and the amount released increased with time (Fig. 4). When synaptosomes (P2) were loaded with 100 µM [³H]L-adenosine (Fig. 4), [³H]L-adenosine release (pmol/mg of protein) at 37°C was 11.7 at time zero and 303 at 600 sec, whereas at 4°C the release was 0 at time zero and 30 at 600 sec. In comparison, at 37°C the amount of [³H]L-adenosine (pmol/mg of protein) remaining in the pellets was found to decrease from 451 at time zero to 119 at 600 sec, and at 4°C was found to be 513 at time zero and 383 at 600 sec. The release increased linearly (r=0.919) from ~ 2 pmol/30 sec at 0.2 mg of brain protein to ~150 pmol/30sec at 1.8 mg of brain protein (data not shown).

The release of [³H]L-adenosine increased with higher concentrations of [³H]L-adenosine (Fig. 5). For example, when synaptosomes (P2) were loaded with 1 µM [³H]L-adenosine, the release (pmol/mg of protein) was 0.72 ± 0.07 at 0 sec and 1.1 ± 0.2 at 30
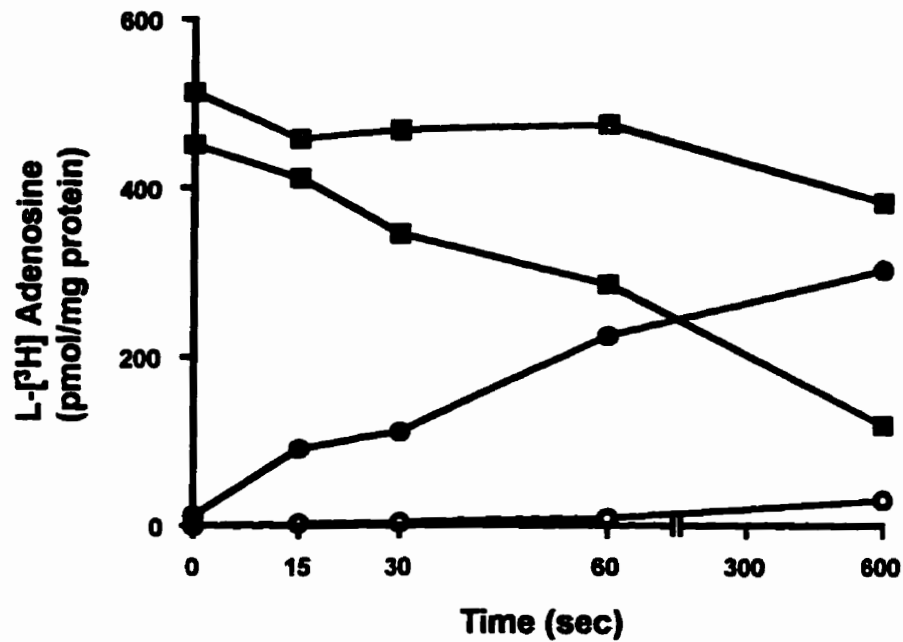
Figure: 4. [³H]L-Adenosine remaining in synaptosomes (squares) or released into supernatant (circles) as a function of incubation time at 4°C (open symbols) or 37°C (closed symbols). Synaptosomes were preloaded with 100 μM [³H]L-adenosine at 37°C for 30 min. Symbols represent mean values obtained from three experiments, each of which was performed in duplicate.
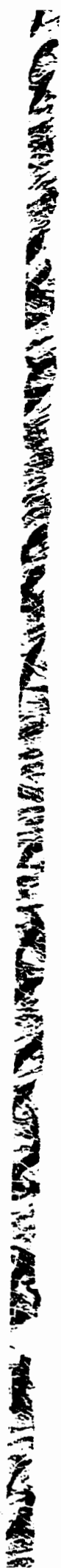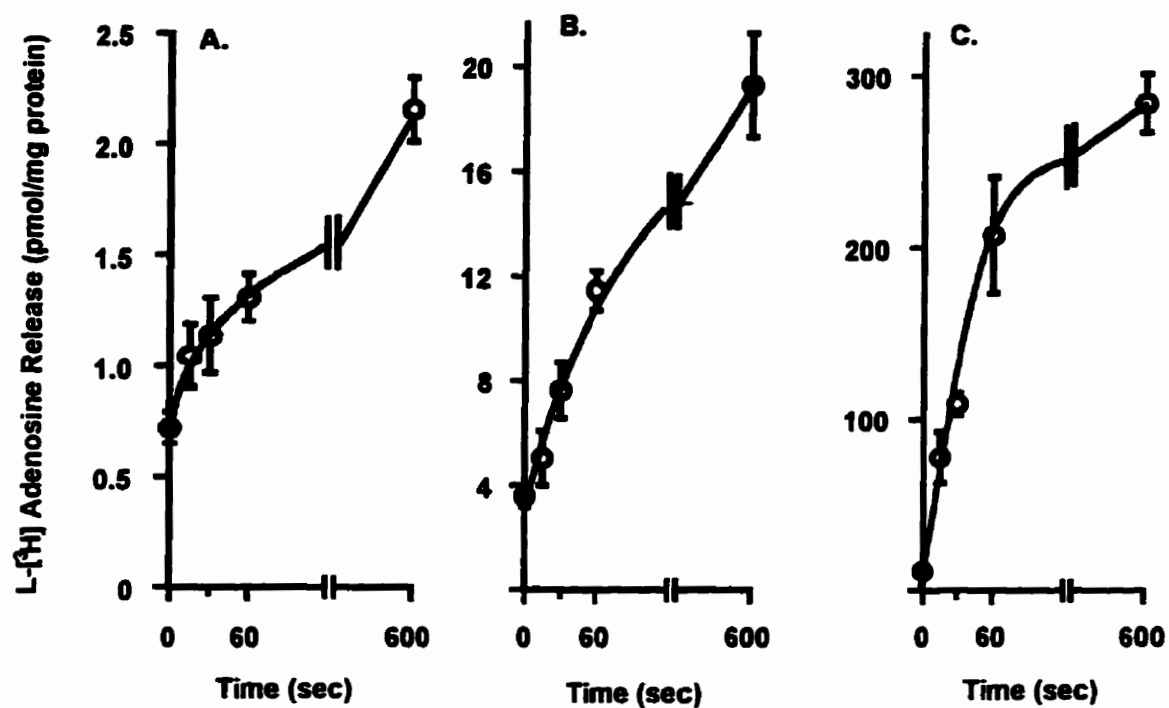
Figure: 5. Release of $[^3H]$L-adenosine from synaptosomes preloaded with $[^3H]$L-adenosine at 1 µM (**A**), 10 µM (**B**), and 100 µM (**C**). Incubations were from 0 to 600 sec at 37°C. Symbols represent mean ± S.E.M. (bars) values from at least four experiments, each of which was performed in duplicate.

sec, 0.38 pmol/mg of protein/30sec; when loaded with 10 μM, the release was 3.6 ± 0.4 at

0 sec and 7.6 ± 1.1 at 30sec, 4.0 pmol/mg of protein/30sec; and when loaded with 100

μM, the release was 11.5 ± 0.5 at 0 sec and 109 ± 6.3 at 30 sec, 97.5 pmol/mg of

protein/30sec. In 30 sec at 37°C, synaptosomes (P2) loaded with 10 μM [^{3}H]L-adenosine

released ~66% of the [^{3}H]L-adenosine accumulated during the preloading period. During

the period of time encompassing the release experiments, >93% of the radiolabel in the

pellet was recovered as [^{3}H]L-adenosine, where as >98% of the released radiolabel was

recovered as [^{3}H]L-adenosine. Our previous work suggests that the most likely

metabolites were phosphorylated derivatives (Gu *et al.*, 1991).

At 100 μM concentrations of the nucleoside transport inhibitors (Fig. 6), release

was significantly (p<0.01) inhibited by 40% with DPR and 52% with NBI relative to a 1%

DMSO vehicle control, and by 49% with dilazep relative to a buffer control. Neither 50

mM KCl nor an isoosmotic amount of sucrose (100 mM) enhanced [^{3}H]L-adenosine

release significantly (Fig. 6). In a separate series of experiments, we found that [^{3}H]L-

adenosine release was inhibited dose-dependently by the nucleoside transport inhibitors

DPR, NBI, and DZP. Statistically significant inhibitions were observed starting at 1 μM

for dilazep (p<0.01) and NBI (p<0.01), and at 10 μM for DPR (p<0.01). At 100 μM

concentrations, the inhibition of release was 41% for DPR, 42% for dilazep, and 77% for

NBI (Fig. 7).

To further test the extent to which [^{3}H]L-adenosine was released through

nucleoside transporters we conducted experiments where the nucleoside transport

substrates L-adenosine, D-adenosine, or uridine were either added to reaction vessels at
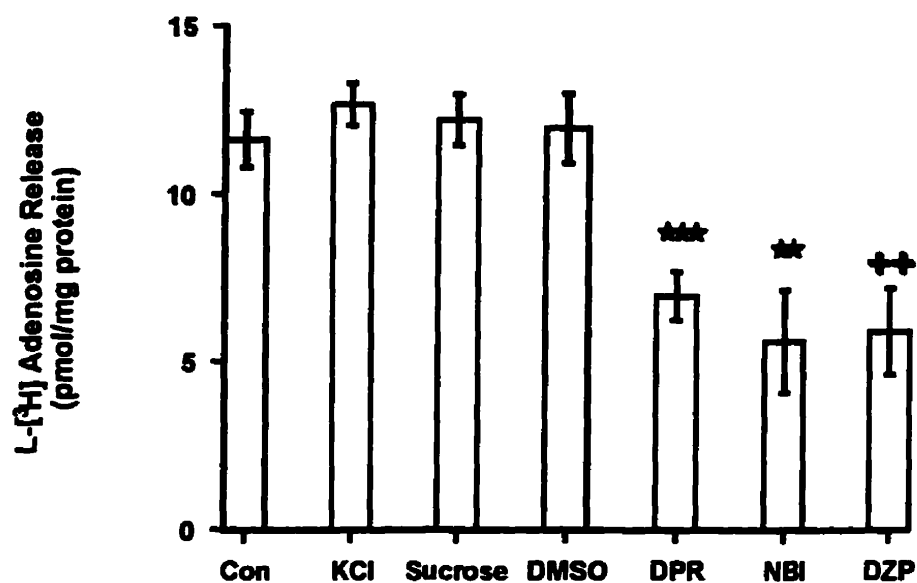
Figure: 6. Release of [^{3}H]L-adenosine from rat brain synaptosomes in the absence (Con=control) or presence of 50 mM KCl, 100 mM sucrose, 1.0% DMSO, 100 μM NBI, or 100 μM dilazep (DZP). Synaptosomes were preloaded with 10 μM [^{3}H]L-adenosine and release was measured at 30 sec. Data are mean ± S.E.M. values from at least five experiments, each of which was performed in duplicate. ***p<0.001, DPR vs. 1.0% DMSO vehicle control; **p<0.01, NBI vs. 1.0% DMSO vehicle control; ++p<0.01, DZP vs. buffer control (Con).
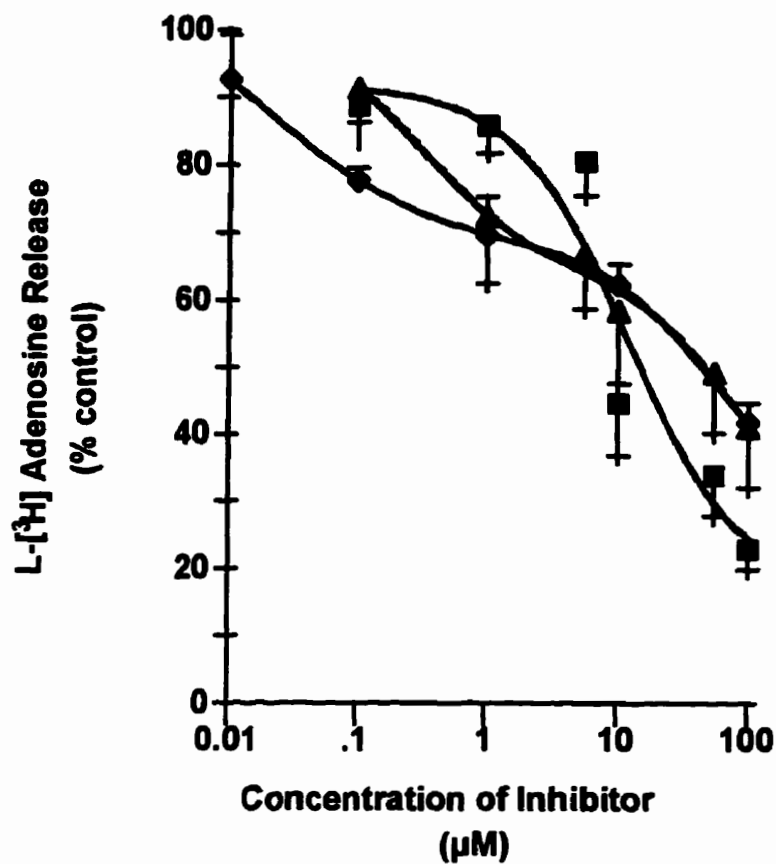
Figure: 7. Release of [³H]L-adenosine from rat brain synaptosomes in the presence of DPR (closed triangles), NBI (closed squared), and dilazep (closed diamonds) at concentrations ranging from 0.01 to 100 μM. Synaptosomes were preloaded with 10 μM [³H]L-adenosine and release was measured at 30 sec. Data are mean ±S.E.M. values from at least five experiments, each of which was performed in duplicate.

the time of initiation of release (*trans* experiment) or were preloaded into synaptosomes

(P2) before the initiation of release (*cis* experiment). For the *cis* experiment, we found

that [³H]L-adenosine release was inhibited 42% by L-adenosine, 69% by uridine, and 81%

by D-adenosine (Fig. 8). In the *cis*-loaded synaptosomal preparations, the amount of

[³H]L-adenosine accumulated by the tissue in the absence of nucleosides (13,620 ± 783

dpm) was not significantly different from samples preincubated with [³H]L-adenosine and

unlabeled L-adenosine (13,338 ± 1,264 dpm), uridine (15,118 ± 884 dpm), or D-

adenosine (14,525 ± 1,382 dpm). For the *trans* experiment, none of the three nucleosides

tested affected release significantly when applied at concentrations of 10 mM (Fig. 9).

DISCUSSION

Increasing extracellular levels of endogenous adenosine through inhibition of its

uptake into cells may effectively enhance the neuroprotective actions of adenosine in a

more site-directed and event-specific manner, and with fewer side effects than might be

expected with, for example, adenosine agonists because adenosine receptors are widely

distributed throughout the body. Accordingly, uptake inhibitors would presumably work

to markedly increase adenosine levels only when and where adenosine was being

produced. However, predicting whether adenosine levels would increase, decrease, or

remain the same after inhibition of nucleoside transporters appears difficult at present and

the following considerations apply.
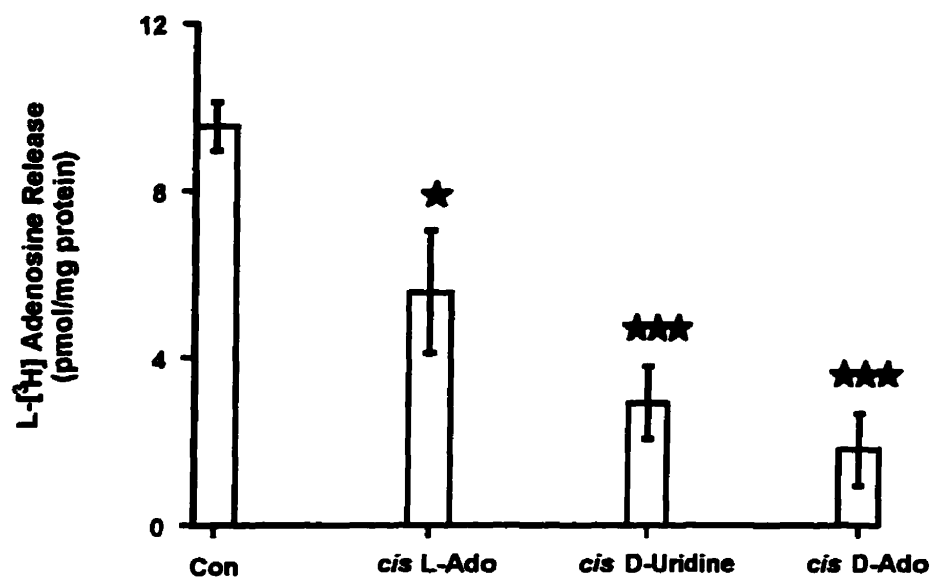
Figure: 8. Release of [³H]L-adenosine from rat brain synaptosomes preloaded with 10 μM [³H]L-adenosine alone (Con=control) or 10 μM [³H]L-adenosine plus 2.5 mM L-adenosine (L-Ado), D-uridine, or D-adenosine (D-Ado). Data are mean ± S.E.M. values from at least five experiments, each of which was performed in duplicate. *p<0.05, L-adenosine vs. control; ***p<0.001, D-uridine and D-adenosine vs. control. There were no statistically significant differences between the L-adenosine, D-uridine, and D-adenosine groups.
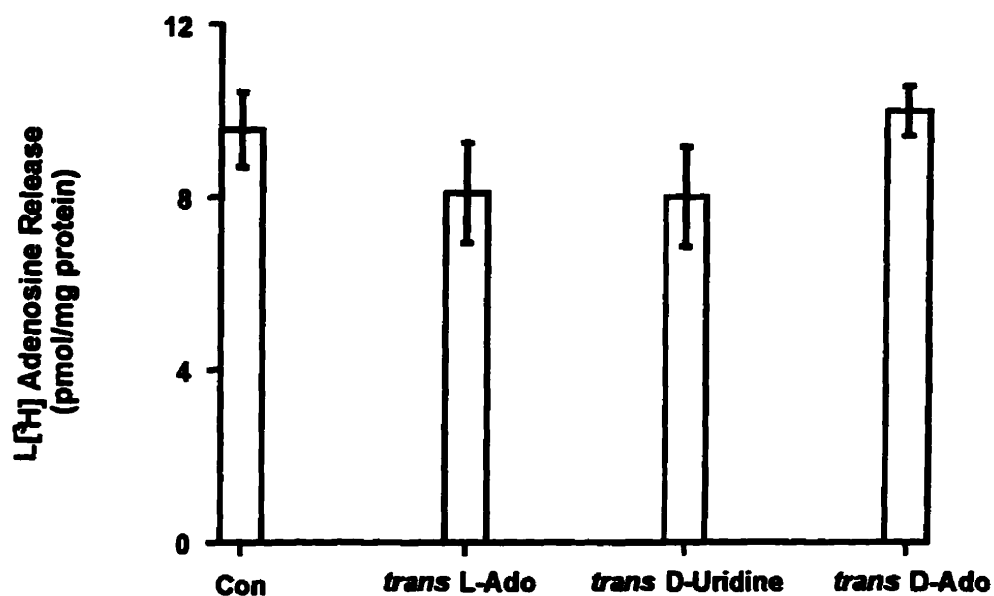
Figure: 9. Release of [^{3}H]L-adenosine from rat brain synaptosomes in the absence (Con=control) or presence of 10 mM L-adenosine, D-uridine, or D-adenosine. Synaptosomes were preloaded with 10 μM [^{3}H]L-adenosine and release was measured at 30 sec. Data are mean ± S.E.M. values from at least three experiments, each of which was performed in duplicate.

First, at least some nucleoside transporters mediate adenosine's influx into as well as its efflux (release) out of cells. Presently, at least three adenosine transporter subtypes have been identified in nervous system tissues (see Geiger and Fyda, 1991); however, little information is available about their roles in mediating adenosine efflux. Therefore, the bidirectional function of the involved adenosine transporter(s) must be determined before one would be able to predict whether an inhibitor was effective in inhibiting influx selectively without affecting efflux.

Second, for each tissue and for each experimental paradigm used, it is necessary to know the degree to which extracellular adenosine originates from adenosine that is released *per se* from intracellular sources or originates from extracellular dephosphorylation of released adenine nucleotides. CNS tissues are known to release adenosine *per se* as well as adenine nucleotides and the ratio of nucleoside/nucleotide release has been found to vary between types of tissue preparation and release stimuli used (White and Hoehn, 1991; Craig and White, 1993). Thus, in the absence of a more complete understanding of bidirectional adenosine transport in the CNS, as well as the sites at which and the conditions under which adenosine is released from neural tissues, development of adenosine transport inhibitors as therapeutic agents may remain a difficult task. To provide such information requires appropriate experimental systems and our description here of the release of [^{3}H]L-adenosine from rat brain synaptosomal preparations represents a step towards that goal.

Up to the present, accurate measurements of nucleoside transporter-mediated release processes were impossible with physiological D-adenosine because it was rapidly

metabolised to its phosphorylated and deaminated products. However, the release process can be determined by alternate substrates for the transporter that have affinities similar to D-adenosine. L-Adenosine (the stereoenantiomer of D-adenosine) appears to fit these criteria in that (1) L-adenosine is more stable metabolically than D-adenosine, (2) L-adenosine is a substrate for CNS adenosine transporters, and (3) the affinity for synaptosomal adenosine transporters for L-adenosine is comparable with that for D-adenosine (Gu *et al.*, 1991; Gu and Geiger, 1992). Although nucleoside transporter permeants such as formycin B and uridine, at least in some tissues, appear to be more stable metabolically than D-adenosine (Plagemann and Woffendin, 1989), their usefulness as permeants of nucleoside transporters in the CNS may be limited by the relatively low affinity with which CNS transporters recognise and carry these nucleoside substrates (Lee and Jarvis, 1988*a*).

Our findings in the present study that [^{3}H]L-adenosine was avidly taken up by synaptosomes agrees with our previous findings with synaptoneurosomes where the uptake of [^{3}H]L-adenosine was blocked up to 65% by the nucleoside transport inhibitor NBI as well as by D-adenosine. Thus, to a large extent uptake was mediated by equilibrative carrier-mediated adenosine transporters (Gu *et al.*, 1991; Gu and Geiger, 1992). In agreement with our previous results with synaptoneurosomes (Gu *et al.*, 1991), [^{3}H]L-adenosine was, in the main, metabolically stable in our synaptosomal preparation; 7% was metabolised in 30 min at 37°C. Knowing this, we began examining conditions under which preloaded [^{3}H]L-adenosine was released. We found that simply resuspending the pellets containing [^{3}H]L-adenosine in a 37°C buffer was sufficient to initiate release;

this indicated that the release resulted from flow down a concentration gradient. The release increased by a factor of 10 when the preloading concentration of $[^3H]$L-adenosine was increased from 1 to 10 μM. However, when the preloading concentration of $[^3H]$L-adenosine was increased to 100 μM, the release was ~24 times that seen at 10 μM. This may indicate that at higher loading concentrations, $[^3H]$L-adenosine release exhibits a more complicated kinetic profile or the involvement of alternative compartments from which and/or mechanisms by which $[^3H]$L-adenosine is released from these preparations.

That this release results from efflux at least partially due to carrier-mediated equilibrative nucleoside transporters was supported by our findings that release was partially blocked by nucleoside transport inhibitors DPR, NBI, and dilazep starting at low micromolar concentrations. Further, release was inhibited by preloaded (*cis* experiment) nucleosides L-adenosine, D-adenosine, and uridine. Of the three nucleosides tested at 2.5 mM concentrations, there was a tendency for D-adenosine and D-uridine to be more effective than L-adenosine at reducing $[^3H]$L-adenosine release. These findings, although not significant statistically, may indicate that at least a portion of the release mechanism is nucleoside stereospecific. The above findings appear consistent with influx studies, where equilibrative nucleoside transporters were identified on rat brain synaptoneurosomes and synaptosomes (Lee and Jarvis, 1988*b*; Gu *et al.*, 1991). The portion of $[^3H]$L-adenosine release that was neither inhibited by transporter inhibitors nor by transporter substrates may represent efflux by simple diffusion. However, further studies are necessary to determine more precisely the nature of the adenosine transporter governing L-adenosine transport and release.

We were not able to demonstrate release of [^{3}H]L-adenosine evoked by 50 mM KCl even though such stimuli were previously shown to cause a modest release of adenosine from rat cortical slices and synaptosomes (MacDonald and White, 1985; White and Hoehn, 1991). We cannot at present rule out the possibility that an enhanced release of [^{3}H]L-adenosine may have been masked by KCl-induced formation of endogenous D-adenosine. After all, we did find that [^{3}H]L-adenosine release could be inhibited by *cis*-loaded D-adenosine.

[^{3}H]L-Adenosine release was not enhanced in the presence of the nucleoside substrates L-adenosine, D-adenosine, or uridine. *Trans* stimulation of nucleoside transport has been observed in only a few cell types such as human, pig, and mouse erythrocytes (Plagmann and Woffendin, 1988). Our findings are consistent with the interpretation that the equilibrative nucleoside transporters responsible for controlling the release of [^{3}H]L-adenosine from rat brain synaptosomes transport substrate equally well when the transporters are either *trans* empty or *trans* loaded.

The test system described here should help to facilitate pharmacological characterisations of adenosine transporters involved with adenosine release in the CNS.

[2]Chapter 2. Nucleoside Transport Mediated Uptake and Release of [^{3}H]L-Adenosine in DDT$_1$ MF-2 Smooth Muscle Cells.

ABSTRACT: [^{3}H]L-Adenosine, an enantiomer of the physiological D-adenosine, was shown previously to be taken up and released, at least in part, through nucleoside transporters in rat brain preparations. In the present study, we used clonal smooth muscle DDT$_1$ MF-2 cells that contain almost exclusively equilibrative inhibitor-sensitive (es) nucleoside transporters to test the hypothesis that L-adenosine is a permeant for these bidirectional nucleoside transporters. DDT$_1$ MF-2 cells accumulated approximately 3-times more [^{3}H]D- than [^{3}H]L-adenosine; 10 μM nitrobenzylthioinosine significantly (p<0.01) inhibited the accumulation of [^{3}H]D-adenosine by 86% and of [^{3}H]L-adenosine by 63%. The IC$_{50}$ values for the nitrobenzylthioinosine-sensitive portions of [^{3}H]L- and [^{3}H]D-adenosine accumulation were 1.6 and 2.0 nM, respectively. [^{3}H]D-Adenosine accumulation was significantly (p<0.05) inhibited by up to 72% with L-adenosine and [^{3}H]L-adenosine accumulation was significantly (p<0.01) inhibited by up to 52% with D-adenosine. Release of accumulated [^{3}H]L-adenosine was temperature- and time-dependent, and was significantly (p<0.05) reduced by 47% with dipyridamole, 39% with dilazep, and 45% with nitrobenzylthioinosine; the apparent IC$_{50}$ value for nitrobenzylthioinosine was less than 1 nM. These data indicate that a significant proportion of [^{3}H]L-adenosine uptake and release in DDT$_1$ MF-2 cells is mediated by es nucleoside transporters.

INTRODUCTION

At least seven nucleoside transporters have been identified to date, and characterised broadly as being either equilibrative or concentrative (see Geiger *et al.*, 1991). These transporters function to regulate the influx of endogenous and exogenous nucleosides as well as nucleoside drugs across plasma membranes into cells (Cass, 1995). Additionally, bidirectional nucleoside transporters that are equilibrative and sensitive to inhibition by nitrobenzylthioinosine, so called *es* transporters, appear to play an important role in the efflux of nucleoside drugs and intracellular nucleosides, the most important of which may be the physiologically active nucleoside adenosine. Thus, nucleoside transporters are important in regulating the extracellular levels and actions of adenosine; these actions are mediated, in the main, by cell surface receptors, and inhibitors of nucleoside transporters continue to be viewed as agents with therapeutic potential.

It is difficult to study the degree to which specific subtypes of nucleoside transporters mediate the efflux of endogenous D-adenosine formed intracellularly in brain because relatively little is known about their types and functions, and because adenosine is so metabolically liable. To gain additional information about mechanisms of adenosine efflux, we showed that [^{3}H]L-adenosine, an enantiomer of the physiological D-adenosine, was transported into and released from heterogeneous rat brain preparations, at least in part, through equilibrative nucleoside transporters (Gu *et al.*, 1995). We showed that [^{3}H]L-adenosine was more metabolically stable than was [^{3}H]D-adenosine, and that it was a permeant of brain nucleoside transporters with values for Michaelis-Menten constants that were similar to those for [^{3}H]D-adenosine (Gu *et al.*, 1991; Gu and Geiger, 1992).

Furthermore, [³H]L-adenosine was released from rat brain preparations in a time- and temperature-dependent manner, and that 52% of the release was blocked by nitrobenzylthioinosine, thus implicating nucleoside transporters in the release of [³H]L-adenosine (Gu *et al.*, 1995).

Determining the type of transporter and the mechanism(s) by which [³H]L-adenosine was being released was made difficult in brain by the heterogeneous nature of the tissue preparations, and the presence of multiple subtypes of nucleoside transporters including *es* transporters and a large proportion of transporters described as being equilibrative and resistant (*i*nsensitive) to inhibition by nitrobenzylthioinosine, so called *ei* transporters (Lee and Jarvis, 1988a; Vijayalakshmi and Belt, 1988). Furthermore, it is unknown the degree to which our previous data was affected by the homogenised nature of the previously used rat brain preparations. To circumvent these potential problems, we used here a clonal cell line DDT₁ MF-2 smooth muscle cells that appear to contain, almost exclusively, *es* transporters (Parkinson *et al.*, 1996) in order to test the hypothesis that [³H]L-adenosine is transported into and released from cells by *es* nucleoside transporters. In agreement with our previous work with rat brain preparations, we found that [³H]D-adenosine and [³H]L-adenosine were transported into and [³H]L-adenosine was released from DDT₁ MF-2 cells, at least in part, by bidirectional *es* nucleoside transporters.

MATERIALS AND METHODS

Materials: [^{3}H]L-Adenosine (13 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA), ^{3}H$_2$O (1.0mCi/g) was from Amersham Canada Ltd. (Oakville, Ontario), [^{14}C]- or [^{3}H]polyethylene glycol (10 mCi/g and 2.0 mCi/g, respectively) and [^{3}H]D-adenosine (61.5 Ci/mmol) were from DuPont Canada (Mississauga, Ontario). Nitrobenzylthioinosine, dipyridamole, and D-adenosine were form Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle Medium (DMEM), RPMI and fetal bovine serum (FBS) were obtained from Gibco BRL. Generous gifts of L-adenosine and dilazep were supplied by Dr. K. Flora of the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute and F. Hoffmann La Roche, Basel, Switzerland, respectively. Syrian hamster smooth muscle DDT$_1$ MF-2 cells were obtained from the American Type Tissue Culture Collection. Statistical analyses were carried out by one way ANOVA with Bonferroni correction, Student's t tests, and nonlinear regressions using Instat and GraphPad PRISM (Biosoft). All data were expressed as mean values and S.E.M. Statistical significance was considered to be at the $p < 0.05$ level.

Cell Culture: DDT$_1$ MF-2 smooth muscle cells [Norris *et al.*, 1974], were grown in suspension, at 37°C in 5% CO$_2$-95% humidified air, and maintained as exponentially proliferating cultures at cell densities of 3×10^4 to 3×10^5 cells/ml in DMEM supplemented with 5% fetal bovine serum, 4.5 g/l glucose and 2 mM L-glutamate [Gerwins *et al.*, 1990]. Cells were harvested by centrifugation (120 g for 10 min), washed

twice (120 g for 5 min) and resuspended in Na⁺ buffer (in mM: Tris, 20; K_2HPO_4, 3; NaCl, 136; $MgCl_2$, 0.5; $CaCl_2$, 0.9; glucose, 20; to pH 7.4 with HCl) to 1 x 10⁶ cells/ml. Cell viability was determined by trypan blue exclusion staining.

[³H]Adenosine Accumulation Assays: [³H]Adenosine accumulation was determined by an oil-stop centrifugation method [Parkinson *et al.*, 1993]. Briefly, a reaction mixture containing 100 μl of [³H]D- or [³H]L-adenosine (5 μCi/ml unless otherwise stated; 1 μM or 2 μM) in Na⁺ buffer was layered over 100 μl oil (85 parts silicon : 15 parts paraffin oil) in a microcentrifuge tube. Uptake was initiated by the addition of 100 μl 1.0 x 10⁶ cells/ml and terminated by addition of ice-cold dilazep (200 μM) and centrifugation at 13,000 g for 30 seconds. Supernatants and oil were aspirated, tubes were washed once with 1 ml Na⁺ buffer (4°C), pellets were digested in Triton X-100 (5%) overnight, and radioactivity was measured by scintillation spectroscopy. Assays were conducted at 25°C unless otherwise indicated. To determine the degree to which adenosine accumulation was inhibited by nitrobenzylthioinosine, cells were pre-treated with nitrobenzylthioinosine at concentrations ranging from 0.1 nM-20 μM, or buffer, for 15 min and incubated for 15 sec with either 2 μM [³H]D- or [³H]L-adenosine. To determine enantiomeric inhibition of uptake, [³H]D-adenosine (1 μM; 15 sec) accumulation was measured in the absence or presence of L-adenosine (100 μM or 1 mM) and [³H]L-adenosine (1 μM; 15 sec) accumulation was measured in the absence or presence of D-adenosine (100 μM or 1 mM).

[^{3}H]L-Adenosine Release Assays: [^{3}H]L-Adenosine (10 µCi/ml; 10 µM) was incubated

with cells (1 x 10^6 cells/ml) at 25°C for one hour unless otherwise stated. Aliquots of 100

µl were added to microcentrifuge tubes, centrifuged at 13,000 g for 20 sec, supernatants

were aspirated, and cell pellets were washed once with 1 ml Na$^+$ buffer (4°C) and kept on

ice until taken for assay. [^{3}H]L-Adenosine release was initiated by resuspension of cell

pellets in 500 µL Na$^+$ buffer (4° or 25 °C) and terminated after 0-300 sec by centrifugation

at 13,000 x g for 20 sec. Cell viability was ≥80%. To determine the degree to which

nucleoside transport inhibitors blocked [^{3}H]L-adenosine release, cells pre-loaded with 10

µM [^{3}H]L-adenosine for one hour were exposed for 15 min to dipyridamole or dilazep

each at 30 µM, nitrobenzylthioinosine at concentrations ranging from 0.3 nM-30 µM, or

buffer prior to centrifugation and resuspension in release buffer alone or release buffer

containing inhibitor.

RESULTS

Accumulation: [^{3}H]D-Adenosine, and to a lesser degree [^{3}H]L-adenosine, was

accumulated in a time- and temperature-dependent manner in DDT$_1$ MF-2 cells.

Accumulations of [^{3}H]D-adenosine were significantly ($p<0.01$) greater than were those of

[^{3}H]L-adenosine at incubations of 15 sec or longer; [^{3}H]D-adenosine accumulations

(pmol/10^5 cells) were, for example, 3.6 ± 1.8 at 15 sec and 8.6 ● 2.4 at 60 sec; [^{3}H]L-

adenosine accumulations were, for example, 1.1 ± 0.2 at 15 sec and 3.2 ± 0.2 at 60 sec

(Fig. 10).   Following preincubation with 10 µM NBI, 60 sec accumulations
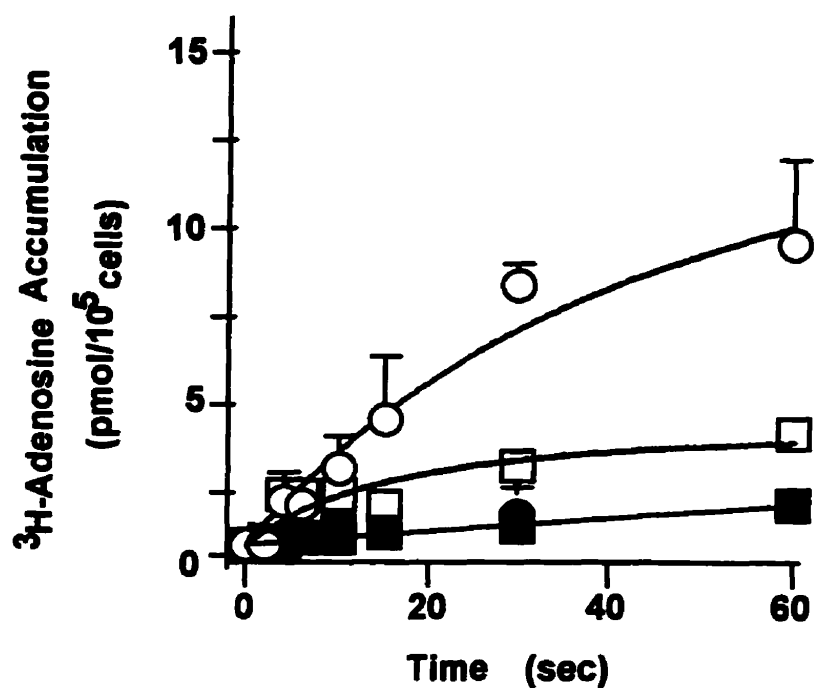
**Figure: 10.** Accumulation of [³H]D-adenosine (circles) and [³H]L-adenosine (squares) by DDT₁ MF-2 cells preincubated for 15 min in the absence (open symbols) or presence (closed symbols) of nitrobenzylthioinosine (10 μM). Incubations with 2 μM [³H]D-adenosine or 2 μM [³H]L-adenosine were for 0 to 60 sec at 25°C. Symbols represent mean ± S.E.M. values from three experiments conducted in triplicate.

were significantly (p<0.01) inhibited by 86% for [^{3}H]D-adenosine and by 63% for [^{3}H]L-adenosine. Residual, NBI-resistant, accumulation of 1.2 ± 0.2 pmol/10^5 cells was exactly the same for [^{3}H]D- and [^{3}H]L-adenosine (Fig. 10).

Significant (p<0.01) dose-dependent inhibition of [^{3}H]L-adenosine accumulation was observed with NBI (Fig. 11). The IC$_{50}$ value for the NBI-sensitive portion of [^{3}H]L-adenosine accumulation was 1.6 ± 0.1 nM (Fig. 11); in comparison, the IC$_{50}$ value for the NBI-sensitive portion of [^{3}H]D-adenosine accumulation was 2.0 ● 0.1 nM (data not shown). [^{3}H]D-Adenosine accumulation of 7.4 ± 0.8 pmol/10^5 cells was inhibited by 29 ± 2% by 100 μM L-adenosine and was significantly (p<0.05) inhibited 72 ● 5% by 1000 μM L-adenosine (Fig. 12). [^{3}H]L-Adenosine accumulation of 1.2 ± 0.2 pmol/10^5 cells was inhibited 31 ● 1% by 100 μM D-adenosine and was significantly (p<0.01) inhibited 52 ± 1% by 1000 μM D-adenosine (Fig. 12).

Release: To initiate release, pelleted cells were resuspended in buffer either at 25°C (Fig. 13A) or 4°C (Fig. 13B). [^{3}H]L-Adenosine in the supernatant increased and that remaining in pellets decreased with time at 25°C (Fig. 13A). The release during the first 5 sec at 4°C and at 25°C was rapid and amounted to 54-55% of the total amount of [^{3}H]L-adenosine loaded. During the 5 sec - 5 min time interval, release at 25°C was greater than at 4°C; of the total amount of [^{3}H]L-adenosine loaded, the amount recovered in the supernatants following 5 min was 88 % and 58 % at 25°C and 4 °C, respectively.
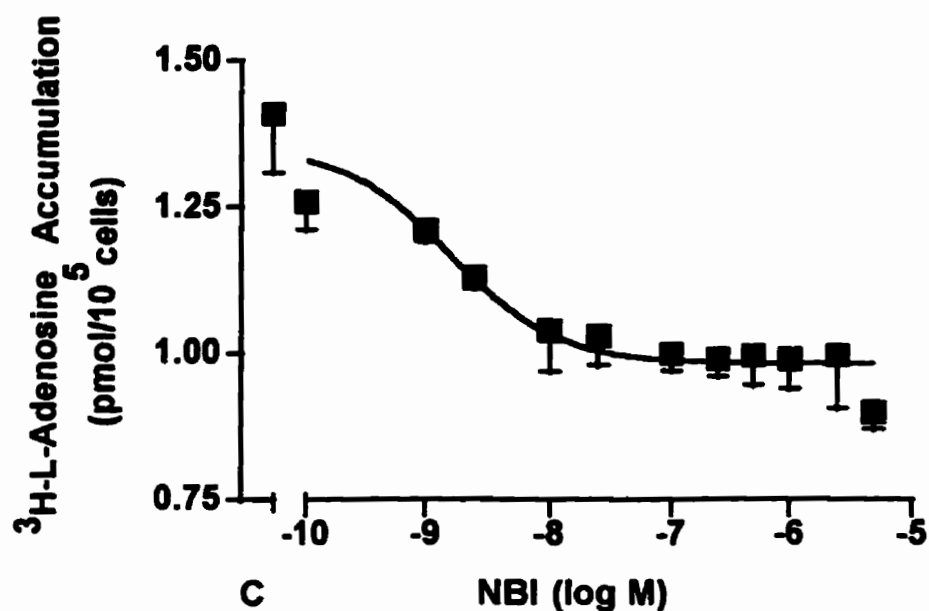
Figure: 11. Accumulation of [^{3}H]L-adenosine by DDT$_1$ MF-2 cells preincubated for 15 min with nitrobenzylthioinosine (NBI) at concentrations ranging from 0.1 nM to 20 µM. Incubations with 2 µM [^{3}H]L-adenosine were for 15 sec at 25°C. Symbols represent mean ± S.E.M. values from three experiments conducted in triplicate. (C= control).
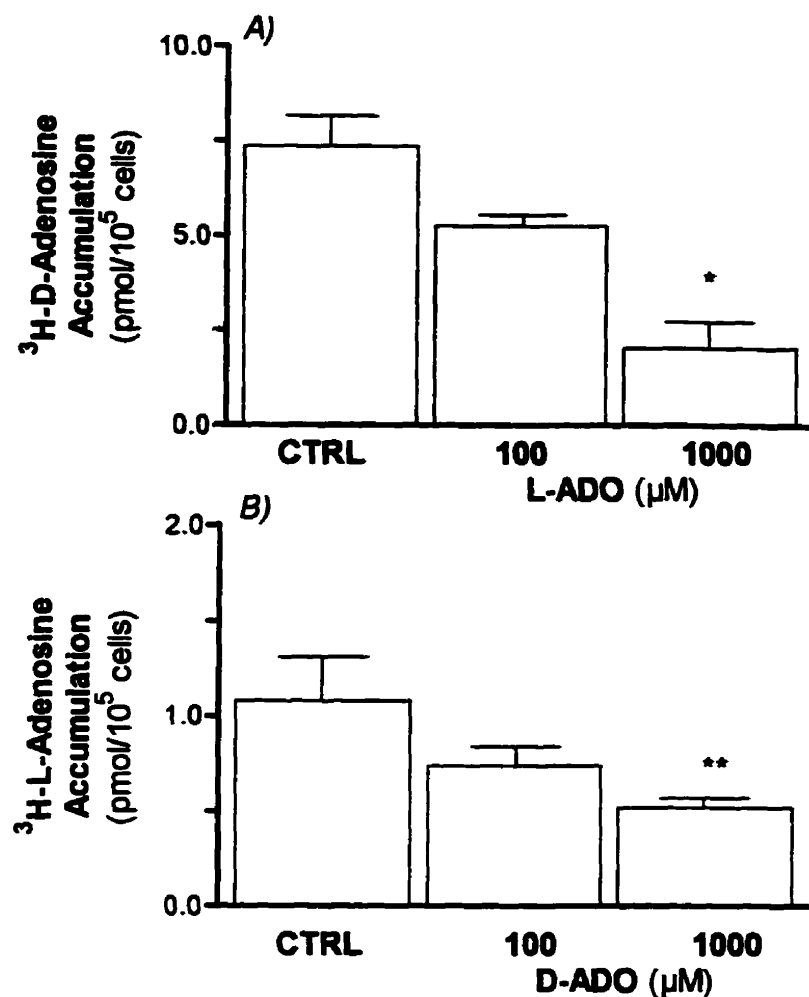
Figure: 12. Accumulation of **A**. [^{3}H]D-adenosine in the absence or presence of 100 µM or 1000 µM L-adenosine (L-ADO), and **B**. [^{3}H]L-adenosine in the absence or presence of 100 µM or 1000 µM D-adenosine (D-ADO). Incubations were with 1 µM [^{3}H]L-adenosine for 15 sec at 25°C. Symbols represent mean ± S.E.M. values from three experiments conducted in triplicate. *p<0.05 and **p<0.01 compared with controls (CTRL).
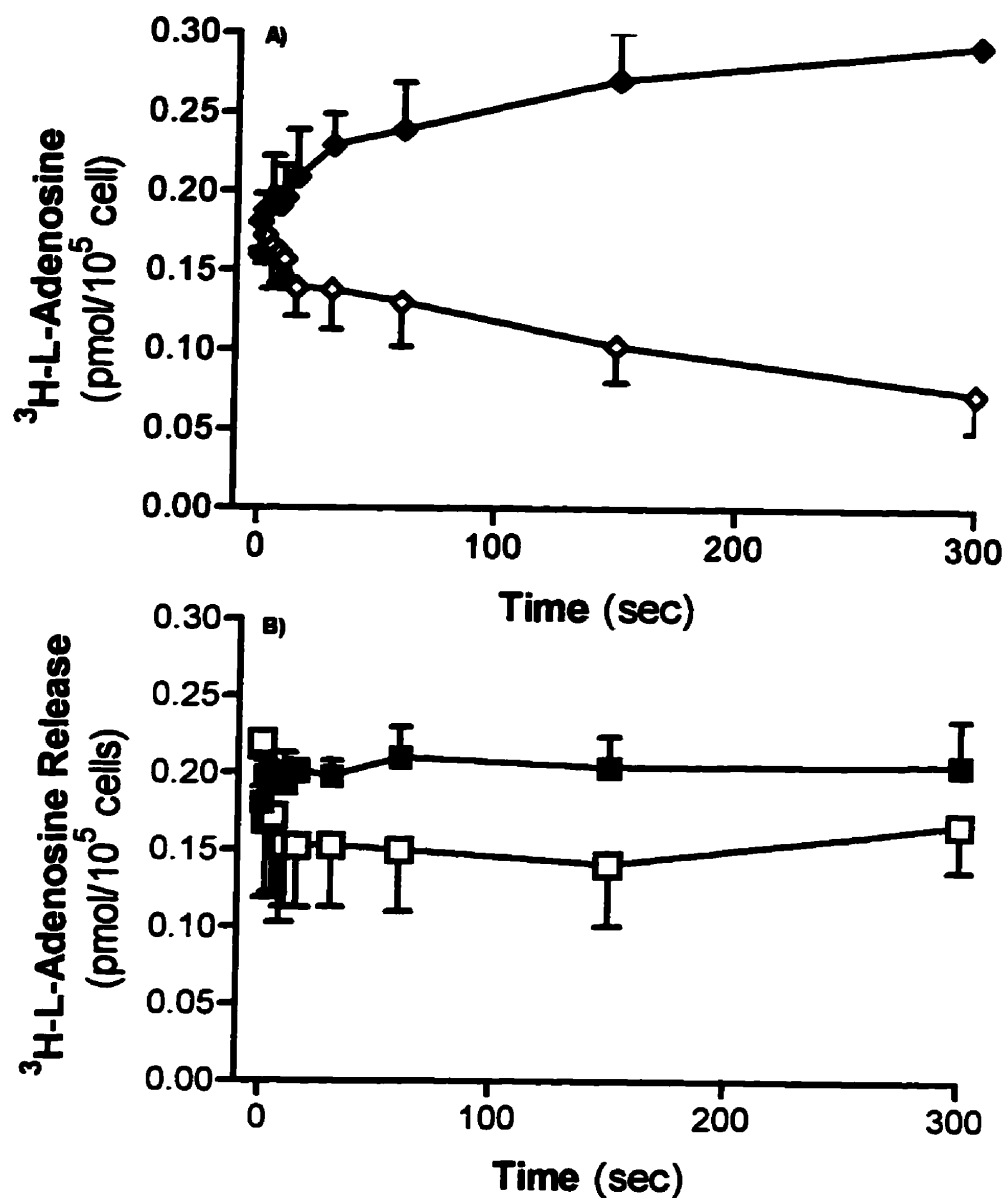
Figure: 13. Time- and temperature-dependent release of [³H]L-adenosine. Cells were incubated with 10 µM [³H]L-adenosine for 60 min and release was initiated following centrifugation and resuspension in buffer. [³H]L-Adenosine was measured in cell pellets (open symbols) and supernatants (closed symbols) for incubations at **A.** 25°C or **B.** 4 °C. Symbols represent mean ± S.E.M. values from three experiments conducted in triplicate.

[^{3}H]L-Adenosine release during 5 min was 43% greater at 25°C than at 4°C. [^{3}H]L-Adenosine release during 5 min was significantly (p<0.05) reduced by 45% with 30 µM NBI and 47% with 30 µM DPR compared to DMSO vehicle, and 39% with dilazep compared to buffer vehicle (Fig. 14). In a separate series of experiments, NBI inhibited 42% of total [^{3}H]L-adenosine release with an apparent IC_{50} value of less than 1 nM (Fig. 15).

DISCUSSION

L-Adenosine, the stereo-enantiomer of the physiological D-adenosine, appears to be a useful probe for the study of nucleoside transport-mediated processes. In rat brain, it was more metabolically stable than D-adenosine, it did not inhibit adenosine kinase activity and only weakly inhibited adenosine deaminase activity, and it was not a substrate for either adenosine kinase or adenosine deaminase (Gu *et al.*, 1991). Further, [^{3}H]L-adenosine was transported into rat brain synaptosomes with rates (Gu *et al.*, 1991) and K_T values (Gu and Geiger, 1992) that were similar to those observed for [^{3}H]D-adenosine. That [^{3}H]L-adenosine transport was mediated, at least in part, by nucleoside transporters was confirmed by showing that nitrobenzylthioinosine and unlabeled D-adenosine blocked uptake by approximately 50% (Gu *et al.*, 1991; Gu and Geiger, 1992). More recently, we found that [^{3}H]L-adenosine was accumulated by and released from crude synaptosomal preparations of rat brain, and that the release of [^{3}H]L -adenosine was inhibited by three structurally dissimilar nucleoside transport inhibitors; inhibition was 40% by dipyridamole,
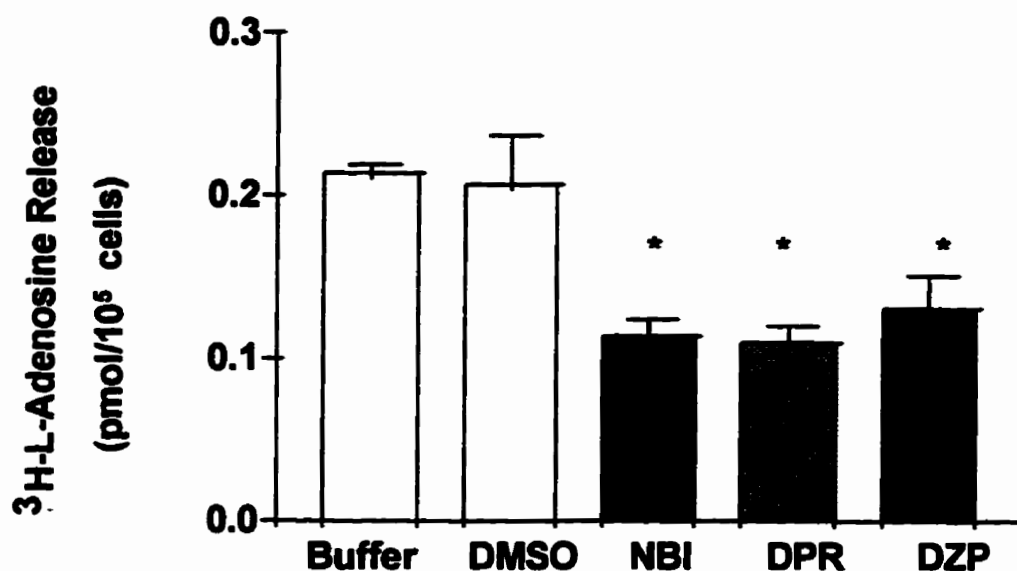
Figure: 14. Inhibition of [³H]L-adenosine release by nucleoside transport inhibitors. Cells were incubated with 10 µl [³H]L-adenosine for 60 min and release was measured over a 5 min period at 25°C in the presence of buffer (vehicle for dilazep [DZP]), 0.3% DMSO (vehicle for nitrobenzylthioinosine [NBI] and dipyridamole [DPR]), 30 µM NBI, DPR or DZP. Bars represent mean ± S.E.M. values from three experiments conducted in triplicate. *p<0.05 compared to respective controls.
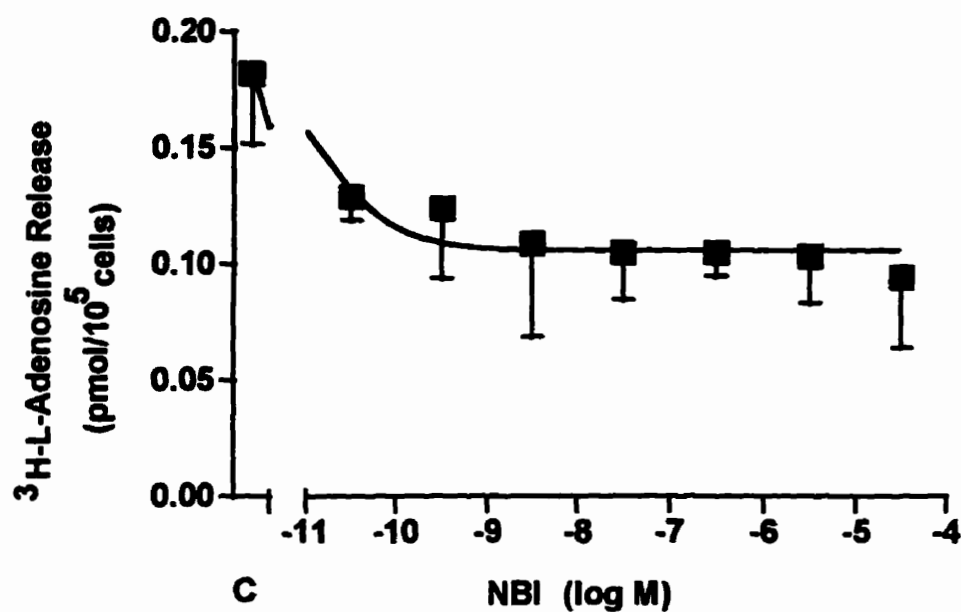
**Figure: 15.** Dose-dependent inhibition of [³H]L-adenosine release by nitro-benzylthioinosine (NBI). Cells were incubated with 10 μM [³H]L-adenosine for 60 min and release was measured over 5 min period at 25°C in the absence (C=control) or presence of NBI at concentrations ranging from 300 pM to 30 μM. Symbols represent mean ± S.E.M. values from three experiments conducted in triplicate.

49% by dilazep, and 52% by nitrobenzylthioinosine (Gu *et al.*, 1995). Thus, the release of [³H]L-adenosine from rat brain preparations was mediated, at least in part, by what appear to be bidirectional equilibrative nucleoside transporters. However, the above studies were conducted with heterogeneous preparations of rat brain derived from homogenised tissue that contained multiple subtypes of nucleoside transporters. Therefore, DDT₁ MF-2 cells representing a homogeneous population of clonal cells that contain almost exclusively *es* type transporters (Parkinson *et al.*, 1996) were used to determine the degree to which [³H]L-adenosine accumulation and release were mediated by specific bidirectional nucleoside transporters.

In rat brain preparations, we found that the rates of [³H]D- and [³H]L-adenosine accumulation were roughly equal (Gu *et al.*, 1991). In contrast, accumulation of [³H]D-adenosine was approximately three-times higher than [³H]L-adenosine in DDT₁ MF-2 cells, although at least part of this difference appeared due to metabolism of D-adenosine to impermeant nucleotides (Parkinson and Geiger, 1996). Thus, the streoselectivity of adenosine transport in DDT₁ MF-2 was less than the stereoselectivity observed in mouse erythrocytes and L-1210/AM cells where the rates of [³H]D-adenosine were 17- to 22-times higher than were the rates for [³H]L-adenosine (Gati *et al.*, 1989). As with rat brain preparations, L-adenosine was a permeant of nucleoside transporters in cultured adrenal chromaffin cells (Casillas *et al.*, 1993). In contrast, L-adenosine was a very poor permeant in mouse erythrocytes, L1210/AM cells, cultured aryworm ovary (Sf9) cells, human erythrocytes, Hela cells, Buffalo Green Monkey cells and parasitic protozoa (Dagnino *et al.*, 1991; Hogue and Cass, 1994; Gati *et al.*, 1989; Upston and Gero, 1995).

Stereoselectivity is also an important and emerging area for the pharmacology of nucleoside transport inhibitors. Pronounced stereoselectivity was observed between R 75321 and its stereo-enantiomers R 88016 and draflazine (Van Belle *et al.*, 1993; Beukers *et al.*, 1994). However, [^{3}H]D-adenosine uptake by DDT$_1$ MF-2 cells was equi-effectively inhibited by propentofylline, a racemic mixture of its hydroxy-metabolite (±) A720287 and its stereoisomers (+) 833791 and (-) 844261 (Parkinson *et al.*, 1996). Thus, it appears clear that stereoselectivity may be another property of nucleoside transporters that varies between species, tissues, cells and drugs (see Geiger and Fyda, 1991).

Following preincubations with 10 μM nitrobenzylthioinosine, accumulation of [^{3}H]D- and [^{3}H]L-adenosine were inhibited by 86% and 63%, respectively to levels indistinguishable between [^{3}H]D- and [^{3}H]L-adenosine. This is similar to rat brain preparations, in which nitrobenzylthioinosine inhibited [^{3}H]L-adenosine accumulation by 52% to 65% (Gu *et al.*, 1991). The IC$_{50}$ value for the nitrobenzylthioinosine-sensitive portion of [^{3}H]L-adenosine accumulation in DDT$_1$ MF-2 cells was 1.6 nM and this value was similar to the IC$_{50}$ value of 2.0 nM for the nitrobenzylthioinosine-sensitive portion of [^{3}H]D-adenosine accumulation found here and to values obtained for *es* transporters in a variety of tissues (Boumah *et al.*, 1994; Jarvis and Young, 1986; Torres *et al.*, 1990). That the accumulation of [^{3}H]L-adenosine was mediated by nucleoside transporters was supported further by findings that [^{3}H]D-adenosine accumulation was inhibited by up to 72% with L-adenosine and [^{3}H]L-adenosine accumulation was inhibited by up to 52% with D-adenosine. The nitrobenzylthioinosine-resistant accumulation of [^{3}H]L-adenosine

may be mediated by passive diffusion or by nucleoside transporters that are resistant to inhibition by nitrobenzylthioinosine. However, our previous findings indicated that DTT_1 MF-2 cells express almost exclusively *es* nucleoside transporters (Parkinson *et al.*, 1996).

To initiate release from DDT_1 MF-2 cells pre-loaded with [^{3}H]L-adenosine, pelleted cells were simply resuspended in fresh buffer and this suggests that [^{3}H]L-adenosine was released down its concentration gradient. Similar findings were made with rat brain synaptosomal preparations (Gu *et al.*, 1995). [^{3}H]L-Adenosine release appeared to be mediated by nucleoside transporters in that the release was inhibited by resuspending cells in ice-cold buffer or by resuspending cells in buffer containing nitrobenzylthioinosine, dipyridamole, or dilazep. The apparent IC_{50} value for the dose-related inhibition of release by nitrobenzylthioinosine was less than 1 nM.

In summary, nitrobenzylthioinosine inhibited a similar proportion of uptake and release of [^{3}H]L-adenosine in DTT_1 MF-2 cells. Furthermore, nitrobenzylthioinosine had similar potencies for inhibiting [^{3}H]L-adenosine uptake and release. This indicates that nucleoside transport inhibitors may inhibit the receptor-mediated effects of adenosine produced intracellularly but potentiate the effects of adenosine extracellularly.

GENERAL DISCUSSION

Tissue levels of endogenous adenosine, controlled by processes related to its production, release, transport and metabolism, play a pivotal role in the regulation of a multitude of physiological functions via the binding of adenosine to extracellular adenosine cell-surface receptors and activation of signal transduction systems. Until recently it was thought that endogenous adenosine at physiological levels entered cells by carrier-mediated nucleoside transport systems, while adenosine at supra-physiological levels entered cells by passive diffusion. More recently, nucleoside transporters have been identified that transport a variety of nucleosides bidirectionally (Plagemann and Wohlueter, 1980). The transporters have been subclassified according to whether nucleosides are transported by equilibrative or sodium-dependent concentrative processes, whether the transporters are sensitive or resistant to nucleoside transport inhibitors, and transporter selectivity for purine or pyrimidine permeants.

Characterization of nucleoside transporters using physiological D-adenosine is often difficult because of its metabolic instability. Our lab focused on the development of an new probe, L-adenosine, for the characterization of bidirectional nucleoside transporters involved in the trans-membrane movement of adenosine following demonstrations by us that [^{3}H]L-adenosine was a metabolically stable enantiomer of adenosine that was taken up by rat brain synaptosomes (Gu *et al.*, 1991; Gu and Geiger, 1992). In the present studies we characterized the uptake and release of L-adenosine in rat brain synaptosomes and in DDT$_1$ MF-2 cells to determine the bi-directional nature of adenosine transporters in these tissue preparations. Our goals were to determine (1) if

[^{3}H]L-adenosine could be released from synaptosomal preparations, (2) the optimal conditions necessary to observe the release, (3) the degree to which this release was mediated by efflux through bidirectional nucleoside transporters, and (4) whether cells grown in culture that appear to express only *es* transporters also exhibit bidirectional transport of [^{3}H]L-adenosine.

Our results showed that L-adenosine was transported into and released from heterogeneous rat brain preparations, at least in part, through equilibrative nucleoside transporters. Due to the heterogeneous cellular origin of the rat brain preparation and the presence of both *es* and *ei* nucleoside transporters (Lee and Jarvis, 1988a), it was important for us to determine whether clonal DDT_1 MF-2 smooth muscle cells which possess almost exclusively *es* transporters (Parkinson *et al.*, 1996) also transport L-adenosine. Importantly, L-adenosine uptake and release, in DDT_1 MF-2 smooth muscle cells as with rat brain synaptosomes, was mediated, at least in part, by *es* transporters.

Pharmacological agents capable of selectively blocking uptake but not the release of adenosine may enhance extracellular levels and actions of endogenous adenosine. L-Adenosine allowed us to characterize adenosine transport in the absence of nucleoside metabolism as well as to characterize the release of adenosine. Such test systems should be useful in characterizing adenosine transporters that mediate, and pharmacological agents that affect, bidirectional transport of adenosine. Ultimately, we wish to identify transport inhibitors and/or release enhancers that mimic therapeutically desirable actions of endogenous adenosine, especially when and where adenosine is being produced/released.

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