CHARACTERIZATION OF ADENOSINE TRANSPORT IN THE CNS

BY

JIANGUO GU

A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

Department of Pharmacology and Therapeutics

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This thesis is dedicated to

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ABSTRACT

Adenosine (Ado) transport systems help control the levels and actions of Ado in the CNS and inhibitors of Ado transport may provide useful therapeutic drugs. This work was aimed 1) to determine factors that may affect measurements of Ado transport in the CNS, 2) to develop suitable approaches for accurate measurements of Ado transporter-mediated influx and efflux, 3) to characterize Ado transport systems in tissues or cells of human and animal CNS, and 4) to determine the effects of Ado transport inhibitors on Ado influx and efflux processes. In this study, zero-trans influx and zero-trans efflux of [^{3}H]Ado were determined using inhibitor-stop/centrifugation or filtration methods. We found that [^{3}H]Ado rapidly entered into CNS tissues where it was extensively metabolized, mainly by Ado kinase (AK). This metabolism caused trapping of Ado in the form of its nucleotides and resulted in an appearance of "concentrative accumulation". When significant amounts of metabolism were present "K_T" values were in the low micromolar range which most likely represented the affinity of AK and not transporters for Ado. When we used short incubations (15 s) coupled with the AK inhibitor 5'-iodotubercidin and the Ado deaminase inhibitor erythro-2-(2-hydroxy-3-nonyl)adenine (EHNA), Ado transport *per se* was measureable. K_T values were 40 μM in rat synaptoneurosomes, 89 μM in human brain synaptoneurosomes, and 179 μM in cultured fetal human astrocytes. Pharmacologically, Ado transport inhibitors completely inhibited Ado transport in human cells. Three structurally distinct nucleoside transport inhibitors nitrobenzylthioinosine, dipyridamole, and dilazep all biphasically inhibited Ado transport in fetal human astrocytes; the potencies of the inhibition (IC_{50} values) were in the nanomolar range for the first phase and in the micromolar range for the second phase. Another approach that we developed for the characterization of Ado transport without metabolic interference was to use a transport probe L-[^{3}H]Ado, a stereoenantiomer of the

physiologically occurring D-Ado. We found that L-[^{3}H]Ado is a metabolically stable substrate for Ado transporters in the CNS. Results from kinetic and pharmacological studies indicated that L-[^{3}H]Ado was transported nearly as well as D-adenosine. Using L-[^{3}H]Ado as a transport probe, we found that CNS Ado transporters were bidirectional in that they mediate both influx and efflux of Ado, and that approximately 50% of Ado transporter-mediated influx and efflux were inhibitable with Ado transport inhibitors. Determination of Ado transporter-mediated influx and efflux processes with L-[^{3}H]Ado may provide an experimental model for identification and development of selective Ado transport inhibitors that may prove to be therapeutically useful.

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ABBREVIATIONS

A1AR	adenosine receptor subtype 1 (cloned)
A2aR	adenosine receptor subtype 2a (cloned)
A2bR	adenosine receptor subtype 2b (cloned)
A3AR	adenosine receptor subtype 3 (cloned)
ADA	adenosine deaminase
ADP	adenosine 5'-diphosphate
AICAR	5-amino-4-imidazole carboxamide ribose
AK	adenosine kinase
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
BMI	bicuculline methiodide
BSA	bovine serum albumin
$[Ca^{2+}]_i$	intracellular calcium
cDNA	complementary deoxyribonucleic acid
CGS 15943A	triazoloquinazoline
CGS 21680	2-(*p*-2-carboxyethyl)phenethylamino)-5'-N-ethylcarboxaminoadenosine
CHA	N^6-cyclohexyladenosine
Ci	Curie
CNS	central nervous system
cyclic AMP	cyclic adenosine 3',5'-monophosphate
DPCPX	1,3-dipropyl-8-cyclopentylxanthine
CPA	N^6-cyclopentyladenosine
CV 1674	2-(p-methoxyphenyl) adenosine
DMEM	Dulbecco's modified Eagle's medium

DCF	2'-deoxycoformycin
D-adenosine	9-β-D-ribofuranosyladenosine
DMPX	3,7-dimethyl-l-propargylxanthine
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPR	2,6-bis (diethanolamino)-4,8 dipiperidinopyridino-(5,4d)-pyrine; dipridamole
DZP	dilazep
ED_{50}	dose at which 50% of maximum effect is reached
EDTA	ethylenediaminetetraacetic acid
EHNA	erythro-2-(2-hydroxy-3-nonyl)adenine
ei	NBI-insensitive (resistant) nucleoside transporter
es	NBI-sensitive nucleoside transporter
FCS	fetal calf serum
fmol	femtomole
g	gram
g	gravity force
GFAP	glial fibrillary acid protein
G-protein	guanine nucleotide-binding protein
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	high performance liquid chromatography
hr	hour
HTL	homocysteine thiolactone
3-IB-MECA	N^6-(3-iodobenzyl)-5'-N-methylcarboxamidoadenosine
IC_{50}	concentration at which 50% of maximum effect is inhibited
IMP	inosine 5'-monophosphate
IP_3	inositol-1,4,5-trisphosphate

Kd	dissociation constant
kDa	kilodalton
Ki	inhibitory constant
Km	Michaelis-Menten constant
K_T	affinity constant of transport
L-adenosine	9-β-L-ribofuranosyladenosine
M	molar
MADO	N^6-methyl adenosine
mg	milligram
min	minute
mm	millimeter
mM	millimolar
mmol	millimole
mRNA	messenger ribonucleic acid
n	number of samples
N1 or cif	Na^+-dependent nucleoside transporter subtype 1
N2 or cit	Na^+-dependent nucleoside transporter subtype 2
N3 or cib	Na^+-dependent nucleoside transporter subtype 3
NBI	nitrobenzylthioinosine
NECA	N-ethylcarboxamidoadenosine
nM	nanomolar
nmol	nanomoles
P1	adenosine receptors
P2	ATP receptors
PD 81,723	(2-amino-4,5-dimethyl-3-thienyl)-[3(trifluoromethyl)-phenyl]methanone
PIA	N^6-phenylisopropyladenosine

pmol	picomoles
r	correlation coefficient
RNA	ribonucleic acid
s	second
S	substrate for Michaelis-Menten equation
SAH	S-adenosylhomocysteine
S.E.M.	standard error of mean
SNST1	Na^+-dependent nucleoside transporter (cloned)
SGLT	Na^+/glucose co-transporter
TLC	thin layer chromatography
µCi	microCurie
µm	micrometer
µM	micromolar
v	velocity
vol	volume
Vmax	maximum velocity
wt	weight
ZMP	acadesine ribotide

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INTRODUCTION

1. Historical Outlook

Adenosine, a purine nucleoside, was first demonstrated to be an endogenous physiologically active substance over 65 years ago when Drury and Szent-Györgyi (1929) observed that adenosine was a cardiovascularly active factor isolated from extracts of heart muscle, brain, kidney, and spleen. With the recognition of the hypotensive and bradycardic effects of adenosine in animals, studies were conducted in humans in attempting to use adenosine as a therapeutic drug for the treatment of cardiovascular diseases (Honey et al., 1930; Jezer et al., 1933; Drury, 1936). These studies were soon abandoned because of the extremely short physiological half life (5 s) of adenosine in the body. The re-emergence of adenosine research in the 1950s was marked by findings that adenosine may function as an endogenous coronary vasodilator (Feldberg and Sherwood, 1954; Winbery et al., 1953; Wolf and Berne, 1956) and a metabolic regulator for local control of blood flow in heart, brain, skeletal muscle, and kidney (Berne, 1963). Newby (1984) later envisioned the physiological role of adenosine to be that of a "retaliatory metabolite", which refers to the tendency of adenosine to provide feedback inhibitory effects on cell functions.

Sattin and Rall (1970) provided the first evidence that the actions of adenosine were receptor-mediated and that these actions could be blocked by the methylxanthines, caffeine and theophylline. This latter finding provided researchers with an effective means with which to study adenosine receptor-mediated effects in a variety of mammalian tissues. Thus began, what may be considered, the modern era of adenosine research.

2. Adenosine Receptors

Sattin and Rall (1970) demonstrated that adenosine, through its actions on cell-surface receptors, stimulated cyclic AMP formation in the brain. Fain et al. (1972) later

found that adenosine inhibited cyclic AMP generation in fat cells. Thus, the stimulation and inhibition of cyclic AMP production provided an early biochemical means with which to identify and classify adenosine receptors. Burnstock (1978) classified purine receptors into two broad categories; adenosine (P1) receptors where cyclic AMP production was affected by receptor activation. and ATP (P2) receptors where cyclic AMP was not involved. P1 receptors were later divided into two subtypes of adenosine receptors, A1 and A2, based upon the ability of adenosine or its analogous to inhibit (through A1 sites) or stimulate (through A2 sites) the activity of adenylate cyclase (Londos and Wolff, 1977; Van Calker et al., 1979). More recently, additional subtypes of adenosine receptors have been identified using pharmacological approaches with more selective adenosine receptor agonists and antagonists as well as by molecular cloning methods. The receptors for adenosine are now known to be widely distributed throughout the nervous system as well as in other tissues where they subserve a variety of functions.

2.1. Pharmacological Classification of Adenosine Receptors

Pharmacological classifications of adenosine receptors are commonly based on the rank order of potencies of adenosine receptor agonists. The most frequently used agonists include N^6-substituted adenosine derivatives such as N^6-phenylisopropyladenosine (PIA), N^6-cyclohexyladenosine (CHA) and N^6-cyclopentyladenosine (CPA), as well as the N-alkylcarboxamino derivative such as 5'-N-ethylcarboxamidoadenosine (NECA). With the agents, the rank order of potency at A1 receptors was found to be R-PIA > adenosine > NECA, and at A2 receptors was NECA > adenosine $\geq$ R-PIA. However, NECA was found to have a high affinity for A1 receptor, which made it difficult to differentiate A2 from A1 receptors. In this regard, the stereoenantiomers of PIA (R-PIA and S-PIA) were used as an additional criterion for the identification of A1 and A2 receptors because R-PIA was much more potent at A1 sites than was S-PIA. More recently, 2-(*p*-2-carboxyethyl)phenethylamino)-5'-N-

ethylcarboxaminoadenosine (CGS 21680), a relatively specific agonist for A2 receptors was introduced for the identification of A2 receptors (Hutchison et al., 1989). Thus, pharmacologically, in rat for example, A1 receptors display a rank order of agonist potencies of CPA > CHA ≥ R-PIA > NECA > S-PIA > CGS 21680, and A2 receptors have a rank order of NECA = CGS 21680 > R-PIA > CHA = CPA > S-PIA.

Adenosine receptor antagonists have also been introduced for the identification of A1 and A2 receptors. 1,3-Dipropyl-8-cyclopentylxanthine (DPCPX) is one of the most commonly used selective antagonists for A1 receptors. Relatively specific antagonists for A2 receptors have also been identified including 3,7-dimethyl-l-propargylxanthine (DMPX) and triazoloquinazoline (CGS 15943A) (Williams et al., 1987; Daly et al., 1988). However, both DMPX and CGS 15943A only have limited selectivity for A2 over A1 receptors.

A2 receptors were further divided into two subclasses, A2a and A2b, based on substantial differences in binding affinities for agonists (Daly et al., 1983; Bruns et al., 1986). N^6-Methyl adenosine (MADO) and 2-(p-methoxyphenyl) adenosine (CV 1674) are two agonist ligands that differentiate between the two A2 receptor subtypes: for A2a receptors, CV1674 is more potent than MADO; for A2b receptors, the order is reversed (Williams, 1990).

A third adenosine receptor subtype, designated as A3, was proposed on the basis of the inhibitory effects by adenosine on Ca^{2+} entry into nerve endings and atrial cells (Ribeiro and Sebastião, 1986). The existence of this kind of A3 receptor remains to be proven. In the mean time, another type of adenosine receptor, designated as an A3 receptor as well, was identified on the basis of molecular cloning (Tucker and Linden, 1993). A selective agonist, 3-IB-MECA (N^6-(3-iodobenzyl)-5'-N-methylcarboxamidoadenosine) has recently been introduced for the cloned A3 receptor (Jacobson et al., 1993).

A novel A4 subtype of adenosine receptor has been recently characterized both pharmacologically and electrophysiologically. This receptor may be involved in the activation of K^+_{ATP} channels (Tucker and Linden, 1993).

2.2. Cloned Adenosine Receptors

A1 receptor: Libert et al. (1989) first cloned two orphan receptors RDC7 and RDC8 from a canine thyroid cDNA library. RDC7 was later identified as the canine A1 adenosine receptor (A1AR) on the basis of [^{3}H]CHA binding results and inhibition of forskolin-stimulated adenylate cyclase activity (Libert et al., 1991). A1AR of rat (Reppert et al., 1991; Mahan et al., 1991), bovine (Tucker et al., 1992; Olah et al., 1992), and rabbit (Bhattacharya et al., 1993) were subsequently cloned. The cloned A1ARs from these three species were found to be 90% homologous. More recently, a human brain hippocampal A1AR has been cloned and activation of the A1AR was found to cause inhibition of adenylate cyclase activity (Libert et al., 1992; Townsend-Nicholson and Shine, 1992).

A2a receptor: The thyroid RDC8 cloned by Libert et al. (1989) was suggested to be an A2a adenosine receptor (A2aR) mainly on the basis of findings that the brain distribution of RDC8, as demonstrated by *in situ* hybridization, was similar to the distribution of A2a receptor binding sites as demonstrated with [^{3}H]NECA and [^{3}H]CGS 21680. RDC8 was further confirmed to be an A2aR based on measurements of adenylate cyclase activity (Maenhaut et al., 1990). Rat brain A2aR was cloned and it shared an 82% homology with that of canine thyroid A2aR (Fink et al., 1992; Chen et al., 1992). More recently, A2aR from human hippocampus was cloned and expressed in a kidney cell line, and CGS 21680 was found to stimulate adenylate cyclase activity (Furlong et al., 1992).

A2b receptors: Stehle et al. (1992) cloned A2b receptor (A2bR) from rat brain. The cloned A2bR shared a 50% homology with A1AR and A2aR. A human hippocampal A2bR was cloned and expressed, and activation of the A2bR increased the activity of adenylate cyclase (Pierce et al., 1992). In another study, activation of A2bR expressed in *Xenopus* oocytes produced increases in Ca^{2+}-dependent chloride conductance (Yakel et al., 1993).

A3 receptors: Meyerhof et al. (1991) cloned an orphan receptor (TGPCR) from rat testes that shared ~50% homology with A1 and A2a receptors. TGPCR was later confirmed to be an unique adenosine receptor and accordingly was designated as an A3 receptor (A3AR) (Zhou et al., 1992). The cloned A3AR is different from the pharmacologically defined A3 receptors as originally envisaged by Ribeiro and Sebastião (Linden 1993). Sheep and human A3ARs have recently been cloned (Linden et al., 1993; Sajjadi and Firestein, 1993) and both share very low degrees of sequence homology to the cloned rat A3AR. The sheep A3AR was found to be widely distributed in different tissues, being most abundant in lung, spleen and pineal gland, and less so in brain, kidney and testes (Linden et al., 1993). The human A3AR was highly expressed in lung, liver, kidney, and heart, but very little expression was found in brain and muscle (Sajjadi and Firestein, 1993). Functionally, A3ARs appear to be involved in the regulation of inositol trisphosphate (IP_3)-induced increases in levels of intracellular Ca^{2+} (Ramkumar et al., 1993).

2.3. Adenosine Receptor Structures

Although the amino acid sequences of adenosine receptor subtypes are different, cloned adenosine receptors do share some common characteristics (Figure 1). Adenosine receptors identified to date are all composed of a single subunit with a molecular mass ranging from 35 to 46 kDa. Structurally, the receptors have seven hydrophobic membrane

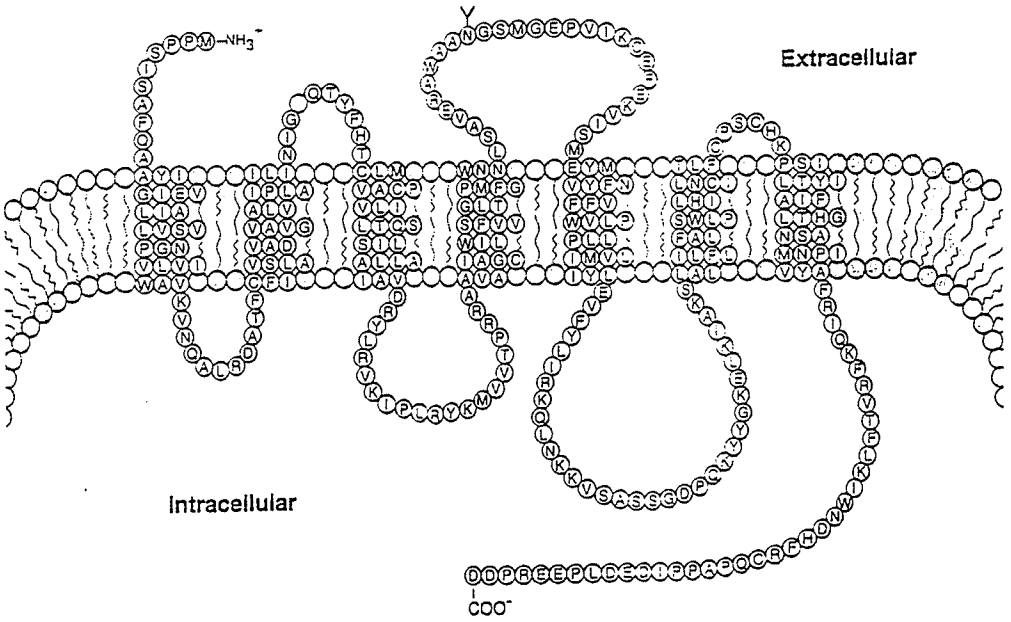

Figure 1. Deduced amino acid sequence and structure of the human A1 adenosine receptor. The receptor is a member of the large of guanine nucleotide-binding protein-coupled receptors that span the plasma membrane seven times. The ligand binding pocket is thought to be formed by amino acids in transmembrane segments 2, 3, and 7. GTP-binding proteins are thought to interact with juxtamembranous of intracellular loop 2 and 3 and carboxyl terminals (Taken from Linden, 1993).

spanning segments (Libert et al., 1989; Linden, 1991). All adenosine receptors contain sites which can interact with guanine nucleotide-binding proteins (G-proteins). Thus, adenosine receptors are members of the large family of G-protein-coupled receptors that includes receptors for other neuroactive substance such as norepinepherine, GABA, opioids, neuropeptide Y, and cannabinoids (Nestler and Duman, 1993).

2.4. Effectors of Adenosine Receptors

The effectors for adenosine receptors are listed in Table 1. G-proteins appear to be the primary effectors. G-proteins exist in multiple families including Gs, Gi, and Go. Gs couples membrane receptors to adenylate cyclase and results in stimulation of adenylate cyclase activity. Gi couples membrane receptors to adenylate cyclase as well, but causes inhibition of adenylate cyclase activity. Gi also couples receptors to several other functional proteins, including ion channels, phospholipase C, and phospholipase A2. Similar to Gi protein, Go couples receptors to ion channel proteins, phospholipase C and phospholipase A2, but Go protein does not couple receptors to adenylate cyclase.

A1 receptors couple to Gi to inhibit adenylate cyclase activity and decrease cyclic AMP production. Through Gi/Go, A1 receptors open K^+ channels and inhibit N-type of Ca^{2+} channels. A1 receptors may be also coupled through Gi/Go to other effectors including phospholipase C, phospholipase A2, phosphodiesterase, glucose transport, Cl^- transport, and Na^+/Ca^{2+} exchange systems (Olsson and Pearson, 1990). A2 receptors (both A2a and A2b) are coupled through Gs to activate adenylate cyclase and increase cyclic AMP generation (Linden, 1993). Effectors for A3 receptors have not been clearly demonstrated. However, from studies on cloned A3 receptors, A3 receptors appear to couple to Gi (Linden, 1993), to inhibit adenylate cyclase activity (Linden, 1993), stimulate phospholipase C activity, increase the generation of IP_3, and increase levels of $[Ca^{2+}]_i$ (Ramkumar et al., 1993).

Table 1. Effectors of adenosine receptors

	Adenosine receptors			
	A1	A2a	A2b	A3
Effectors	Gi/Go proteins	Gs proteins	Gs proteins	Gi proteins
	↓ Adenylate cyclase	↑ Adenylate cyclase	↑ Adenylate cyclase	↓ Adenylate cyclase
	↑ K^+ Channels			↑ $[Ca^{2+}]_i$
	↓ Ca^{2+} Channels			
	↑↓phospholipase C			

(Taken from Linden, 1993 with modification).

2.5. Distribution of Adenosine Receptors in the CNS

Radioligand binding assays and receptor autoradiography with radiolabeled adenosine receptor agonists and antagonists (Geiger and Nagy, 1990; Fastbom et al., 1986) as well as *in situ* hybridization experiments with radiolabelled receptor cDNA probes (Reppert et al., 1991) have demonstrated that adenosine receptors are heterogeneously distributed among brain regions of human and several species of animals (Table 2). A1 receptors are widely distributed in the brain. The A1 sites are most abundant in the CA1 subregion of the hippocampus (Mahan et al., 1991) yet are also enriched in cerebellum, cortex, striatum, and thalamus (Fastbom et al., 1986; Fastbom and Fredholm, 1990; Fastbom et al., 1987). A2 receptors are located on smooth muscle and endothelial cells of cerebral vessels (McBean et al., 1988; 1989), as well as structures encompassing the basal ganglia (Martinez-Mir et al., 1991). A2a receptors are predominantly located in the striatum where they are co-localized with dopamine D2-receptors, as well as in the nucleus accumbens and the olfactory tuburcles (Fink et al., 1992). A2b receptors are concentrated in the pars tuberalis of pituitary gland (Linden, 1993). A3 receptors were found to be present at low levels in the cortex, striatum, and olfactory bulbs (Linden, 1993).

2.6. Receptor-Mediated Adenosine Functions in the CNS.

In the brain, adenosine is generally thought to regulate neuronal activity and cerebral blood flow. Under normal and resting conditions, adenosine appears to produce a "basal adenosinergic tone" which subtly regulates normal neuronal functions (Rainnie et al., 1994). "Basal adenosinergic tone" has been demonstrated in behavioral (Katimes et al., 1983; Snyder et al., 1981; Dunwiddie and Worth, 1982) and electrophysiological studies (Dunwiddie and Hoffer, 1980; Hass and Greene, 1988; Rainnie et al., 1994) where adenosine receptor antagonists or adenosine deaminase (ADA) produced effects opposite to those produced by adenosine. Under adverse conditions such as epilepsy, hypoxia or

Table 2. Brain distribution of adenosine receptors

Receptors	Distribution
A1	Hippocampus, cerebellum, cortex, striatum, thalamus
A2a	Striatum, nucleus accumbens, olfactory bulb
A2b	Pars tuberalis
A3	(Low levels) cortex, striatum, cerebellum, olfactory bulb

(Taken from Linden, 1993 with modification).

hypoglycemia, adenosine can function as a retaliatory metabolite (Newby, 1984) to prevent tissue damage and provide injured tissues with an environment for recovery (Haas et al., 1993). Thus, adenosine maintains CNS homeostasis between the metabolic demands of and energy supplies to cells and tissues.

2.6.1. A1 receptors

Activation of A1 receptors appear to depress neuronal firing as a result of plasma membrane hyperpolarization (opening of K^+ channel), and to inhibit neurotransmitter release through inhibition of Ca^{2+} channels. The functional consequences of these effects may differ depending on the brain region being studied. For example, Rainnie et al. (1994) have shown that the electroencephalographic arousal produced by mesopontine cholinergic neuronal discharge was tonically inhibited by adenosine. Their results provide the basic mechanism that the arousal effects of coffee (caffeine) and tea (theophylline) may be through blockade of adenosine receptors in mesopontine. In hippocampus, where adenosine A1 receptors are highly expressed, it has been proposed that A1 receptor activation may increase the effective signal-to-noise ratio for neuronal transmission and thus regulate neuronal plasticity (Mitchell et al., 1993). At the presynaptic level, activation of A1 receptors suppress Ca^{2+}-dependent release of various neurotransmitters including glutamate, norepinephrine, dopamine, serotonin, and acetylcholine (Fredholm et al., 1983).

2.6.2. A2a receptors

Neuronal cells: The behavioral effects of methylxanthines such as increased animal locomotor activity are similar to those caused by dopamine receptor agonists (Fuxe and Ungerstedt, 1974) and are thought to be mediated through blockade of A2a receptors co-localized with D2 receptors in the striatum. A2a receptors interact with D2 receptors on postsynaptic membranes and under normal conditions endogenous adenosine through

activation of A2a receptors is believed to maintain an inhibitory influence on D2 receptors. Methylxanthines which are inhibitors of A2a receptors can thus reverse the effects of endogenous adenosine, increase the affinity of D2 receptors for dopamine, and lead to activation of D2 receptors (Ferré et al., 1992; 1993a,b).

Cerebral arterioles: A2 receptors, presumably A2aRs, are distributed on brain parenchymal arteriole smooth muscle cells and are thought to mediate vessel dilation in response to adenosine and its analogs (Ngai and Winn, 1993). Endogenous adenosine, upon activation of A2aRs, may help maintain CNS homeostasis between the metabolic demands and energy supplies of cells and tissues. For example, under hypoxic conditions ATP is broken down and adenosine is subsequently released, causing cerebral vasodilatation. This dilation leads to an increase in the delivery of oxygen, which in turn decreases the degradation of ATP and thus returns adenosine concentrations to normal levels (Meno, et al., 1993).

2.6.3. A2b receptors

The physiological functions of A2b receptors remain unclear. In recent studies it was suggested that A2b receptors may participate in the regulation of neurotransmitter release (Simpson et al., 1992; O'Regan et al., 1992). In rat ischemic cerebral cortex, NECA, a non-selective adenosine receptor agonist, biphasically affected excitatory amino acid transmitter release (Simpson et al., 1992); NECA at low concentrations (10^{-8} M) inhibited release through activation of A1 receptors, whereas at high concentrations (10^{-5} M) NECA increased release through activation of A2b receptors (O'Regan et al., 1992).

2.6.4. A3 receptors

The newly developed A3 selective agonist 3-IB-MECA was found to depress locomotor activity in mice (Jacobson et al., 1993). 3-IB-MECA also caused other behavioral

changes such as rapid scratching behavior (Jacobson et al., 1993). These effects of A3 receptor activation appear to be associated with their expression in cerebellum and striatum.

3. Endogenous Adenosine

3.1. Levels of Endogenous Adenosine

The extracellular levels of endogenous adenosine in the CNS have been determined biochemically (Clark and Dar, 1988), pharmacologically (Dunwiddie and Diao, 1994), and through microdialysis (Van Wylen, 1988; Ballarin et al., 1991) and cortical cup sampling techniques (Phillis et al., 1988, 1989). Under basal (resting) conditions, adenosine concentrations range variably from nanomolar to low micromolar depending on the techniques used. Using biochemical methods and freeze-blowing or microwave irradiation to rapidly inactivate brain metabolism, levels of 0.5-10 μM were measured (Lewin and Bleck, 1981; Clark and Dar, 1988; Schrader et al., 1980; Nordstrom et al., 1977; Winn et al., 1980; Wojcik and Neff, 1982; Dunwiddie et al., 1981; Fredholm et al., 1984; Newman and McIlwain, 1977). In microdialysis studies, the levels of extracellular adenosine ranged from 40 nM to 2 μM (Hagberg et al., 1987; Dux et al., 1990; Zetterstorm et al., 1982; Ballarin et al., 1991; Van Wylen et al., 1988; Sciotti and Van Wylen, 1993a; 1993b). Using the cortical cup technique, lower levels of extracellular adenosine concentrations (15-50 nM) were noted (Phillis et al., 1988; 1989; 1993). Extracellular adenosine levels of 140-200 nM were estimated on the basis of dose-response relationships for adenosine inhibition of evoked neuronal excitatory responses (Dunwiddie and Diao, 1994). Adenosine concentrations in the hundreds of nanomolar range are now generally believed to represent basal extracellular adenosine concentrations (Table 3).

Under conditions where there is either an increase in neuronal activity or a decrease in metabolic supply the levels of extracellular adenosine consistently increase.

Table 3. Adenosine levels in brain

Intervention	Species	Adenosine (μM)	
		Basal	Stimulated
Cerebral concussion	Rat	0.2[a]	14[a]
K+ depolarization	Rat	0.9-1.1	2
Bicuculine seizures	Pig	0.5	3.5
Hypoxia	Rat	1-2	5
	Pig	1.3	2.1
Global ischemia	Rat	1.9	40
	Rat	0.3[a]	5.5[a]
Middle cerebral artery occlusion	Rat	0.1[a]	2.7[a]
Basal (chronic implantation)	Rat	0.05-0.2	-

Basal levels of adenosine after chronic implanation of microdialysis probe represent real basal adenosine levels. [a]Correction for recovery not clearly stated. Values should probably be multiplied by a factor of 3-10 (Taken from Rudolphi et al.,1992a).

Table 3 lists the extracellular adenosine levels in brain before and after different interventions (Rudolphi et al., 1992a,b). For example, under conditions of bicuculine-induced seizures, extracellular adenosine concentrations increased by 7-fold from a resting level of 0.5 μM to 3.5 μM. Brain hypoxia resulted in a 5-fold increase from 1 to 5 μM. The most pronounced increase of extracellular adenosine was found in global brain ischemia where 20-fold increases to 40 μM were observed (Table 3).

3.2. Sources of Endogenous Adenosine

3.2.1. Intracellular formation and release of adenosine

Adenosine release has been studied *in vitro* using such preparations as brain slices, synaptosomes, and cultured brain cells. Depolarizing agents, electrical stimulation, and excitatory amino acids have all been found to increase adenosine release (Pons et al., 1980; Jonzon and Fredholm, 1985; MacDonald and White, 1985; Hoehn and White, 1990a,b; Pedata et al., 1990). Although adenosine release has been shown to be at least partially Ca^{2+}-dependent, there is no evidence that adenosine is located within synaptic vesicles and released by exocytosis (White and MacDonald, 1990).

Adenosine release may be initiated when the levels of adenosine are higher intracellularly than extracellularly. Intracellular adenosine then flows down the concentration gradient. The increases of intracellular adenosine concentrations may occur under a variety of pathological conditions including seizures (During and Spencer, 1992), glucose deprivation, sustained hypotension and hypoxia, as well as cerebral ischemia (Daval et al., 1991; Park et al., 1988). The major source of intracellular adenosine appears to be break down of intracellular ATP to AMP, and AMP may further degrade to adenosine by the action of cytosolic 5'-nucleotidase enzymes (Figure 2). Another potentially significant metabolic pathway (Figure 2) involved in the production of adenosine is hydrolysis of S-adenosylhomocysteine (SAH) to adenosine by the enzyme SAH hydrolase (Snyder, 1985). However, adenosine produced from this pathway is

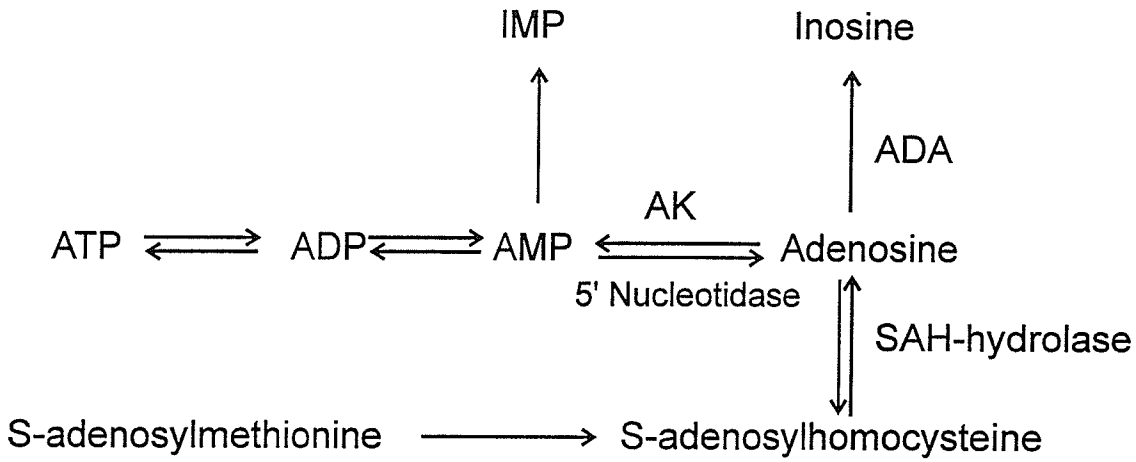

Figure 2. Major pathway of intracellular adenosine production and metabolism. (ADA) adenosine deaminase; (AK) adenosine kinase; (SAH-hydrolase) S-adenosylhomocysteine hydrolase.

limited because the levels of SAH are low and strictly controlled in tissues (Snyder, 1985). Although of lesser importance to intracellular adenosine levels, other possible pathways include *de novo* synthesis of adenosine from 5'-IMP through succinate synthetase and lyase, adenosine synthesis from adenine through adenine phosphoribosyl transferase, adenosine formation from 3'-AMP derived from the breakdown of RNA, the condensation of adenine with ribose-1-phosphate through purine nucleoside phosphorylase, and breakdown of DNA or cyclic AMP (Geiger and Nagy, 1990).

3.2.2. Formation of adenosine from released nucleotides

ATP may be released from purinergic neurons as a co-transmitter together with other neurotransmitters (Burnstock, 1972; Burnstock, 1986). The released ATP may then be metabolized by ecto-5'-nucleotidase to adenosine. The degree to which extracellular adenosine is formed from released ATP is variable and depends on the ecto-5'-nucleotidase activity of the cells and on the stimulation paradigm used. Evidence that this mechanism contributes to the extracellular levels of adenosine includes findings that induction of ecto-5'-nucleotidase activity with phenobarbital resulted in increased levels of extracellular adenosine (Jensen and Jacobsen, 1987). Hypothyroidism enhanced brain 5'-nucleotidase activity thereby enhancing adenosine formation from released ATP (Mazurkiewicz and Saggerson, 1989). Inhibition of ecto-5'-nucleotidase resulted in decreased levels of extracellular adenosine (MacDonald and White, 1985; Richardson et al., 1987). Auto-inhibitory cholinergic cortical terminals that lack ecto-5'-nucleotidase could not form adenosine from released ATP (Richardson et al., 1987). The age-related changes of ecto-5'-nucleotidase in different brain regions of rats may account for differences in the source of extracellular adenosine (Fuchs et al., 1991). With regard to the effects of different stimulation types on the sources of extracellular adenosine, it was found that adenosine was released as such rather than by metabolism of released nucleotides when synaptosomes were depolarized with 50 mM K^+. However, about half

of the veratridine-evoked increases in extracellular adenosine were due to metabolism of released nucleotides (MacDonald and White, 1985).

3.2.3. Regulation of endogenous adenosine levels

The extracellular levels of endogenous adenosine are regulated by two major mechanisms, metabolism and transport. Each of these mechanisms plays a role with temporal and/or spatial differences.

Adenosine is metabolized intracellularly by several enzymes (Figure 2), including adenosine kinase (AK), adenosine deaminase (ADA), and S-adenosylhomocysteine hydrolase (SAH-hydrolase). AK is a cytosolic enzyme that phosphorylates adenosine to adenine nucleotides including AMP, ADP and ATP. AK has an affinity (Km) for adenosine in the low micromolar range and is saturated at near physiological concentrations of adenosine (Phillips and Newsholme, 1979; Schrader, 1983; Mistry and Drummond, 1986). At higher concentrations of adenosine, substrate inhibition of AK activity has been observed (Fisher and Newsholme, 1984). Physiologically, adenosine phosphorylation appears to play an important role in purine salvage mechanism. ADA exists in two forms, cytosolic ADA and ecto-ADA. Cytosolic ADA deaminates adenosine to inosine, a metabolite that is virtually inactive at adenosine receptors. The Km of ADA for adenosine is about 40 μM (Lupidi et al., 1992; Geiger and Nagy, 1987; Centelles et al., 1988). Accordingly, it has been proposed that adenosine metabolism through deamination may be predominant only when adenosine concentrations are in the supra-physiological range. Thus, in conditions such as ischemia, when large amounts of adenosine are formed as a result of ATP hydrolysis, deamination of adenosine results in increased levels of inosine. Ecto-ADA has been found in a few cell types where it may function to metabolize adenosine to inosine extracellularly, but in the CNS the presence of ecto-ADA has not been clearly demonstrated and it was suggested that extracellular metabolism of adenosine may play a very minor role in the regulation of extracellular

levels of endogenous adenosine in the CNS (Andy and Kornfeld, 1982; Hellewell and Pearson, 1983; Meghji et al., 1988). Another pathway of adenosine metabolism is through condensation with homocysteine to form S-adenosylhomocysteine (SAH). The enzyme responsible for this reaction, SAH-hydrolase, is intracellularly located and widely distributed throughout the CNS (Schatz et al., 1977; Broch and Ueland, 1980; Gharib et al., 1983). Adenosine metabolism through this pathway appears to be limited by the availability of intracellular L-homocysteine: increases in homocysteine concentration greatly enhanced adenosine metabolism through this pathway and decreased levels of endogenous adenosine (Sciotti and Van Wylen, 1993b; Lloyd et al; 1993). The intracellular metabolism of extracellular adenosine decreases levels of intracellular adenosine and thus decreases the adenosine available for release.

Adenosine transporters appear to mediate both uptake and release of adenosine. Therefore the transporters may participate in the regulation of adenosine levels. This notion is strongly supported by findings that adenosine transport inhibitors can increase adenosine levels by inhibiting adenosine influx or decrease adenosine levels by inhibiting adenosine efflux. Details on the role of adenosine transporters in adenosine influx will be discussed in section 5.1 and 6, and in efflux will be discussed in section 10.

4. Adenosine-Based Therapeutic Strategies

4.1. Adenosine Receptor Agonists

In animal models, adenosine and its analogs have been tested as possible treatments for seizures, neuronal injury secondary to cerebral ischemia, anxiolytic diseases, and other CNS disorders (Marangos et al., 1990; Zhang et al., 1993; Von Lubitz et al., 1988; Von Lubitz and Marangos, 1990; Daval et al., 1991). Use of adenosine receptor analogs, especially the receptor subtype specific agents, appears to be the most direct and powerful approach of all adenosine-based therapeutics (discussed below). However, the clinical applications of adenosine agonists for CNS diseases are limited

because of the potential peripheral side effects carried by adenosine agonists when administered parenterally and because most adenosine derivatives have only a limited ability to penetrate the blood brain barrier (Brodie et al., 1987).

4.2. Adenosine Kinase and Deaminase Inhibitors

Inhibition of adenosine metabolism could theoretically raise intracellular adenosine concentrations thus allowing more adenosine to be "released". 5'-Iodotubercidin, an AK inhibitor, was found to significantly increase basal levels of adenosine and cerebral blood flow (Sciotti et al., 1993). The AK inhibitors 5'-amino-5'-deoxyadenosine and 5'-iodotubercidin were highly efficacious as anticonvulsants with ED_{50} values in the nanomolar range when administered via intracranial microinjection (Zhang et al., 1993). The ADA inhibitor erythro-2-(2-hydroxy-3-nonyl)adenine (EHNA) also increased basal levels of adenosine and cerebral blood flow (Sciotti et al., 1993; Meno et al., 1993). Under hypoxia/ischemia conditions, the ADA inhibitors 2'-deoxycoformycin (DCF) and EHNA both potentiated adenosine "release" (Phillis et al., 1988). DCF was shown to effectively reduce ischemia-induced damage in the hippocampus of Mongolian gerbils (Phillis and O'Regan, 1989) and to protect against focal ischemic brain injury in rat (Lin and Phillis, 1992).

Acadesine (5-amino-4-imidazolecarboxamide ribose, AICAR), a purine precursor, was found to increase adenosine levels near the sites where, and during the periods when, ATP breaks down as a result of ischemia or seizure activity (Gruber et al., 1989; Marangos et al., 1990). In rats, AICAR prevented cerebral ischemia (Clough-Helfman and Phillis, 1990) and inhibited homocysteine thiolactone elicited-seizures (Marangos et al., 1990). The mechanism by which acadesine increases adenosine levels may be related to its ability to inhibit AK, ADA, or AMP deaminase, however acadesine only weakly inhibited ADA and AK; Ki values were 0.46 and 2.3 mM, respectively (Baggott et al., 1986). The acadesine metabolite, acadesine ribotide (ZMP), was a weak inhibitor of AMP

deaminase (Ki ~ 1 mM) (Baggott et al., 1986). As such, ZMP blocked the conversion of AMP to inosine monophosphate (IMP) and channeled more ATP catabolism through AMP to adenosine (Mullane, 1993).

The use of adenosine metabolizing enzyme inhibitors may be expected to be somewhat less entangled by the global side-effects seen with the direct use of adenosine agonists. However, since purine enzymes are multi-functional and are involved in maintaining intermediary metabolism, the long term effects on cellular functions should be considered in the overall evaluation of this therapeutic approach (Marangos, 1991). Indeed, tubercidin and 2'-deoxycoformycin at high concentrations have been found to be cytotoxic (Cass et al., 1992).

4.3. Adenosine Transport inhibitors

Adenosine transport inhibitors (Figure 3), such as dipyridamole (2,6-bis (diethanolamino)-4,8 dipiperidinopyridino-(5,4d)-pyrine, DPR), nitrobenzylthioinosine (6-[(4-nitrobenzyl) mercapto]purine ribonucleoside, NBI or NBMPR) and dilazep (1,4-bis-[3-(3,4,5,-trimethoxybenzyl-oxy)propyl]perhydro-1,4-diazepine) have been found to increase extracellular levels of adenosine under pathological conditions (Phillis et al., 1989; Fowler, 1993; Meno et al., 1993) and less so under "normal" conditions (Phillis et al., 1989; Fowler, 1993). These findings suggest that adenosine transport inhibitors may be identified that selectively increase adenosine levels where (tissue selectivity) and during those time when (temporal selectivity) adenosine is being produced as a result of cellular stress or injury (Geiger and Fyda, 1991). In experimental animal models, adenosine transport inhibitors have been found to decrease cerebral ischemic injury, to prevent seizures, and to improve the "quality" of sleep (Wauquier et al., 1987). Adenosine transport inhibitors such as NBI, DPR, and dilazep were found not to cause cytotoxicity and may have less untoward side effects than may be observed with adenosine metabolizing enzyme inhibitors (Cass et al., 1992).

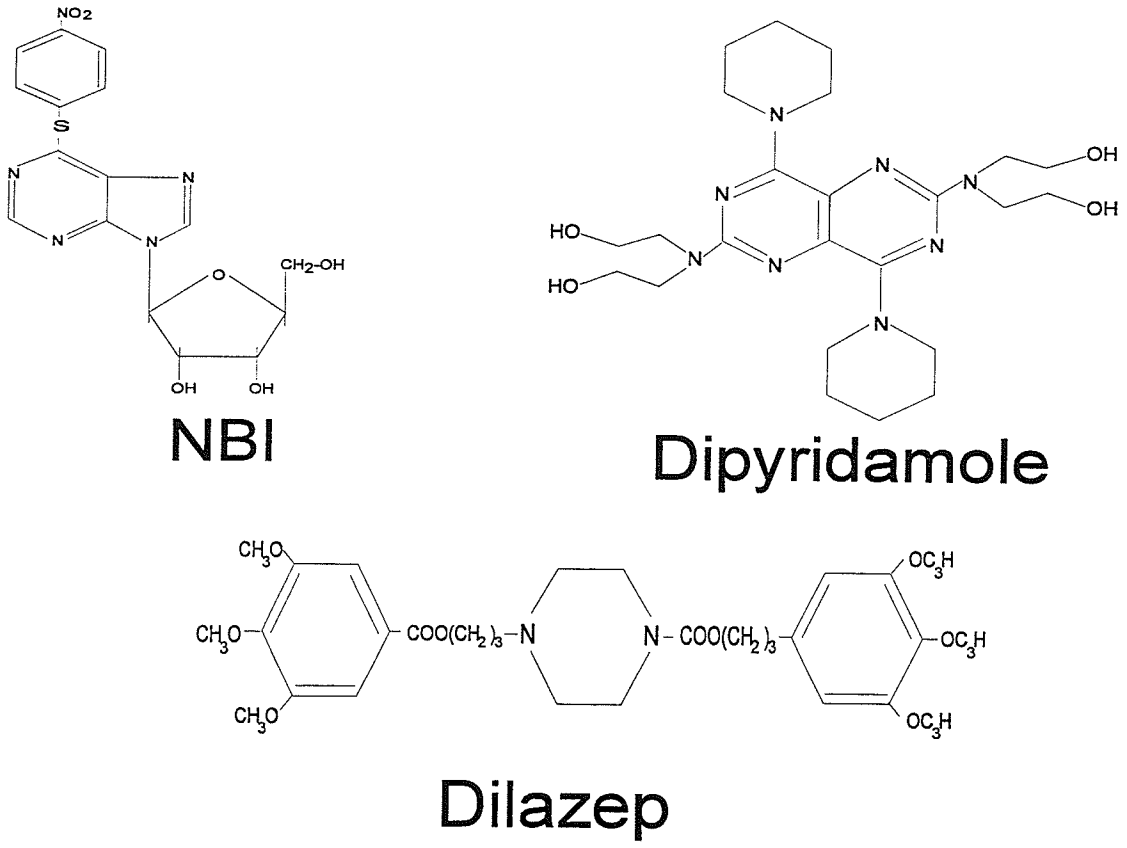

Figure 3. Chemical structures of adenosine transport inhibitors

4.4. Other Possible Approaches

A1 adenosine receptors have been shown to be allosterically modified such that the binding of ligands to A1 receptors was enhanced by (2-amino-4,5-dimethyl-3-thienyl)-[3(trifluoromethyl)-phenyl]methanone (PD 81,723) (Bruns and Fergus, 1990; Janusz et al., 1991). Functionally, PD 81,723 enhanced the ability of adenosine to inhibit neuronal excitability and epileptiform bursting in the hippocampal slice (Janusz et al., 1991; Janusz and Berman, 1993). Adenosine binding enhancers may represent a new strategy, but it is too early to predict whether this is a viable approach to the development of adenosine pharmaceuticals.

5. Adenosine Transport Systems

5.1. General Considerations

The extracellular levels of many neurotransmitters such as dopamine, norepinepherine, serotonin, glutamate, GABA and glycine are primarily controlled by their re-uptake from synaptic spaces into cells through specific transport systems. It is now accepted that adenosine transport systems are also an important mechanism by which the actions of extracellular adenosine are terminated. Such a concept for the role of adenosine transport systems is consistent with findings that (a) membrane adenosine transport systems are present in CNS tissues, (b) removal of adenosine from extracellular spaces occurs as a result of translocation of extracellular adenosine into the cells through adenosine transport systems, (c) inhibition of adenosine transport enhances extracellular adenosine levels, (d) most of the enzymes responsible for adenosine metabolism are located intracellularly and therefore elimination of adenosine by metabolism is secondary to adenosine transport, and (e) the removal of extracellular adenosine through extracellular metabolism is not significant because of the limited capacity of the ecto-enzymes. With the recognition of adenosine as an important CNS neuromodulatory substance whose actions are controlled by adenosine transport systems interest in

adenosine transporters has increased over the past 15 years. However, research in this area has progressed slowly because of the complexities associated with their characterization (Geiger and Fyda, 1991).

5.2. Strategies for Characterization of Adenosine Transport Systems

Adenosine transport systems have been characterized according to three basic research strategies: transport kinetics, binding studies, and molecular biology. Transport kinetic studies determine the rates and capacities of adenosine transport systems, as well as the ability of transport inhibitors to block the translocation of adenosine across plasma membranes. Therefore, this approach provides functional information of adenosine transport systems. Binding studies provide an alternative way to characterize the transporters with emphasis on locations and pharmacological subtypes of adenosine transporters. This approach is helpful in determining potencies with which various agents interact with binding sites on adenosine transporters. Molecular biology studies are focused to determine the structures of adenosine transporters and provide insights into mechanisms. All three approaches are inter-related, however this thesis is focused on kinetic characterization of adenosine transport in the CNS.

5.3. Kinetic Characterization of Adenosine Transport

5.3.1. Definition of transport

Transport denotes the transfer of an unmodified/unmetabolized substrate across the cell membrane as mediated by a specific transporter. It reflects the activity of the transporter carrying the substrate. Transporter-mediated substrate flux is different from simple diffusion of substances in that it is saturable and specific. Transport is also different from uptake in that transport only measures unmodified substrate accumulation while uptake measures the accumulation of substrate as well as any form of its metabolites.

5.3.2. Initial transport rates

In order to characterize adenosine transport, initial rates of transport must be measured. The initial transport rates denotes the velocity of unidirectional flux of substrate under conditions where there is no subsequent metabolism of substrate. Three strategies are commonly used. First, for cells capable of metabolizing adenosine, initial rates of substrate flux (transport rates) are measured using reaction periods as short as possible so that intracellular metabolism is insignificant. Second, cells are manipulated by pharmacological or genetic means to render cells incapable of metabolizing adenosine. Third, to use substrates for adenosine transporters that are metabolically stable nucleosides yet are recognized by the transporters with an affinity similar to adenosine. All strategies carry some disadvantages. For example, inhibitors that block adenosine metabolism may also interact directly with adenosine transporters and thereby interfere with the measurement of adenosine transport rates. The usefulness of other nucleosides as probes for determining adenosine transport activity in the CNS may be limited because nucleoside transporters appear to recognize adenosine with greater affinity over other nucleosides. The measurements of initial rates of adenosine influx in the CNS are often complicated by rapid influx of adenosine across cell membranes and subsequent metabolism by enzymes including AK, ADA, and S-adenosylhomocysteine hydrolase. Thus, sometimes the measured "apparent" rates for cellular accumulation of an isotope derived from labeled adenosine may not just reflect adenosine transport through the transporters, but in addition 1) passive diffusion through the plasma membrane, 2) metabolic trapping by conversion of adenosine to membrane impermeable nucleotides, and 3) efflux of adenosine and its deaminated products such as inosine or hypoxanthine. Where metabolism predominates, influx measurements reflect uptake (transport plus metabolism) rather than transport alone.

5.3.3. Experimental approaches to measurement of nucleoside transport kinetics

Initial rates of nucleoside transport in peripheral and CNS tissues are most commonly measured with radiolabeled substrate under conditions of zero-trans influx. The 'zero-trans' refers to the flux of radiolabeled substrate when the levels of substrate (labeled and unlabeled) present on the opposite side (the trans side) of the membrane are infinitely low compared to the side (cis-side) where substrate is added (Figure 4). In influx (transport) studies, cells are incubated with variable concentrations of radio-labeled substrates and intracellular accumulations of substrate are subsequently measured.

5.3.4. Transport kinetic parameters

When initial rates of zero-trans flux are measured, nucleoside transporter-mediated influx appears to be consistent with an "ordinary carrier model" (Krupka, 1989) and thus the transport process can be described by the following Michaelis-Menten equation.

$$v = Vmax[S]/([S] + K_T)$$

As in enzyme kinetics, Vmax is the maximum velocity, which reflects the maximal capacity of a transporter for a substrate, and K_T equals the substrate concentration at which the flux rate is half of Vmax. Experimentally, K_T and Vmax are determined by measuring initial rates (v) of a unidirectional (zero-trans) flux of a substrate at various concentrations (S). The Michaelis-Menten equation can be transformed into the following equation for ease of illustration as a double-reciprocal plot (Lineweaver-Burke plot).

$$1/v = 1/Vmax + K_T/Vmax[S]$$

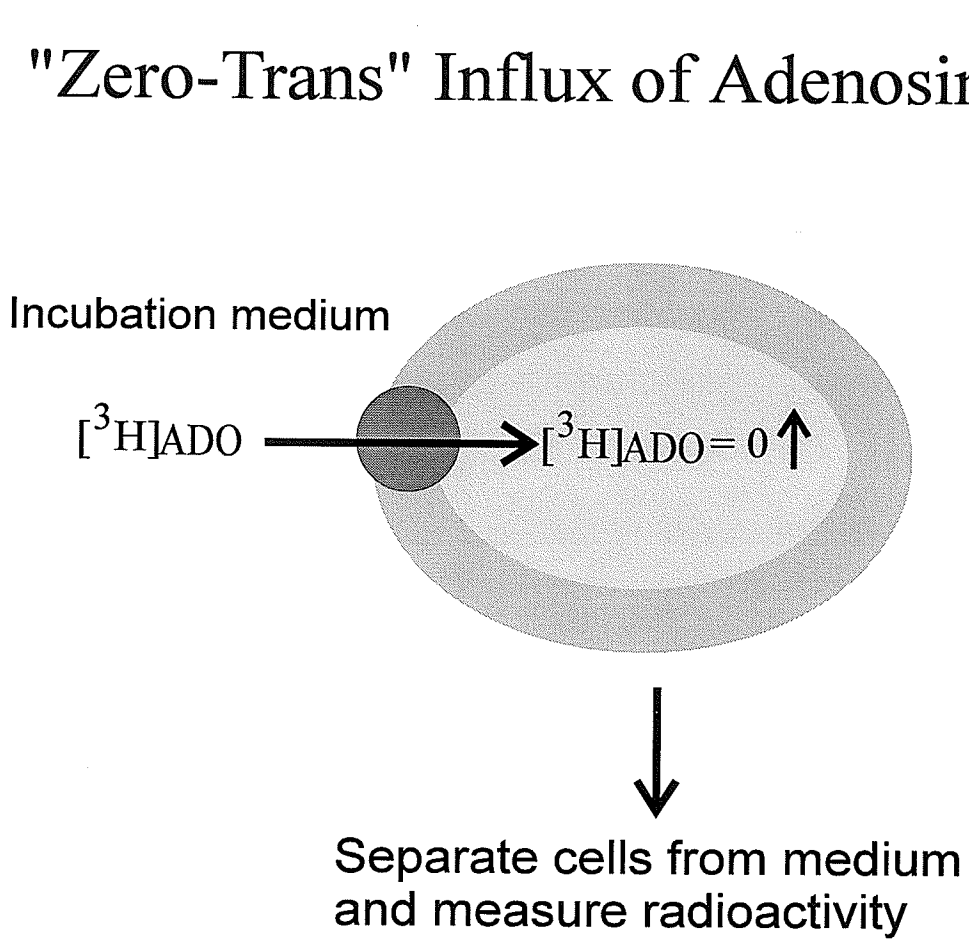

Figure 4. Measurement of "Zero-trans" influx of adenosine. Cells are incubated in medium containing radio labeled adenosine. Reactions are terminated by inhibitor-stop/centrifugation method. Initial rates of [^{3}H]adenosine influx are obtained with very short incubation times and under conditions where there is little metabolism of intracellular adenosine.

Plots of 1/v vs 1/[S] yield linear relationships and facilitate determinations of Vmax and K_T values.

6. Characteristics of Adenosine Transport Systems

6.1. Classification

Nucleoside transporters can be generally classified into two categories (Table 4), carrier-mediated equilibrative (facilitated) systems (Plagemann et al., 1988; Geiger and Fyda, 1991) and concentrative Na^+-dependent transporters (Johnston and Geiger, 1989; 1990; Plagemann et al., 1990a; Williams and Jarvis, 1991; Belt et al., 1993). Carrier-mediated equilibrative systems mediate substrate flux along concentration gradients, and zero net fluxes are reached when intracellular and extracellular nucleoside concentrations are equivalent. Equilibrative transport systems for nucleosides can be further classified into two subclasses, equilibrative NBI-sensitive ("es") and equilibrative NBI-insensitive (resistant) transporters ("ei"), according to their relative sensitivities to inhibition by the adenosine transport inhibitor NBI (Plagemann et al., 1988). For the NBI-sensitive transporter, influx of nucleosides is strongly inhibited by nanomolar concentrations of NBI. For the NBI-insensitive transporter, influx of nucleosides is only inhibited by micromolar concentrations of NBI. The Na^+-dependent nucleoside transporters mediate concentrative accumulations of substrates against a concentration gradient with Na^+ supplying the driving force. The concentrative Na^+-dependent transporters for nucleosides have been further classified into at least three subtypes, N1, N2, and N3, on the basis of substrate specificity. N1 (cif) is generally purine-selective; formycin B (7-hydroxy-3-ß-ribofuranosylpyrazolo(4,3-d)pyridine) serves as a model substrate (Vijayalakshmi and Belt, 1988; Crawford et al., 1990a). N2 (cit) is generally pyrimidine-selective; thymidine serves as a model substrate (Vijayalakshmi and Belt, 1988). N3 (cib) is not selective and accepts a broad range of purine and pyrimidine nucleosides as substrates (Wu et al., 1992; Huang et al., 1993). All nucleoside transporters accept

Table 4. Classification and properties of the nucleoside transporters

	Nucleoside transporters				
	Equilibrative		Concentrative		
	es	ei	N1	N2	N3
Na+-dependence	-	-	+	+	+
Inhibition by NBI	+	-	-	-	-
Inhibition by dipyridamole	+	+	-	-	-
Probable substrates:					
Adenosine	+	+	+	+	+
Formycin B	+	+	+	-	+
Inosine	+	+	+	-	+
Guanosine	+	+	+	-	+
Deoxyadenosine	+	+	+	+	+
Tubercidin	+	+	-	-	+
Uridine	+	+	+	+	+
Thymidine	+	+	-	+	+
Cytidine	+	+	-	+	+

(NBI) nitrobenzylthioinosine; (es) equilibrative sensitive; (ei) equilibrative insensitive (Taken from Linden et al., 1993)

adenosine as a substrate, thus nucleoside transporters are adenosine transporters (Table 4).

6.2. Equilibrative Transporters

The "es" and the "ei" have been identified to be products of separate genes (Belt and Noel, 1988), however, the two transporters exhibit similar substrate specificities and kinetic properties for permeation of different nucleosides (Plagemann and Wohlhueter, 1984). Of the two equilibrative transporter systems, the "es" system has been best characterized in mammalian peripheral cells. Some types of cells, such as human erythrocytes and S49 mouse lymphoma cells, express only "es" transporters (Plagemann and Woffendin, 1988; Plagemann and Wohlhueter, 1984), whereas other cells, such as Novikoff N1S1-67 rat hepatoma cell lines, express only "ei" transporters (Plagemann and Wohlhueter, 1984). For the majority of cells, such as Ehrlich ascites-tumor cells and mouse leukemia L1210 cells, both "es" and "ei" are present albeit in different proportions (Hammond and Johnstone, 1989).

In the CNS, equilibrative transport systems are present in many different cells and tissue preparations, including dissociated brain cells (Geiger et al., 1988), cultured mammalian CNS cell lines (Belt, 1983), cerebral cortical synaptosomes (Lee and Jarvis, 1988a, b; Shank and Baldy, 1990; Jones and Hammond, 1992), and cerebral cortical slices (Davies and Hambley, 1986). Equilibrative transport systems were found to account for ~ 80% of total adenosine transport in CNS cells (Bender et al., 1980; Banay-Schwartz et al., 1980). In rat and guinea-pig brain synaptosomes, about 40%-60% was found to be mediated by "es" system and the rest was through "ei" (Lee and Jarvis, 1988a; 1988b)

6.2.1. Substrate specificity

In some tissues, equilibrative transporters appear to display broad substrate specificities in that they can accept a variety of nucleosides as substrates (Plagemann and Wohlhueter, 1980). In other tissues such as human erythrocytes and some cultured cells the transporters appear to prefer adenosine over other nucleoside substrates (Paterson et al., 1984); in hepatoma cells the apparent affinities for adenosine were 1.5 to 2-fold higher than that for inosine and thymidine (Plagemann and Wohlhueter, 1980). In the CNS, adenosine transport in chick cerebral neurons has a relative affinity for adenosine that is 10 to 20-fold higher than that for inosine and thymidine, respectively. Nucleoside transporter selectivity for adenosine has also been demonstrated in cerebral cortical synaptosomes (Bender et al., 1980; Wu and Phillis, 1984), dissociated brain cells (Geiger et al., 1988), and cerebral microvessels (Stefanovich, 1983). The high selectivity of CNS nucleoside transporters for adenosine appears to be consistent with the concept that adenosine transporters are one of the major mechanisms responsible for controlling the extracellular levels and actions of adenosine .

6.2.2. Inhibitor sensitivity

As mentioned above, both "es" and "ei" transporter systems have been classified on the basis of their divergent sensitivities to inhibition by NBI (Morgan and Marangos, 1987b). However, structurally distinct transport inhibitors, including dipyridamole, dilazep and lidoflazine, block adenosine transport through "es" almost equipotently with inhibition of "ei" (Plagemann and Wohlhueter, 1984; Plagemann and Woffendin, 1988).

6.2.3. Kinetics

Affinity constants for nucleoside transport through equilibrative transport systems range from values in the tens to hundreds of micromolar depending on the type of cell studied. When adenosine was used as a substrate, human erythrocytes had K_T values of 60-100 μM and Vmax values of ~ 25 μM/s (Berlin and Oliver, 1975; Steck et al., 1969;

Mizel and Wilson, 1972; Lemkin and Hare, 1973; Quilan and Hochstadt, 1974; Strauss et al., 1977; Li and Hochstadt, 1976; Miras-Portugal, 1976; Trimble and Coulson, 1984; Kolassa et al., 1977; Plagemann et al., 1985; 1990b). When uridine was used as a substrate, erythrocytes were found to have K_T values of ~ 70 μM and Vmax values of ~ 40 μM/s (Plagemann and Wolffendin, 1989a; Plagemann et al., 1990b). Thus, the values of K_T and Vmax for nucleoside transport in erythrocytes are similar regardless of the substrate used. However, using formycin B as a substrate resulted in a higher K_T value of 223 μM while Vmax values of 29 μM/s remained similar to those obtained with adenosine and uridine (Plagemann et al., 1990b). In contrast, "es" transporters for adenosine had K_T values of 400 μM and Vmax values of 4.9 μM/s in mouse spleen lymphocytes (Plagemann et al., 1990a), and K_T values of 16 μM and Vmax values of 9.5 pmol/μl water/s in L1210/B23.1, a mutant cell line derived from mouse leukemia cell that only express "es" transporters. In human placental membrane vesicles from brush-border cells, K_T and Vmax values were found to be 142 μM and 1.9 pmol/mg protein/s, respectively.

Kinetic values for "ei"-mediated transport have been only rarely reported (Belt and Noel, 1985; Plagemann and Wohlhueter, 1980). When uridine was used as a substrate, "ei" transport in Walker 256 cells had a K_T value of 196 μM and a Vmax value of 21 pmol/s/10^6 cells (Belt and Noel, 1985). Transport kinetics of "ei" for uridine in Novikoff N1S1-67 rat hepatoma were similar to those reported above for Walker 256 cells (Plagemann and Wohlhueter, 1980).

Many of the studies on adenosine accumulation in CNS tissues were done with very long incubation times (>30 s) (Bender et al., 1980; 1981a, b; Barberis et al., 1981; Phillis et al., 1981; Phillis and Wu, 1983; Gonzales and Leslie, 1985) and as a result the measured kinetic parameters most likely represented adenosine uptake rather than transport. Accordingly, the "K_T" values found to be in the low micromolar range most

likely represented the affinity of metabolic enzymes and not transporters for adenosine (Table 5).

Where adenosine transport *per se* was measured (Thampy and Barnes, 1983a, b; Geiger et al., 1988; Johnston and Geiger, 1990), equilibrative nucleoside transport systems on neurons from primary cultures of chick brain were found to contain a high affinity component for adenosine with K_T values of 13 μM and Vmax values of 0.15 nmol/min/mg protein (Thampy and Barnes, 1983b), while on chick brain glia cells a low affinity system for adenosine with K_T values of 370 μM and Vmax values of 10.3 nmol/min/mg protein were measured (Thampy and Barnes, 1983a). Johnston and Geiger (1988) found that dissociated rat brain cells expressed a high affinity component with K_T values of 1.8 μM and Vmax values of 9.9 pmol/mg protein/15 s, and a lower affinity component with K_T values of 582 μM and Vmax values of 2213 pmol/mg protein/15 s.

The kinetic studies of nucleoside transport through "ei" can be measured in tissues treated with low nanomolar concentrations of NBI to block "es" system. Using this method, in rat cerebral cortical synaptosomes, uridine influx by "ei" component had K_T values of 214 μM and Vmax values of 16 pmol/s/mg protein and by "es" component had K_T values of 300 μM and Vmax values of 12 pmol/s/mg protein (Lee and Jarvis, 1988a). Similarly, in guinea pig cerebral cortical synaptosomes, kinetic values for adenosine influx were similar for both "es" and "ei" systems; for "es" and "ei" K_T values were 17 and 68 μM and Vmax values were 2.8 and 6.1 pmol/mg protein/s, respectively (Lee and Jarvis, 1988b).

6.3. Na^+- Dependent Transporters

Sodium-dependent transport of nucleosides has been demonstrated mainly in peripheral tissues (Belt et al., 1993). N1 transporters have been observed in mouse intestinal epithelial cells (Vijayalakshmi and Belt, 1988), L1210 mouse leukemia cells (Crawford et al., 1990a), mouse spleen lymphocytes (Plagemann and Woffendin, 1989b),

Table 5. Kinetic parameters for adenosine accumulation in preparations of nervous system tissues

Tissue preparation	Adenosine uptake		Assay condition		Reference
	K_m (μM)	V_{max}[a]	Time (s)	Temperature (°C)	
Rat					
Synaptosomes	0.9	10.5	60	37	Bender *et al.*, 1981
Synaptosomes	9	6	600	37	Premont *et al.*, 1979
Synaptosomes ($K_{T(H)}$)	1.0	1.7	900	37	Bender *et al.*, 1980
($K_{T(L)}$)	5.3	6.8	900	37	Bender *et al.*, 1980
Blood–brain barrier	18	NA	NA	NA	Cornford and Oldendorf, 1975
Capillaries	4.7	2.1	600	NA	Wu and Phillis, 1982
Dissociated cells ($K_{T(H)}$)	0.8	24.8	15	37	Geiger *et al.*, 1988
($K_{T(L)}$)	259	8868	15	37	Geiger *et al.*, 1988
Mouse					
Brain slices	140	75[b]	3600	37	Banay-Schwartz *et al.*, 1980
Astrocytes	3.4	360	3600	37	Hertz, 1978
Astrocytes	6.5	160	1800	37	Bender and Hertz, 1986
Neurones	6.1	110	1200	37	Bender and Hertz, 1986
Endothelium	5	1150	300	37	Beck *et al.*, 1983a
Smooth muscle	10	950	300	37	Beck *et al.*, 1983b
Dissociated cells ($K_{T(H)}$)	0.34	14	15	37	Johnston and Geiger, 1990
($K_{T(L)}$)	407	2452	15	37	Johnston and Geiger, 1990
Guinea-pig					
Synaptosomes	21	2573	1–30	30	Barberis *et al.*, 1981
Synaptosomes	17[c]	168	5	22	Lee and Jarvis, 1988b
Synaptosomes	68[d]	366	5	22	Lee and Jarvis, 1988b
Brain slices	19	NA	600	37	Shimizu *et al.*, 1972
Dissociated cells ($K_{T(H)}$)	1.5	18.4	15	37	Johnston and Geiger, 1990
($K_{T(L)}$)	488	7168	15	37	Johnston and Geiger, 1990
Other					
Torpedo electric organ	2	300	1800	22	Zimmermann *et al.*, 1979
Torpedo electric organ	2.4	17.3	1800	22	Meunier and Morel, 1978
Astrocytoma	1.0	140	600	NA	Lewin and Bleck, 1979
LRM55 Astroglia	1200	NA	30	NA	Shain and Madelian, 1987
LRM55 Astroglia	254	NA	600	NA	Shain and Madelian, 1987
LRM55 Astroglia	46	NA	1800	NA	Shain and Madelian, 1987
Chromaffin cells	1.0	~13[e]	60	22	Torres *et al.*, 1987
Chromaffin cells	4.6	66.7[e]	60	37	Delicado *et al.*, 1990
Bovine microvessels	1.9	0.2	600	37	Stefanovich, 1983
Chick glia					
(+ inhibitors)	370	10300	<25	37	Thampy and Barnes, 1983b
(− inhibitors)	12	340	600	37	Thampy and Barnes, 1983b
Chick neurones					
(+ inhibitors)	13	150	<25	37	Thampy and Barnes, 1983a
(− inhibitors)	6.4	160	<25	37	Thampy and Barnes, 1983a
Spiny lobster olfactory organ	7.1	0.6	900	22	Trapido-Rosenthal *et al.*, 1987

NA = data not available.
[a] All V_{max} values are expressed in units of pmol/mg protein/min unless otherwise indicated.
[b] μmol/ml tissue H_2O/min.
[c] Inhibitor-sensitive nucleoside transport system.
[d] Inhibitor-resistant nucleoside transport system.
[e] pmol/10^6 cells/min.

(Taken from Geiger and Fyda, 1991)

and peritoneal macrophages (Plagemann and Aran, 1990). N2 transporters have thus far been observed only in kidney and intestine (Vijayalakshmi and Belt, 1988; Williams and Jarvis, 1991). N3 was found to be present in human colon carcinoma cells and rabbit choroid plexus (Belt et al., 1991; Wu et al., 1991).

In the CNS, Na^+-dependent nucleoside transport which accounts for about 20-25% of the total amount of adenosine transported (Bender et al., 1980; Meunier and Morel, 1978) has been noted in, for example, cultured neurons and astrocytes of rat brain and spinal cord (Hösli and Hösli, 1988; Hertz and Matz, 1989), and dissociated brain cell preparations (Johnston and Geiger, 1989; 1990). In dissociated brain cell preparations from rat brain, Na^+ increased adenosine accumulations, and this Na^+-dependent transport was inhibited by ouabain, a Na^+-K^+/ATPase inhibitor, and by 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation. These results suggest that this transport system was an energy-dependent active transport process. Further characterization of these Na^+-dependent transporters has not yet been accomplished.

6.3.1. Na^+-Nucleoside stoichiometry

Na^+-dependent nucleoside transport systems are secondary active systems that require Na^+ as a co-substrate. N1 and N2 Na^+-dependent nucleoside transporters have Na^+:nucleoside stoichiometries of 1:1, and N3 has a 2:1 Na^+/nucleoside-coupling ratio (Gutierrez and Giacomini, 1993).

6.3.2. Substrate preference

The subtypes of Na^+-dependent nucleoside transport systems display substrate selectivity for nucleosides: N1 is purine selective, N2 is pyrimidine selective, and N3 accepts both purines and pyrimidines. However, adenosine and uridine are recognized with high affinity by all Na^+-dependent nucleoside transport systems identified to date. In the CNS, only adenosine was tested as a substrate of Na^+-dependent nucleoside transport

systems. Whether these transporters recognize other nucleosides remains to be investigated.

6.3.3. Inhibitor sensitivity

In general, Na^+-dependent nucleoside transport are not inhibited by nucleoside transport inhibitors at concentrations < 1 μM. In fact, adenosine transport inhibitors such as dipyridamole or NBI were found even to enhance concentrative accumulations of nucleosides when both Na^+-dependent and equilibrative transporters were co-localized on the same cells (Plagemann et al., 1990a; Crawford et al., 1990b). This enhancement was attributed to inhibition of nucleoside efflux through dipyridamole and NBI-sensitive equilibrative transporters while the nucleoside was pumped into the cells by the Na^+-dependent transporters (Plagemann et al., 1990b; Crawford et al., 1990b). However, the Na^+-dependent nucleoside transporter can be inhibited with high concentrations of phloridzin, an inhibitor of Na^+-linked glucose transport and Na^+/nucleoside co-transporters (Huang et al., 1993).

6.3.4. Kinetics

Only a few mutant cell lines, such as L1210/MA27.1 murine leukemia cells possess a single type of Na^+-dependent transporter (Crawford et al., 1990a). For the majority of cells, Na^+-dependent transport and equilibrative transporters co-exist on the same cells. Thus, the kinetic parameters of Na^+-dependent nucleoside transport are usually determined by two indirect methods. In one method, the transport rates at various concentrations of nucleosides are measured both in the presence and absence of NaCl, and the rate of Na^+-dependent transport is taken as the difference between the rates observed in the two conditions. Alternatively, Na^+-dependent nucleoside transport is measured with cells pretreated with high concentration of DPR to block Na^+-independent nucleoside transport (Plagemann et al., 1990a).

N1 transporters were the first Na^+-dependent transporters to be identified and have now been relatively well characterized. Using adenosine as a substrate, N1 transporters in L1210/AM27.1 cells exhibited K_T values of 9.4 μM (Dagnino et al., 1991). When formycin B was used as a substrate, N1 transporters had K_T values of 9 μM and Vmax values of 0.42 pmol/μl cell water/s in mouse spleen cells, K_T values of 45 μM and Vmax values of 4.4 pmol/10^6 cells/s in mouse intestinal epithelial cells, and K_T values of 6 μM in peritoneal rat macrophages. K_T values of 200 μM were measured in mouse spleen cells when uridine was used as substrate (Plagemann and Woffendin, 1989b). N2 Na^+/nucleoside transporters are less well characterized. In one study N2 of rabbit intestine was expressed in *Xenopus laevis* oocytes and kinetics was studied with thymidine as a substrate; K_T values of 36 μM and Vmax values of 15 pmol/oocyte per h were measured (Jarvis and Griffith, 1991). For N3 Na^+/nucleoside transporter, the kinetics remain to be investigated

In the CNS, Johnston and Geiger (1989, 1990) demonstrated that sodium significantly decreased the K_T in rat and left unchanged the Vmax values for both the high- and low-affinity adenosine transport systems. This study did not differentiate Na^+-dependent transport from equilibrative transport and thus the kinetic parameters obtained reflected integrated values of both Na^+-independent (equilibrative) and Na^+-dependent kinetics for adenosine transport. However, it can be predicted from this study that the K_T value for Na^+-dependent adenosine would be less than the integrated K_T value.

Both K_T and Vmax values of Na^+-dependent transporters are usually lower (5-10-times) than those of equilibrative transporter (Plagemann et al., 1990a). Thus, at high concentrations, nucleoside influx may be primarily through the equilibrative transporter. At low physiological concentrations, nucleosides may enter mainly through active transport system (Plagemann et al., 1990a).

7. Binding Studies with [^{3}H]NBI and [^{3}H]Dipyridamole

7.1. Adenosine Transport Sites on Cells

Partially because of the difficulties in directly measuring initial rates of adenosine transport, an alternative approach to the study of adenosine transport has been to use radioligand markers such as [^{3}H]NBI and [^{3}H]DPR for the pharmacological characterization of adenosine transport sites. NBI appears to bind at or near the extracellular permeant sites of "es" nucleoside transporters (Agbanyo et al., 1988) which are distinct from cell surface adenosine receptors (Hammond and Clanachan, 1983). The affinities of "es" transporter sites for [^{3}H]NBI are very similar for most cells or tissues studied to date (Plagemann and Woffendin, 1988). For example, the binding of [^{3}H]NBI to cerebral cortical membrane preparations from human, dog, guinea pig, rat, and mouse displayed similar Kd values between 0.16 - 0.44 nM and Bmax values between 128 - 196 fmol/mg protein. In most species such as human, dog, guinea pig, and rabbit, [^{3}H]NBI bound with affinities similar to its potencies for inhibiting nucleoside influx (Verma and Marangos, 1985; Hammond and Clanachan, 1984). Pharmacologically, the potencies with which other compounds inhibit [^{3}H]NBI binding correlated well with their ability to inhibit nucleoside transport (Hammond et al., 1983). Some adenosine transport inhibitors such as hexobendine and papaverine appear to inhibit adenosine influx by occupying high affinity sites for NBI while other transport inhibitors such as clonazepam and dipyridamole appear to inhibit adenosine influx through interaction at different sites (Wu and Phillis, 1982).

[^{3}H]DPR has been found to bind to cortical membranes of guinea pig with high affinity, but, in rat cortical membranes only very low affinity [^{3}H]DPR binding was observed (Marangos et al., 1985; Marangos and Deckert, 1987; Geiger and Nagy, 1990). [^{3}H]DPR bound to a greater number of sites than did [^{3}H]NBI, and [^{3}H]DPR binding was biphasically and incompletely inhibited by NBI (Marangos et al., 1985; Marangos

and Deckert, 1987). These findings suggest that [^{3}H]DPR may interact with both NBI sensitive and insensitive nucleoside transporters (Marangos and Deckert, 1987).

The usefulness of ligand markers especially [^{3}H]NBI for characterization of nucleoside transport sites in CNS cells has been questioned based on findings that the concentrations of NBI required to inhibit adenosine influx (~ 1 μM) was much greater than the concentrations used to saturate 50% of the binding sites (0.1 - 1 nM) (Phillis and Wu, 1983; Wu and Phillis, 1984). Furthermore, the potencies with which some adenosine transport inhibitors competed for [^{3}H]NBI did not agree closely with their potencies to inhibit nucleoside influx (Ogbunude and Baer 1990). Recently, new radioligands such as [^{3}H]dilazep and [^{3}H]R75231 ((±)-2-(aminocarbonyl)-N-4-amino-2,6-dichlorophenyl)-4-[5,5-bis(4-fluorophenyl)pentyl-1-piperazineacetamide, a lidoflazine analogue) have been synthesized and used for binding studies of nucleoside transporters (Gati and Paterson, 1989; IJzerman et al., 1992), but it is unclear whether these new radioligands represent better probes for CNS nucleoside transporters.

7.2. Distribution of adenosine transporters in the Brain

Through the use of autoradiography, the regional distribution of adenosine transport sites in brain has been determined using [^{3}H]NBI and [^{3}H]dipyridamole. Adenosine transport sites were found to be heterogeneously distributed in different brain regions with [^{3}H]DPR or [^{3}H]NBI as ligands (Geiger and Nagy, 1984; Nagy et al., 1985; Deckert et al., 1988a,b,c). Heterogeneity of adenosine transporters was also found in spinal cord (Geiger and Nagy, 1985). The distribution of adenosine transporters appears to coincide fairly closely with the regional distribution of ADA reactive neurons (De Carvalho et al., 1992; Geiger and Nagy, 1984). However, a close correspondence between the distribution of adenosine transporters and adenosine receptors was not observed (Deckert et al., 1988a).

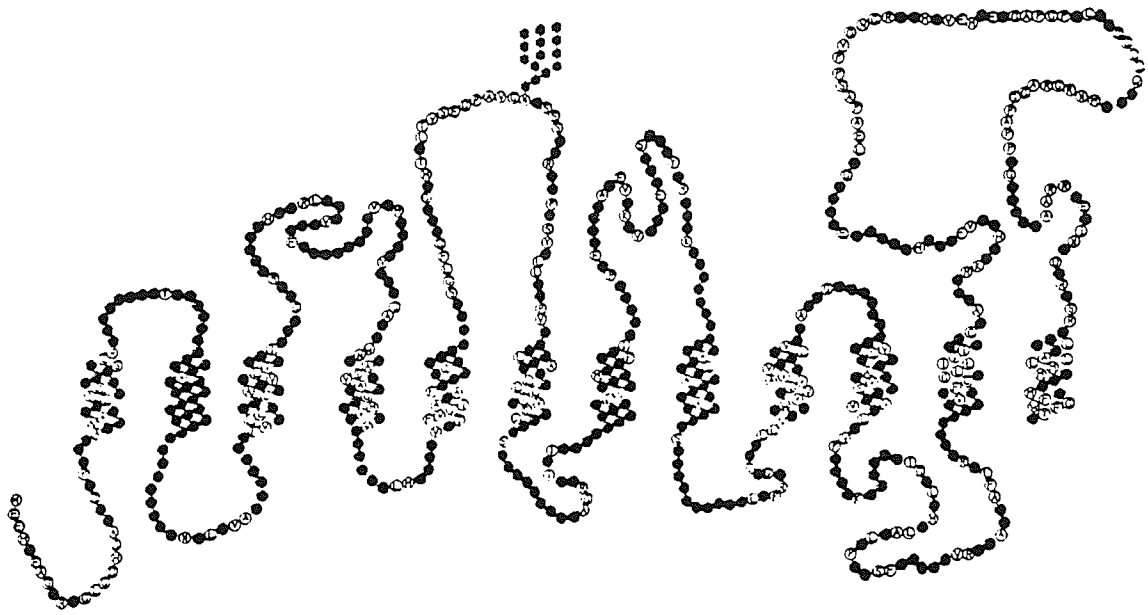

Figure 5. Predicted secondary structure model of SNST1 based on Kyte-Doolittle analysis. The model predicts 12 transmembrane spanning domains. Intracellular residues are shown below and extracellular residues above the putative transmembrane helices. Residues identical with SGLT1 are filled circles (Taken from Pajor and Wright, 1992).

Regional distribution of Na^+-dependent nucleoside transporters has not been studied due to the lack of a suitable ligand for these types of transporters. Recent advances in molecular cloning of Na^+-dependent transporters (see below, Pajor and Wright, 1992) may soon allow for determination of their regional distribution.

8. Molecular biology of nucleoside transporters

To date, one type of nucleoside transporter, the Na^+-dependent nucleoside transporter (SNST1), has been cloned (Pajor and Wright, 1992). SNST1 is a member of the Na^+/glucose co-transporter (SGLT) family. SNST1 shares 61% identity and 80% similarity with SGLT1. The transporter encodes a protein of 672 amino acids (Figure 5). On the basis of Kyte-Doolittle analysis (Kyte and Doolittle, 1982), it was predicted that the transporter had 12 transmembrane domains. Thus, the topology of the transporter is similar to transporters for dopamine, noradrenaline, and 5-HT (Amara, 1993). The mRNA of SNST1 was found in rabbit heart and kidney. The expression of this transporter in *Xenopus* oocytes resulted in Na^+-dependent [^{3}H]uridine influx (Pajor and Wright, 1992). Although not characterized in detail, the substrate selectivity of SNST1 showed similarities to the N3 activity expressed in oocytes microinjected with rat jejunal mRNA (Pajor and Wright, 1992; Huang et al., 1993).

Other Na^+-dependent nucleoside transporters have been functionally expressed in *Xenopus laevis* oocytes following microinjection of polyadenylated [polyA$^+$] mRNA isolated from both rat and rabbit intestinal cells (Huang et al., 1993; Griffith, 1991; Terasaki, et al., 1993). The transporters expressed from rat intestinal mRNA were characterized as N1 and N3 subtypes (Huang, et al., 1993), whereas, N2 subtype transporters were expressed when rabbit intestinal mRNA was injected into oocytes (Jarvis and Griffith, 1991; Terasaki et al., 1993). The expressed transporters were found to be functionally similar to their correspondent transporters on intact enterocytes and

membrane vesicles in terms of substrate selectivity, transport kinetics, and pharmacological properties.

The characteristics of "es" transporter protein have been studied using [^{3}H]NBI photoaffinity labelling techniques (Young and Jarvis, 1983; Wu and Young, 1984; Kwong et al., 1987). The "es" transporters on erythrocytes, leukemia cells, lung, heart and brain of several species have been isolated and identified as having apparent molecular weights of ~ 55000 (Wu and Young, 1984; Shi et al., 1984; Jarvis and Ng, 1985; Crawford et al., 1990c). The transporters appear to be commonly present as glycosylated forms (Hammond and Johnstone, 1989; Kwong et al., 1993). Purified "es" transporters reconstituted onto artificial membrane exhibited transport kinetic and pharmacological characteristics similar to the characteristics observed in intact cells (Crawford et al., 1990c).

9. Species Differences in Adenosine Transport

In the CNS, inter-species differences have been demonstrated for other adenosinergic components including adenosine receptors (Fastbom et al., 1986), ADA (Yamamoto et al., 1987) and 5'-nucleotidase (Lee et al., 1986). Species differences in adenosine transport systems in the CNS have been noted in terms of kinetics, inhibitor sensitivities, and ligand binding affinities.

9.1. Kinetics

Johnston and Geiger (1990) demonstrated species differences in adenosine transport on dissociated brain cell preparations from mouse, guinea-pig and rat (Table 6). For the high affinity equilibrative adenosine transporters (experiment without sodium), the rank order of K_T values for brain cells of these animals was guinea pig > rat > mouse, while the rank order of Vmax values was rat > guinea pig > mouse. In the same study,

Table 6. Kinetic Values for [^{3}H]Adenosine Transport in Dissociated Brain Cells of Mouse, Guinea-Pig and Rat

		K_T (μM)		Vmax (pmoles/mg protein/15 sec)	
Species	Sodium	High	Low	High	Low
Mouse	With	0.34 ± 0.1	407 ± 234	3.5 ± 0.7	613 ± 136[d]
	Without	0.31 ± 0.1	447 ± 206	2.7 ± 0.8	648 ± 197
Guinea-Pig	With	1.5 ± 0.5[a]	488 ± 259	4.6 ± 1.6	1792 ± 880
	Without	3.4 ± 1.6	474 ± 258	4.8 ± 2.1	1788 ± 860
Rat	With	0.9 ± 0.2[b]	313 ± 100	8.9 ± 1.6[c]	3428 ± 1054
	Without	1.8 ± 0.4[e]	582 ± 205	9.9 ± 3.0	2213 ± 772

[a] $P < 0.01$ guinea-pig v. mouse

[b] $P < 0.05$ rat v. mouse

[c] $P < 0.05$ rat v. guinea-pig or mouse

[d] $P < 0.05$ mouse v. guinea pig or rat

[e] $P < 0.05$ with sodium v. without sodium

Values represent mean ± SEM values from experiments conducted six times. Each experiment was performed in triplicate (Taken from Johnston and Geiger, 1990).

kinetic parameters measured in the presence of Na^+ were also found to vary among these species (Johnston and Geiger, 1990).

9.2. Inhibition of Nucleoside Influx by Transport inhibitors

In the CNS, NBI was approximately equipotent in inhibiting adenosine accumulation in, for example, rat and guinea pig synaptosomes (Morgan and Marangos, 1987). However, DPR, dilazep, and mioflazine showed significant species differences in their inhibitory potencies for adenosine and, as well, other nucleosides (Shank and Baldy, 1990). DPR was 6-fold more potent in inhibiting adenosine accumulation by synaptosomes prepared from guinea pig than from rat (Morgan and Marangos, 1987). These results are consistent with studies in peripheral cells. Uridine transport in human, pig, and rabbit cells was up to 10-times more sensitive to inhibition by DPR (IC_{50} ~ 50 nM) and dilazep (IC_{50} ~ 5 nM) than in mouse and rat cells. Uridine transport in human erythrocytes was strongly inhibited by lidoflazine (IC_{50} 10 nM), whereas that in both mouse and rat cells was highly resistant to inhibition (IC_{50} > 10, 000 nM) (Plagemann and Woffendin, 1988).

9.3. Ligand Binding

The species differences of adenosine transport systems were further revealed by binding studies (Verma and Marangos, 1985). Although the affinity and capacity of [^{3}H]NBI binding was similar for most cells or tissues (Plagemann and Woffendin, 1988; Verma and Marangos, 1985), inhibitory potencies of transport inhibitors exhibited remarkable species differences. For example, in human cerebral cortical membranes the inhibitory equilibrium dissociation rate constant, Ki, for DPR inhibition of [^{3}H]NBI binding was reported to be 2.7 nM, while Ki values in brain membranes from guinea pig, mouse and rat brains were reported to be 6.3, 82.9 and 1135 nM, respectively (Verma and Marangos, 1985). Thus, rat and mouse cortical membranes appear to contain a class of

high-affinity NBI binding sites that display a low affinity for DPR and other adenosine transport inhibitors (Hammond and Clanachan, 1985; Verma and Marangos, 1985; Marangos et al., 1982). The low potency of DPR as an inhibitor of the [^{3}H]NBI binding in rat does not appear to be restricted to rat brain tissues as the same finding was observed for rat heart (Williams et al., 1984) and lung (Shi et al., 1984)

10. Adenosine Efflux

Exocytosis from synaptic vesicles is an important mechanism by which neurotransmitters are released. The release of adenosine, however, is more complicated since adenosine may be not only released via exocytosis (although direct evidence of exocytotic release of adenosine is lacking), but also may be released through bidirectional nucleoside transport systems. Direct characterization of adenosine release is difficult because D-adenosine is metabolically unstable which prevents "loading" cells with adenosine and subsequently determining release characteristics. However, adenosine transporter-mediated release process may be studied by measuring zero-trans efflux of a metabolically stable substrate for nucleoside transporters (Figure 6).

10.1. Adenosine Efflux and Nucleoside Transport Systems

The "es" and "ei" transporters of many peripheral cells are bidirectional symmetric nucleoside transporters such that the transporters can mediate both influx and efflux of substrates at the same rate (Plagemann and Woffendin, 1989a). In human erythrocytes, efflux of uridine (Plagemann et al., 1982), formycin B (Plagemann and Woffendin, 1989a), and adenosine (Plagemann et al., 1990b) was found to be mediated through "es" transporters. In mouse spleen lymphocytes, where "es", "ei", and Na^+-dependent transporters were present, efflux occurred mainly through "es" with lesser involvement of "ei" transporters (Plagemann et al., 1990a). It appears that equilibrative transport systems for adenosine function not only to remove extracellular adenosine,

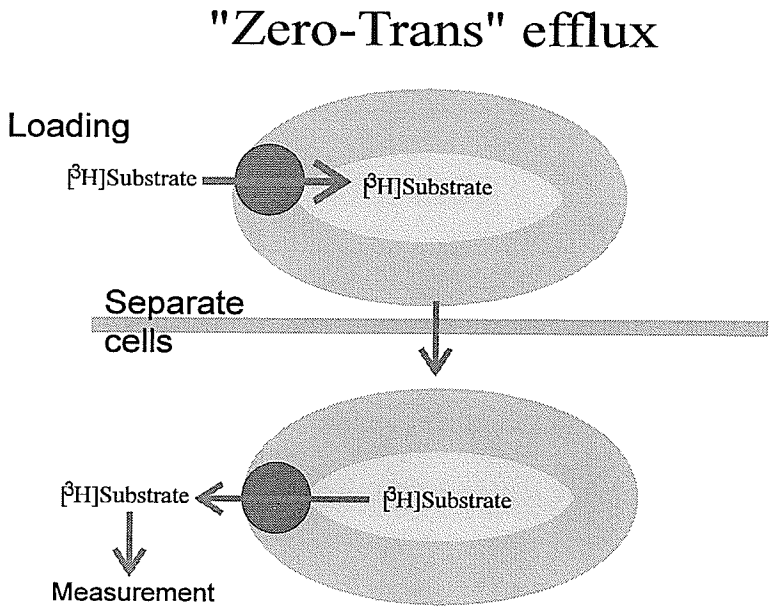

Figure 6. Measurement of "Zero-trans" efflux. Transporter-mediated efflux can be studied with cells preloaded with [^{3}H]-labelled substrate. Cells are then separated from loading medium and resuspended in buffer containing no substrate (zero trans). The subsequent release of [^{3}H]-labelled substrate is measured.

thereby terminating its effects and salvaging the purine, but also to provide a means of releasing adenosine, where it can exert its modulatory effects at adenosine receptors.

10.2. Effects of Adenosine Transport Inhibitors on Adenosine Efflux and Adenosine Levels

In peripheral cells, efflux of nucleosides through "es" transporters can be blocked with low concentrations of the nucleoside transport inhibitors DPR and NBI (Jarvis and Young, 1986; Plagemann et al., 1990a,b). In contrast, efflux through "ei" transporters requires concentrations of transport inhibitors several order of magnitudes higher (Jarvis and Young, 1986). In the CNS, nucleoside transport inhibitors decreased adenosine release and this suggests that adenosine efflux may be through equilibrative ("es" or "ei") transporters (Fredholm and Jonzon, 1981; Jonzon and Fredholm, 1985).

Since adenosine transport is bidirectional and adenosine transport inhibitors block both influx and efflux, the effects of adenosine transport inhibition on levels of extracellular adenosine are complicated (Figure 7). If transport inhibitors block influx to a greater degree than efflux (release), then adenosine transport inhibitors can enhance extracellular levels of endogenous adenosine. In many studies, it has been shown that adenosine transport inhibitors indeed increased adenosine levels (Pazzagli et al., 1993; Ballarin et al., 1991; Park and Gidday, 1990; Phillis et al., 1989). If adenosine transport inhibitors block equally well the influx and efflux of adenosine, and the major source of extracellular adenosine originates from released ATP, then adenosine transport inhibitors may still increase extracellular adenosine levels (White and Hoehn, 1991). If transport inhibitors block efflux to a greater degree than influx, and adenosine is mainly formed intracellularly, then adenosine transport inhibitors may decrease extracellular levels of endogenous adenosine (Fredholm and Jonzon, 1981; Jonzon and Fredholm, 1985). Finally, if significant amounts of adenosine are transported through Na^+-dependent transporters, then inhibition of "es" nucleoside transporters will favor the accumulation of

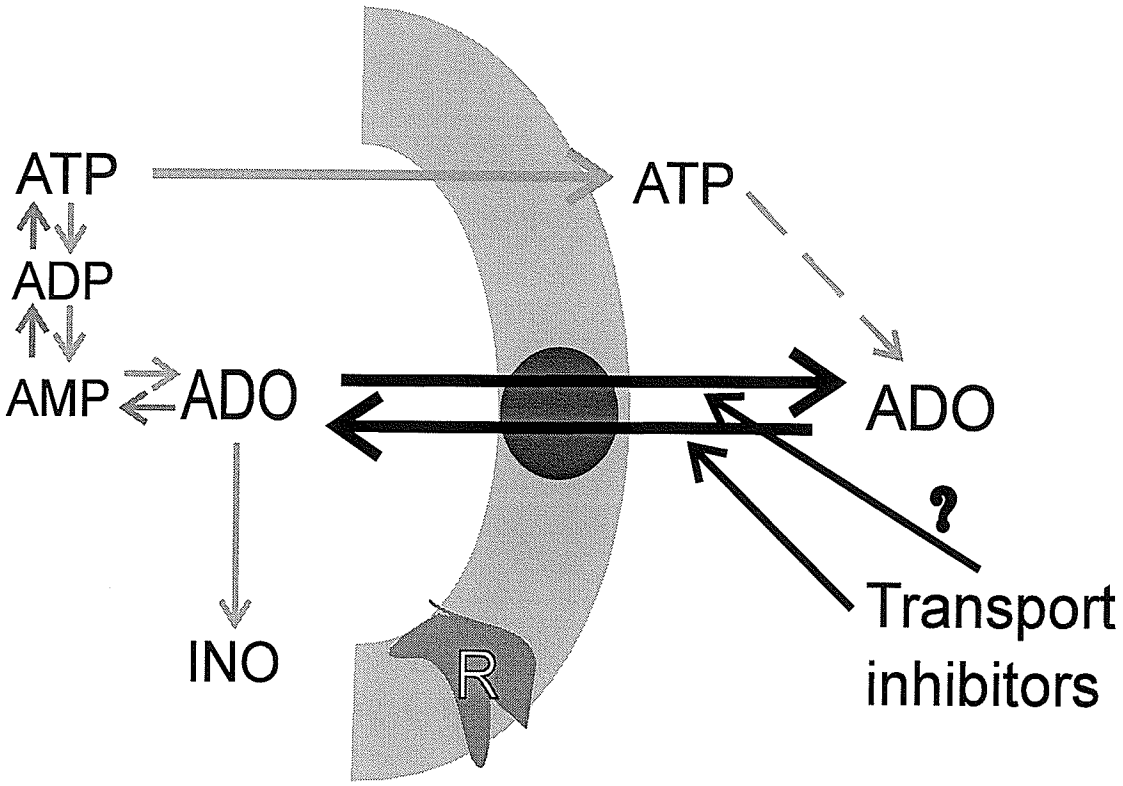

Figure 7. Bidirectional adenosine transport and effects of adenosine transport inhibitors. Adenosine transport inhibitors may enhance extracellular levels of endogenous adenosine if transport inhibitors block influx to a greater degree than efflux (release), or if adenosine transport inhibitors block equally well the influx and efflux of adenosine and the major source of extracellular adenosine originates from released ATP. Adenosine transport inhibitors may decrease extracellular adenosine levels if the inhibitors block efflux to a greater degree than influx, or if significant amounts of adenosine are transported through Na^+-dependent transporters (not illustrated).

adenosine inside of cells and thus decrease extracellular adenosine levels (Plagemann et al., 1990a). Currently, the development of adenosine transport inhibitors as therapeutic agents appears to be progressing in the absence of knowledge of how CNS nucleoside transporters mediate efflux of adenosine and whether adenosine transport inhibitors can allow adenosine efflux yet selectively prevent its uptake.

11. Therapeutic Applications of Adenosine Transport Inhibitors

DPR was introduced about 30 years ago as an anti-thrombotic and anti-ischemic drug (Emmons et al., 1965; Rajah et al., 1977). Initially the actions of DPR were thought to be due to inhibition of cyclic AMP phosphodiesterase. It was later found that the effects of DPR were due to its inhibition of adenosine transport systems on endothelial cells and erythrocytes (Ohisalo, 1987). Through inhibition of adenosine transport, NBI and dilazep potentiated the inhibitory effects of adenosine on vessel dilation and platelet aggregation (Donck, 1991; Lerman et al., 1989). Nucleoside transport inhibitors have also been shown to be useful adjuncts with antitumor drugs in cancer therapy, to reverse multidrug-resistance to antitumor drugs (Chen et al., 1993; Morgan et al., 1991), and to enhance the therapeutic index of antitumor drugs both in vitro and in vivo (Belt et al., 1993, Cass et al., 1992; Asoh et al., 1989; Cao and Zhen, 1989).

In the CNS, adenosine transport inhibitors have been tested for possible clinical uses as enhancers of the extracellular levels and thereby the actions of endogenous adenosine. Based on the functional effects of adenosine transport inhibitors, one of the possible clinical applications of adenosine transport inhibitors is for the treatment of seizures. Dilazep significantly decreased the incidence and severity of bicuculline methiodide (BMI)-induced convulsions (Murray et al., 1993) and kainic acid-induced seizures (Zhang et al., 1990). Simultaneous administration of the adenosine transporter inhibitor mioflazine with purine precursor 5-amino-4-imidazole carboxamide riboside (AICAR) suppressed homocysteine thiolactone (HTL) induced-seizures in mice

(Marangos et al., 1990). The adenosine transport inhibitor papaverine had anticonvulsant effects on kindled seizures (Dragunow et al., 1985). Further, many clinically used anticonvulsant drugs such as benzodiazepines have inhibitory effects on adenosine transport (Phillis et al., 1981; Johnson et al., 1991).

Another potential clinical application of adenosine transport inhibitors is to improve cerebral blood circulation and thus to protect brain cells from ischemic damage. Intravenous administration of dipyridamole to conscious rabbits gradually and persistently increased cerebral blood flow in different brain regions (Puiroud et al., 1988). Dipyridamole and papaverine potentiated the anoxia-induced increases in CBF (Phillis et al., 1984). Lidoflazine and flunarizine, two bi-fluorophenyl-piperazine derivatives with high potencies to inhibit adenosine transport, both potentiated reactive hyperemia elicited by a brief anoxic challenge (Phillis et al., 1985). Propentofylline enhanced extracellular adenosine levels (Dux et al., 1990; Andine et al., 1990), increased blood flow to ischemic brain regions, decreased glutamate release in the gerbil hippocampus (Miyashita et al., 1992), and decreased ischemia-induced neuronal and vascular degeneration in animal brains (Dux et al., 1990; Andine et al., 1990). Furthermore, in acute ischemic stroke patients, propentofylline protected against glucose depletion in infarct and noninfarct regions and propentofylline-treated patients appeared to have a trend towards clinical improvement (Huber et al., 1993).

Adenosine transport inhibitors may be useful for the treatment of sleep disorders (Wauquier et al., 1987). The adenosine transport inhibitor mioflazine was reported to be an effective sleep promotor in dogs (Wauquier et al., 1987) and humans (Hoppenbrouwers and Vanden Bussche, 1989). Mioflazine's effect differed from hypnotic drugs in that a physiological sleep was produced. Adenosine transport inhibitors also may be a useful treatment for psychiatric diseases since many psychotropic agents such as phenothiazines, butyrophenoes, and spiperone as well as anxiolytic agents such as

hydroxyzine and tracazolate were found to potently inhibit adenosine uptake in CNS tissues (Phillis and Wu, 1982; Snyder, 1985).

Chapter 1. L-[^{3}H]Adenosine, A New Metabolically Stable Enantiomeric Probe for Adenosine Transport Systems in Rat Brain Synaptoneurosomes

ABSTRACT: The stereoenantiomers D-[^{3}H]adenosine and L-[^{3}H]adenosine were used to study adenosine accumulation in rat cerebral cortical synaptoneurosomes. L-Adenosine very weakly inhibited rat brain adenosine deaminase (ADA) activity with a K_i value of 385 μM. It did not inhibit rat brain adenosine kinase (AK) activity, nor was it utilized as a substrate for either ADA or AK. The rate constants (fmol/mg of protein/s) for L-[^{3}H]adenosine accumulation measured in assays where transport was stopped either with inhibitor-stop centrifugation or with rapid filtration methods were 82 ± 14 and 75 ± 10, respectively. Using the filtration method, the rates of L-[^{3}H]adenosine accumulation were not significantly different from the value of 105 ± 15 fmol/mg of protein/s measured for D-[^{3}H]adenosine transport. Unlabeled D-adenosine and nitrobenzylthioinosine, both at a concentration of 100 μM, reduced the levels and rates of L-[^{3}H]adenosine accumulation by > 44%. These findings suggest that L-adenosine, a metabolically stable enantiomeric analog, and the naturally occurring D-adenosine are both taken up by rat brain synaptoneurosomes by similar processes, and as such L-adenosine may represent an important new probe with which adenosine uptake may be studied.

INTRODUCTION

The transport of adenosine into cells of the CNS is believed to be one of the most important mechanisms that regulate the levels of adenosine available for binding to cell surface adenosine receptors, through which most of the actions of adenosine are thought to be mediated (Wu and Phillis, 1984; Geiger and Nagy, 1990; Williams, 1989). Adenosine is transported in CNS tissues by passive-diffusion and what have been

described as transporter mediated equilibrative diffusion as well as sodium-dependent processes (Geiger et al., 1988; Johnston and Geiger, 1989; Lee and Jarvis, 1988a; b). Measurements of adenosine transport in CNS tissues are more difficult than in, for example, human erythrocytes, because there are fewer transport sites. The existence of fewer sites necessitates the use of longer incubation times to ensure adequate accumulation:blank ratios. However, because the transport rates in CNS tissues, in analogy to better characterized systems in peripheral tissues and cultured cells (Wohlhueter and Plagemann, 1989), are believed to be faster than the metabolism of intracellular adenosine by either adenosine deaminase (ADA) or adenosine kinase (AK), initial rates of substrate translocation across the plasma membrane are not measured under conditions of prolonged incubations. These problems could be bypassed by using a nucleoside that is not deaminated by ADA to inosine which may itself be a substrate for the bi-directional nucleoside transporter or one that is not phosphorylated by AK to 5'-AMP, which would be trapped in intracellular compartments. However, nucleoside substrates, such as uridine, which are more metabolically stable than adenosine, may not, in all tissues, be recognized by various identified subtypes of adenosine transporters (Lee and Jarvis, 1988b; Geiger et al., 1988).

Recently, L-adenosine (9-β-L-ribofuranosyladenosine), the enantiomer of naturally occurring D-adenosine (9-β-D-ribofuranosyladenosine) became available commercially. While the present studies were being conducted, it was reported that L-[^{3}H]adenosine was a substrate for passive and, to a far lesser extent, equilibrative adenosine transport systems that displayed a high degree of stereoselectivity in that very little L-[^{3}H]adenosine was accumulated by the mouse erythrocytes and L-1210 cells studied (Gati et al., 1989). Here we report that L-adenosine is metabolically stable and that it is accumulated almost equally as well as D-adenosine by synaptoneurosomes prepared from rat cerebral cortex.

MATERIALS AND METHODS

Animals and Preparation of Synaptoneurosomes

Male Sprague-Dawley rats weighing 300 ± 20 g were obtained from the University of Manitoba Central Animal Care breeding colony and were sacrificed by decapitation. Brains were removed, placed on ice-cold plates, and cerebral cortices were dissected out as previously described (Geiger and Nagy, 1984; 1986). Synaptoneurosomes were prepared essentially as described by Hollingsworth et al. (1985) by gently homogenizing cerebral cortices in a teflon-glass homogenizer (0.125 mm clearance) in 7 volumes (wt/vol) of a physiological-HEPES buffer consisting of 110 mM NaCl, 25 mM glucose, 68.3 mM sucrose, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, and 20 mM HEPES (pH 7.4, 336-340 mosmol). Homogenates were filtered by gravity through a triple layer of 80-μm-pore-size-nylon netting (Thompson, Montreal) and by pressure filtration through 10-μm-pore-size filters (Millipore LCWP-047) loaded in Swinnex holders. The filtrate was centrifuged at 1000 x *g* for 15 min at 4°C, and the pellets were resuspended in 30 volumes of the HEPES buffer and recentrifuged. The final pellet was resuspended in 15 volumes of buffer necessary to yield 3 mg protein/ml. All procedures were conducted at 4°C and all assays with the synaptoneurosomes were completed within two hours of their preparation.

Adenosine Kinase Assay

Adenosine kinase activity was measured according to the method of Lin et al. (1988). As a source for AK, samples of rat cerebral cortex were homogenized in 10 volumes of buffer with a Polytron (setting 6, three 10-s bursts) and following centrifugation at 135,500 x *g* for 1hr, buffer was added to supernatant to reach a 1 to 800 (wt/vol) dilution. In a final volume of 50 μl, reaction tubes contained 0.1 % (wt/vol) bovine serum albumin, 0.5 μM erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), 5 %

glycerol (vol/vol), 50 mM KCl, 1.6 mM $MgCl_2$, 50 mM 2-mercaptoethanol, 1.2 mM ATP, and 1.0 μM (0.1 μCi) [^{3}H]adenosine. Enzyme reactions were started by addition of [^{3}H]adenosine and following incubations for 5 min at 37°C, reactions were stopped by heating reaction vessels for 5 min at 85°C. After cooling to room temperature, 20 μl were spotted on to DE 81 ion-exchange filter discs (Whatman, Clifton, N.J.). The filters were dried at room temperature, placed in 5 ml scintillation vials and washed twice for 5 min with 1 mM ammonium formate, twice in distilled water, and once in 95 % ethanol. Phosphorylated derivatives of adenosine were eluted from the discs by the addition of 0.5 ml of a solution consisting of 0.1 M HCl and 0.4 M KCl, and following the addition of 4.5 ml of Beckman Ready Safe were counted by scintillation spectroscopy at an efficiency of 38 to 42 %. The ability of L-adenosine to act as a substrate for or an inhibitor of AK was determined at concentrations ranging from 0.5 to 1000 μM. Protein was assayed by the method of Lowry et al. (1951) using bovine serum albumin as the standard. Results were expressed as nmoles product formed per 5 min per milligram of protein.

Adenosine Deaminase Assay

Measurements of ADA activity were performed by a previously described HPLC method [Geiger and Nagy, 1986]. Samples of rat cerebral cortex were homogenized with a Polytron (setting 6, three 10-s bursts), and 50 μl aliquots were added to reaction vessels containing 500 μM D-adenosine in a total volume of 100 μl of buffer. Incubations were for 30 min at 37°C, and reactions were stopped by addition of 10 μl of 20% trichloroacetic acid. Samples were neutralized with a tri-n-octylamine/Freon mixture (45:155 vol/vol) and 25 μl aliquots of the aqueous phase were chromatographed using reverse-phase HPLC. The activity of ADA was calculated as nanomoles of product formed (inosine plus hypoxanthine) per milligram of protein per 30 min. In order to test whether L-adenosine inhibits ADA activity, concentrations of L-adenosine were varied

from 100 to 100,000 μM. The ability of L-adenosine to act as a substrate for rat brain ADA was determined with concentrations of L-adenosine that ranged from 10 to 10,000 μM.

Adenosine Transport Assays

Reaction vessels in a total volume of 1.0 ml HEPES buffer contained 3% (wt/vol) bovine serum albumin and about 0.3 mg of synaptoneurosome protein. Tissues were preincubated for 20 s at 37°C, and transport was initiated by addition of 1.0 μM L-[^{3}H]adenosine (33 Ci/mmol, Moravek Biochemicals, Brea, CA, U.S.A.) or D-[^{3}H]adenosine (50.2 Ci/mmol, New England Nuclear, Montreal) at specific activity concentrations of about 0.1 Ci/mmol. Incubations were for 5 to 60 s and reactions were terminated by one of two methods: filtration or by inhibitor-stop centrifugation.

In the centrifugation method, the adenosine transport inhibitors dilazep and dipyridamole at final concentrations of 1000 and 100 μM, respectively, were added and reaction tubes were centrifuged immediately at 13,000 x *g* for 15 s. The supernatants were discarded, pellets were gently dislodged from the walls of the centrifuge tubes with 0.5 ml of ice-cold HEPES buffer containing 100 μM dilazep, and following recentrifugation, pellets were digested in 0.3 ml of 0.1 M NaOH. Assay blanks were handled as above except that the incubations were carried out at 4°C and, before addition of L-[^{3}H]adenosine, synaptoneurosomes were preincubated for 20 s with 100 μM L-adenosine and the nucleoside transport inhibitors dilazep (1 mM) and dipyridamole (100 μM).

In the filtration method, transport assays were terminated by the addition of 4 ml ice-cold HEPES buffer to the reaction vessel and filtration through glass fiber filters (No. 32; Schleicher and Schuell, Keene, NH, U.S.A), which were washed one additional time with 5 ml of ice-cold HEPES buffer.

L-[^{3}H]Adenosine was not accumulated by nor was there any measureable L-[^{3}H]adenosine binding to mechanically disrupted synaptoneurosomes (data not shown). Radioactivity was determined by scintillation spectroscopy at an efficiency of 38 to 45 % using Ready Safe (Beckman) scintillation fluid. In studies of D-adenosine competition for L-[^{3}H]adenosine transport, 100 μM D-adenosine was added simultaneously with the ligand. For studies of nitrobenzylthioinosine (NBI) competition for L-[^{3}H]adenosine transport, tissue was preincubated with 100 μM NBI for 2 min before addition of L-[^{3}H]adenosine. Levels of transport were expressed as pmoles accumulated per milligram of protein. Rate constants, expressed as femtomoles per milligram of protein per second, were calculated by linear regression and compared statistically with Student's *t* tests where significance was considered at the $p < 0.05$ level.

Materials

D-[2,8,5'-^{3}H]Adenosine (50.2 Ci/mmol) was purchased from New England Nuclear (Montreal). L-Adenosine was purchased from Moravek Biochemicals (Brea, California) and was generously donated by Dr. Karl Flora, Pharmaceutical Resources Branch of the National Cancer Institute. Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA-HCl) was generously supplied by Dr. S. Grossman of Burroughs Wellcome Co. (Research Triangle Park, NC, U.S.A.). Dilazep was provided by Drs. Kutscher and Schumacher of ASTA Pharma (Frankfurt, F.R.G.). 2-Mercaptoethanol was purchased from Kodak Chemical Co. (Rochester, NY, U.S.A.). All other chemicals were of analytical grade and were obtained from standard sources. NBI and dipyridamole were dissolved in dimethyl sulfoxide (DMSO) and diluted with buffer to achieve final concentrations of DMSO levels no greater than 1% (vol/vol).

RESULTS

To test for the suitability of L-adenosine (see Figure 8 for chemical structure) in studies of adenosine transport processes in CNS tissues, experiments were conducted first to determine whether L-adenosine was an inhibitor of or a substrate for the adenosine-metabolizing enzymes ADA and AK. The deamination of 500 μM D-adenosine, 59.4 ± 7.2 nmoles per 30 min per mg protein, was inhibited weakly by L-adenosine; K_i was 385 μM. The phosphorylation of 1 μM D-[^{3}H]adenosine, 12.7 ± 0.9 nmol/5 min/mg of protein, was unaffected by up to 10 mM concentrations of L-adenosine. L-Adenosine was not deaminated nor was L-[^{3}H]adenosine phosphorylated under the assay conditions used.

Using an inhibitor-stop centrifugation method (Geiger, 1987; Geiger et al., 1988; Johnston and Geiger, 1989), L-[^{3}H]adenosine accumulation (pmol/mg of protein) at 5 s was 1.4 ± 0.4 and appeared to be linear (r=0.964) for incubations up to 60 s, when the level was 5.6 ± 1.1 (Figure 9). The rate constant for these experiments (n=6), expressed as femtomoles per milligram protein per second, was 82 ± 14. The accumulation of L-[^{3}H]adenosine was found to be slightly higher by filtration, accumulation levels (pmol/mg protein) at 5 s were 2.6 ± 0.6 and at 60 s were 6.6 ± 0.8, but the rate constant of 74 ± 10 (n=6, r=0.999) was not significantly different from those obtained in the centrifugation experiments. The transport of D-[^{3}H]adenosine, as determined by the same filtration assay method, ranged from 2.4 ± 0.6 at 5 s to 8.1 ± 0.8 pmol/mg of protein at 60 s and was only slightly higher than that for L-[^{3}H]adenosine (Figure 10). The rate constant for D-[^{3}H]adenosine, 106 ± 15 fmol/mg of protein/s (n=4, r=0.994) was not significantly different from the rate constant for L-[^{3}H]adenosine.

Inhibition studies were conducted to determine if D-adenosine and NBI, an adenosine transport inhibitor, would inhibit the transport of L-[^{3}H]adenosine. D-Adenosine and NBI each at 100 μM significantly reduced the amounts of L-

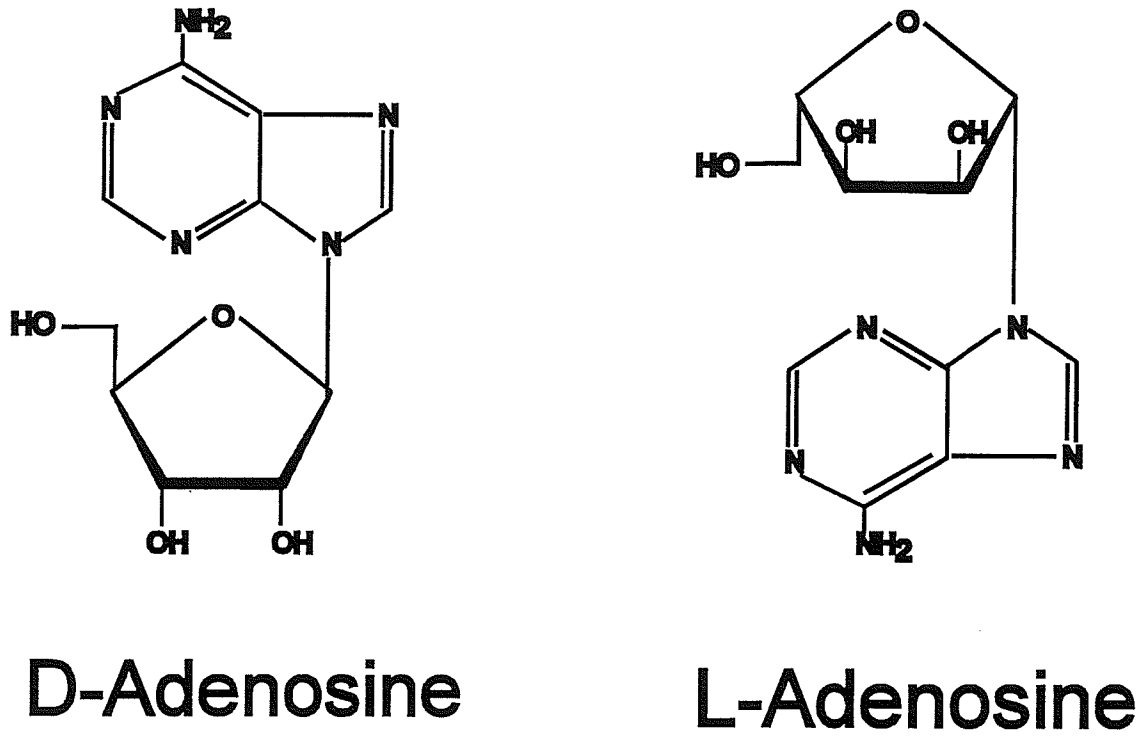

Figure 8. Structures of D- and L-adenosine

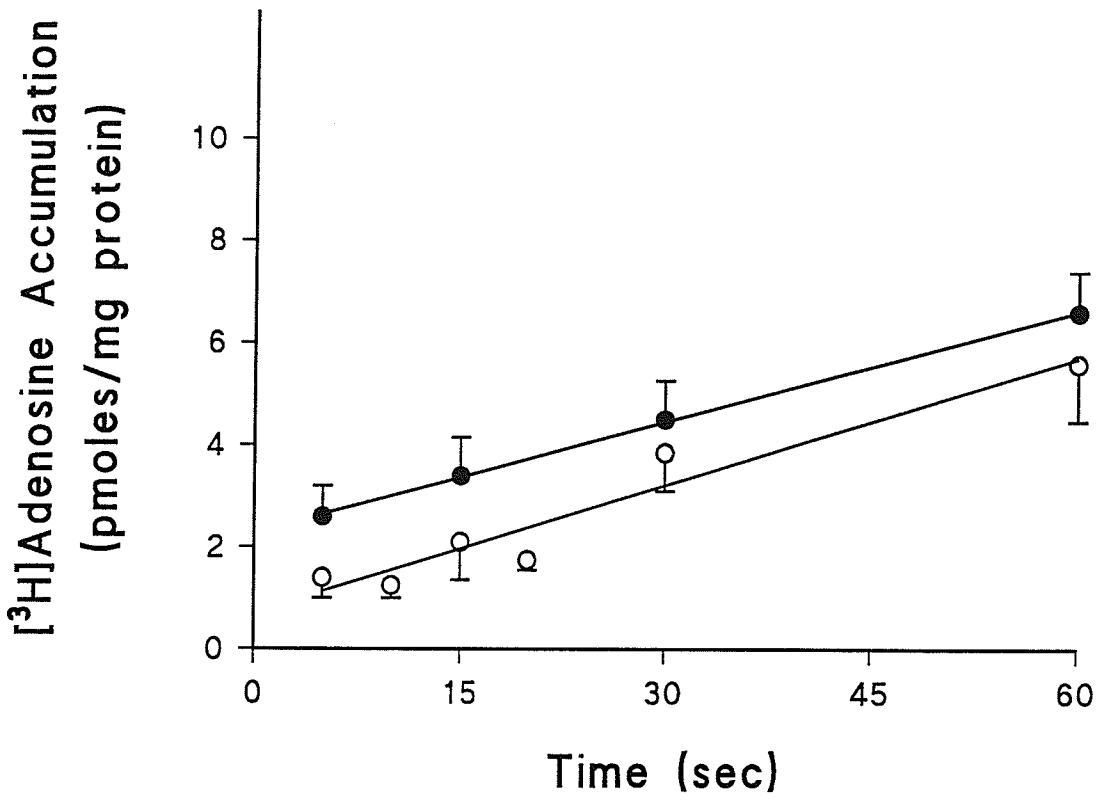

Figure 9. Transport of L-[^{3}H]adenosine expressed as a function of different incubation times at 37°C determined by either a filtration (n=6, closed circles) or an inhibitor-stop centrifugation (n=6, open circles) method. Symbols represent mean ± S.E.M. values.

[^{3}H]adenosine accumulated at each of the incubation times examined (Figure 10). For example, at 5 s the levels were 2.6 ± 0.6 pmol/mg of protein in the absence of D-adenosine or NBI and were 0.6 ± 0.2 and 0.9 ± 0.2 pmol/mg in the presence of D-adenosine and NBI, respectively. At 60 s the levels were 6.6 ± 0.8 pmol/mg in the absence of D-adenosine or NBI and were 3.0 ± 0.3 and 3.2 ± 0.7 pmol/mg in the presence of D-adenosine and NBI, respectively. The rate constant (fmol/mg of protein/s) for experiments conducted with D-adenosine of 42 ± 3 (n = 4, r = 0.974) and with NBI of 42 ± 10 (n = 4, r = 0.987) were significantly ($p < 0.05$) less than control (absence of D-adenosine or NBI) values of 74 ± 10. The levels of L-[^{3}H]adenosine accumulated in the presence of 100 μM D-adenosine were virtually identical to those in the presence of 100 μM NBI.

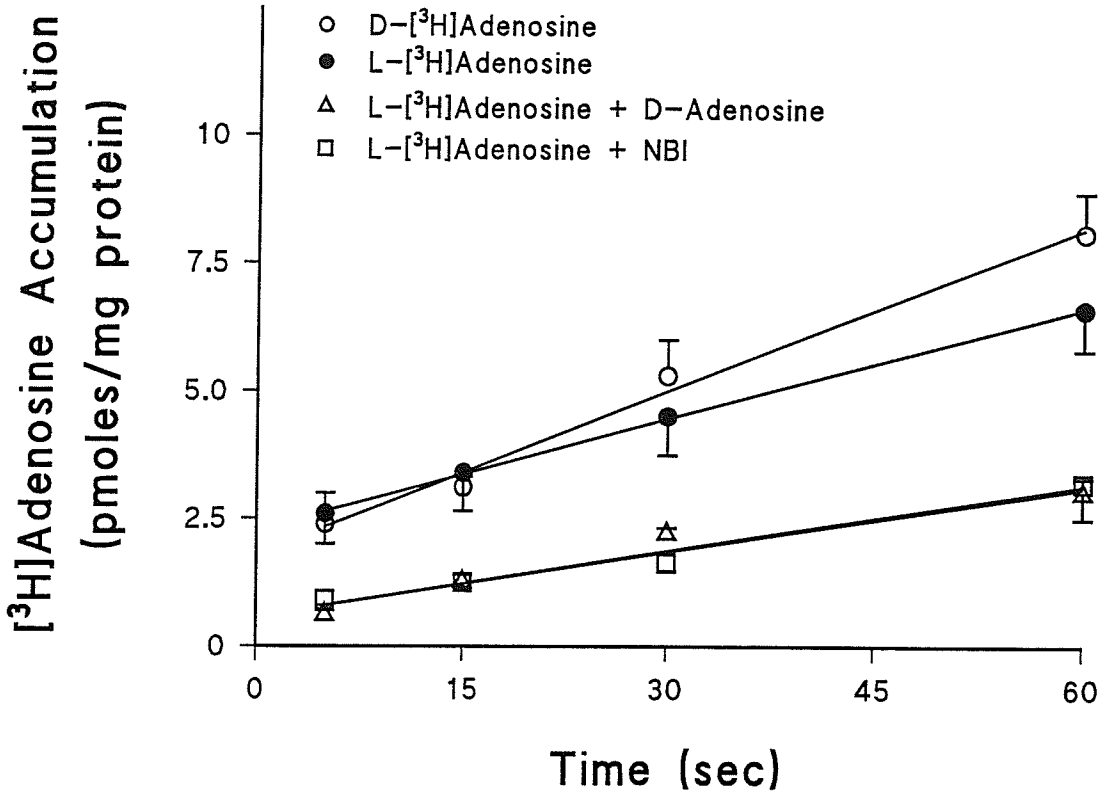

Figure 10. Transport of 1 μM L-[^{3}H]adenosine (n=6, closed circles) in the presence of either 100 μM D-adenosine (n=4, open triangle) or 100 μM NBI (n=4, open square), and 1 μM D-[^{3}H]adenosine (n=4, open circles) as a function of different incubation times at 37°C determined by a rapid filtration method. Symbols represent mean ± S.E.M. values.

DISCUSSION

Investigators who study adenosine transport systems in CNS tissues using D-[^{3}H]adenosine as the substrate must overcome some important methodological problems due to its metabolic instability [Wohlhueter and Plagemann, 1989]. D-Adenosine can be metabolized by ADA and this is important because under other than zero-trans conditions, accumulated adenosine and/or inosine may be transported back out of the cell. In addition, because the K_m of AK for adenosine is lower than that of ADA, low levels of accumulated adenosine would be immediately phosphorylated to 5'-AMP, which would then accumulate, and because 5'-AMP can not freely exit the cell, adenosine could not reach an equilibrium. Therefore, 'steady state' conditions would be reached where the influx of radioactive label would equal that trapped inside the cell. As a consequence, accumulation of phosphorylated derivatives could lead to the incorrect identification of concentrative processes. When studying D-adenosine transport in cells where these enzymes are present, initial transport conditions may be approached by using very short incubation intervals. However, this is difficult in tissues with low rates of adenosine transport capabilities, like the CNS, because of very low levels of accumulated radioactivity during brief incubation intervals. Therefore, identification of a substrate that is selectively recognized by adenosine transport system(s), and that is not a substrate for ADA or AK, would represent a major technological advance for investigators in this field.

L-Adenosine, and other L-ribose substituted nucleobases, were first synthesized starting in 1964 with the intention that new, more metabolically stable analogs might be identified that would facilitate the study of nucleic acids and increase the therapeutic usefulness of nucleosides such as 6-mercaptopurine (Acton et al., 1964). Metabolically, the L-isomer of adenosine was found not to be metabolized by ADA from *Aspergillus oryzae* nor was the L-isomer of 5'-AMP dephosphorylated by 5'-nucleotidase from

Trimeresurus flavoviridis (Asai et al., 1967). In the present study L-adenosine was not found to be metabolized by rat brain AK or ADA. Biologically, D-adenosine, but not L-adenosine, was able to support the growth of mammalian cells in culture (Asai et al., 1967). In addition, L-enantiomers of adenosine, AMP, ADP, ATP, and 5'-(N-ethylcarboxamido)adenosine were found to be inactive at pharmacologically relevant concentrations in tests of human platelet aggregation (Cusack et al., 1979; Cusack and Hourani, 1981) and relaxation of guinea-pig taenia coli (Cusack and Planker, 1979). Thus, where the question has been studied, adenosine receptors have a strict requirement for D-ribose, and therefore the deaminase- and phosphorylation-resistant L-adenosine may be used for transport studies without concern for possible binding to and activation of adenosine receptors.

While the present studies were being conducted, adenosine transport systems were described on mouse leukemia L-1210/AM cells and mouse erythrocytes that displayed 17- and 22-fold higher rates of transport of D-adenosine in comparison to L-adenosine (Gati et al., 1989). These findings contrast sharply with those described here in that the transport rates of D-adenosine were comparable to those of L-adenosine in rat cerebral cortex synaptoneurosomes. Our findings suggest that adenosine transporters on rat synaptoneurosomes do not display a marked stereoselectivity for the ribose moiety of adenosine. This lack of stereospecificity might be expected if D-and L-adenosine were both transported not by a carrier-mediated system but rather by passive diffusion. This possibility is, however, highly unlikely because NBI and D-adenosine both significantly inhibited the transport of L-[^{3}H]adenosine by > 44%. Although adenosine accumulation was measured by a method different from that employed by Gait et al. (1989) it is likely that the different characteristics of L-adenosine transport found here were due to differences among species and tissues.

In mouse erythrocytes, 200 μM D-adenosine and 5 μM NBI (NBMPR) inhibited the transport of L-[^{3}H]adenosine by 60 and 85 %, respectively; the inhibitor-resistant

component of L-[^{3}H]adenosine accumulation was thought to be mediated by passive diffusion (Gati et al., 1989). If the same conclusion can be made for rat synaptoneurosomes then it would follow that over 50% of D-[^{3}H]adenosine accumulated even at physiological concentrations occurs by way of passive diffusion. Furthermore, this would be consistent with finding from various laboratories that > 50% of D-[^{3}H]adenosine transport is resistant to the blocking actions of nucleoside transport inhibitors (Geiger et al., 1988; Lee and Jarvis 1988a,b; Morgan and Marangos, 1987; Phillis and Wu, 1983). Further studies are now being conducted to characterize more fully the relative contributions of passive and equilibrative transport processes in cells and tissues of the CNS using L-[^{3}H]adenosine, a new metabolically stable probe for these systems.

Chapter 2. Transport and Metabolism of D-[^{3}H]Adenosine and L-[^{3}H]Adenosine in Rat Cerebral Cortical Synaptoneurosomes

ABSTRACT: The relationship between transport and metabolism in synaptoneurosomes was examined to determine the metabolic stability of rapidly accumulated D-[^{3}H]adenosine and L-[^{3}H]adenosine and the degree to which metabolism of the accumulated purines affected measurements of apparent K_T and V_{max} values for adenosine transport. For D-[^{3}H] adenosine, high and low affinity accumulation processes were present. For the high affinity system an inverse relationship was found between transport reaction times and K_T and V_{max} values. For incubations of 5, 15 and 600 s, which corresponded to 24, 32 and 76% phosphorylation of accumulated D-[^{3}H]adenosine to nucleotides, apparent K_T values were 9.4, 8.4 and 4.5 μM, respectively, and V_{max} values were 850, 70 and 12 pmol/min/mg protein, respectively. Pretreatment with 10 μM erythro-9-(2-hydroxy-3-nonyl)adenine, an adenosine deaminase inhibitor, and 5'-iodotubercidin, an adenosine kinase inhibitor, decreased the phosphorylation of accumulated D-[^{3}H]adenosine to 6% with 5-s and 9% with 15-s incubations. This resulted in significantly higher K_T (μM) values; 36 at 5 s and 44 at 15 s. At 10-minute incubations in the presence of these inhibitors, metabolism of accumulated D-[^{3}H]adenosine was 32%, and apparent K_T and V_{max} values at this time were not significantly different from those obtained without inhibitors. For L-[^{3}H]adenosine, apparent K_T and V_{max} values for 20 s incubations were 38.7 μM and 330 pmol/min/mg protein, respectively. Metabolism (mainly phosphorylation) of accumulated L-[^{3}H]adenosine was observed only at incubations longer than 30 s. Taken together, these results demonstrate that adenosine transport is significantly faster than subsequent metabolism, that accumulated D-adenosine is rapidly incorporated into and trapped intracellularly as adenine nucleotides thereby affecting measured kinetic parameters for

adenosine transport and giving an `appearance' of concentrative accumulations, and that the apparent K_T value of 39 μM for D-adenosine transport conducted in the presence of the enzyme inhibitors was the same as the apparent K_T value for L-adenosine transport.

INTRODUCTION

The actions of adenosine in the CNS are well known and are believed to be coupled to various intracellular second messenger systems, including cyclic AMP, inositol triphosphate, and calcium, through activation of specific cell surface receptors (Williams, 1989; Stone, 1991). Once produced, extracellular levels of endogenous adenosine are controlled by both transport (re-uptake) and enzymatic processes (Geiger and Nagy, 1990). The physiological relevance of these processes in regulating the levels and thus the actions of adenosine is supported by findings that inhibitors of adenosine transport or adenosine deaminase (ADA), an adenosine catabolic enzyme, raise tissue levels of adenosine and potentiate the effects of endogenous and exogenously administered adenosine (Geiger et al., 1991; Geiger and Fyda, 1991; Ballarin et al., 1991). Although both processes appear to function in this regard, it has been suggested that adenosine transport systems represent the primary means by which adenosine in extracellular spaces is removed from the vicinity of its receptors (Wu and Phillis, 1984). However, much of what we infer about CNS adenosine transporters comes from research conducted on non-nervous system tissues, and it remains uncertain the extent to which these data can be extrapolated to CNS tissues of animals or humans (Geiger and Fyda, 1991).

Measuring initial rates of adenosine accumulation, i.e., transport, is difficult because of the rapid transfer of adenosine across cell membranes and its metabolism by enzymes such as adenosine kinase (AK), ADA and S-adenosylhomocysteine hydrolase which all have high (and in some cases, higher) affinity for adenosine and slower

maximal velocities than do adenosine transporters. Therefore, in cultured cells and peripheral tissues, where adenosine influx through adenosine transporters is more rapid than its subsequent intracellular metabolism, D-adenosine transport across plasma membranes of cells capable of metabolizing this substrate must be measured using very brief reaction intervals or ATP-depleted or adenosine metabolic enzyme-inhibited cells or tissues. If, for example, phosphorylation of accumulated adenosine is allowed to occur, then the radiolabel would be trapped intracellularly, values for V_{max} would be grossly underestimated, and the apparent K_T value for accumulated adenosine would reflect the activity of AK (Wohlhueter and Plagemann, 1989). Kinetic measurements of adenosine transport under conditions where initial rates of accumulation have not been measured have been considered to be invalid (Young and Jarvis, 1983). The following studies were performed to determine the kinetic parameters for adenosine transport and the extent to which such measurements are affected by metabolism of accumulated D- and L-adenosine in rat cerebral cortical synaptoneurosomes.

MATERIALS AND METHODS

Animals and Preparation of Synaptoneurosomes

Male Sprague-Dawley rats weighing 300 ± 20 g, obtained from the University of Manitoba Central Animal Care breeding facility, were sacrificed by decapitation. Brains were removed and placed on ice-cold plates, and cerebral cortices were dissected free of subcortical tissue. Synaptoneurosomes were prepared as previously described and used within 2 hr of preparation (Gu et al., 1991). In briefly, a Teflon-glass homogenizer (0.125 mm clearance) was used to homogenize cerebral cortical tissue in 7 volumes (wt/vol) of buffer consisting of 110 mM NaCl, 25 mM glucose, 68.3 mM sucrose, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, and 20 mM HEPES (pH 7.4, 336-340 mosmol). Homogenates were filtered through a triple layer of nylon mesh (pore size, 80 μm;

Thompson, Montreal, Quebec, Canada) and then pressure-filtered through Millipore LCWP- 047 filters (pore size, 10 μm). Filtrates were then centrifuged at 1,000 x g for 10 min at 4°C, and pellets were resuspended in buffer to yield a concentration of ~3 mg of protein/ml. All procedures for tissue preparation were conducted at 4°C.

D- and L-[^{3}H]Adenosine Transport Assays

Reactions were conducted in a total volume of 1.0 ml buffer containing 3% (wt/vol) BSA and ~0.3 mg of synaptoneurosome protein. Synaptoneurosomes were preincubated for 10 min at 37 °C in the absence or presence of 10 μM erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) plus 10 μM 5'-iodotubercidin to inhibit ADA and AK, respectively. Transport reactions were initiated by addition of D-[^{3}H]adenosine, the specific activity of which was varied between 1 to 5 μCi/μmol (50.2 Ci/mmol; New England Nuclear) or L-[^{3}H]adenosine, which had a specific activity of ~0.1 μCi/μmol (33 Ci/mmol; Moravek Biochemicals Inc., Brea, CA, U.S.A.). For determination of apparent K_T and V_{max} values, final concentrations of D-adenosine and L-adenosine were varied from 1.0 to 2500 and from 1.0 to 250 μM, respectively. Incubations were conducted at 37 °C for intervals that varied from 5 s to 10 min for transport assays and for measurements of metabolism of accumulated [^{3}H]-labeled purines.

In D-[^{3}H]adenosine transport assays, accumulation was terminated by the addition of 100 μl of an inhibitor cocktail containing the nucleoside transport inhibitors (final concentrations) dilazep (1.0 mM) and dipyridamole (0.1 mM), as well as EHNA (0.1 mM) and 5'- iodotubercidin (10 μM). This cocktail was effective in preventing adenosine uptake (data not shown) and metabolism. Reaction vessels were centrifuged at 13,000 x g for 30 s, supernatants were aspirated, and the pellets were gently dislodged from the walls of the centrifuge tubes with 0.5 ml of ice-cold buffer containing 100 μM dilazep and recentrifuged as above. Supernatants were aspirated, and the pellets were digested in 0.3 ml of 0.1M NaOH. The digestate was added to 4.5 ml of Beckman Ready

Safe scintillation cocktail, and radioactivity was determined by scintillation spectrophotometry. Assay blanks were handled as described above except that incubations were carried out at 4°C and, before addition of D-[^{3}H]adenosine, synaptoneurosomes were preincubated for 10 min at 37°C with the inhibitor cocktail described above. The final concentration of dimethyl sulfoxide used to dissolve dipyridamole was always less than 1%, a level at which no effects on adenosine transport were observed.

In L-[^{3}H]adenosine accumulation assays, incubations were for 20 s at 37 °C, and reactions were terminated by addition of 4 ml of ice-cold buffer followed by rapid filtration through GF/B glass fiber filters (Whatman), which were washed under reduced pressure with 5 ml of ice-cold buffer. Values of L-[^{3}H]adenosine accumulation were the same regardless of whether assays were terminated by centrifugation or filtration (Gu et al., 1991).

The apparent K_T and V_{max} values were calculated using computerized non-linear regression of saturation data, and results were illustrated as Lineweaver-Burke plots. Protein contents were measured according to the method of Lowry et al. (1951). Unless otherwise indicated, all values represent means ± S.E.M. Statistical analyses were performed by using an analysis of variance, and significance was considered at the $p<0.05$ level.

Metabolism of Accumulated D- and L-[^{3}H]Adenosine

Accumulated adenosine and its metabolites were isolated and quantified using TLC. Adenosine transport assays for D- and L-[^{3}H]adenosine were performed as described above except that a higher specific activity of radioactivity was used (5 μCi/μ mol), and incubations were for 5, 15, 30, 60 s, and 10 min. [^{3}H]-Labeled purines were extracted by adding 100 μl of ice-cold trichloroacetic acid (5%, wt/vol) and disrupting the pellet by trituration. Samples were kept on ice for 20 min and were centrifuged at 13,000

x g for 5 min. Aliquots of 20 µl were taken for measurements of total purine accumulation, and 50 µl aliquots were spotted, along with authentic purines as standards, on Polygram cellulose precoated plastic sheets (Machray Nagel, FRG), which were developed in a solvent composed of 1-butanol/acetone/glacial acetic acid/concentrated ammonium hydroxide/distilled water (14:10:6:1:8; by volume). Adenosine and adenosine metabolites were identified on TLC plates under UV light (254 nm), cut out, and placed into scintillation vials containing 0.5 ml of 0.1 M HCl, and vials were vortex-mixed. Samples were counted in 4.5 ml of scintillation fluid.

Intracellular Volume Measurements

Synaptoneurosomes (100 µl aliquots) were placed into reaction vessels containing 800 µl buffer and 100 µl of ^3H_2O (1.0 µCi/ml, Amersham) plus [U-^{14}C]-sucrose (0.5 µ Ci/ml, 6 mCi/mmol; Sigma). Following equilibration for 5 min at 37 ^oC, samples were centrifuged for 30 s at 13,000 x *g* through 250 µl of an oil mixture consisting of dinonyl phthalate and n-butyl phthalate (1:4, vol/vol). The pellets were digested with 0.1 M NaOH and counted by scintillation spectroscopy. The difference between ^3H and ^{14}C was used to calculate intracellular volume. Data were expressed as microliters per milligram of protein.

RESULTS

D-[^{3}H]Adenosine was rapidly transported into rat cerebral cortical synaptoneurosomes and incorporated predominantly into adenine nucleotides (AMP, ADP, and ATP) (Figure 11). In the absence of EHNA and 5'-iodotubercidin, at 5 s incubations, 70 ± 4% of the total accumulated [^{3}H]-labeled D-purines were recovered unchanged as D-[^{3}H]adenosine, whereas 24 ± 3% were recovered as radiolabeled adenine nucleotides (Figure 11). The rate of metabolism increased linearly for incubations up to 1

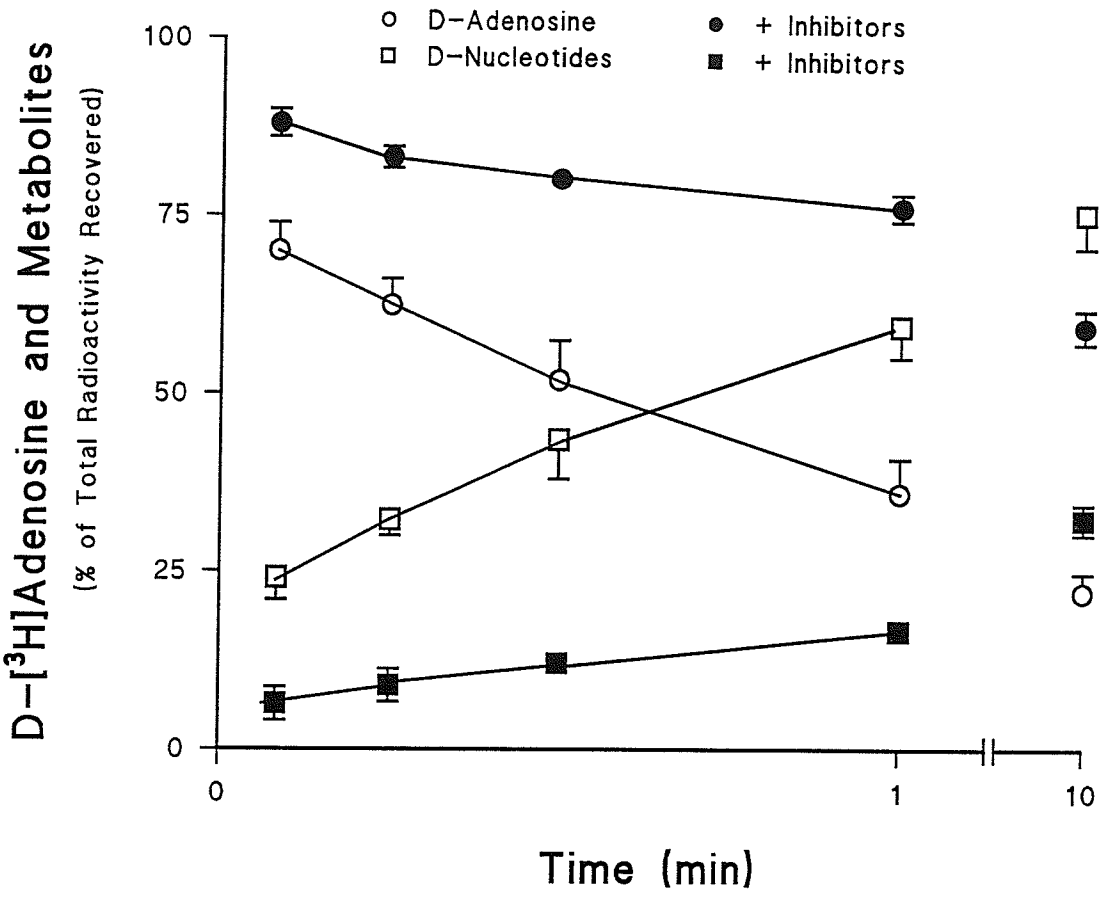

Figure 11. Metabolism of accumulated D-[^{3}H]adenosine in rat cerebral cortical synaptoneurosomes in the absence (open symbols) and presence (solid symbols) of 10 μM EHNA and 10 μM 5'-iodotubercidin. Following addition of 1 μM D-[^{3}H]adenosine tissues were incubated at 37 °C for 5 to 600 s. D-[^{3}H]Adenosine (circles), AMP + ADP +ATP (squares) and inosine + hypoxanthine (not shown) were separated by TLC and data were expressed as a percentage of total radioactivity accumulated. Values are mean ± S.E.M. from 6 experiments.

min, at which time 40 ± 5% of accumulated [^{3}H]-labeled D-purines were in the form of D-[^{3}H]adenosine and 60 ± 4% were metabolized to adenine nucleotides. In the presence of EHNA and 5'-iodotubercidin, the metabolism of accumulated D-[^{3}H]adenosine to labeled adenine nucleotides (expressed as a percentage of total [^{3}H]-labeled D-purines accumulated) was significantly reduced to 6 ± 2% at 5 s, 17 ± 1% at 1 min and 32 ± 2% at 10 min (Figure 11). The amount of radiolabel that remained unchanged as accumulated D-[^{3}H]adenosine was 88 ± 2% at 5 s, 76 ± 1% at 1 min and 60 ± 2% at 10 min. In both the absence or the presence of EHNA and 5'-iodotubercidin, the amount of deamination products (inosine and hypoxanthine) represented a small percentage (~5%) of total accumulated [^{3}H]-labeled D-purines (data not shown).

Intra-synaptoneurosomal volumes were 3.0 ± 0.2 μl/mg of protein (n=7); a value identical to that determined by Cash and Subbarao [1989]. The accumulation of D-[^{3}H]adenosine and its metabolites (total accumulated [^{3}H]-labeled D-purines), expressed as μM, as a function of incubation time in the absence or presence of EHNA and 5'-iodotubercidin is shown in Figure 12. At incubations longer than 5 s, D-[^{3}H]purine accumulation in synaptoneurosomes pretreated with enzyme inhibitors was significantly less than when inhibitors were absent (Figure 12A). In the absence of enzyme inhibitors, equilibration of accumulated purines with extracellular D-[^{3}H]adenosine (1 μM) was reached at 15 s (0.98 ± 0.6 μM). At 10 min incubations, the concentration of labeled purines in the synaptoneurosomes was 13.0 ± 1.3 μM. In the presence of enzyme inhibitors, the concentration of accumulated purines reached equilibration by 60 s (1.2 ± 0.1 μM); at 10 min, intracellular purine concentration reached 2.7 ± 0.3 μM (Figure 12A). When accumulated purines were separated by TLC and the data were represented as amount of D-[^{3}H]adenosine accumulated as a function of time, a different profile emerged (Figure 12B). In the absence of enzyme inhibitors, equilibration of intracellular with extracellular D-[^{3}H]adenosine was now reached at 60 s (1.0 ± 0.2 μM), and concentrations of D-[^{3}H]adenosine at 10 min were 2.9 ± 0.4 μM. In the presence of ADA

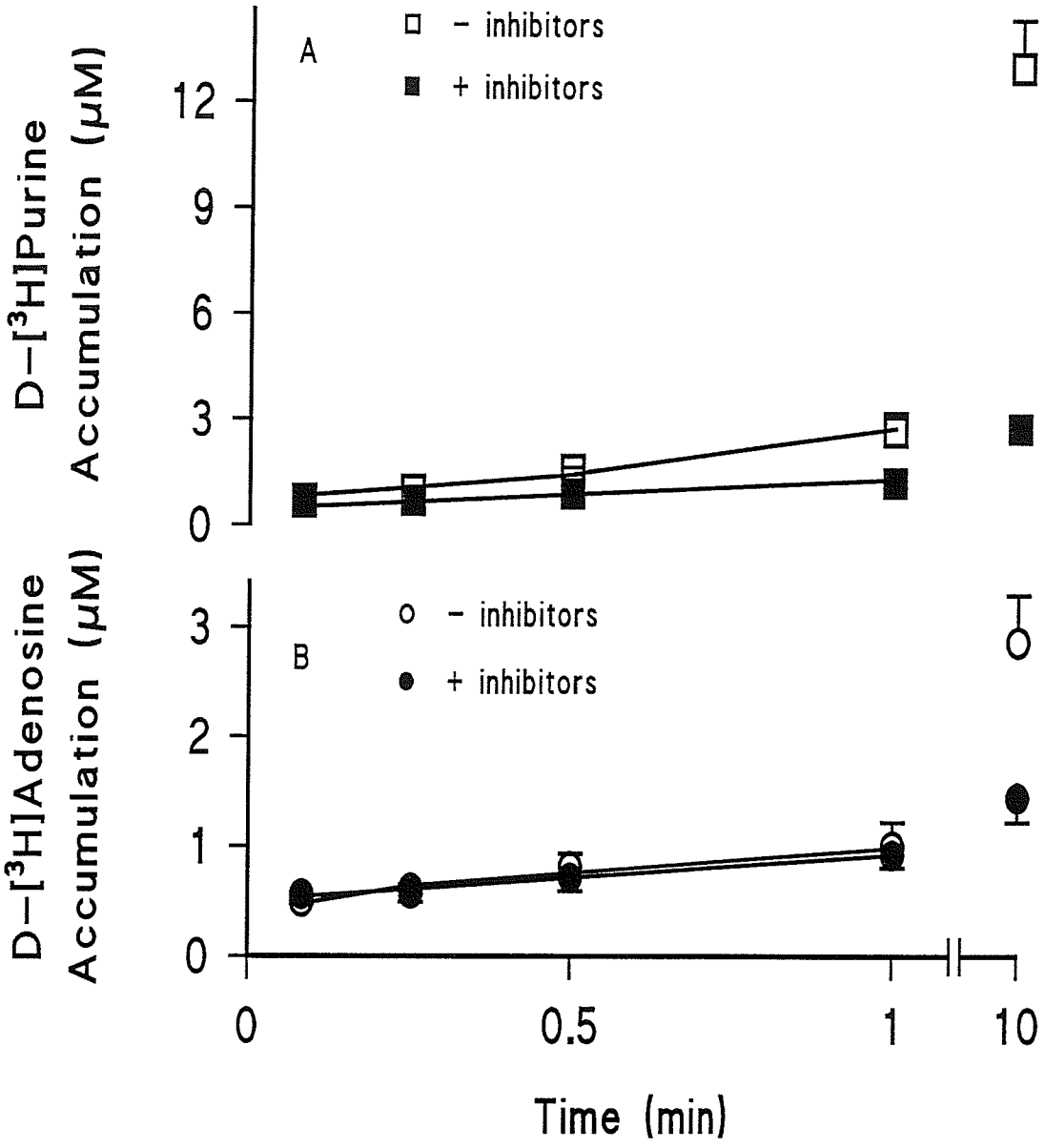

Figure 12. Accumulation of D-[^{3}H]purines (A., squares) and D-[^{3}H]adenosine (B., circles) in rat cerebral cortical synaptoneurosomes in the absence (open symbols) or presence (closed symbols) of 10 μM EHNA and 10 μM 5'-iodotubercidin. Tissues were incubated with 1 μM D-[^{3}H]adenosine at 37°C for 5 to 600 s. Purines, expressed in μM, represent accumulated adenosine plus adenine nucleotides and deaminated products. Values are mean ± S.E.M. from 6 experiments.

and AK inhibitors, accumulations of D-[^{3}H]adenosine again reached equilibration concentrations at 60 s incubations, but under these conditions accumulations remained relatively constant for up to 10 min (1.4 ± 0.2 μM). It is clear that intracellular concentrations of [^{3}H]-labeled D-purines and D-[^{3}H]adenosine higher than the extracellular concentration of 1 μM were observed when significant phosphorylation and trapping of substrate was present (Figure 12B).

D-[^{3}H]Adenosine, in the absence and presence of enzyme inhibitors, was transported into synaptoneurosomes by both high and low affinity processes regardless of whether the incubation periods were for 5 s (Figure 13), 15 s (data not illustrated), or 10 min (data not illustrated). In the absence of enzyme inhibitors there was a clear trend towards an inverse relationship between incubation interval and magnitude of apparent K_T and V_{max} values; the high affinity process was saturable with apparent K_T values (μ M) of 9.4 ± 4.2 at 5 s, 8.4 ± 2.3 at 15 s, and 4.5 ± 0.6 at 10 min. The apparent V_{max} values were 850 ± 420, 70 ± 15, and 12 ± 2 pmol/min/mg of protein at 5 s, 15 s, and 10 min, respectively. In the presence of EHNA and 5'-iodotubercidin, the K_T values (μM) at 5 s of 36.4 ± 0.6 (Figure 13.) and at 15 s of 43.6 ± 13.4 were significantly higher than values obtained in the absence of the enzyme inhibitors. At 10-min incubations, the apparent K_T value of 15.8 ± 4.7 (μM) obtained from experiments conducted with enzyme inhibitors was not significantly higher than the value for non-inhibited tissues. The V_{max} values of 712 ± 68, 182 ± 52, and 8 ± 2 pmol/min/mg of protein at 5 s, 15 s, and 10 min, respectively, were not significantly different from values obtained in the absence of the ADA and AK inhibitors. Although present, low affinity accumulations of D-[^{3}H]adenosine did not appear to be saturable, and derived affinity constant values of 4 ± 1 mM at 5 s, 3 ± 1 mM at 15 s, and 7 ± 2 mM at 10-min incubations may represent estimates for passive diffusion. The apparent K_T and V_{max} values of the low affinity process were not significantly affected by pre-treatment of the synaptoneurosomes with ADA and AK inhibitors.

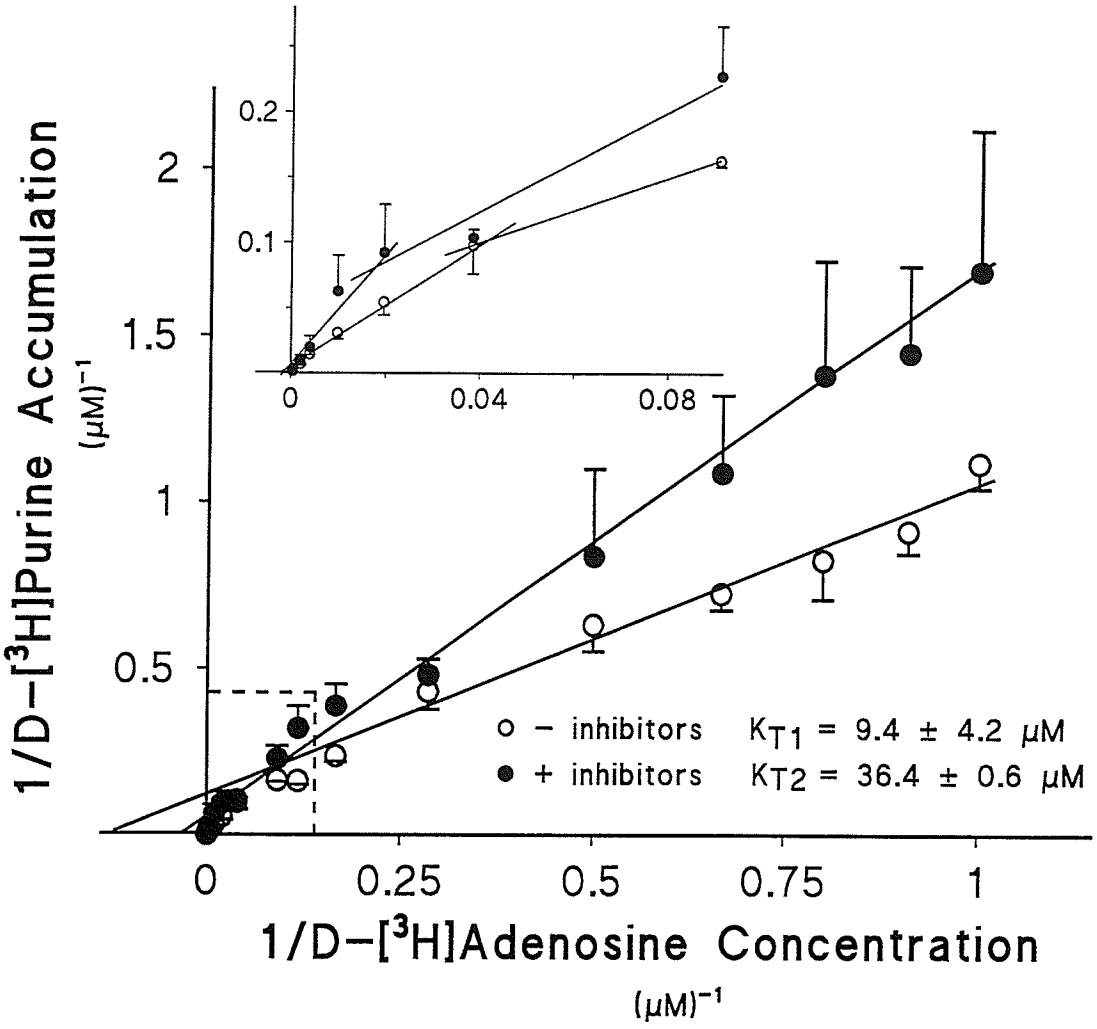

Figure 13. Accumulation of D-[^{3}H]purines in rat cerebral cortical synaptoneurosomes in the absence (open circles) and presence (closed circles) of 10 μM EHNA and 10 μM 5'-iodotubercidin. Synaptoneurosomes were incubated with 1 to 2500 μM D-adenosine 5 s at 37°C. K_T values for the high affinity process were 9.4 ± 4.2 and 36.4 ± 0.6 μM in the absence or presence of the enzyme inhibitors, respectively. K_T values for the low affinity process were 4 ± 1 and 11 ± 3 mM in the absence or presence of the enzyme inhibitors, respectively. Values are mean ± S.E.M. from 6 experiments.

Previously, we reported that L-adenosine was not a substrate for nor an inhibitor of rat brain homogenate ADA and AK (Gu et al., 1991). Although L-adenosine is much more metabolically stable than D-adenosine, statistically significant metabolism (mainly phosphorylation) began to occur at incubations longer than 30 s (Figure 14). At 5 s, 15 s, 30 s, 60 s and 10 min the percentage of total accumulated [^{3}H]-labeled L-purine found unchanged as L-[^{3}H]adenosine was 94 ± 2, 94 ± 1, 92 ± 1, 88 ± 2 and 65 ± 1, respectively, and that found as labeled nucleotides was 2.5 ± 1.4, 4.8 ± 1.0, 5.7 ± 2.3, 9.8 ± 2.5 and 31 ± 2.1, respectively (Figure 14). No L-[^{3}H]adenosine was metabolized by ADA to inosine and hypoxanthine. The accumulation of L-[^{3}H]adenosine was saturable and computerized least squares analysis of data illustrated as Lineweaver-Burke plots (Figure 15) yielded values for K_T of 39 ± 7 μM and V_{max} of 330 ± 71 pmol/min/mg protein. EHNA and 5'-iodotubercidin, at 10 μM concentrations, did not inhibit accumulations of L-[^{3}H]adenosine (data not shown).

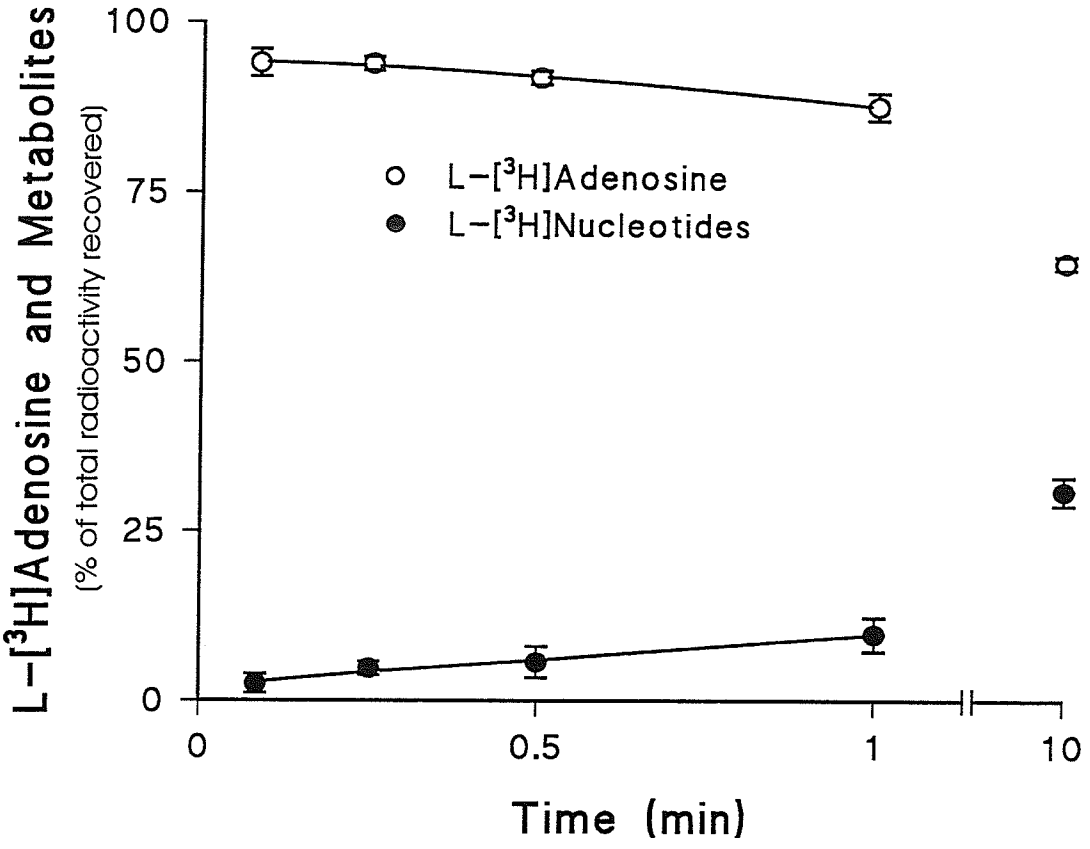

Figure 14. Metabolism of accumulated L-[^{3}H]adenosine in rat cerebral cortical synaptoneurosomes. Following addition of 1 μM L-[^{3}H]adenosine tissues were incubated at 37°C for 5 to 600 s. L-[^{3}H]Adenosine (open circles), L-AMP + L-ADP +L-ATP (solid circles) and L-inosine + L-hypoxanthine (not shown) were separated by TLC and data were expressed as a percentage of total radioactivity accumulated. Values are mean ± S.E.M. from 6 experiments.

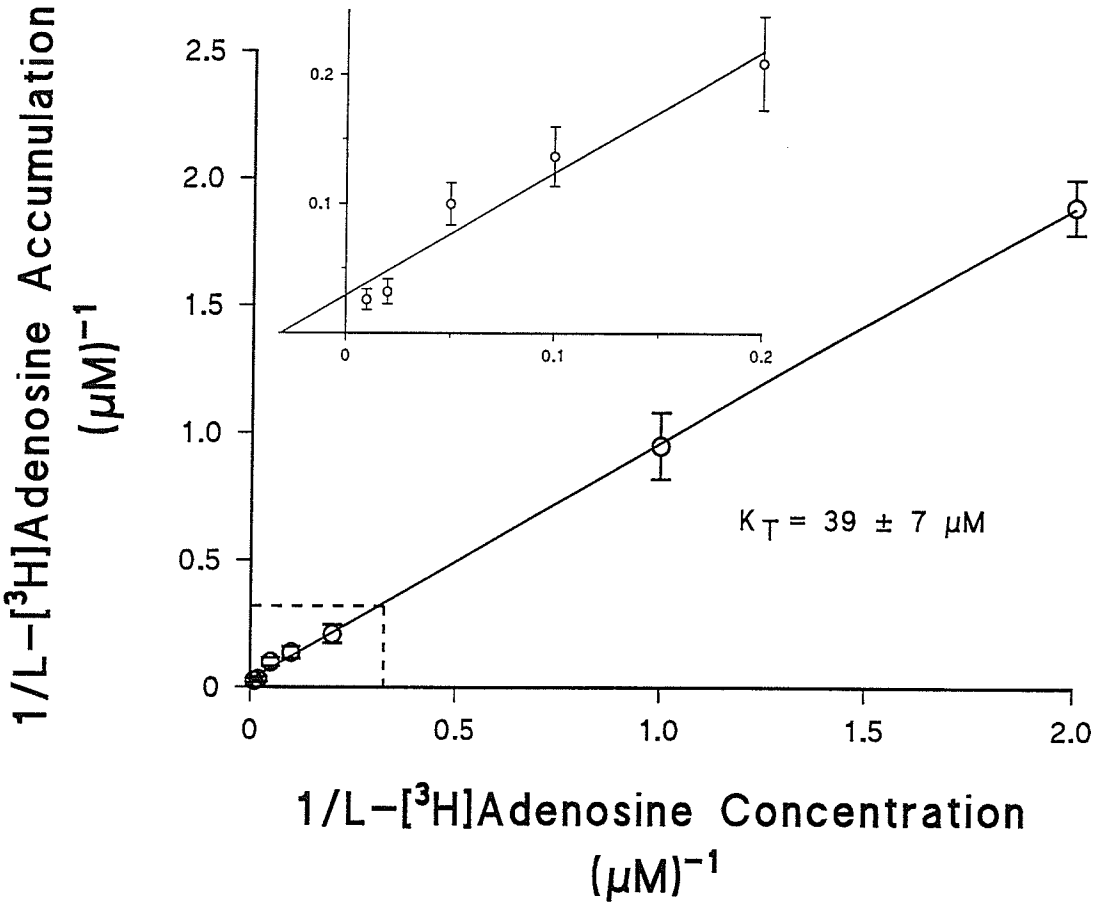

Figure 15. Kinetic analysis of L-[^{3}H]adenosine transport in rat cerebral cortical synaptoneurosomes. Synaptoneurosomes were incubated at 37°C for 20 s with L-[^{3}H]adenosine at concentrations ranging from 1.0 to 250 μM. The accumulation of L-[^{3}H]adenosine was saturable (isotherm not shown). Data are mean ± S.E.M. from 6 experiments.

DISCUSSION

The present studies were directed at determining whether some of the tenets thought basic to peripheral tissues and cultured cells with respect to adenosine transport hold true for rat cerebral cortical synaptoneurosomes. Our findings indicate (a) that accumulated D-[^{3}H]adenosine was rapidly metabolized (phosphorylated), mainly to adenine nucleotides, and that L-[^{3}H]adenosine was more metabolically stable, (b) that the transport of D-adenosine into synaptoneurosomes was significantly faster than its subsequent metabolism, (c) that an inverse relationship exists between the extent of metabolism of accumulated D-adenosine and the measured apparent K_T and V_{max} values for high affinity adenosine transport, and (d) that inhibition of ADA and AK activity increased apparent K_T values of the transport system for D-adenosine to 39 μM, a value virtually identical to that when L-adenosine was used.

Adenosine transport, in most cell types, is faster than the intracellular metabolism of accumulated adenosine (Young and Jarvis, 1983). Therefore, accurate measurements of adenosine influx into cells capable of metabolizing this substrate can only be obtained under conditions where initial rates of accumulation are measured in the absence of subsequent metabolism. However, this important underlying principle, although shown in non-CNS tissues, has not, to our knowledge, been tested for CNS tissues or cells. Under conditions where initial rates of adenosine accumulation are not measured, accumulations of radiolabel may reflect metabolic trapping of impermeable adenine nucleotides, efflux (backflux) of adenosine or of adenosine's deaminated products, or entry and exit of purine nucleosides and nucleobases via non-carrier-mediated processes. We have shown here that the V_{max} at short (5-s) incubation intervals was significantly greater than at longer (15-s and 10-min) incubation intervals and thus adenosine influx into synaptoneurosomes is more rapid than its subsequent metabolism, a finding similar to that of transporters in peripheral tissues and some cultured cells (Wohlhueter and Plagemann, 1989). Based on

the above findings, under conditions other than initial rates of accumulation, measured kinetic values for influx would be affected by the subsequent intracellular metabolism, and this is consistent with the inverse relationship found here between apparent K_T and V_{max} values and incubation times. Thus, when phosphorylation of accumulated adenosine occurs, the kinetic values of accumulation begin to approximate the K_m and V_{max} values of AK (see Geiger and Fyda, 1991). However, in the absence of significant metabolism, the K_T value of 39 μM found here is similar to those obtained from experiments of initial (zero-trans) rates of nucleoside translocation performed with peripheral tissues and cultured cells (Plagemann and Wohlhueter, 1980; Young and Jarvis, 1983). Therefore, inhibition of ADA and AK activity can be used to produce K_T values that approximate those obtained when initial rates of accumulation are measured.

In addition to concerns regarding incubation conditions, it has been found that metabolism and thus measurements of transport kinetics of accumulated adenosine can also be markedly affected by the level of substrate used. AK has a higher affinity for D-adenosine than does ADA (Geiger and Nagy, 1990). Furthermore, AK is a low capacity enzyme subject to substrate inhibition at near physiological levels of adenosine. Therefore, it is likely that the predominance of AK over ADA in the metabolism of accumulated D-adenosine would be observed mainly when physiological levels (1 μM) of D-adenosine are used, but not at higher levels (5 to 10 μM) (Lum et al., 1979; Plagemann et al., 1985). Our findings that transported D-adenosine was mainly phosphorylated to nucleotides, that 5'-AMP is the major product (data not shown), and that very little D-adenosine was deaminated into inosine and hypoxanthine are consistent with this concept and with previous findings (see Geiger and Nagy, 1990; Bender et al., 1981; see Wu and Phillis, 1984). However, the use of prolonged incubation conditions (greater than initial rates) and high levels of substrate may help explain, at least in part, previous findings that showed extensive deamination of accumulated adenosine in primary cultures of mouse astrocytes (Hertz and Matz, 1989).

During short incubation periods, EHNA and 5'-iodotubercidin, competitive inhibitors of ADA and AK, respectively, effectively decreased the metabolism of accumulated D-adenosine yet did not inhibit D-adenosine accumulation. This is consistent with our findings in synaptoneurosomes and with previous findings in non-CNS tissues that these inhibitors were ineffective in blocking nucleoside transport (Fernandez-Rivera-Rio and Gonzales-Garcia, 1985; Kroll et al., 1989; LeHir and Dubach, 1985). The present findings that EHNA and 5'-iodotubercidin increased the apparent K_T values (decreased apparent affinity) strongly suggests that these enzyme inhibitors were exerting their effects through the elimination of metabolic trapping, thereby unmasking more accurate measurements of kinetic values for adenosine transport. The effects of metabolic trapping on accumulated purine levels (Figure 12) indicate that intracellular concentrations of purines reached levels higher than the levels of substrate used when extensive phosphorylation of D-adenosine occurred. These findings are consistent with the notion that prolonged incubations can give an illusion of "concentrative" transport, which upon closer examination is actually metabolic trapping of adenine nucleotides. That the apparent K_T values for adenosine transport obtained in the presence of EHNA and 5'-iodotubercidin accurately reflected the transport processes was further supported by our findings that the K_T values for L-adenosine, under conditions where significant metabolism (phosphorylation) occurred only at incubations longer than 30 s, were virtually identical to those obtained for D-adenosine in the presence of the enzyme inhibitors. The metabolic stability of L-adenosine, its high affinity for adenosine transporters, and its inability to activate adenosine receptors (Cusack et al., 1979; Cusack and Planker, 1979; Cusack and Hourani, 1981) would thus enable it to be used as a probe for the study of adenosine transport processes in the CNS under conditions where metabolism of D-adenosine is prevalent and where the use of metabolic inhibitors might be inappropriate.

Where measurements of nucleoside transport in peripheral and CNS tissues, and cultured cells have been made in the absence of interference by metabolism of the accumulated nucleoside, K_T values have all been substantially higher (tens to hundreds of μM) than the presumed endogenous nucleoside levels. For example, in rat cerebral cortical synaptosomes, the K_T values of uridine for inhibitor-sensitive and inhibitor-resistant transport components were 300 and 214 μM, respectively (Lee and Jarvis, 1988a). The K_i values of adenosine for these two components were 62 and 54 μM, respectively (Lee and Jarvis, 1988a). In guinea pig cerebral cortical synaptosomes, the K_T values of adenosine for inhibitor-sensitive sites was 17 μM and for inhibitor-insensitive sites was 68 μM (Lee and Jarvis, 1988b). The K_T value for adenosine accumulation into ATP-depleted primary cultures of chick brain neurons was 13 μM (Thampy and Barnes, 1983b) and into astrocytes was 370 μM (Thampy and Barnes, 1983a). It is remarkable that when non-ATP-depleted astrocytes were used, K_T values of 12 μM were obtained (Thampy and Barnes, 1983a). This last finding is consistent with our present findings and with previous studies showing that where extensive metabolism has occurred, lower K_T values were observed (see Geiger and Fyda, 1991; Geiger and Nagy, 1990).

Based on the K_T value of 39 μM and the apparent nanomolar levels of adenosine in the CNS (Phillis et al., 1991; Ballarin et al., 1991), the physiological relevance of such a low affinity transport system might be questioned. However, the physiological significance of these higher K_T systems becomes more clear when it is considered that because the V_{max} of transport at 5-s incubations (little if any metabolism present) is up to 50 times higher than the Vmax for uptake at 10 min incubations (accumulated adenosine largely metabolized), Michaelis-Menton kinetics dictates that a high K_T system would predominate over a low K_T system for concentrations of substrate ranging from 0.1 to 100 μM (data not shown). Furthermore, owing to the complex nature of purine metabolism, the rapid breakdown of ATP to adenosine during the sampling

process, and the difficulty in measuring the intracellular levels of adenosine, it is not possible at present to state with confidence what the intra- and extracellular levels of adenosine are. Thus, it is clear that a low affinity (K_T 39 μM) adenosine transport system is present in synaptoneurosomes but that it is not measurable when significant amounts of metabolism, mainly phosphorylation, is present.

Chapter 3. [^{3}H]Adenosine Transport in Synaptoneurosomes of Postmortem Human Brain

ABSTRACT: [^{3}H]Adenosine transport was characterized in cerebral cortical synaptoneurosomes prepared from postmortem human brain using an inhibitor-stop/centrifugation method. The adenosine transport inhibitors dipyridamole and dilazep completely and rapidly blocked transmembrane fluxes of [^{3}H]adenosine. For 5-s incubations, two kinetically distinguishable processes were identified, i.e., a high-affinity adenosine transport system with K_T and Vmax values of 89 μM and 0.98 nmol/min/mg protein, respectively, and a low-affinity adenosine transport system that did not appear to be saturable. For incubations with 1 μM [^{3}H]adenosine as substrate, intrasynaptoneurosomal concentrations of [^{3}H]adenosine were 0.26 μM at 5 s and 1 μM at 600 s. Metabolism of accumulated [^{3}H]adenosine to adenine nucleotides was 15% for 5-s, 23% for 15-s, 34% for 30-s, 43% for 60-s and 80% for 600-s incubations. The concentrations (μM) of total accumulated [^{3}H]purines ([^{3}H]adenosine plus metabolites) at these times were 0.3, 0.5, 1.0, 1.3 and 5.6, respectively. These results indicate that in the presence of extensive metabolism the intrasynaptoneurosomal accumulation of [^{3}H]purines was higher than the initial concentration of 1 μM [^{3}H]adenosine in the reaction medium. For 5-, 15-, 30-, 60-, and 600-s incubations in the presence of the adenosine deaminase inhibitor EHNA and the adenosine kinase inhibitor 5'-iodotubercidin, metabolism of the transported [^{3}H]adenosine was 14, 14, 16, 14, and 38%, respectively. During these times, total [^{3}H]purine accumulation was 0.3, 0.5, 0.5, 0.7, and 1.8 μM, respectively. Thus, the apparently "concentrative" accumulation of [^{3}H]purines can be prevented by inhibition of adenosine metabolism and, taken together, these results suggest that adenosine transport in at least synaptoneurosomes prepared from postmortem human brain is via a non-concentrative and equilibrative system.

INTRODUCTION

It has been increasingly appreciated that in the CNS the physiological actions of adenosine, which are expressed through specific subtypes of adenosine receptors (Stone, 1991), may be exploited for therapeutic benefit (Williams, 1989). Adenosine and its analogues have been tested for their ability to inhibit seizures, decrease neuronal injury secondary to ischemic and hypoxic insults, and protect against neurodegenerative diseases (Daval et al., 1991). However, because adenosine receptor agonists do not readily cross the blood brain barrier, and because of their undesirable peripheral side effects and their lack of site and event specificity, alternative agents have been developed which are capable of controlling endogenous levels of adenosine (Daly, 1982). One target of this pharmaceutical approach is adenosine transport (Wu and Phillis, 1984; Geiger and Fyda, 1991). Inhibitors of adenosine transport may exert their effects primarily at those sites where and during those times when adenosine is being produced. In this way adenosine transport inhibitors would maintain extra-neuronal/extracellular adenosine at high levels thereby indirectly enhancing adenosine receptor-mediated responses. Indeed, several selective adenosine transport inhibitors have been shown to, for example, improve the `quality' of sleep (Wauquier et al., 1987), prevent seizures (Zhang et al., 1990), and increase cerebral blood flow (Phillis et al., 1989).

In order to understand how adenosine transport systems in the CNS control extra-neuronal/extracellular adenosine levels and how adenosine transport inhibitors affect the transport systems, adenosine transport in CNS tissues of animals has been characterized (Geiger and Fyda, 1991). Inhibitor-sensitive and -resistant equilibrative adenosine transport systems as well as sodium-dependent concentrative systems have been described (Johnston and Geiger, 1990). Research on adenosine transport systems in animals, which has been ongoing for over two decades, has identified significant differences among species (Johnston and Geiger, 1990; Morgan and Marangos, 1987).

Given those differences and despite the current interest in the development of adenosine transport inhibitors as therapeutic agents, adenosine transporters in human brain have not yet been studied nor characterized. Here, using synaptoneurosomes prepared from postmortem human cerebral cortex, we report on the kinetic characteristics of adenosine transport.

MATERIALS AND METHODS

Preparation of synaptoneurosomes from postmortem human cerebral cortex

Postmortem human brains were obtained from 7 males and 5 females (average age, 79 ± 4 years). All patients were without history of neurological or psychiatric diseases and died as a consequence of peripheral vascular or pulmonary diseases. Following death, bodies were transferred to the hospital's morgue where they were refrigerated before autopsy. Postmortem delay times ranged from 4 to 24 hours. Brains were removed and kept on ice while they were bisected; left hemispheres were taken for neuropathological analyses and right hemispheres were dissected to obtain cerebral cortical gray matter. Synaptoneurosomes were prepared as described previously (Hollingsworth et al., 1985; Gu et al., 1991). In brief, a Teflon-glass homogenizer (0.125 mm clearance) was used to homogenize tissues in 7 volumes (wt/vol) of a buffer consisting of 110 mM NaCl, 25 mM glucose, 68.3 mM sucrose, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, and 20 mM HEPES (pH 7.4). Homogenates were filtered by gravity through a triple layer of 80-μm nylon netting (Thompson, Montreal) and then pressure filtered through 10 μm filters (Millipore LCWP-047) loaded in Swinnex holders. The filtrate was centrifuged at 1,000 x *g* for 15 min at 4°C and the synaptoneurosomal pellets were suspended in buffer to yield a protein concentration of 3 mg/ml. Protein was measured according to the method of Lowry et al. (1951). All procedures were conducted

at 4°C unless otherwise indicated and all assays were completed within three hours of synaptoneurosome preparation.

[^{3}H]Adenosine transport assay

Reactions were conducted in a total volume of 1.0 ml buffer that contained 3% (wt/vol) BSA, and ~ 0.3 mg of synaptoneurosome protein (unless otherwise indicated). Synaptoneurosomes were preincubated for 10 min at 37°C and transport was initiated by addition of [^{3}H]adenosine (5 mCi/μmol, NEN). Incubations were conducted at 37°C for intervals ranging from 5 to 600 s and reactions were terminated by the addition of 100 μl inhibitor-stop cocktail containing the nucleoside transport inhibitors dilazep and dipyridamole together with the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenosine (EHNA) and the adenosine kinase inhibitor 5'-iodotubercidin at final concentrations (mM) of 1.0, 0.1, 0.1, and 0.01, respectively. The inhibitor-stop cocktail contained 1% dimethyl sulfoxide (DMSO). EHNA, 5'-iodotubercidin, and DMSO at the concentration used had no effect on adenosine transport (Gu and Geiger, 1992; Fernanadez-Rivera-Rio and Gonzales-Garcia, 1985; Kroll et al., 1989; LeHir and Dubach, 1985). The reaction vessels were immediately centrifuged at 13,000 *g* for 30 s and the supernatants were aspirated. Pellets were gently dislodged from the walls of the centrifuge tubes with 0.5 ml of ice-cold buffer containing 100 μM dilazep. After re-centrifugation, supernatants were aspirated, and pellets were digested in 0.3 ml of 0.1 M NaOH and added to 4.5 ml of Beckman Ready Safe scintillation cocktail. Radioactivity was determined by scintillation spectrophotometry. In assay blanks, synaptoneurosomes were preincubated with the inhibitor-stop cocktail for 10 min at 37°C, placed on ice, and after a 10 min delay, [^{3}H]adenosine was added and the reaction vessels were processed as described above.

Two conditions were used in determining whether adenosine transport could be terminated completely and instantaneously by the inhibitor-stop cocktail. First,

synaptoneurosomes were pretreated with inhibitor-stop cocktail for 10 min and then 1 μM [^{3}H]adenosine was added. Second, the inhibitor-stop cocktail and 1 μM [^{3}H]adenosine were added simultaneously to the reaction medium containing synaptoneurosomes. In both experiments, incubation times were 0, 5, 15, 30, and 60 s. The results were compared with experiments where transport was initiated by the addition of 1 μM [^{3}H]adenosine and terminated with the addition of the inhibitor-stop cocktail. To determine the protein dependence of [^{3}H]adenosine transport, synaptoneurosome protein concentrations ranging from 0.15 to 0.6 mg/ml were incubated with 1 μM [^{3}H]adenosine for 5 s. To determine the accumulations of [^{3}H]adenosine and its metabolites, synaptoneurosomes were incubated with 1 μM [^{3}H]adenosine (5 mCi/μmol) for 5, 15, 30, 60 s and 10 min in the absence or presence of 10 μM EHNA plus 10 μM 5'-iodotubercidin. To determine the kinetic values for adenosine transport (K_T and Vmax values), [^{3}H]adenosine accumulation (5-s incubations) was initiated by additions of [^{3}H]adenosine at final concentrations ranging from 1 to 2500 μM. In an attempt to determine the effects of postmortem delay on [^{3}H]adenosine transport, male Sprague Dawley rats (250-350 g) were decapitated, heads were stored in 4^{o}C for 0, 1, 4, and 24 hrs, and synaptoneurosomes were prepared. Transport was conducted as above with 40 μ M [^{3}H]adenosine and incubations were for 5 s.

To determine the accumulations of [^{3}H]adenosine and its metabolites, the pellet was washed, supernatant aspirated, and 100 μl of ice-cold aqueous 5% trichloroacetic acid (TCA) was added. Samples were placed on ice for ~20 minutes and centrifuged. Aliquots of 20 μl were taken to measure the accumulation of total [^{3}H]purine ([^{3}H]adenosine and its metabolites). Aliquots of 50 μl were spotted on Polygram cellulose precoated plastic TLC sheets (Machray Nagel, F.R.G.) and developed in a solvent composed of 1-butanol/acetone/glacial acetic acid/concentrated ammonium hydroxide/distilled water (14:10:6:1:8 by volume). Individual spots of [^{3}H]adenosine and its metabolites were identified under ultraviolet light (254 nm), cut out, and placed in

scintillation vials along with 0.5 ml of 0.1 M HCl. Samples were vortex mixed and allowed to sit at room temperature for an additional 20 minutes prior to the addition of 4.5 ml of scintillation fluid.

Intrasynaptoneurosomal water was measured and used in the calculation of the intrasynaptoneurosomal concentration of accumulated [^{3}H]adenosine and its metabolites (Gu and Geiger, 1992). Kinetic values of [^{3}H]adenosine transport were determined by computerized non-linear regression analyses of the data and results were illustrated on Lineweaver-Burke plots. Unless otherwise indicated, all values represent means ± S.E.M. Statistical analyses were performed by using a Student's *t* test and significance was considered at the $p<0.05$ level.

RESULTS

The accumulation of [^{3}H]purines derived from transported [^{3}H]adenosine was completely and apparently instantaneously terminated by our inhibitor-stop cocktail. No significant accumulation of [^{3}H]purines occurred during incubations of ≤ 30 s in experiments where synaptoneurosomes were pretreated with inhibitor-stop cocktail for 10 min or where inhibitor-stop cocktail and 1 μM [^{3}H]adenosine were added simultaneously (Figure 16). [^{3}H]Adenosine accumulation increased linearly (r=0.997) with increased synaptoneurosomal protein concentrations over the range of 0.15-0.6 mg/ml (Figure 17). The accumulation rate was ~2 pmol/mg protein/5 s. In synaptoneurosomes prepared from postmortem human brain, [^{3}H]adenosine accumulations were not significantly changed in a time dependent manner by postmortem delays that ranged from 4 to 24 hour (data not shown). Although rats were not handled in a manner strictly analogous to human corpses, in our experiments to determine possible effects of postmortem delay on adenosine transport, we found that [^{3}H]adenosine accumulations in rat brain synaptoneurosomes were not significantly affected by postmortem delays that ranged from 0 to 24 hours. The

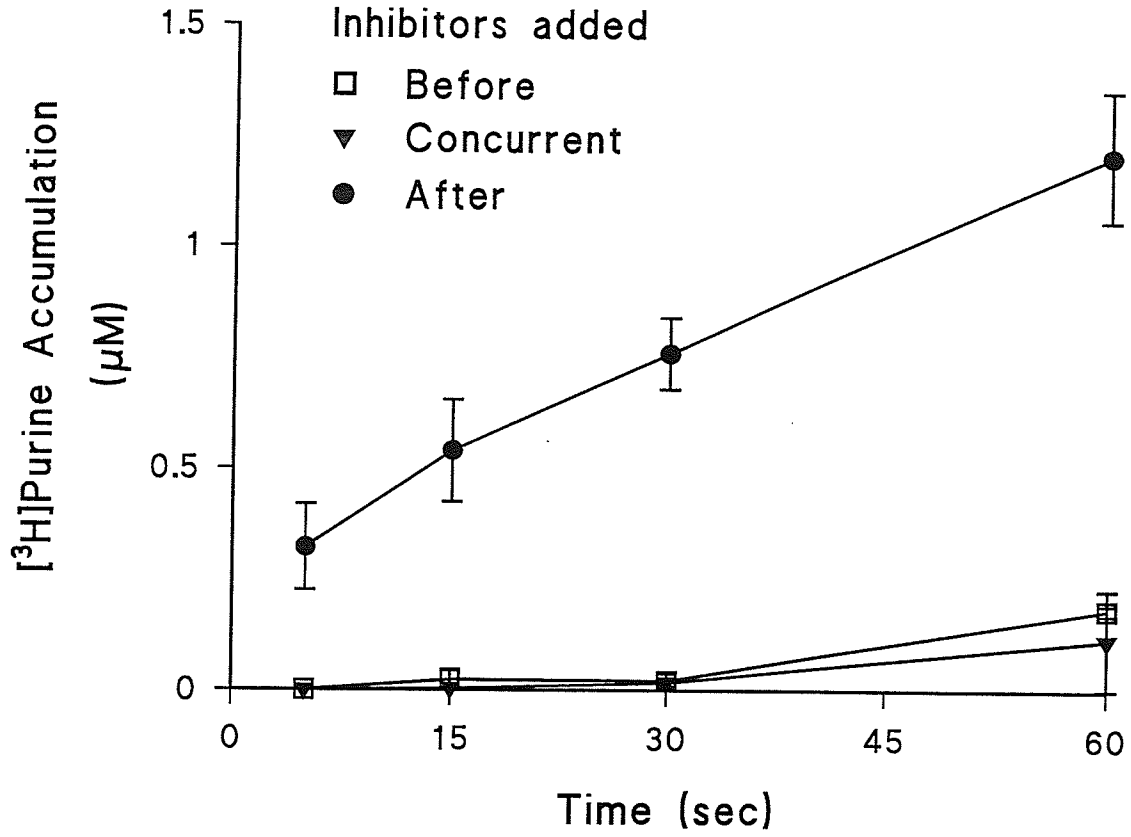

Figure 16. Instantaneous and complete termination of [^{3}H]adenosine transport. Inhibitor-stop cocktail (1 mM dilazep, 0.1 mM dipyridamole, 0.1 mM EHNA, and 10 μM 5'-iodotubercidin) was added before (open squares), at (solid triangles), and after (solid circles) initiation of accumulation with 1 μM [^{3}H]adenosine. Values represent means ± S.E.M. of the results from 2 experiments conducted in triplicate.

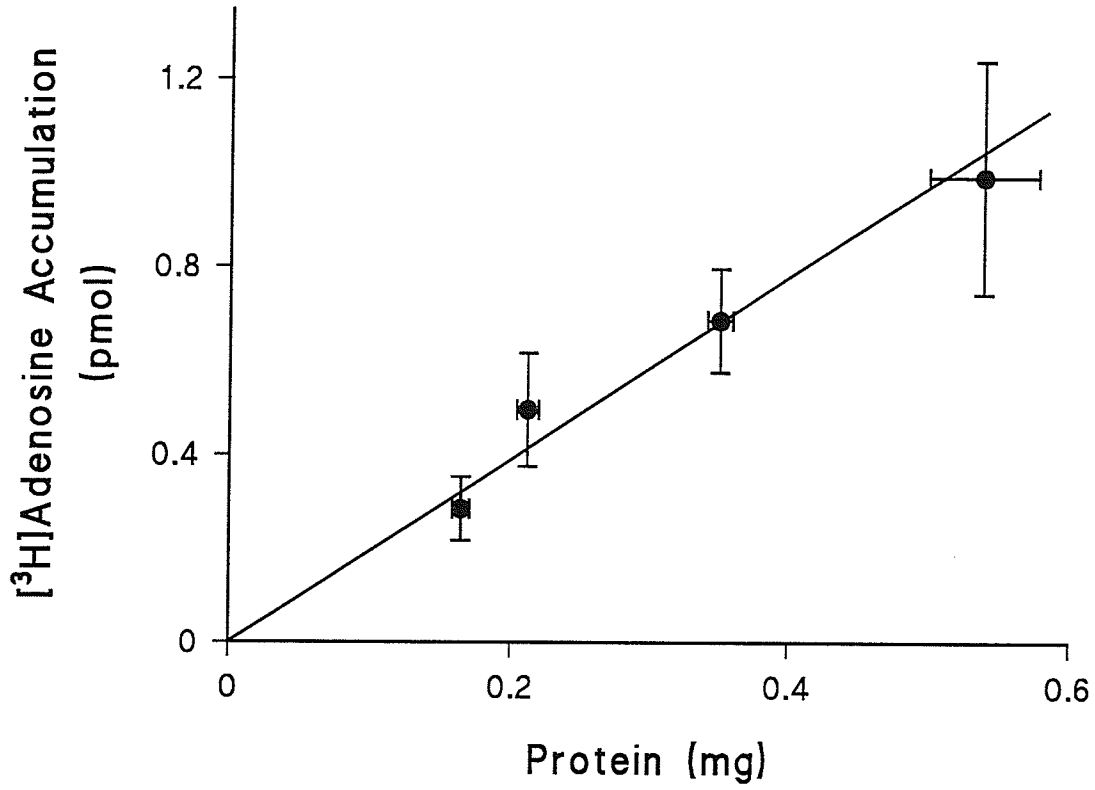

Figure 17. [^{3}H]Adenosine accumulation expressed as a function of synaptoneurosomal protein. Synaptoneurosomes were incubated with 1 μM. Values represent mean ± S.E.M. of the results from 5 experiments conducted in duplicate.

accumulations (pmol/mg protein/5 s) of [^{3}H]adenosine in rat synaptoneurosomes were 61 ± 10, 61 ± 13, 76 ± 23, and 57 ± 2 for 0-, 1-, 4-, and 24- h postmortem delays (n=3, for each time point), respectively.

[^{3}H]Adenosine metabolism in synaptoneurosomes increased with longer incubation times (Figure 18, 19). At incubation times of 5, 15, 30, 60, and 600 s, metabolism of accumulated [^{3}H]adenosine mainly to adenine nucleotides was 15, 23, 34, 43, and 80%, respectively (Figure 19). In the presence of 10 μM EHNA and 10 μM 5'-iodotubercidin, metabolism of accumulated [^{3}H]adenosine was approximately 14% at 5 to 60 s incubations and 38% at 600-s incubation (Figure 18). Except at 5-s incubation time, EHNA and 5'-iodotubercidin significantly inhibited [^{3}H]adenosine metabolism (Figure 18, 19).

[^{3}H]Purine ([^{3}H]adenosine plus [^{3}H]metabolites) accumulation increased with longer incubation times. Considering the intrasynaptoneurosomal volume as determined experimentally through measurements of water content (3 μl/mg protein), [^{3}H]purine concentrations (μM) in synaptoneurosomes were found to be 0.3, 1.3, and 5.6 after 5-, 60-, and 600-s incubations, respectively. [^{3}H]Purine concentrations after 60- and 600-s incubations were significantly higher than the initial [^{3}H]adenosine concentration in the reaction medium (1 μM). In the presence of 10 μM EHNA and 10 μM 5'-iodotubercidin, [^{3}H]purine concentrations were 0.3, 0.7, and 1.8 μM after 5-, 60-, and 600-s incubations. [^{3}H]Purine concentrations in synaptoneurosomes were significantly lower in the presence of the inhibitors than in the absence of the inhibitors after 60- and 600-s incubations (Figure 20).

[^{3}H]Adenosine accumulation in synaptoneurosomes increased with longer incubation times. [^{3}H]Adenosine concentrations (μM) in synaptoneurosomes were 0.25 and 1.1 at 5 and 600-s incubation times, respectively. For 5-s incubation, the concentration was only 25% of that in the reaction medium (1 μM). At 600-s incubation, the concentration of 1.1 μM was not significantly different from the initial [^{3}H]adenosine

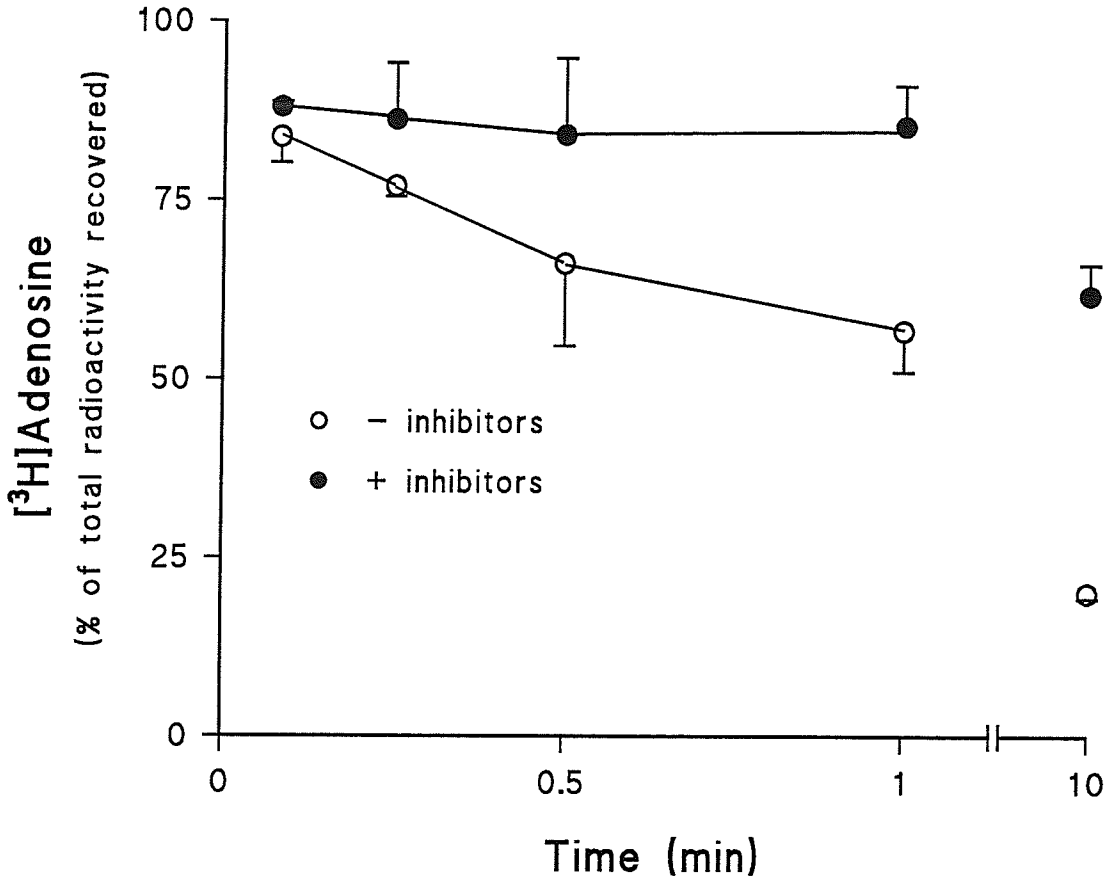

Figure 18. Metabolism of accumulated [^{3}H]adenosine. Synaptoneurosomes were incubated with 1 μM [^{3}H]adenosine in the absence (open circles) or presence (solid circles) of 10 μM EHNA and 5'-iodotubercidin. [^{3}H]Adenosine in synaptoneurosomes was separated by TLC. Values represent means ± S.E.M of the results from 4 experiments conducted in duplicate.

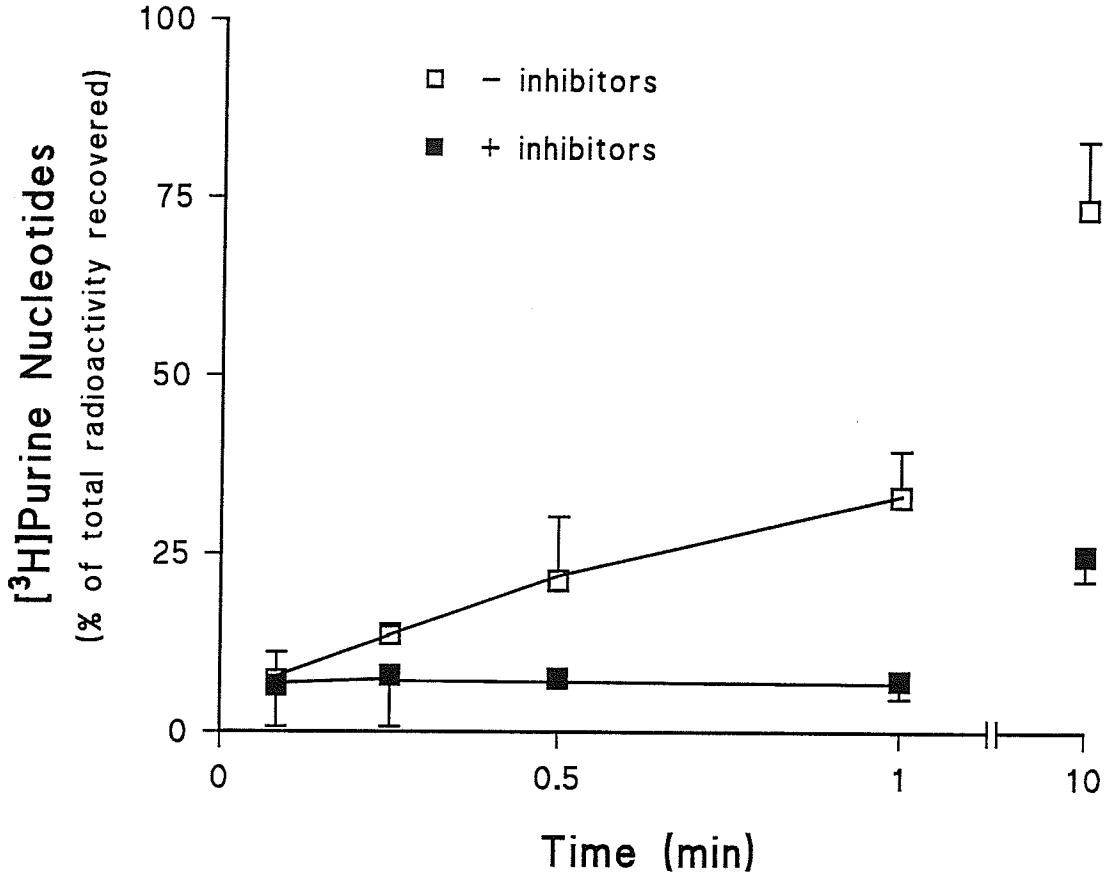

Figure 19. Metabolism of accumulated [^{3}H]adenosine to [^{3}H]purine nucleotides. [^{3}H]Purine nucleotide production was measured after synaptoneurosomes were incubated with 1 μM [^{3}H]adenosine in the absence (open squares) or presence (solid squares) of 10 μM EHNA and 5'-iodotubercidin. [^{3}H]Purine nucleotides in synaptoneurosomes were separated by TLC. Values represent means ± S.E.M of the results from 4 experiments conducted in duplicate.

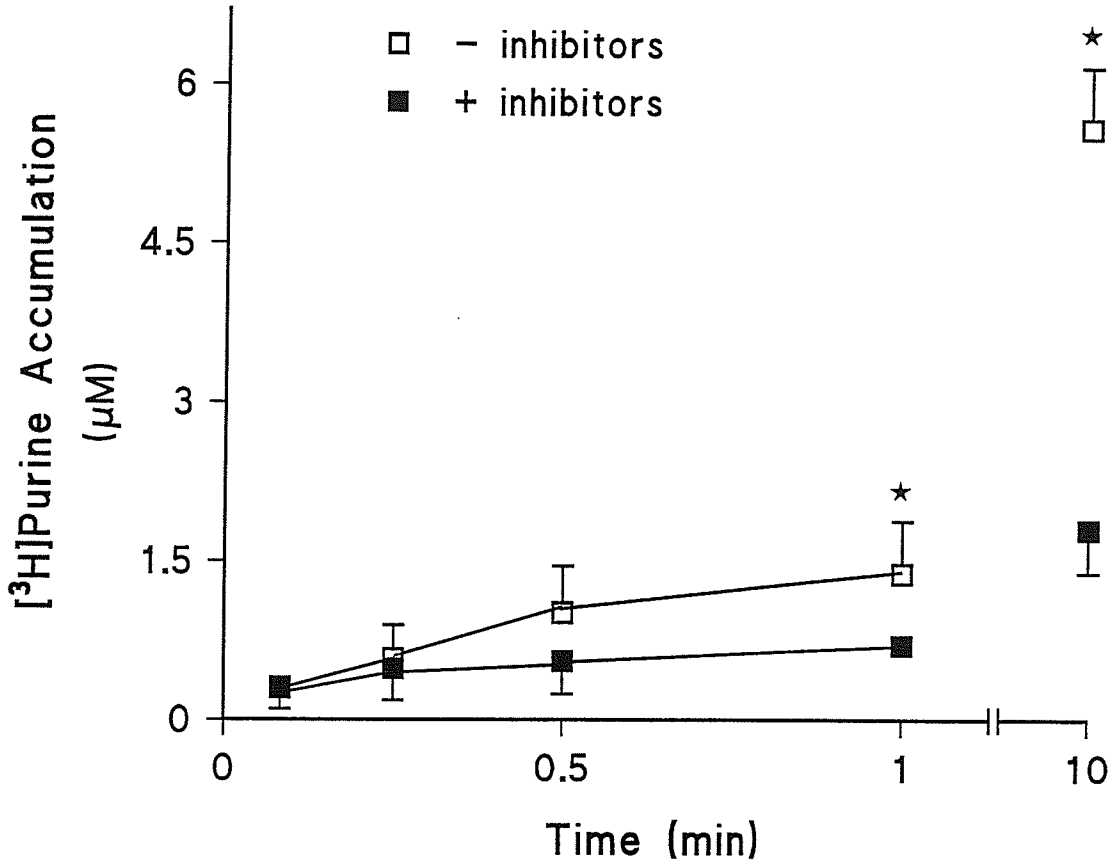

Figure 20. [^{3}H]Purine ([^{3}H]adenosine plus metabolites) accumulation. Synaptoneurosomes were incubated with 1 μM [^{3}H]adenosine in the absence (open squares) or presence (solid squares) of 10 μM EHNA and 5'-iodotubercidin. Values represent mean ± S.E.M. of the results from 4 experiments conducted in duplicate.

concentration in the reaction medium (1 μM). The accumulations of [^{3}H]adenosine in synaptoneurosomes were not significantly affected by 10 μM EHNA and 10 μM 5'-iodotubercidin (Figure 21).

[^{3}H]Adenosine was transported into synaptoneurosomes by both high- and low-affinity processes at 5-s incubations (Figure 22). Apparent K_T and Vmax values in the high affinity state were 89 ± 16 μM and 0.98 ± 0.2 nmol/min/mg protein, respectively. Although present, low-affinity accumulations of [^{3}H]adenosine did not appear to be saturable and may represent passive diffusion.

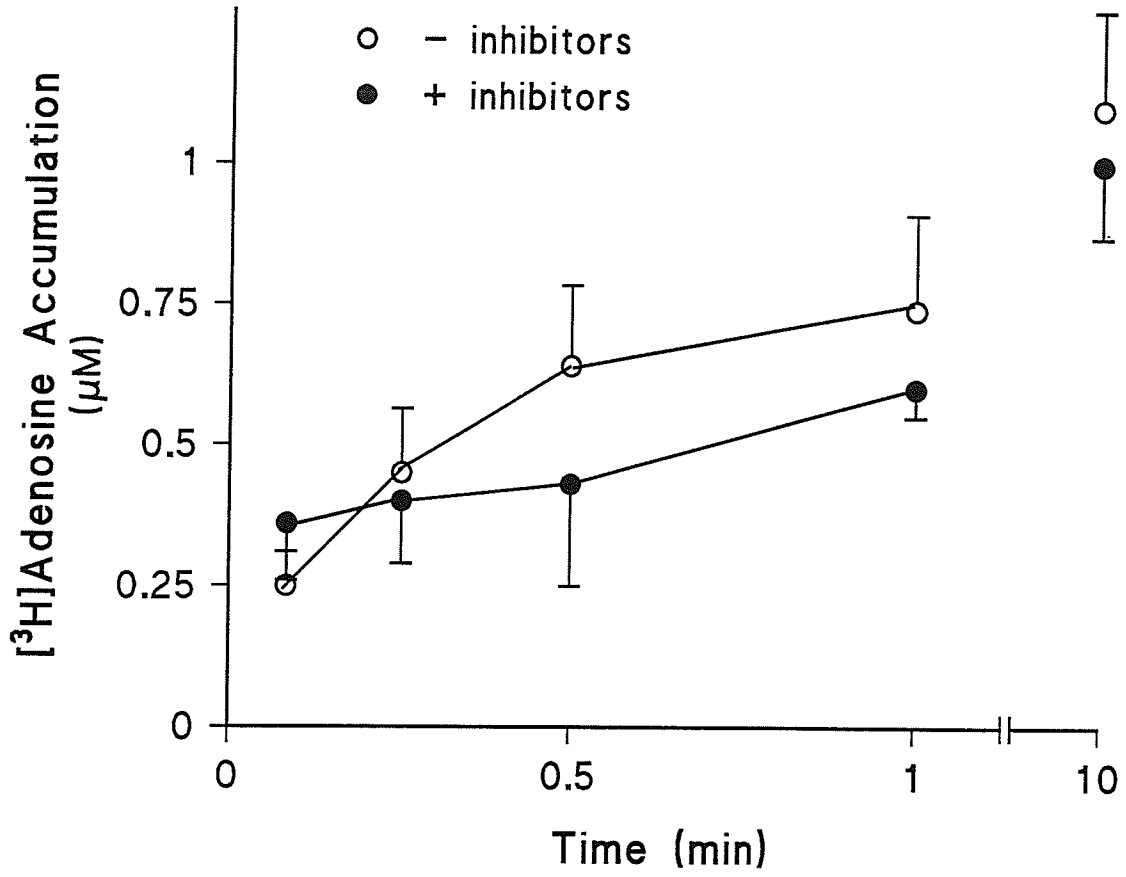

Figure 21. [^{3}H]Adenosine accumulation. Synaptoneurosomes were incubated with 1 μM [^{3}H]adenosine in the absence (open circles) or presence (solid circles) of 10 μM EHNA and 5'-iodotubercidin. [^{3}H]Adenosine was separated from its metabolites in synaptoneurosomes by TLC. Values represent mean ± S.E.M. of the results from 4 experiments conducted in duplicate.

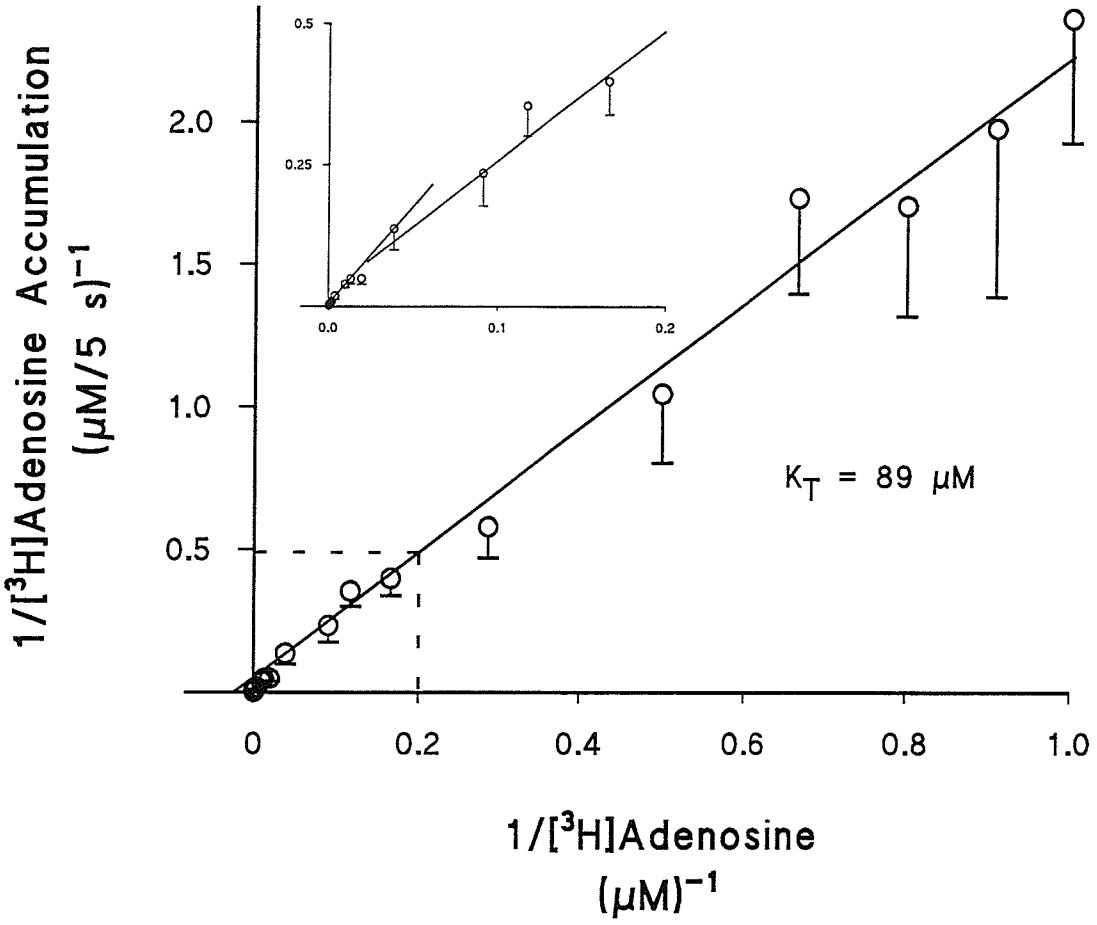

Figure 22. Lineweaver-Burke plot of [^{3}H]adenosine transport. Synaptoneurosomes were incubated with 1 to 2500μM [^{3}H]adenosine for 5 s. Apparent K_T and Vmax values were 89 μM and 0.98 nmol/min/mg protein for the high affinity site and were 4.5 mM and 15.2 nmol/min/mg protein for the low affinity site, respectively. Values represent means ± S.E.M. of the results from 5 experiments conducted in duplicate.

DISCUSSION

Adenosine transport has been studied extensively in animal CNS, in cultured cells, and in human peripheral tissues. The present report, to our knowledge, represents the first determination of the kinetic characteristics of adenosine transport in human CNS. This is an issue of some importance because basic characterization of adenosine transport in humans would seem to be a logical prerequisite to pharmaceutical development of adenosine transport inhibitors as therapeutic agents. In synaptoneurosomes prepared from postmortem human brain, we showed that [^{3}H]adenosine transport is completely and virtually instantaneously terminated by an inhibitor-stop cocktail containing the adenosine transport inhibitors dipyridamole and dilazep, that at long incubation times transported adenosine is extensively metabolized to its nucleotides, that the metabolism of transported adenosine caused an apparently `concentrative' purine accumulation, and that adenosine transport appears to be via a non-concentrative and equilibrative system.

A major complicating factor in making measurements of initial rates of adenosine transport in CNS tissues of experimental animals is the presence of adenosine transporters that are both equilibrative and inhibitor-resistant. This insensitivity to transport inhibitors renders inhibitor quench/stop methods relatively ineffective. In the absence of complete and instantaneous stop by an inhibitor-stop cocktail, [^{3}H]adenosine and its deaminated metabolites would continue to flow into and out of tissues and thus measured accumulation would not represent transport rates at selected incubation times (Geiger and Fyda, 1991). This potential source of error would tend to be most severe when accumulations were measured over shorter incubation periods (Geiger et al., 1988). Therefore, a prerequisite to making accurate measurements of [^{3}H]adenosine transport in human brain synaptoneurosomes was to demonstrate that the inhibitor-stop cocktail used in these studies could completely and virtually instantaneously block transmembrane fluxes of [^{3}H]adenosine. This was accomplished through pretreatment of

synaptoneurosomes with inhibitors and through the simultaneous additions of the stop cocktail with substrate adenosine.

Another complicating factor is that intracellular enzymes which metabolize adenosine recognize adenosine more slowly and with higher affinity compared with adenosine transporters. This results in metabolic trapping of adenosine in the form of adenine nucleotides. Previously, we demonstrated in rat brain synaptoneurosomes that metabolic trapping could shift the apparent K_T value of adenosine transport to a value affected by adenosine kinase activity, and could cause an appearance of concentrative transport (Gu and Geiger, 1992). Here, a similar effect was found in that metabolism of accumulated [^{3}H]adenosine to adenine nucleotides caused trapping of extrasynaptoneurosomal [^{3}H]adenosine as indicated by findings that the accumulation of [^{3}H]purines ([^{3}H]adenosine plus metabolites) was higher in the absence of the adenosine kinase inhibitor 5'-iodotubercidin and the adenosine deaminase inhibitor EHNA than in the presence of these inhibitors. For short incubation periods, the accumulation of [^{3}H]adenosine was shown to be equilibrative in that intrasynaptoneurosomal levels of [^{3}H]adenosine were never higher than those in the reaction medium. Therefore, adenosine transport in synaptoneurosomes prepared from postmortem human brain did not appear to be mediated by a concentrative process.

In light of the above findings, apparent K_T and Vmax values were determined using 5 s incubations. Under these conditions metabolism and metabolic trapping was very low (even without adenosine kinase and adenosine deaminase inhibitors), and the intrasynaptoneurosomal [^{3}H]adenosine concentration was much less than the adenosine concentration in reaction medium. Thus, the requirements apparently were met for a valid study of adenosine transport kinetics (Gu and Geiger, 1992). The maximum velocities of [^{3}H]adenosine transport in synaptoneurosomes from human brain were not significantly different from those values obtained previously in synaptoneurosomes from rat brain (Gu and Geiger, 1992). However, the transport affinity in human brain synaptoneurosomes

was about half of that in rat brain synaptoneurosomes. The kinetic properties of adenosine transport in human brain appear to be very similar to those in, for example, human erythrocytes where equilibrative and inhibitor-sensitive adenosine transporters were found with apparent K_T values of 98 μM (Paterson et al., 1984) and 62 μM (Plagemann et al., 1985). Two possible reasons for the difference between our findings in human and rat include postmortem effects and species differences between humans and rats. The first possibility is discounted somewhat by our findings of no correlation between the measured K_T values in postmortem human brain and the human postmortem delay times that ranged from 4 to 24 hours (data not shown). Furthermore, postmortem effects were not observed between binding affinity of [^{3}H]nitrobenzylthioinosine to adenosine transporters in postmortem human brain with postmortem delay ranging from 3 to 12 hours (Kataria and Harik, 1988). Moreover, in an attempt to estimate possible postmortem effects on adenosine transport, parallel studies of adenosine transport in postmortem rat brain were conducted with postmortem delay times ranging from 0 to 24 hours and we found that adenosine accumulation was not significantly affected by the postmortem delay. Thus, our findings suggest that postmortem delay did not have a major confounding effect on adenosine transport kinetics and, therefore, we are left with the possible suggestion of species differences.

Chapter 4. Primary Cultures of Fetal Human Astrocytes Express Low Affinity Adenosine Transporters that are Inhibitor-Sensitive and Inhibitor-Resistant

ABSTRACT: In cultured fetal human astrocytes, we determined kinetic characteristics of [^{3}H]adenosine transport, the extent to which accumulated [^{3}H]adenosine was metabolized, the effects such metabolism had on measurements of apparent kinetic values of K_T and Vmax, and sensitivities with which nucleoside transport inhibitors blocked [^{3}H]adenosine transport. For incubations with 10 μM [^{3}H]adenosine as substrate, accumulated [^{3}H]adenosine was metabolized by 23% (15 s incubations) and by 60% (60 s incubations) to the adenine nucleotides AMP, ADP and ATP. At the longer incubation time of 60 s, phosphorylation of accumulated adenosine caused an appearance of "concentrative" uptake in that the intracellular levels of [^{3}H]purines (adenosine plus its metabolites) were 1.4-fold higher than in the medium. The intracellular accumulation of [^{3}H]adenosine, on the other hand, was not concentrative; levels were 4.7 μM (15 s incubation) and 6 μM (60 s incubation). Pretreatment with 10 μM erythro-9-(2-hydroxy-3-nonyl)adenine, an adenosine deaminase inhibitor, and 10 μM 5'-iodotubercidin, an adenosine kinase inhibitor, decreased the metabolism of accumulated [^{3}H]adenosine to 4% at 15-s incubations and 24% at 60 s incubations. Kinetically, [^{3}H]adenosine accumulations at 15-s incubations, consisted of a high affinity component with apparent K_T and Vmax values of 6.2 μM and 0.15 nmol/min/mg protein, and a low affinity site with K_T and Vmax values of 2.6 mM and 21 nmol/min/mg protein, respectively. [^{3}H]Adenosine accumulations at 15 s incubations in the presence of 10 μM EHNA and 10 μM 5'-iodotubercidin, where less than 4% of accumulated adenosine was metabolized, provided a measure of transport *per se* and the data were best described by a one component kinetic model with K_T and Vmax values of 179 μM and 5.2 nmol/min/mg protein, respectively. The nucleoside transport inhibitors dipyridamole (DPR), nitrobenzylthioinosine (NBI) and dilazep biphasically inhibited [^{3}H]adenosine transport;

for the inhibitor-sensitive component the IC_{50} (nM) values were 1.8 for DPR, 4.3 for NBI, and 4.8 for dilazep, and for the inhibitor-resistant component the IC_{50} (μM) values were 3.0 for DPR, 7.3 for NBI, and 2.1 for dilazep. Astrocytes cultured from fetal human brain express low affinity and high capacity adenosine transporters that are both inhibitor-sensitive and -resistant.

INTRODUCTION

Re-uptake of adenosine into cells that have released it or uptake into adjacent cells effectively limits the amount of adenosine available to interact with cell surface adenosine receptors [Geiger and Fyda, 1991, Wu and Phillis, 1984]. In this way, the physiological actions of adenosine can be regulated through control of its levels in the microenvironment surrounding these receptor sites. Further, for cells such as those found in the CNS where *de novo* synthesis of nucleotides is believed to be somewhat limited, nucleoside uptake has the added importance of helping to maintain cellular energy charge. The increase in interest surrounding the physiological actions of adenosine and the potential therapeutic advantage obtained through manipulations of the levels and actions of adenosine has focused attention on adenosine transport systems as potential targets for agents designed to increase levels of adenosine in the vicinity of its receptor sites. However, relatively little is known about adenosine transport systems in distinct types of CNS cells and less still is known about such systems in human CNS cells.

Over the past decade at least 6 different adenosine transporters have been discovered and characterized to various degrees based on being either equilibrative or energy requiring, sensitive or resistant to the blocking actions of certain nucleoside transport inhibitors, and substrate preference [Geiger and Fyda, 1991; Belt et al., 1993]. Expression of these transporters is not uniform among cell types; some cells such as human erythrocytes only express equilibrative/inhibitor-sensitive nucleoside transporters

termed "es", while other cells express to varying degrees "es", equilibrative/inhibitor-resistant ("ei"), as well as Na^+-dependent concentrative nucleoside transporters [Plagemann and Wohlhueter, 1980; Crawford et al., 1990].

For cells of CNS origin, some comparative data is available about the accumulation and metabolism of adenosine by neurons and astrocytes derived from mouse and chick brain. Thampy and Barnes [1983a.b] found that adenosine transporters in chick astrocytes exhibited a lower affinity but much higher capacity for adenosine than did chick neurons. Similarly, the uptake of adenosine in mouse astrocytes was greater than that observed in neurons [Bender and Hertz, 1986]. In light of findings that enzymes and transporters for adenosine are different among species [Johnston and Geiger, 1990; Plagemann and Woffendin, 1988] we have been focusing our efforts on characterizing adenosine transporters in human brain tissues and cells. Here, we report on studies conducted to determine the metabolism, transport kinetics, and pharmacology of adenosine transport in human fetal astrocytes.

MATERIALS AND METHODS

Primary cultures of fetal human astrocytes: All procedures related to the acquisition and use of human fetal tissue were approved by the Human Ethics Committees of the Health Sciences Centre Hospital and the University of Manitoba Faculty of Medicine. Fetal tissue was obtained and astrocytes were cultured according to procedures previously described [Furer et al., 1993]. Fetal brain tissue from 15 to 17 week old fetus' were separated from meninges and blood vessels, and washed in DMEM culture media containing 10% fetal calf serum (FCS), 1% L-glutamine and 0.2% antibiotic solution consisting of 1000 units/ml of penicillin G, 10 mg/ml streptomycin and 25 μg/ml amphotericin B in 0.9 % NaCl. Following repeated trituration through a 20 gauge needle, cells were centrifuged at 270 x g for 10 min, suspended in culture medium, plated in 75

cm^2 flasks, and incubated in 5% CO_2 at 37°C. Fresh media was added every 3 to 4 days. Seven day old cultures, representing a mixture of fetal cell types, were placed on a rotatory shaker for 2 hours at 330 rpm at room temperature, the supernatant was discarded, and the remaining cells, mainly astrocytes, were exposed for 10 min to a solution consisting of 0.05% trypsin plus 0.53 mM EDTA. Cells were collected, centrifuged at 270 x g for 10 min, resuspended in DMEM media containing 10% FCS, and placed into 75 cm^2 culture flasks. After 30 min, decanted cells, which were >98% astrocytes, were plated onto 35 mm culture dishes at a density of 10,000 cells/ml and allowed to grow to confluency before being taken for adenosine transport assays. Cultures of astrocytes routinely exhibited ≥ 95% positive staining for glial fibrillary acid protein.

Adenosine Transport Assays: All adenosine transport assays were conducted on attached cells in 35 mm culture dishes. After removal of culture medium, the cells were washed twice with 1 ml HEPES buffer containing (in mM) 110 NaCl, 25 glucose, 68.3 sucrose, 5.3 KCl, 1.8 $CaCl_2$, 1.0 $MgS0_4$, and 20 HEPES. Cells were preincubated with 0.9 ml HEPES buffer for 5 min, and unless otherwise indicated, transport was initiated by addition of 10 μM, 0.1 Ci/mmol [^{3}H]adenosine (50.2 Ci/mmol, New England Nuclear). Unless indicated otherwise, incubations were for 15 s at 37°C. Transport was terminated by addition of 1.0 ml ice-cold inhibitor stop cocktail containing 1 mM dilazep, 0.1 mM dipyridamole, and 1 mM adenosine. Following aspiration of the medium, cells were washed one additional time with the inhibitor stop cocktail. Cells were exposed to 0.5 ml of 5% TCA, and after 12 hours at 4°C, cells were scraped off from dishes and placed into 1.5 ml centrifuge tubes. Following centrifugation at 13,000 x g for 5 min, 50 μl of supernatant were spotted onto Polygram cellulose precoated plastic TLC plates (Machray Nagel, Germany) and radiolabeled adenosine and its metabolites were separated and quantitated as previously described [Gu and Geiger, 1992]. Pellets were digested with 1.0 N NaOH and protein was determined by the method of Lowry et al. [1951].

To determine the accumulation of [^{3}H]adenosine and its metabolites as a function of incubation time, incubations were varied from 5 to 600 s. To determine the effects of adenosine deaminase (ADA) and adenosine kinase (AK) inhibition on adenosine transport, cells were preincubated with 10 μM erythro-9-(2-hydroxyl-3-nonyl)adenine (EHNA) and 10 μM 5'-iodotubercidin to inhibit ADA and AK, respectively. To determine affinity (K_T) and turnover rate (V_{max}) values of [^{3}H]adenosine transport, concentrations of [^{3}H]adenosine were varied from 0.1 to 2500 μM. To determine the inhibitory potencies of the nucleoside transport blockers dipyridamole (DPR), dilazep, and nitrobenzylthioinosine (NBI), cells were preincubated for 5 min with the blockers at concentrations ranging from 0.1 nM to 10 μM.

Intracellular Volume Measurements: Astrocytes were detached from culture dishes following a brief, 1 minute, incubation at room temperature with trypsin (0.5 mg/ml) and EDTA (0.5 mM). The removed astrocytes were placed into centrifuge tubes containing 800 μl of buffer, 100 μl of $^3H_20$ (1.0 μCi/ml) and [U-^{14}C]sucrose (0.5 μCi/ml, 6 mCi/mmol). Following equilibration for 5 min at 37^{o}C, samples were centrifuged for 30 s at 13,000 x g through 250 μl of an oil mixture consisting of dinonyl phthalate and n-butyl phthalate (1:4 vol/vol). The pellets were digested with 0.1 M NaOH and counted for radioactivity by scintillation spectroscopy. The ratio of ^{3}H to ^{14}C was used to calculate the intracellular volume which was expressed as μl/mg protein.

The apparent K_T and V_{max} values were calculated using computerized nonlinear regression of saturation data, and results were illustrated as Lineweaver-Burke plots. IC_{50} values for adenosine transport inhibitors were calculated by nonlinear curve fitting with GraphPAD (GraphPAD Software Inc.). Data represent mean ± S.E.M. values. Statistical analyses were performed by using analysis of variance, and significance was considered at the $p<0.05$ level.

RESULTS

The primary cultures of fetal human astrocytes used in our studies were typically >95% pure as demonstrated by positive staining for GFAP (data not shown). The astrocytes were found to avidly take up and metabolize [^{3}H]adenosine in a time- and temperature-dependent manner. [^{3}H]Purine ([^{3}H]adenosine plus [^{3}H]metabolites) accumulation increased in relation to longer transport assay incubation times (Figure 23). Intracellular concentrations of [^{3}H]purines (μM), calculated on the basis of measured intracellular volumes of 5 μl/mg protein, were 4.2 at 5 s, 5.4 at 15 s, 7.0 at 30 s, and 13.9 at 60 s incubations. Intracellular concentrations of [^{3}H]purines at 60 s incubations were significantly higher ($p < 0.05$) by approximately 1.4-fold than the initial 10 μM [^{3}H]adenosine concentrations used in the transport assay.

To determine whether the "concentrative" accumulation of [^{3}H]purines was due to metabolism and intracellular trapping of transported [^{3}H]adenosine in the form of its nucleotides we first determined the extent to which [^{3}H]adenosine was metabolized as a function of incubation time. The amount of accumulated radiolabeled purines recovered as [^{3}H]adenosine decreased and as nucleotides and the deaminated products inosine and hypoxanthine increased with prolonged incubation periods. Metabolism of accumulated [^{3}H]adenosine, mainly to adenine nucleotides, was 6% at 5 s, 23% at 15 s, 29% at 30, and 60% at 60 s (Figure 24). Less than 5% of accumulated [^{3}H]adenosine was metabolized to inosine and hypoxanthine even at incubations of 60 s. In lieu of findings that extensive metabolism of accumulated [^{3}H]adenosine profoundly affects measurements of adenosine transport we determined the effectiveness with which EHNA an inhibitor of adenosine deaminase and 5'-iodotubercidin an inhibitor of adenosine kinase could block the intracellular metabolism of adenosine.

In the presence of 10 μM EHNA and 10 μM 5'-iodotubercidin, metabolism of accumulated [^{3}H]adenosine, expressed as percent of total radioactivity recovered, was

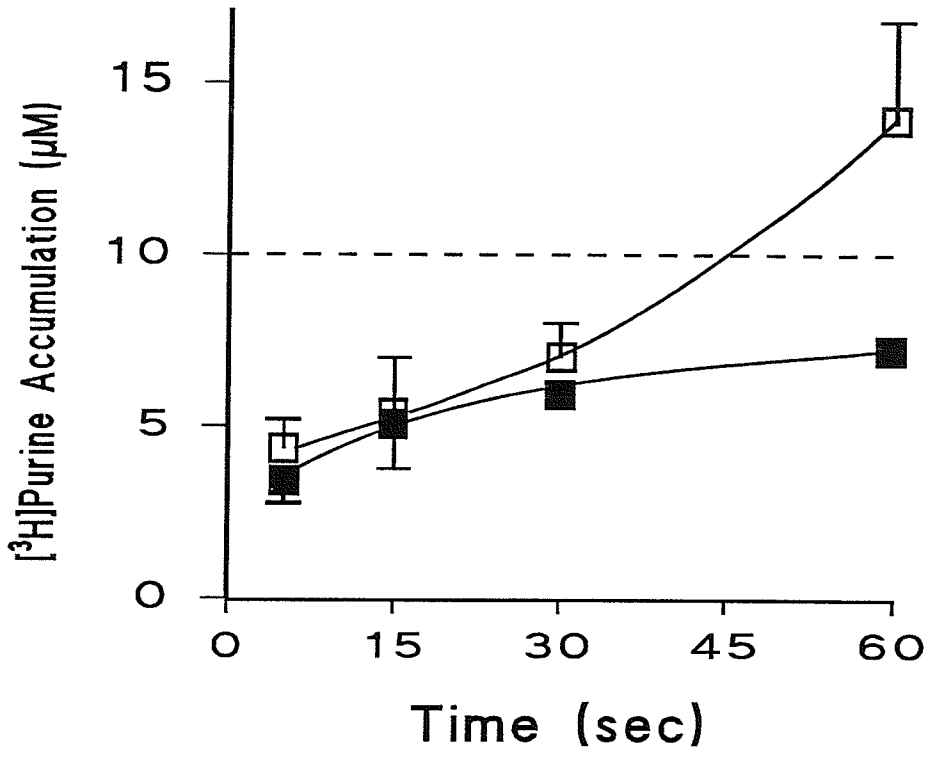

Figure 23. Acumulation of radiolabeled purines (adenosine + inosine + hypoxanthine + AMP + ADP + ATP) in the absence (open squares) and presence (closed squares) of inhibitors of ADA (EHNA) and AK (5'-iodotubercidin) for incubation times ranging from 5 to 60 s. Data represent mean ± S.E.M. from 4 experiments.

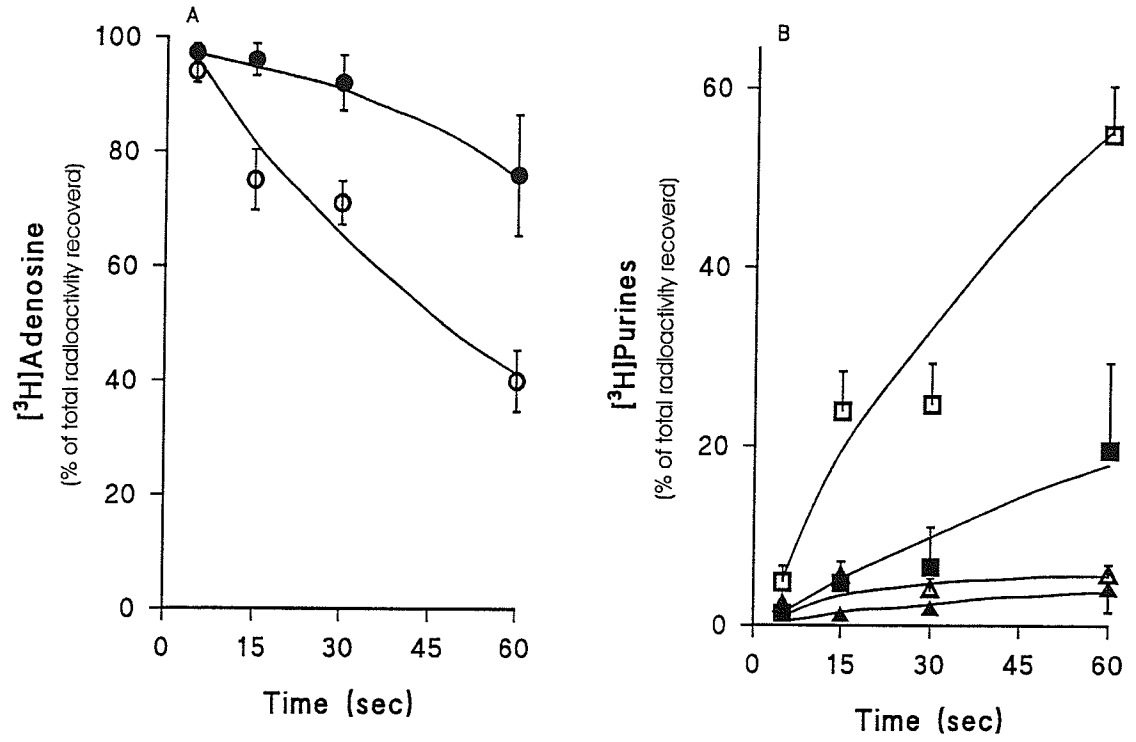

Figure 24. Metabolism of [^{3}H]adenosine (A, circles) to nucleotides (B, squares) and hypoxanthine/inosine (B, triangle) in the absence (open symbols) and presence (closed symbols) of inhibitors of ADA (EHNA) and AK (5'-iodotubercidin). Metabolism was expressed as percentage of total radioactivity recovered. Data represent mean ± S.E.M. from 4 experiments.

significantly inhibited ($p < 0.05$) from 6% to 3% at 5 s, from 23% to 4% at 15 s, from 29% to 8% at 30 s, and from 60% to 24% at 60 s incubations (Figure 24). In the presence of 10 μM EHNA and 10 μM 5'-iodotubercidin, [^{3}H]purine concentrations (μM) were slightly reduced from 4.2 to 3.5 at 5 s, from 5.4 to 5.3 at 15 s, from 7.0 to 6.0 at 30 s, and were significantly reduced ($p < 0.05$) from 13.9 to 7.4 at 60 s incubations (Figure 23). In the presence of 10 μM EHNA and 10 μM 5'-iodotubercidin, [^{3}H]adenosine concentrations (μM) were not significantly affected; 3.8 to 3.4 at 5 s, 4.7 to 5.0 at 15 s, 5.0 to 5.4 at 30 s, and 6.0 to 5.7 at 60 s incubations (Figure 23).

Kinetically, [^{3}H]adenosine accumulation into astrocytes measured with 15 s incubations in the absence of 10 μM EHNA and 10 μM 5'-iodotubercidin consisted of a high affinity component with apparent Kt and Vmax values of 6.2 ± 1.3 μM and 0.15 ± 0.05 nmol/min/mg protein, and a low affinity component with apparent Kt and Vmax values of 2.6 ± 1.2 mM and 21 ± 9 nmol/min/mg protein (Figure 25). In the presence of 10 μM EHNA and 10 μM 5'-iodotubercidin, adenosine transport data were best fit as one component kinetic process with Kt and Vmax values of 179 ± 67 μM and 5.2 ± 1.9 nmol/min/mg protein.

Three structurally dissimilar adenosine transporter inhibitors, dipyridamole, nitrobenzylthioinosine and dilazep, each dose-dependently inhibited the transport of 10 μM [^{3}H]adenosine at 15 s incubations (Figure 26). At 10 μM concentrations, [^{3}H]adenosine transport was inhibited by greater than 85% by dipyridamole, and greater than 95% by nitrobenzylthioinosine and dilazep. At concentrations of inhibitors lower than 10 μM, the inhibition curves for all three inhibitors were biphasic. One component of the transport was highly sensitive to DPR, dilazep, and NBI with IC_{50} values of 1.8 nM, 4.3 nM and 4.8 nM, respectively. The IC_{50} values for the inhibitor resistant components were 3.0 μM for DPR, 7.3 μM for dilazep, and 2.1 μM for NBI.

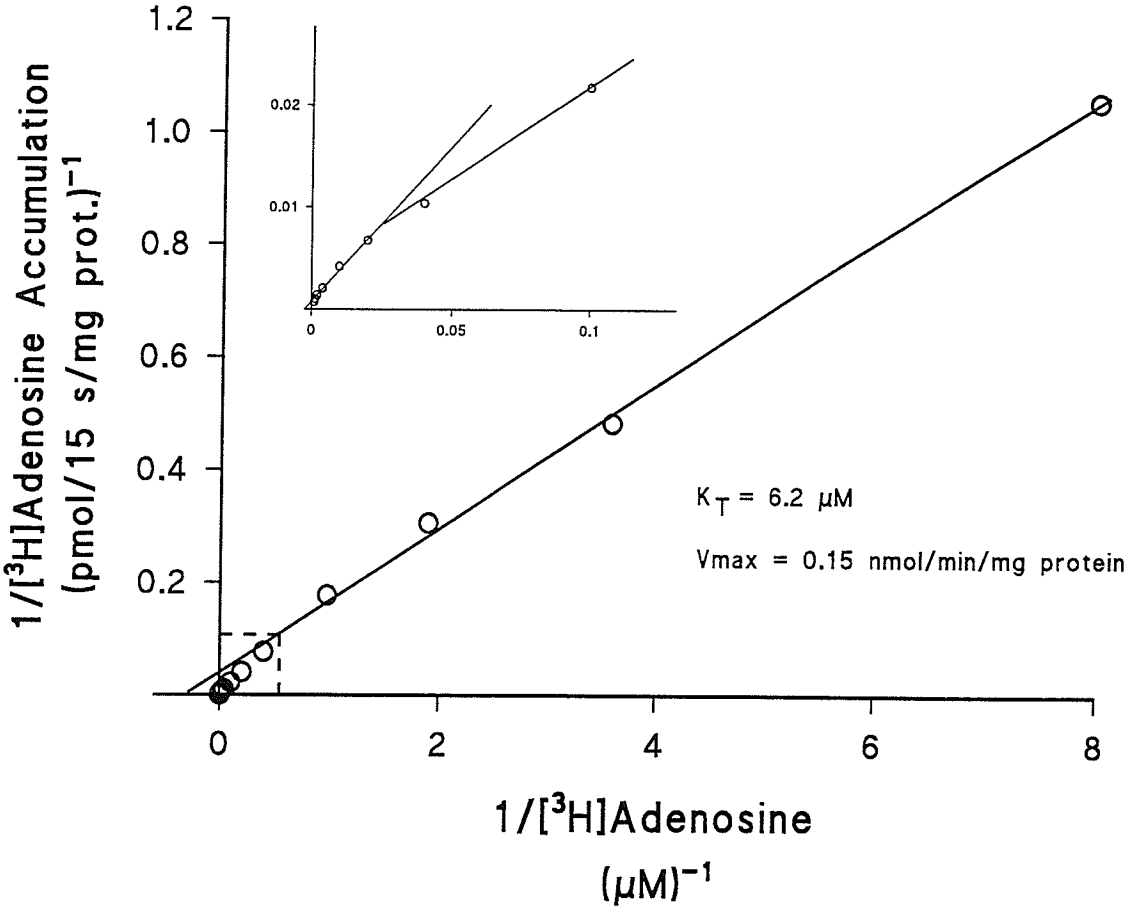

Figure 25. Double reciprocal plot of adenosine accumulation in fetal human astrocytes. Two kinetically distinguishable adenosine uptake systems were present in the absence of ADA (EHNA) and AK (5'-iodotubercidin) inhibitors. The high affinity state K_T and Vmax values were 6.2 ± 1.3 μM and 150 ± 50 pmoles/min/mg protein, respectively. The low affinity state K_T and Vmax values were 2.6 ± 1.2 μM and 21 ± 9 nmol/min/mg protein, respectively. Data represent mean ± S.E.M. values from 5 experiments (mean values were plotted).

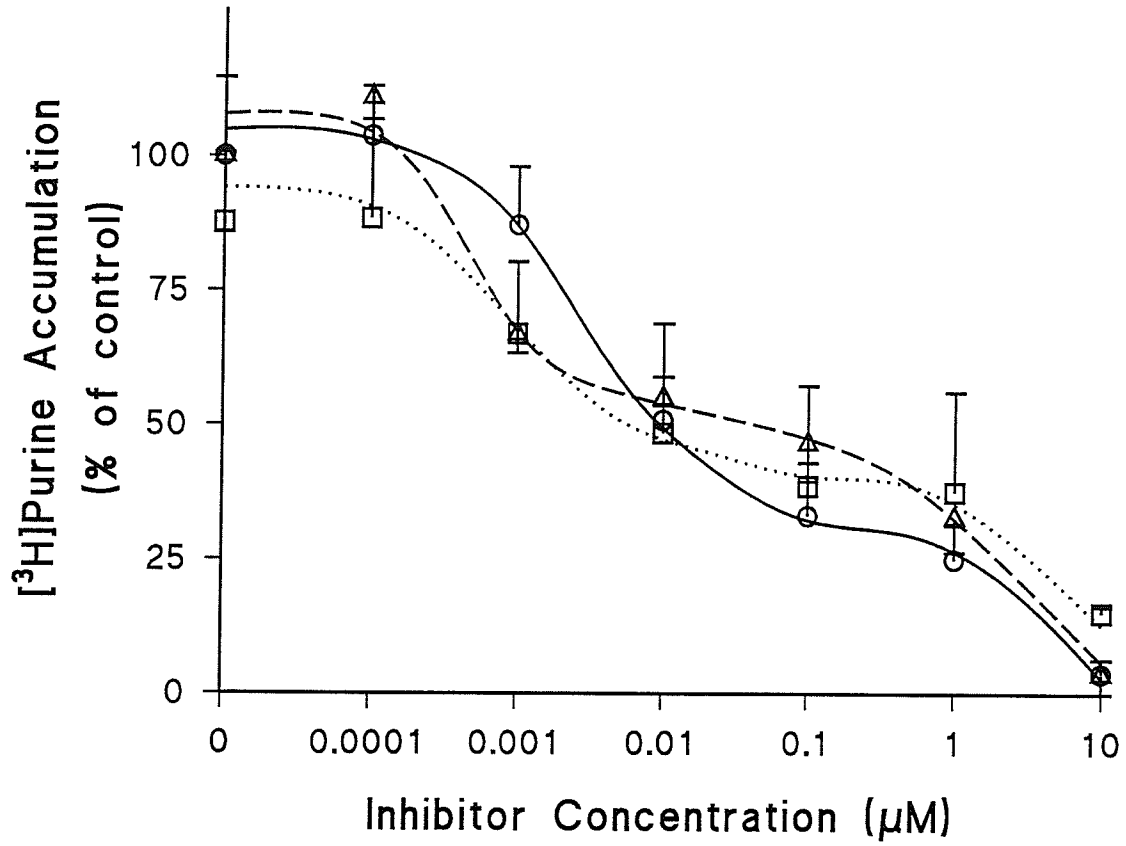

Figure 26. Inhibition of adenosine transport by the nucleoside transport inhibitors dipyridamole (open squares), dilazep (open circles) and nitrobenzylthioinosine (open triangles). All three compounds inhibited adenosine uptake biphasically. The IC_{50} values for the high and low affinity inhibition were 1.8 nM and 3.0 μM for dipyridamole, 4.3 nM and 7.3 μM for dilazep, and 4.8 nM and 2.1 μM for nitrobenzylthioinosine, respectively. Data represent mean ± S.E.M. values from at least 6 experiments.

DISCUSSION

Nucleoside transporters function to control the intracellular and extracellular levels of adenosine in the CNS (see Wu and Phillis, 1984; Geiger and Fyda, 1991). Inhibitors of adenosine transport have been shown to increase the extracellular levels and the adenosine receptor-mediated actions of adenosine in the CNS (see Geiger and Fyda, 1991). Therefore, nucleoside transport inhibitors have been increasingly thought of as potential therapeutic agents to enhance adenosine-mediated effects including protection against the onset and severity of seizures, and against ischemic neuronal injury. However, to date very little information is available concerning adenosine transporters in human CNS and here we describe some biochemical and pharmacological properties of adenosine transporters on cultured astrocytes from fetal human brain.

Astrocytes are the most numerous cell type in the CNS. Our findings that astrocytes cultured from fetal human brain avidly take up and metabolize adenosine agrees with previous work conducted with astrocytes cultured from chick and mouse brain (Bender and Hertz, 1986; Thampy and Barnes, 1983). Adenosine accumulated by fetal human astrocytes was rapidly metabolized by adenosine kinase (AK) to its phosphorylated derivatives (i.e. adenine nucleotides) and the derived affinity constant for this accumulation process of 6.2 μM reflects this metabolism. Previously, we demonstrated in rat and human brain synaptoneurosomes that metabolism by AK subsequent to adenosine transport produced kinetic values for adenosine uptake that were strongly influenced by AK's affinity for adenosine which is higher than that of adenosine transporters (Gu and Geiger, 1992; Gu et al., 1993). Previously, it was shown that under conditions where adenosine metabolism was not inhibited, adenosine uptake in cultured mouse astrocytes exhibited Km values of 6.1 μM and Vmax values of 0.11 nmol/min/mg protein (Bender and Hertz, 1986), and in cultured chick glial cells had Km values of 12 μM and Vmax values of 0.34 nmol/min/mg protein (Thampy and Barnes, 1986). Thus,

our findings of Km values of 6.2 μM and Vmax values of 0.15 nmol/min/mg protein are similar to previous reported values for adenosine transport in the presence of extensive substrate metabolism (i.e. adenosine uptake).

In contrast, under conditions where adenosine metabolism was prevented through the use of the AK inhibitor 5'-iodotubercidin and the ADA inhibitor EHNA, we demonstrated that adenosine transport in astrocytes cultured from fetal human brain exhibited a single kinetic process which had a low affinity (K_T = 179 μM) and high capacity (Vmax = 5.2 nmol/min/mg) for adenosine. These kinetic properties of adenosine transport are consistent with values for K_T of 370 μM and for Vmax of 10.3 nmol/min/mg protein obtained in a previous study with cultured glial cells from chick embryo brain where the transport experiments were conducted in cells depleting of ATP thus effectively blocking metabolism of adenosine by AK (Thampy and Barnes, 1983).

The functional importance of this low affinity/high capacity adenosine transport system identified in astrocytes may be revealed through the use of the Michaelis-Menten equation. At concentrations of endogenous adenosine ranging from around 100 nM under basal condition to 100 μM under pathological conditions, the transport rates for adenosine, as calculated using the equation, would be several-fold faster in astrocytes than in, for example, synaptoneurosomes prepared from rat or post-mortem human brain (Gu and Geiger, 1992; Gu et al., 1993). Similarly, adenosine transport rates would be faster in chick glial cells than in chick neuronal cells (Thampy and Barnes, 1983a,b). The low affinity/high capacity transport systems in astrocytes would enable astrocytes to accumulate adenosine under a broad range of endogenous levels and thus astrocytes may provide the brain with an effective means by which to provide spatial confinement for the neuro-regulatory actions of adenosine.

Pharmacologically, we demonstrated that adenosine transport in cultured astrocytes of fetal human brain were inhibited bi-phasically by three structurally distinct nucleoside transport inhibitors; nitrobenzylthioinosine, dipyridamole, and dilazep. The

values of IC_{50} for the inhibitor-sensitive phase were nanomolar and for the inhibitor-resistant phase were micromolar. Our findings are similar to those reported previously for cultured chick astrocytes (Thampy and Barnes, 1983), guinea pig cortical synaptosomes (Lee and Jarvis, 1988), and in many peripheral cells (Plagemann and Wohlhueter, 1980; Crawford et al., 1990) and suggest that pharmacologically distinct inhibitor-sensitive and inhibitor-resistant adenosine transporters are present in human brain. These subtypes of adenosine transporters in astrocytes may serve as important targets for nucleoside transport inhibitors whereby the levels of endogenous adenosine are increased and the neuroprotective actions of extracellular adenosine are potentiated.

Chapter 5. L-[^{3}H]Adenosine Release Through Bidirectional Adenosine Transporters on Rat Brain Synaptosomes: a Novel System for Determining Bidirectional Nucleoside Transport Characteristics.

ABSTRACT: Adenosine transport inhibitors as enhancers of extracellular levels of endogenous adenosine would, presumably, only be effective if (a) the inhibitors block influx to a greater degree than efflux (release) of intracellular adenosine or (b) 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 synaptosomes pre-loaded with L-[^{3}H]adenosine, our objectives here were to determine (a) if L-[^{3}H]adenosine, a substrate for adenosine transporters that is more metabolically stable than physiological D-adenosine, was released from synaptosomes, (b) the optimal conditions necessary to observe the release, and (c) the degree to which this release was mediated by efflux through nucleoside transporters. L-[^{3}H]Adenosine release was found to be concentration- and time-dependent, temperature-sensitive, and linear with synaptosomal protein. L-[^{3}H]Adenosine release was inhibited 40% by dipyridamole, 52% by nitrobenzylthioinosine, and 49% by dilazep, each at 100 μM. Following loading with L-[^{3}H]adenosine alone or L-[^{3}H]adenosine plus unlabeled L-adenosine, D-adenosine or uridine, L-[^{3}H]adenosine release was inhibited 42% by L-adenosine, 69% by uridine, and 81% by D-adenosine. The inhibition of L-[^{3}H]adenosine release from synaptosomes 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 in synaptosomal preparations.

INTRODUCTION

The uptake of adenosine into neural cells by nucleoside (adenosine) 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 adenosine transport may increase the extracellular levels of endogenous adenosine. In so doing, adenosine's actions, through binding to and activation of cell surface adenosine 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 approach was successful, adenosine transport inhibitors may be identified that could enhance some of the neuroprotective properties of adenosine (Rudolphi et al., 1992a,b; Williams, 1989; Geiger and Fyda, 1991).

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 6 classes of transporters have been identified and classified on the basis of inhibitor sensitivity, substrate specificity, and sodium ion dependence (Parkinson et al., 1993; Geiger and Fyda, 1991; Vijalakshmi and Belt, 1988; Jones and Hammond, 1992; William and Jarvis, 1991). At least some of these nucleoside transporters have been shown to be bidirectional such that the transporters can mediate both influx and efflux of substrates (Plagemann and Woffendin, 1989a).

To our knowledge, it is presently unknown whether the best characterized 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 transporters, it is

unknown whether, or the degree to which, adenosine transport inhibitors can selectively block influx or efflux. Moreover, depending on the 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 would appear 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 or 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 bidirectional functions of adenosine transporters and in determining effects of pharmacological agents on bidirectional transport of adenosine centers around the study of adenosine efflux. The reason for this is because D-adenosine is metabolically unstable which prevents "loading" cells with adenosine and subsequently determining release characteristics. We recently showed that L-adenosine, a more metabolically stable stereoenantiomer of physiological adenosine (D-adenosine), was transported into synaptosomal preparations, at least in part, by nucleoside transporters (Gu et al. 1991; Gu and Geiger, 1992). This suggested to us that L-adenosine may be used as a probe to study nucleoside transporter-mediated release processes. Here, we report some characteristics of L-[^{3}H]adenosine release from rat brain synaptosomes and show that this release appears to be due mainly to efflux through adenosine transporters. This system may find usefulness in characterizing the bidirectional function of CNS adenosine transporters and in identifying drugs capable of increasing extracellular levels of adenosine via selective inhibition of adenosine influx.

MATERIALS AND METHODS

Animals and preparation of synaptosomes

Male Sprague Dawley rats weighing 300 ± 25 g obtained from the University of Manitoba Central Animal Care breeding facility were sacrificed 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./vol.) of 0.32 M sucrose. Homogenates were centrifuged at 1800 x *g* for 5 min and supernatants were centrifuged at 13,000 x *g* for 20 min. Pellets were resuspended in HEPES buffer (110 mM NaCl, 25 mM glucose, 68.3 mM sucrose, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, and 20 mM HEPES, pH 7.4) and centrifuged at 13,000 x *g* for 20 min. All procedures were conducted at 4°C unless otherwise indicated.

L-[^{3}H]Adenosine release assay

Synaptosomal pellets (about 0.8 mg/100 µl) were resuspended in HEPES buffer containing, unless otherwise indicated, 10 µM L-[^{3}H]adenosine, 0.5 µCi/mmol (Moravek Biochemicals, Brea, California, 33 Ci/mmol). Synaptosomes were incubated at 37°C for 30 min and 100 µl aliquots were transferred to 1.5 ml microcentrifuge tubes and centrifuged at 13,000 x *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. Without disruption of the pellets, 1 ml of ice-cold HEPES buffer was added to each centrifuge tube followed by immediate aspiration. Release was initiated by vortex mixing following addition of 1 ml prewarmed (37°C) HEPES buffer or buffer plus various agents including nucleoside substrates or nucleoside transport inhibitors. Unless otherwise indicated, reactions were carried out at 37°C for 30 s. L-[^{3}H]Adenosine release was terminated by centrifugation at 13,000 x *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; half of the digestate was taken for

measurement of radioactivity and the remaining half was used to measure protein according to the method of Lowry et al. (1951).

Release as a function of synaptosomal protein concentration was determined using 0.2 to 2 mg of synaptosomal protein loaded with 100 μM L-[^{3}H]adenosine. L-[^{3}H]Adenosine release as a function of the length of incubation period was determined at two temperatures, 37°C and 4°C, at times that ranged from 0 to 600 s. Synaptosomes loaded with L-[^{3}H]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 on L-[^{3}H]adenosine release were determined by adding these agents simultaneously with buffer during resuspension of the final pellet. 1% DMSO which was used to dissolve DPR and NBI had no significant effects on L-[^{3}H]adenosine release. In order to determine whether nucleoside transport substrates could affect L-[^{3}H]adenosine release from the *cis* side of synaptosomal membrane, synaptosomes were loaded with 10 μM L-[^{3}H]adenosine plus 2.5 mM of L-adenosine, D-adenosine, or uridine. For the *cis* loaded experiments, the amount of radioactivity accumulated by the synaptosomes in control samples was not significantly different from samples incubated with nucleoside substrates.

L-[^{3}H]Adenosine metabolism was determined by a previously described TLC method (Gu and Geiger, 1992). Unless otherwise indicated all data represent mean ± SEM values. Statistical analyses were performed by using analysis of variance and Student's *t* tests. Statistical significance was considered to be at the $p < 0.05$ level.

RESULTS

L-[^{3}H]Adenosine was accumulated by (data not shown) as well as released from rat brain synaptosomes in a concentration-dependent and temperature-sensitive manner. Synaptosomes pre-loaded with L-[^{3}H]adenosine released more L-[^{3}H]adenosine at 37^{o}C than at 4^{o}C: the amount released increased with increased reaction times (Figure 27). When synaptosomes were loaded with 100 μM L-[^{3}H]adenosine, L-[^{3}H]adenosine release (pmol/mg protein) at 37^{o}C was 11.7 at time zero and 264 at 600 s while at 4^{o}C the release was 0 at time zero and 60 at 600 s. In comparison, at 37^{o}C the amount of L-[^{3}H]adenosine (pmol/mg protein) remaining in the pellets was found to decrease from 670 at time zero to 140 at 600 s, and, at 4^{o}C was found to be 704 at time zero and 510 at 600 s. The release increased linearly (r=0.919) with the concentration of synaptosomal protein that ranged from 0.2 to 2 mg (Figure 28). The release of L-[^{3}H]adenosine increased with higher loading concentrations of L-[^{3}H]adenosine (Figure 29). For example, when synaptosomes were loaded with 1 μM L-[^{3}H]adenosine, the release (pmol/mg protein) was 0.7 ± 0.1 at 0 s and 1.1 ± 0.2 at 30 s; when loaded with 10 μM, the release was 3.6 ± 0.4 at 0 s and 7.6 ± 1.1 at 30 s; and when loaded with 100 μM, the release was 11.5 ± 0.5 at 0 s and 109 ± 6.3 at 30 s. In 30 s at 37^{o}C, synaptosomes loaded with 10 μM L-[^{3}H]adenosine released approximately 66 % of the L-[^{3}H]adenosine accumulated during the pre-loading period. During the period of time encompassing the release experiments, only approximately 7% of the L-[^{3}H]adenosine remaining in the pellets was metabolized while approximately 2% of the L-[^{3}H]adenosine in the supernatant was metabolized. No attempt was made to identify the metabolites.

L-[^{3}H]Adenosine release was inhibited by 100 μM concentrations of the nucleoside transport inhibitors DPR, NBI, and dilazep (Figure. 30); release was significantly 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. Treatment with

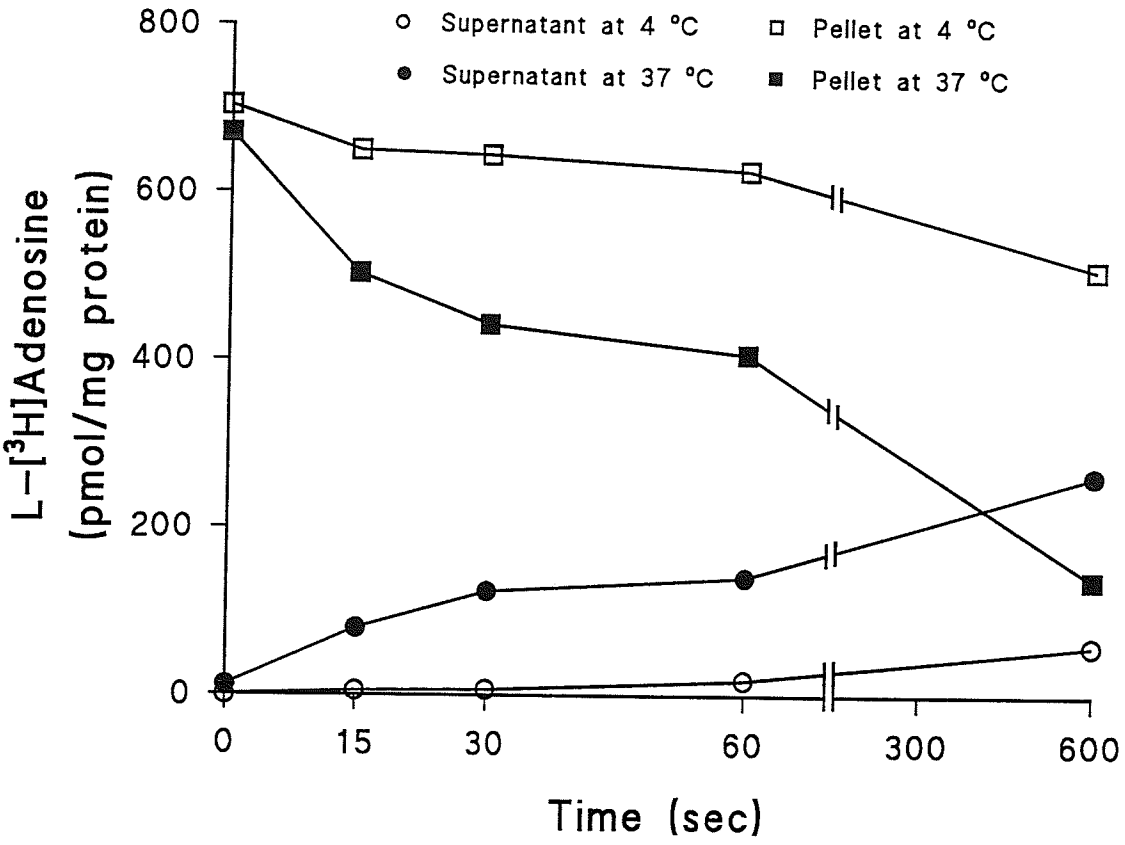

Figure 27. L-[^{3}H]Adenosine remaining in synaptosomes (squares) or released into supernatant (circles) as a function of incubation time at 4°C (open symbols) or 37°C (closed symbols). Synaptosomes were preloaded with 100 μM L-[^{3}H]adenosine at 37°C for 30 min. Symbols represent values obtained from a typical experiment performed in duplicate and replicated two additional times with similar results.

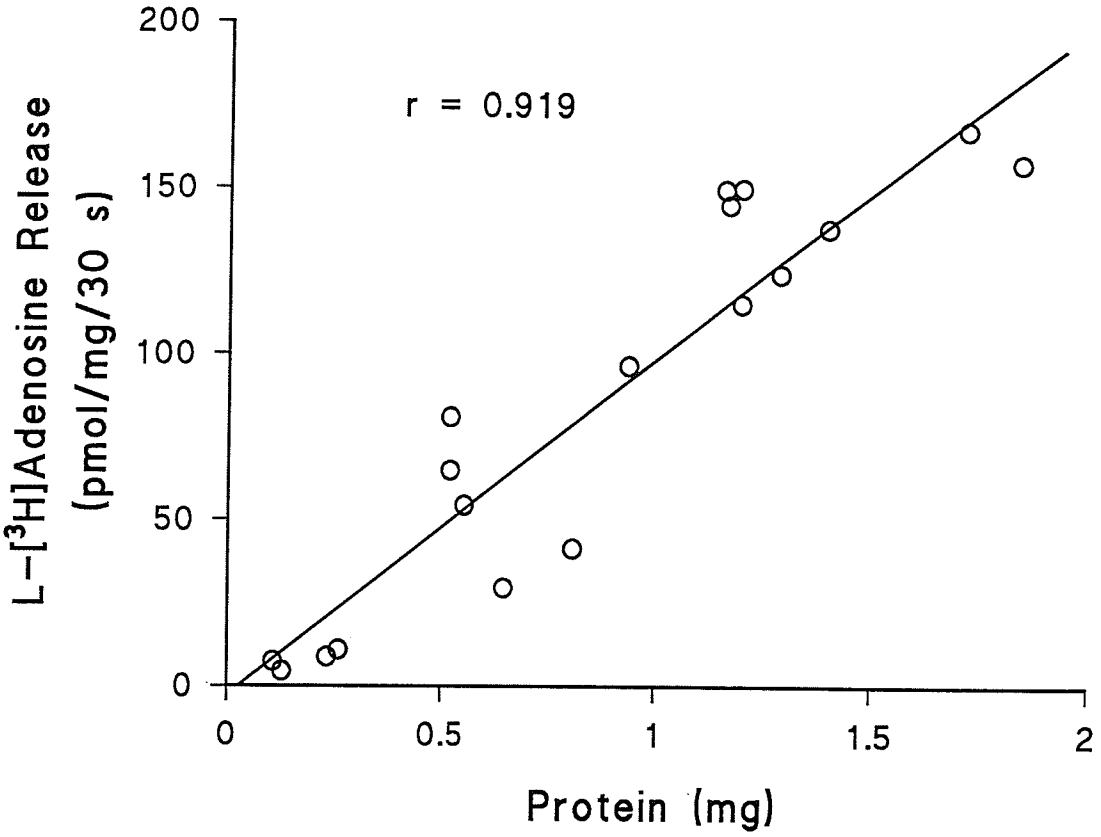

Figure 28. Release of L-[^{3}H]adenosine expressed as a function of synaptosomal protein. Synaptosomes at protein concentrations ranging from 0.2 to 2.0 mg were loaded with 100 μM L-[^{3}H]adenosine and incubations were for 30 s at 37°C. Correlation coefficient for the computer-generated linear regression line was 0.919. Each symbol represents a single determination made in duplicate.

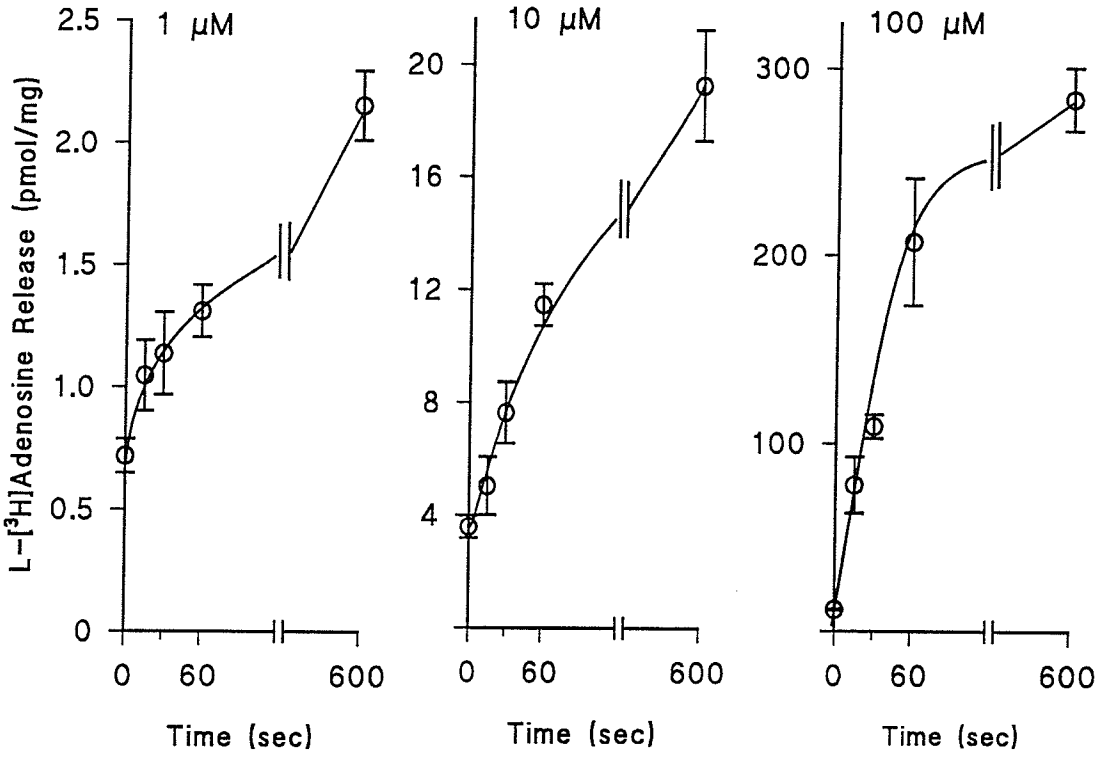

Figure 29. Release of L-[^{3}H]adenosine from synaptosomes pre-loaded with L-[^{3}H]adenosine at 1 μM, 10 μM and 100 μM. Incubations were from 0 to 600 s at 37°C. Symbols represent mean ± SEM (bars) values from at least four experiments each of which was performed in duplicate.

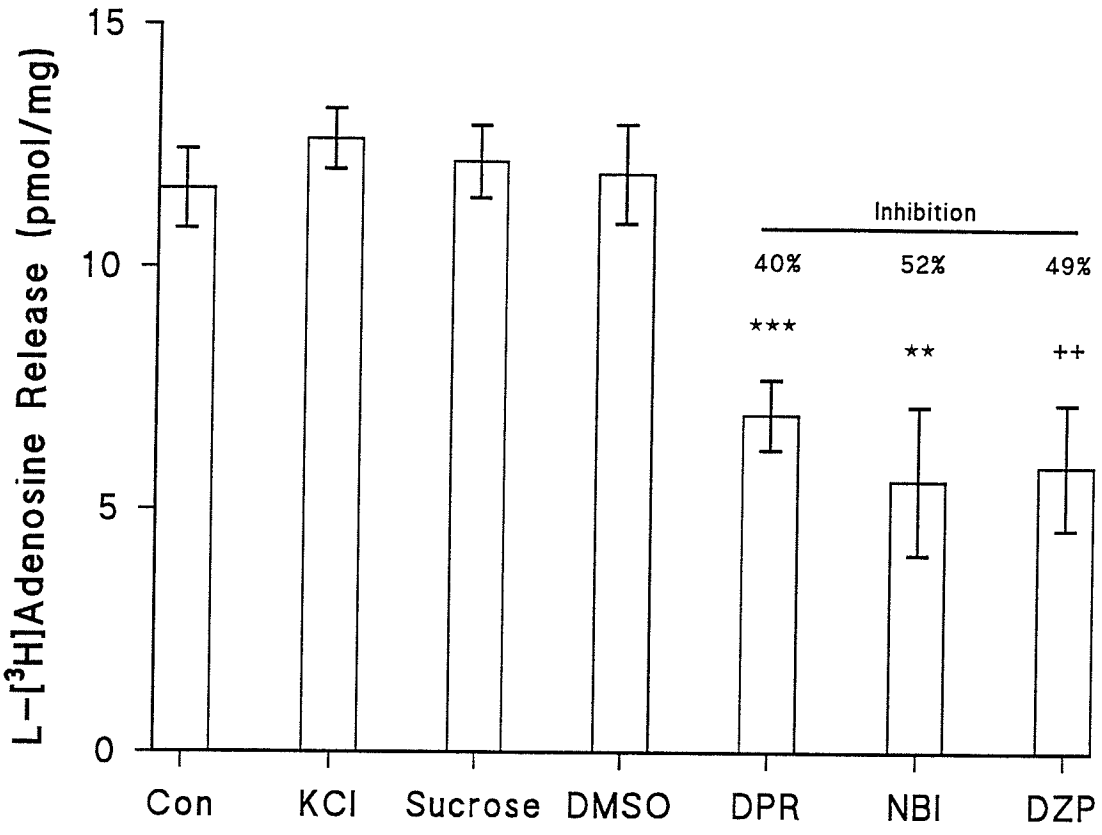

Figure 30. Release of L-[^{3}H]adenosine from rat brain synaptosomes in the absence (Con = control) or presence of 50 mM KCl, 100 mM sucrose, 1.0 % DMSO, 100 μM dipyridamole (DPR), 100 μM nitrobenzylthioinosine (NBI) or 100 μM dilazep (DZP). Synaptosomes were preloaded with 10 μM L-[^{3}H]adenosine and release was measured for 30 s. Data are mean ± SEM values from at least 5 experiments each of which was performed in duplicate. *** $p < 0.001$ DPR versus 1.0% DMSO vehicle control. ** $p < 0.01$ NBI versus 1.0% DMSO vehicle control. ++ $p < 0.01$ DZP versus buffer control (Con).

50 mM KCl or an iso-osmotic amount of sucrose (100 mM) did not significantly enhance L-[^{3}H]adenosine release (Figure 30).

To further test the extent to which L-[^{3}H]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 the time of initiation of release (*trans* - experiment) or were preloaded into synaptosomes prior to the initiation of release (*cis* - experiment). For the *cis* - experiment, we found that L-[^{3}H]adenosine release was inhibited 42% by L-adenosine, 69% by uridine, and 81% by D-adenosine (Figure 31). For the *trans* - experiment, none of the three nucleosides tested significantly affected release when applied at concentrations of 10 mM (Figure 32).

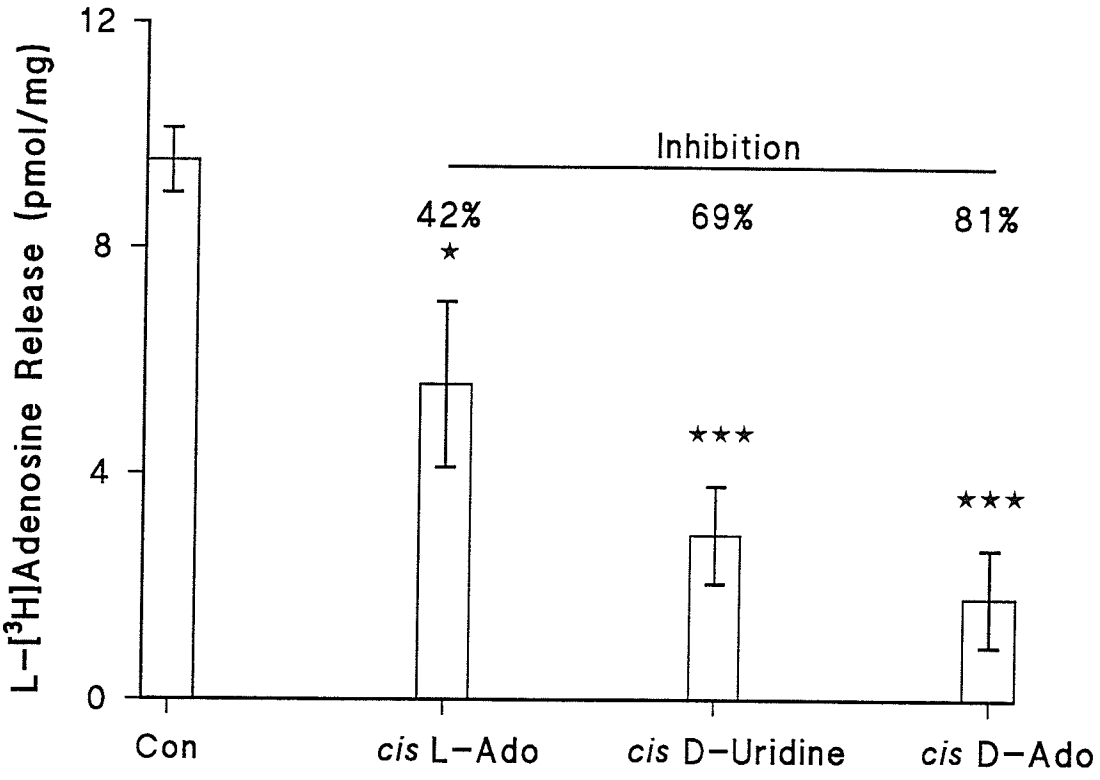

Figure 31. Release of L-[^{3}H]adenosine from rat brain synaptosomes pre-loaded with 10 μM L-[^{3}H]adenosine alone (Con = control) or 10 μM L-[^{3}H]adenosine plus 2.5 mM L-adenosine (L-Ado), D-uridine or D-adenosine (D-Ado). Data are mean ± SEM values from at least 5 experiments each of which was performed in duplicate.* $p < 0.05$ L-adenosine versus control. *** $p < 0.001$ D-uridine and D-adenosine versus control.

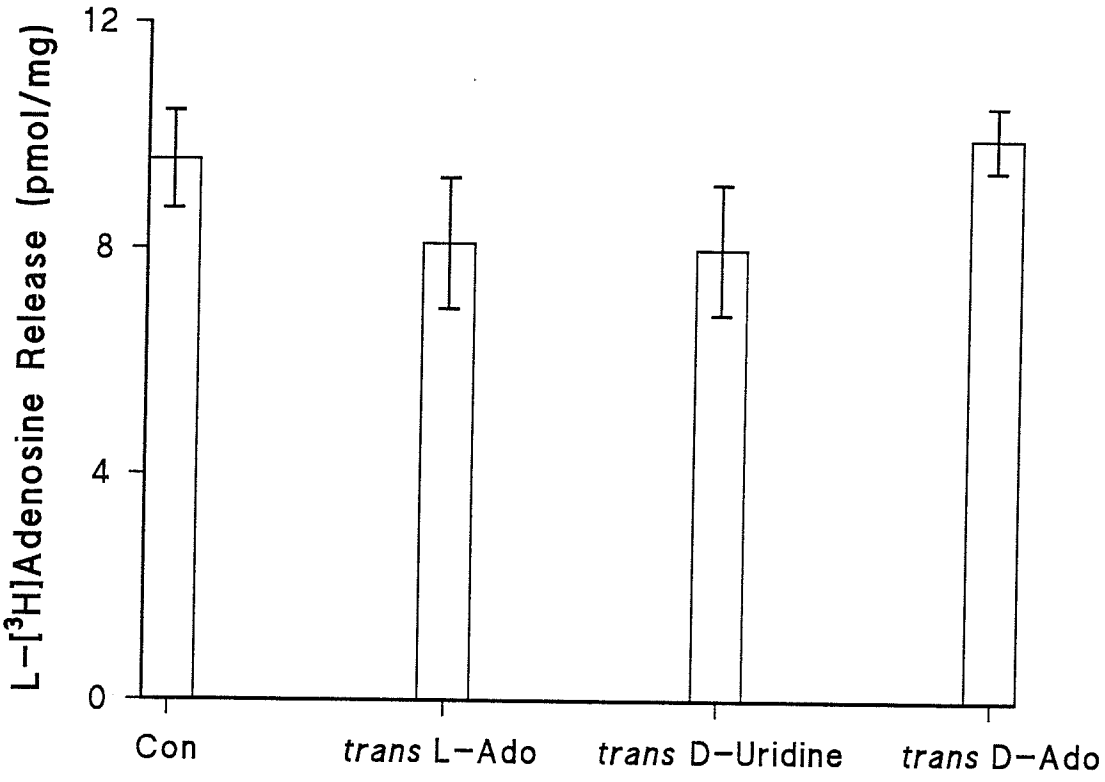

Figure 32. Release of L-[^{3}H]adenosine from rat brain synaptosomes in the absence (Con = control) or presence of 10 mM L-adenosine, D-uridine or D-adenosine. Synaptosomes were preloaded with 10 μM L-[^{3}H]adenosine and release was measured for 30 s. Data are mean ± SEM values from at least 3 experiments each of which was performed in duplicate.

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 since adenosine receptors are widely distributed through out the body. Accordingly, uptake inhibitors would presumably work to increase adenosine levels only when and where adenosine was being produced. However, predicting whether adenosine levels would increase, decrease or remain the same following inhibition of nucleoside transporters appears difficult at present and the following considerations apply.

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 role(s) 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 selectively inhibiting influx 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 (Craig and White, 1993; White and Hoehn, 1991).

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 L-[^{3}H]adenosine through rat brain synaptosome adenosine transporters is 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 metabolized to its phosphorylated and deaminated products. However, the release process can be determined by alternate substrates for nucleoside transporters which have affinities similar to D-adenosine. L-Adenosine (the stereoenantiomer of D-adenosine), appears to fit these criteria in that (a) L-adenosine is more metabolically stable than D-adenosine, (b) L-adenosine is a substrate for CNS adenosine transporters, and (c) the affinity of synaptosomal adenosine transporters for L-adenosine is comparable to that for D-adenosine (Gu et al., 1991; Gu and Geiger, 1992). Although nucleoside transport permeants such as formycin B and uridine, in at least some tissues, appear to be more metabolically stable than D-adenosine (Plagemann and Woffendin, 1989a,b), their usefulness as permeants of nucleoside transporters in the CNS is limited by the relatively low affinity with which CNS transporters appear to recognize and carry these nucleoside substrates (Lee and Jarvis, 1988a,b; Jones and Hammond, 1992).

Our findings in the present study that L-[^{3}H]adenosine was avidly taken up by synaptosomes agrees favorably with our previous findings with synaptoneurosomes where the uptake of L-[^{3}H]adenosine was blocked up to 65% by the nucleoside transport inhibitor NBI as well as by D-adenosine. Thus, uptake was most likely mediated by equilibrative carrier-mediated adenosine transporters (Gu et al., 1991; Gu and Geiger, 1992). In agreement with our previous results (Gu et al., 1991), L-[^{3}H]adenosine was metabolically stable in synaptoneurosomes; 7% metabolized in 30 min at 37^{o}C. Knowing this, we began examining conditions under which pre-loaded L-[^{3}H]adenosine was

released. We found that simply resuspending the pellets containing L-[^{3}H]adenosine in a 37^{o}C buffer was sufficient to initiate release. This was the first indication that the release resulted from flow down a concentration gradient. That this release resulted from efflux through carrier-mediated equilibrative nucleoside transporters was supported by our findings that release was at least partially blocked by the nucleoside transport inhibitors DPR, NBI and dilazep as well as by the pre-loaded (*cis* experiment) nucleosides L-adenosine, D-adenosine and uridine. These finding seem to be consistent with influx studies where equilibrative nucleoside transporters were identified on rat brain synaptoneurosomes and guinea pig brain synaptosomes (Gu et al., 1991; Lee and Jarvis, 1988b). The portion of L-[^{3}H]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 transporters governing L-adenosine release.

We were not able to demonstrate 50 mM KCl-evoked release of L-[^{3}H]adenosine even though such stimuli were previously shown to evoke the release of endogenous adenosine albeit in small amounts from rat cortical slices and synaptosomes (White and Hoehn, 1991; MacDonald and White, 1985). However, this apparent lack of release may have been masked by competitive inhibition of L-[^{3}H]adenosine release by KCl-induced intra-synaptosomal formation of endogenous D-adenosine. This suggestion is supported by our findings that L-[^{3}H]adenosine release could be inhibited by *cis*-loaded D-adenosine.

Trans-stimulation of nucleoside transport has been observed in a few cell types such as human, pig, and mouse erythrocytes (Plagemann et al., 1988). We found that L-[^{3}H]adenosine release was not enhanced following "*trans*-stimulation" with the nucleoside substrates L-adenosine, D-adenosine or uridine. Thus, our findings are consistent with the interpretation that L-[^{3}H]adenosine release from rat brain

synaptosomes occurs equally well whether the transporters are either *trans*-empty or *trans*-loaded.

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

GENERAL DISCUSSION

These studies stemmed from the concept that CNS adenosine transport systems may be the primary mechanisms by which extracellular levels of endogenous adenosine are controlled and that inhibition of adenosine transport may enhance the levels of and thereby the actions of adenosine. The aims of this work were, 1) to determine the factors that affect measurements of CNS adenosine transport, 2) to develop suitable approaches for accurate measurement of adenosine transporter-mediated influx and efflux, 3) to characterize CNS adenosine transport systems in human and rat CNS tissues, and 4) to determine the effects of adenosine transport inhibitors on adenosine influx and efflux processes.

In the experiments presented here, we first addressed whether and the extent to which intracellular metabolism of adenosine by adenosine kinase (AK), was a major problem inherent in studies of adenosine transport in the CNS. We found that physiologically occurring D-adenosine was rapidly metabolized to and trapped intracellularly as adenine nucleotides. The consequence of this metabolic trapping was to give an "appearance" of concentrative uptake. Furthermore, we found that there was an inverse relationship between the degree of adenosine metabolism and the magnitude of apparent K_T values; the apparent K_T values became close to Km values for AK when adenosine was extensively metabolized. Having determined the effects of adenosine metabolism on measurements of adenosine transport, the subsequent study was aimed at circumventing these metabolism complications in order to obtain better measures of adenosine transport in the CNS. In this regard, we used inhibitors of adenosine kinase and adenosine deaminase as well as very short incubation times. Under these conditions, we measured K_T values of 36 μM and Vmax values of 712 pmol/min/mg protein in rat synaptoneurosomal preparations, K_T values of 89 μM and Vmax values of 980

pmol/min/mg protein in human synaptoneurosomal preparations, and K_T values of 179 μM and Vmax values of 5196 pmol/min/mg protein in cultured fetal human astrocytes. Further, for the studies on human brain synaptoneurosomes and astrocytes, we determined the effects of adenosine transport inhibitors on adenosine accumulation. We found that adenosine transport in human synaptoneurosomes could be completely inhibited by DPR and dilazep. On the other hand in astrocytes, adenosine transport was inhibited biphasically by DPR (IC_{50} = 1.8 nM and 3.0 μM), dilazep (IC_{50} = 4.3 nM and 7.3 μM), and NBI (IC_{50} = 4.8 nM and 2.1 μM).

Simultaneous with the above studies, we tested the suitability of L-[^{3}H]adenosine for its use as a probe for studies of adenosine transporters in the CNS. We found that L-[^{3}H]adenosine was a metabolically stable substrate for adenosine transporters in rat brain preparations. Kinetically, L-[^{3}H]adenosine was transported nearly as well as D-[^{3}H]adenosine and transport kinetic parameters for both were virtually identical. We also tested the possible utility of L-adenosine as a probe for the study of nucleoside transporter-mediated efflux (release). We found that L-[^{3}H]adenosine efflux was mediated at least in part by adenosine transporters and that the efflux was partially inhibited by transport substrates and inhibitors. This experimental system may provide a useful system for evaluating the effects of pharmacological agents on bidirectional transport of adenosine in brain preparations.

Metabolism and Transport

In these studies we emphasized the impact of metabolism on the measurement of adenosine transport kinetics. As mentioned in the INTRODUCTION, many earlier studies on adenosine transport in the CNS measured adenosine uptake rather than transport. Measuring adenosine transport *per se* rather than uptake is very important. Physiologically, it lets us understand the characteristics of adenosine transport systems. Pharmacologically, it is the basis upon which potencies of adenosine transport inhibitors

should be evaluated. Under conditions where adenosine transport *per se* is not measured, inhibitory potencies of adenosine transport inhibitors may be underestimated. It was demonstrated for example that the IC_{50} value for dipyridamole was 350 nM with long incubation times (Bender and Hertz, 1986); 76 nM with shorter incubation times (Morgan and Stone, 1986); and 1.3 nM when transport *per se* was measured (Geiger et al., 1988). Furthermore, if uptake rather than transport *per se* is measured, a compound that inhibits AK activity may be incorrectly characterized as a transport inhibitor.

Measurement of Adenosine Transport Kinetics Without Metabolism

In some studies, problems of measuring transport complicated by metabolism of the permeant were circumvented by depleting cells of ATP thus inhibiting adenosine kinase activity (Plagemann and wohlhueter, 1980; Plagemann et al., 1988). This approach, however, may potentially cause alterations of membrane properties thus directly or indirectly affecting the function of the adenosine transporters (Plagemann and Wohlhueter, 1980). Alternatively, metabolic conversions of adenosine by AK and ADA can be inhibited by the inhibitors of AK and ADA such as 5'-iodotubercidin and EHNA, respectively (Plagemann and Wohlhueter, 1980; Plagemann et al., 1985; 1988). However, these inhibitors should be used cautiously because at low doses these inhibitors may be ineffective and at high doses they may interfere with adenosine transporters. Genetic manipulation has been applied to render some cell lines incapable of metabolizing transport permeants (Plagemann et al., 1980). However, this technique has not been exploited for CNS cells.

In most of our experiments, the AK inhibitor 5'-iodotubercidin and the ADA inhibitor EHNA were used to inhibit intracellular adenosine metabolism. In view of the uncertainties mentioned above with the use of these two inhibitors, it was necessary to have other alternative approach(s) for the characterization of adenosine transport systems.

Probes for Characterization of Adenosine Transport Systems

Various nucleoside permeants have been used in the study of adenosine transport systems. [^{3}H]-5'-Deoxyadenosine has been used as a probe to study transport in several cell lines of lymphoid origin where it is not metabolized (Plagemann and Wohlhueter, 1980). However, in cells that possess 5'-methythioadenosine phosphorylase 5'-deoxyadenosine is extensively phosphorylated and thus its usefulness is limited (Plagemann et al., 1988). Formycin B is a substrate for both the equilibrative and Na^+-dependent transporters in mammalian cells, but it is not a substrate for cellular metabolic enzymes so that its accumulation by cells can be measured unimpeded by metabolic conversions (Plagemann and Aran, 1990; Plagemann and Woffendin, 1989a). However, formycin B was found to bind to intracellular components and thus may cause an appearance of "concentrative accumulation" by the cells (Plagemann and Woffendin, 1989a). Uridine was also used as a probe for the characterization of nucleoside transport. In the CNS, uridine has been tested for its usefulness as a probe in the characterization of adenosine transporter-mediated influx process. This probe appear to be metabolically stable and is a substrate for adenosine transporters in CNS tissues including synaptosomal preparations from rat, guinea pig and rabbit brains (Jones and Hammond, 1992; Lee and Jarvis, 1988a,b). However, the affinities of CNS adenosine transport systems appear to be lower for uridine than for adenosine (Lee and Jarvis, 1988a,b).

Considering the problems with those previously used transporter probes, we explored the use of L-adenosine, a stereoenantiomer of physiologically occurring D-adenosine, as a probe for the characterization of adenosine transport systems in the CNS. L-Adenosine appears to be a better probe than uridine and formycin B for characterization of both influx and efflux processes in the CNS. This probe enables the study of the directionally symmetry as well as inhibitor selectivity and potency.

Stereoselectivity of CNS Adenosine Transporters

Most neurotransmitter transporters exhibit stereoselectivity for substrates. Adenosine transporters in normal mouse erythrocytes and L1210 cell line appear highly selective to D-adenosine over L-adenosine (Gati et al., 1989; Dagnino et al., 1991a,b). However, CNS adenosine transport systems appear not to be as stereoselective. Lack of stereoselectivity for adenosine transport has also been observed in mouse erythrocytes infected with the malarial parasite *Plasmodium yoelii.* The infected erythrocytes were found to express a transporter-like entity that mediated a non-stereoselective equilibrative flux of D-adenosine and L-adenosine (Gati et al., 1990). Whether the CNS adenosine transporters are similar to those transporters remains to be studied.

Adenosine Transport in Synaptoneurosomes vs Astrocytes

In this study, human brain synaptoneurosomes were found to contain a higher affinity transport system for adenosine (K_T 89 μM) than did astrocytes (K_T 179 μM). On the other hand, it is important to note that astrocytes have a greater capacity to accumulate adenosine (Vmax values of 5196 pmol/min/mg protein) than do the nerve terminals (Vmax 980 pmol/min/mg protein). According to Michaelis-Menton equation, it appears that at physiological and pathologically high levels of adenosine, astrocytes would transport more adenosine than would neuronal cells. Higher capacity of adenosine transport in astrocytes may provide a restriction such that adenosine mainly acts to modulate neuronal functions in the regions where neuronal activity is high. However, at the time adenosine is just released from nerve terminals, the local levels of adenosine should be much higher around neurons than around astrocytes. Under this condition, adenosine transport rates could be higher in neurons than in astrocytes. Thus, both neuronal cells and astrocytes may serve as targets for adenosine transport inhibitors to enhance levels and actions of endogenous adenosine.

Significance of these studies

These studies clarified the general confusion in the concepts between adenosine uptake and adenosine transport in the CNS. We indicated that the adenosine "transport" characteristics described by many earlier studies actually measured uptake and that only under conditions described by our studies adenosine transport *per se* is measured . Thus, CNS adenosine transport systems are not high affinity (K_T values of low micromolar) systems, they are low affinity systems that are similar to those observed in peripheral tissues.

The study on bidirectional CNS adenosine transport systems with L-adenosine, the adenosine transport probe that we developed, initiates a new research direction toward discovering drugs that can selectively inhibit adenosine influx.

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