Regulation of Extracellular Adenosine Levels: Role of Nucleoside Transporters, Purine Metabolism and the Blood-Brain-Barrier

By

Christopher J.D. Sinclair

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
Faculty of Medicine
University of Manitoba

© August, 2001

National Library of Canada

Acquisitions and Bibliographic Services

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada

Acquisitions et services bibliographiques

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-62667-9

Canada

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES
*****
COPYRIGHT PERMISSION PAGE

Regulation of Extracellular Adenosine Levels: Role of Nucleoside Transporters, Purine Metabolism and the Blood-Brain-Barrier

BY

Christopher J.D. Sinclair

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

of

Doctor of Philosophy

CHRISTOPHER J.D. SINCLAIR ©2001

Permission has been granted to the Library of The University of Manitoba to lend or sell copies of this thesis/practicum, to the National Library of Canada to microfilm this thesis and to lend or sell copies of the film, and to University Microfilm Inc. to publish an abstract of this thesis/practicum.

The author reserves other publication rights, and neither this thesis/practicum nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

I would like to dedicate this thesis to my parents, Jim and Geri Sinclair. Without their love, encouragement and guidance, I would not have been able to become the person that I am today. They have provided me with everything that a person could ask for. I love them with all my heart. Their love and support are the reason for this thesis and any success I have had.

Table of Contents

Table of Contents .... *i*

Acknowledgements .... *iv*

Abstract .... *vi*

List of Figures .... *viii*

List of Tables .... *x*

Abbreviations .... *xi*

Chapter 1: Introduction to Purinergic Research **1**

1.0 History of Purinergic Research **1**

2.0 Purine Receptors **2**

2.1 P_2 Purine Receptors **3**

2.2 P_1 Purine Receptors **4**

2.2.1 Adenosine A_1 Receptors 5

2.2.2 Adenosine A_{2A} Receptors 6

2.2.3 Adenosine A_{2B} Receptors 9

2.2.4 Adenosine A_3 Receptors 11

2.2.5 Limitations of Adenosine Agonists/Antagonists Therapeutically 13

3.0 Regulation of Extracellular Adenosine in the CNS **14**

3.1 Extracellular Adenine Nucleotides **15**

3.1.1 Release of Adenine Nucleotides 16

3.1.2 Metabolism of Adenine Nucleotides and Nucleosides 18

3.1.2.1 Ecto-5'Nucleotidase 18

3.1.2.2 Ecto-ATPase 21

3.1.2.3 Ecto-Adenosine Deaminase 22

3.1.2.4 Other enzymes 23

3.2 Nucleoside Transporters 23

3.2.1 Equilibrative Nucleoside Transporters 24

3.2.1.1 *es* Nucleoside Transporter 26

3.2.1.2 *ei* Nucleoside Transporter 32

3.2.2 Na^+-dependent Nucleoside Transporters 34

3.2.2.1 CIF Nucleoside Transporters 35

3.2.2.2 Other Concentrative Nucleoside Transporters 38

3.3 Adenosine Kinase 39

3.3.1 Purification and Molecular Biology 40

3.3.2 Endogenous Activity 42

3.3.3 Pharmacological Regulation 46

3.4 Adenosine Deaminase 48

3.4.1 Purification and Molecular Biology 49

3.4.2 Endogenous activity 51

3.4.3 Pharmacological Regulation 52

3.5 Cytosolic 5'Nucleotidase 53

3.5.1 5'AMP-preferring 5'nucleotidase 53

3.5.1.1 Molecular Biology and Purification 54

3.5.1.2 Endogenous Activity 55

3.5.1.3 Pharmacological Regulation 56

3.5.2 5'IMP-preferring 5'nucleotidase 56

3.5.2.1 Molecular Biology and Purification 57

3.5.2.2 Endogenous Activity 58

3.5.2.3 Pharmacological Regulation... 59

3.6 Other Intracellular Metabolic Pathways 59

3.7 Adenosine Transport Across the Blood-Brain Barrier.................................. 61

4.0 Investigating the Regulation of Extracellular Adenosine 62

4.1 Adenosine uptake 62

4.2 Adenosine Release 63

5.0 Hypotheses.. 69

Chapter 2: Adenosine permeation of a dynamic *in vitro* blood-brain barrier inhibited by dipyridamole. 72

Chapter 3: Stimulation of Nucleoside Efflux and Inhibition of Adenosine Kinase by A_1 Adenosine Receptor Activation. 81

Chapter 4: Nucleoside Transporter Subtype Expression: Effects on Potency of Adenosine Kinase Inhibitors... 103

Chapter 5: Purine uptake and Release in Rat C6 Glioma Cells: Nucleoside transport and Purine Metabolism under ATP Depleting conditions 126

Chapter 6: Release of [^{3}H]purines from rat primary cortical neurons and astrocytes during treatment with hypoxia, iodoacetate or sodium cyanide. 157

Chapter 7: General Discussion 191

Acknowledgements

Dr Fiona Parkinson- Without Dr Parkinson's acceptance of this "lost" student into her lab, I would never have had the chance to finish to get to this point. She has given me the ultimate in supervision: providing me with guidance and support for my thesis project and allowed me the flexibility and time to engage in activities outside of my work in her lab. Her door was always open for my questions and excited moments after one experiment. I have the utmost respect for her and I thank her for all the effort she has put forth with me.

Dr Jonathan Geiger and his laboratory- When I began in the department, I feared Dr Geiger and his straightforward, no-nonsense approach to students. As the years went on, he provided me with support for my thesis project and other endeavors. I would thank to thank him for all the time and effort he has put into my development over the years, including the examination of this thesis. I would also to thank the other members of the Geiger lab with special thanks going out to Dr Mike Mayne and Nick Shepel for their camaraderie and candid discussions over the years.

Dr Gary Glavin- I would like to thank Gary for all the support that he has given to me, whether it be letters of recommendation, verbal support when I needed guidance or a much needed beer. No matter how busy he was, there was always time for students. Thank you Gary for everything you have done for me, and it is more than you know.

My examining committee- I would like to sincerely thank Drs Labella, Tuor and Walz for taking the time and effort it took to assess and examine my thesis

.

Members of the Parkinson lab- All the members of the Parkinson lab have given me support in their own way over the years. Stephanie Borgland helped to initiate me into the lab while Wei Xiong always was there to give me a hand with any task I may have had. Timothy, April, Christian, and Oliver were there during the later years to ask me questions and help me to grow in my ability to communicate and provide some leadership. Last but not least, Chris Anderson was very important to me develop as a student and a scientist. He provided friendship and leadership to me that helped me to get through the not-so-easy times of being a graduate student.

NSERC, MHRC, Faculty of Graduate Studies, and the Department of Pharmacology- I would like to thank each of these groups that have at one time or another provided me with financial support during my time as a graduate student.

The love of my life, Christina Keys- Last and most importantly, she is the person that has meant the most to me during my graduate training. Christina has given me all the love and support that one person can give another during the long and arduous times that I spent in the Chown building. No matter when or for how long I had to go to the lab, Christina understood and supported me. Her love has kept me going at times when things weren't going right, when I needed her most. Christina, without the love I have with you, this would not have been done.

Abstract

Adenosine is an important neuromodulator in brain and, through activation of membrane bound receptors, it has been reported to have neuroprotective properties during strokes or seizures. The levels of adenosine have been reported to increase up to 100-fold during cerebral ischemia; however, the pathways involved in regulating intra- and extracellular concentrations of adenosine are poorly characterized. This thesis work has investigated how nucleoside transporters, purinergic enzymes and the blood-brain barrier (BBB) are involved in the regulation of extracellular adenosine levels. The first study demonstrated that lumenal administration of the nucleoside transport inhibitor dipyridamole inhibited the movement of [^{14}C]adenosine from the interstitial space into the lumen of a dynamic *in vitro* model of the BBB without permeating the BBB. This demonstrates that nucleoside transport inhibitors may be able to regulate CNS adenosine levels even without entering the brain parenchyma. The next study demonstrated that activation of the adenosine A_1 receptor during ATP depleting conditions in DDT_1 MF-2 cells increased the cellular release of [^{3}H]adenosine via a PKC-dependent pathway. These results demonstrate the autoregulatory effects of adenosine receptor activation on adenosine production. In a third study, expression of the rENT1 nucleoside transporter in rat C6 glioma cells facilitated the cellular permeability of the adenosine kinase inhibitor iodotubercidin. This demonstrates that the site selective effects of adenosine kinase inhibitors may be determined by nucleoside transporter distribution *in vivo*. The rat C6 glioma cells were also used to demonstrate release of [^{3}H]hypoxanthine but not [^{3}H]adenosine during ATP depleting conditions. This result indicates that astrocytes may be more important as a site of salvage, rather than a source, of adenosine during

conditions such as hypoxia/ischemia . To investigate this further, primary rat cortical astrocytes and neurons were subjected to hypoxia, iodoacetate or sodium cyanide to deplete the cells of ATP. The results indicate that adenosine is produced by an intracellular pathway in neurons and by an extracellular pathway in astrocytes. The overall conclusion of this thesis work is that the regulation of extracellular adenosine levels is complex and varies among cell types.

List of Figures

Figure 1: Schematic diagram of G-protein coupled receptors

Figure 2: Basic overview of the adenosinergic System

Figure 3: Schematic diagram of nucleoside transporters

Figure 4: Mechanism of ATP depletion

Figure 5: Effect of lumenal administration of dipyridamole on [^{3}H]adenosine or [^{14}C]adenosine permeation of the DIV-BBB

Figure 6: Effect of glycolytic inhibition on release of [^{3}H]purines from DDT_1 MF-2 cells loaded with [^{3}H]adenine

Figure 7: Effects of adenosine receptor agonists and antagonists on IAA-stimulated efflux of [^{3}H]purines from DDT_1 MF-2 cells loaded with [^{3}H]adenine

Figure 8: The effects of PLC and PKC activation on purine efflux induced by IAA with or without CHA

Figure 9: The effects of PKC inhibition on CHA or PMA mediated increases in [^{3}H]purine nucleoside efflux

Figure 10: The effects of adenosine receptor agonist or antagonist treatment of DDT_1 MF-2 cells on cytosolic adenosine kinase activity

Figure 11: Effect of ITU or NH_2dAdo on AK activity or 1 μM [^{3}H]adenosine uptake in C6 cells

Figure 12: Determination of nucleoside transporter subtypes in wt-C6, rENT1-C6, rCNT2-C6 and vector-C6 rat glioma cells by inhibition of nucleoside accumulation and RT-PCR

Figure 13: Effect of ITU or NH_2dAdo on [^{3}H]uridine uptake in wt-C6, rENT1-C6 and rCNT2-C6 cells

Figure 14: Effect of ITU or NH_2dAdo on [^{3}H]adenosine uptake in wt-C6, rENT1-C6 and rCNT2-C6 cells

Figure 15: Accumulation of 1 μM [^{3}H]adenosine or 10 μM [^{3}H]formycin B in C6 cells

Figure 16: Determination of equilibrative nucleoside transporter subtypes in C6 rat glioma cells by RT-PCR and inhibition of nucleoside accumulation

Figure 17: [^{3}H]Formycin B release from C6 cells

Figure 18: Effect of nucleoside transport inhibitors on purines released from C6 cells during or following treatment with IAA and NaCN

Figure 19: Schematic diagram of IAA/NaCN-induced [^{3}H]purine pathways in rat C6 glioma cells

Figure 20:Effect of glucose-free buffer, hypoxic-buffer, 100 μM NaCN or 500 μM IAA upon [^{3}H]purine release from rat primary cortical neurons (A) or astrocytes (B).

Figure 21: Effects of ITU, EHNA, BCX-34 and DPR on the release of [^{3}H]purines from glucose-free buffer treated rat primary cortical neurons (A) or astrocytes (B).

Figure 22: Effects of ITU, EHNA, BCX-34 and DPR on the release of [^{3}H]purines from hypoxic buffer treated rat primary cortical neurons (A) or astrocytes (B).

Figure 23: Effects of ITU, EHNA, BCX-34 and DPR on the release of [^{3}H]purines from 100 μM NaCN treated rat primary cortical neurons (A) or astrocytes (B).

Figure 24: Effects of ITU, EHNA, BCX-34 and DPR on the release of [^{3}H]purines from 500 μM IAA treated rat primary cortical neurons (A) or astrocytes (B).

Figure 25: Schematic Diagram of Purine Cycle between Neurons and Astrocytes

List of Tables

Table 1: Profile of adenosine receptor subtypes

Table 2: Endogenous regulators of purine metabolism

Table 3: IAA-induced release of adenosine or total purines from DDT_1 MF-2 cells

Table 4: Effects of CHA and PMA on adenosine kinase activity

Table 5: TLC analysis of purines released (pmol/mg protein) from rat C6 glioma cells

Table 6: Effect of inhibition of adenosine deaminase and AMP deaminase or purine nucleoside phosphorylase on IAA/NaCN-evoked [^{3}H]purine release (pmol/mg protein or % control)

Abbreviations

µM- micromolar

ADA-adenosine deaminase

ADP- adenosine diphosphate

AK- adenosine kinase

AMP- adenosine monophosphate

AMPDA- AMP deaminase

ATP- adenosine triphosphate

B_{max}- maximum binding of radiolabeled compound

BAEC- bovine adrenal endothelial cells

BBB- blood brain barrier

BCX-34- 2-Amino-1,5-dihydro-7-(3-pyridinylmethyl)-4H-pyrrolo[3,2,-d]pyridin-4-one

BPG- bisphosphogylcerate

cAMP- cyclic adenosine monophosphate

CHA- N^6-cyclohexyladenosine

cib- concentrative, inhibitor insensitive, broadly selective nucleoside transporter

cif- concentrative, inhibitor insensitive, formycin B selective nucleoside transporter

cit- concentrative, inhibitor insensitive, thymidine selective nucleoside transporter

CNS- central nervous system

CNT- concentrative nucleoside transporter

CPA- N^6-cyclopentyladenosine

CPT- cyclopentyltheophylline

CSC- chlorostyrylcaffeine

CSF- cerebrospinal fluid

DCF- deoxycoformycin

DIV-BBB- dynamic *in vitro* blood brain barrier

DPCPX- 1,3-dipropyl-8-cyclopentylxanthine

DPR- dipyridamole

DZP- dilazep

EHNA- erythro-9-(2-hydroxy-3-nonyl) adenine

ei- equilibrative nucleoside transporter insensitive to NBMPR inhibition

ENT- equilibrative nucleoside transporter

es- equilibrative nucleoside transporter sensitive to NBMPR inhibition

GFAP- glial fibrillary acidic protein

HGPRT- hypoxanthine-guanine phosphopribosyltransferase

IMP- inosine monophosphate

IP_3- inositol-1,4,5-trisphosphate

ITU- iodotubericidin

K_i- inhibitory constant

K_m- Michaelis Menton constant

LTD- long term depression

LTP- long term potentiation

mRNA- messenger ribonucleic acid

MW- molecular weight

NBMPR- nitrobenzylmercaptopurine riboside

NECA- 5;-N-ethyl-carboxamidoadenosine

NH_2dAdo- 5'-amino-5'deoxyadenosine

nM- nanomolar

P_i- inorganic phosphate

PKA- protein kinase A

PKC- protein kinase C

PLA_2- phospholipase A_2

PLC- phospholipase C

PLD- phospholipase D

PNP- purine nucleoside phosphorylase

R-PIA- N^6-(R-phenylisopropyl)-adenosine

RBC- red blood cell

ROS- reactive oxygen species

RT-PCR- reverse transcriptase polymerase chain reaction

SAH- s-adenosyl homocysteine

SAHH- s-adenosyl homocysteine hydrolase

UDP-uridine diphosphate

UTP- uridine triphosphate

V_{max}- maximum velocity of permeant influx

Chapter 1: Introduction to Purinergic Research

1.0 History of Purinergic Research

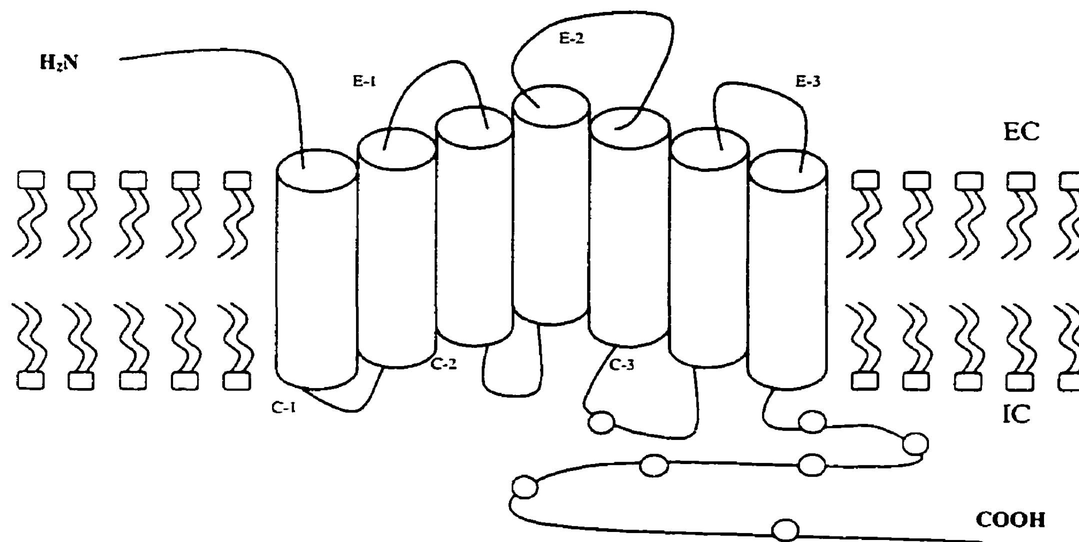
Adenosine is a purine nucleoside found in all cells. It is involved in numerous physiological processes ranging from adenine nucleotide precursor and building block of RNA to membrane-bound receptor-mediated cellular effects. In 1929, Drury and Szent-Gyorgi were the first to report that an endogenous substance, which could be extracted from numerous tissues, was capable of producing bradycardia (Drury and Szent-Gyorgi, 1929). In the following years, Reis discovered that adenosine could be produced from hydrolysis of 5'AMP (Reis, 1934). These discoveries led to attempts at the use of adenosine therapeutically during the 1930s for cardiovascular conditions. The use of adenosine was soon abandoned because of poor clinical efficacy (Jezer et al., 1933), possibly due to the rapid termination of action upon administration (see fig 2). From 1936 when Drury reported the effects of adenosine on blood flow (Drury, 1936), until 1960, purinergic research was predominantly focused on cardiovascular effects of adenine nucleotides (Arch and Newsholme, 1978). During the 1960s, adenosine was reported to be involved in regulation of blood flow in the heart, brain, kidney, spleen, and skeletal muscle. At this point it became apparent that adenosine was biologically active and an important regulator of blood delivery to tissues, especially when energy demand was exceeding supply. Until the late 1960s, adenosine's mechanism of action was largely unknown. Receptor-mediated effects of adenosine leading to decreased cellular cAMP production that could be blocked by methylxanthines such as caffeine and theophylline was reported (Sattin and Rall, 1970). This discovery allowed researchers to use pharmacological techniques to investigate adenosine-mediated effects outside of the

cardiovascular system. During the ensuing 20 years, adenosine was reported to modulate immune responses, lipolysis, and gastroesophageal function. Effects of adenosine in the nervous system were first documented in 1970. The majority of adenosine's CNS properties have been regarded as 'inhibitory' such as anti-convulsant, anti-nociceptive and decreased excitatory neurotransmission. As adenosine in the CNS does not meet the classical definition of neurotransmitter, it has been known as a 'neuromodulator'; more commonly, although not correctly, an inhibitory neuromodulator. Adenosine has also been regarded as a "retaliatory metabolite", due to its inverse relationship to ATP levels and its primarily inhibitory receptor-mediated effects (Newby, 1984). The receptor-mediated effects of adenosine (see section 2.2) in the CNS are being researched extensively, with interest in developing treatments for various CNS disorders. This research must be combined with a better understanding of the endogenous regulation of adenosine (see section 3), in order for potential therapeutic goals to be met.

2.0 Purine Receptors

Burnstock originally classified purine receptors as P_1 or P_2 purinergic receptors in 1977 (Burnstock, 1977). These classifications were based on studies, which demonstrated P_1 receptors were sensitive to inhibition by methylxanthines, activated by adenosine and modulated adenylate cyclase activity while P_2 receptors were insensitive to methylxanthine antagonism, activated by adenine nucleotides and induced prostanoid synthesis.

Figure 1: Schematic diagram of G-protein coupled receptors



Schematic structure of seven transmembrane spanning G-protein coupled adenosine and P_{2Y} purinergic receptors. Although the basic structure is similar, there are significant differences in the intra- and extra-cellular loops and the transmembrane domains between the different receptors. EC-extracellular, IC-intracellular, E-extracellular loops, C-intracellular loops, O- conventional regulatory sites.

2.1 P_2 Purine Receptors

Extracellular adenine nucleotides have been reported to activate cell surface receptors in the CNS and many peripheral tissues. These receptors have been broadly classified into two categories based upon signaling properties of the receptor: P_{2X} and P_{2Y} . The P_{2X} receptor subtypes are transmitter-gated channels. Seven different P_{2X} receptors have been characterized and cloned from human and rat tissues. Functional P_{2X} receptors are homo- or hetero-multimers of individual P_{2X} subunits. Upon activation, these receptors allow transmembrane fluxes of Na^+, K^+ or Ca^{2+} depending the receptor subtype. The P_{2Y} receptor subtypes are G-protein coupled 7-transmembrane domain receptors, much like

the P_1 receptors. Six P_{2Y} subtypes have been characterized and cloned, with 5 of these receptors coupling to $G_{q/11}$ G-proteins and the other to $G_{i/o}$ G-proteins. The receptors were originally classified based on the rank order of potency of ADP, ATP, UDP and UTP to increase levels of intracellular Ca^{2+}. Although these receptors are of importance to purinergic systems, this thesis will deal only with extracellular ATP and other adenine nucleotides as potential sources of extracellular adenosine.

2.2 P_1 Purine Receptors

Soon after the original classification of P_1 receptors, two independent laboratories proposed subtypes of these receptors. These reports indicated adenosine analogues either stimulated or inhibited cAMP formation while adenosine was capable of producing either response. This led to the initial classification of adenosine A_1 and A_2 purinergic receptors by van Calker and co-workers (van Calker et al., 1979). These were also characterized as R_A and R_I adenosine receptors by Londos and co-workers (Londos et al., 1980). The adenosine A_2 receptor was subsequently divided into A_{2A} and A_{2B} receptors based on the potency of adenosine and 2-substituted adenosine analogues to stimulate cAMP production (Braun and Levitzki, 1979; Daly et al., 1982). The most recent P_1 purinergic receptor to be identified was the A_3 adenosine receptor, which was discovered by cloning and expression screening (Zhou et al., 1992). An A_4 adenosine receptor has been proposed based on agonist binding profiles (Cornfield et al., 1992); however the existence of this receptor remains in doubt (Luthin and Linden, 1995). All 4 cloned adenosine receptor subtypes are members of the rhodopsin-like 7-transmembrane G-protein coupled receptor family (see fig 1). This thesis will describe the function,

distribution and pharmacological potential of these adenosine receptor subtypes with emphasis on the CNS properties (see table 1).

2.2.1 Adenosine A_1 Receptors

Adenosine A_1 receptors couple to pertussis-toxin sensitive G-proteins (G_{i1-3}/G_o), which in most cells inhibit AC activity thereby leading to decreased cellular cAMP (Fredholm, 1995). In addition to inhibition of AC, adenosine A_1 receptor activation has been reported to increase K^+ and decrease Ca^{2+} currents and activate PLC. The adenosine A_1 receptor was initially cloned from canine tissues (Libert et al., 1989) and has been subsequently cloned from many other mammalian species including rat (Mahan et al., 1991; Reppert et al., 1991) and human (Libert et al., 1992; Townsend-Nicholson and Shine, 1992). The recombinant adenosine A_1 receptor contains 326 amino acids (AAs), which produce a 36.7 kDa protein. The adenosine A_1 receptor appears to be highly conserved as there is >90 % AA sequence homology between species. There are numerous "selective" adenosine A_1 receptor agonists that have been used, including CPA, CHA, R-PIA and S-PIA, that have nanomolar affinity for the adenosine A_1 receptor. Adenosine, *per se*, and NECA show similar potencies as agonists for the adenosine A_1 and A_{2A} receptors. Methylxanthines such as caffeine and theophylline were used as adenosine receptor antagonists in the initial P_1 receptor studies, however, these compounds do not differentiate between adenosine receptor subtypes and are not potent. DPCPX is likely the most widely used A_1 receptor antagonist and has a K_i of ~1nM. During the past five years, there have been a number of reports of other potent and selective adenosine A_1 receptor agonists and antagonists. However, the majority of

research that has been published to date has used the compounds described above to investigate the adenosine A_1 receptor. The molecular biology and pharmacological advances in the A_1 receptor field have enabled researchers to gain further insight into the distribution and function of these receptors in the CNS. The distribution of A_1 receptors in human and rat brains has been widely investigated. Although species differences exist and differing results have been reported using autoradiography and immunohistochemistry, a general pattern of CNS A_1 receptor distribution is apparent. The highest levels of receptors are seen in the hippocampus (CA1 and CA2/3 regions), cortex (layers III and IV) and thalamus. Species differences have been reported in the striatum (high in human, low in rat) and cerebellum (low in human, high in rat).

2.2.2 Adenosine A_{2A} Receptors

Adenosine A_{2A} receptors couple primarily to G_S G-proteins. Activation of the A_{2A} receptor by adenosine or other analogues will produce an increase in intracellular cAMP. The A_{2A} receptor has also been reported to produce changes in MAP kinase (Hirano et al., 1996; Sexl et al., 1997) and protein phosphatase (Revan et al., 1996; Svenningsson et al., 2000) activity via a cAMP-independent mechanism. Similar to the A_1 receptor, the A_{2A} receptor was initially cloned from canine tissues (Maenhaut et al., 1990), then subsequently from other species including rat (Chern et al., 1992) and human (Furlong et al., 1992; Peterfreund et al., 1996). The chromosomal location of the A_{2A} receptor gene in humans has been localized to 22q11.2 (Le et al., 1996; Libert et al., 1991). The cloned A_{2A} receptor has 410-412 AAs and has a MW of ~45 kDa, the largest of the cloned

Table 1: Profile of Adenosine Receptor Subtypes

	Adenosine A_1	Adenosine A_{2A}	Adenosine A_{2B}	Adenosine A_3
Tissue Distribution[1]	Brain, spinal cord >fat >testis >uterus >heart >kidney >>skeletal muscle, liver, intestine, stomach	Brain> fat, thymus, heart, lung > spleen, testis, esophagus also found in blood vessels	large intestine =bladder = caecum >>lung > brain	Species dependent; rat testis > lung, heart, kidney > brain; human lung, liver > aorta, brain> testis
Species Cloned	canine, rat, human, guinea pig, bovine, rabbit, mouse	canine, rat, human, guinea pig, mouse	rat, human, mouse	rat, human, sheep
Amino acids	326-328	409-412	332	317-320
Protein size	36.7 kDa	45 kDa	36.4 kDa	36.6 kDa
Activated by adenosine at	3-30 nM	1-20 nM	5-20μM	>1μM
Selective Agonists[2]	CCPA > CPA > CHA = R-PIA	HE-NECA> CGS 21680> APEC	none	APNEA, N^6-benzyl NECA, Cl-IB-MECA
Selective Antagonists	DPCPX, WRC0571, 8-CPT	ZM 241385, SCH 58261, CSC, KF 17837	none;alloxazine slightly selective	MRS 1220 MRS 1460 MRS 1191
G-protein	$G_{i(1-3)}$, G_o, $G_{q/11}$	G_s	G_s	$G_{i(2,3)}$, $G_{q/11}$
Effector system	↓cAMP ↑IP_3/DAG ↑I_{K+}, ↓I_{Ca2+}	↑cAMP	↑cAMP	↓cAMP ↑IP_3/DAG

Table is based upon the IUPHAR Compendium of Receptor Characterization and Classification [Fredholm, 1998 #1300].
[1]Based upon Northern Blot, *in situ* hybridization and RT-PCR
[2]Rank order of potency is based on different assay systems in different species

adenosine receptors (Ongini and Fredholm, 1996). The A_{2A} receptor has been reported to have >90% sequence similarity at the AA level across species. The diversity of pharmacological tools available for the A_{2A} receptor is comparable to the A_1 receptor, as

there are numerous selective and potent agonists and antagonists available (see table 1). The most widely used of the A_{2A} receptor agonists is CGS 21680, which has a k_d of ~1 nM. In addition to its use as an agonist, CGS 21680 has been widely used as a radiolabeled marker for the A_{2A} receptor in binding studies. As stated in section 2.1.1, adenosine and NECA have similar potencies as agonists for the A_{2A} receptor compared to the A_1 receptor. More recently demonstrated selective and potent A_{2A} receptor agonists include HE-NECA (Klotz et al., 1998) and ATL146e (Linden, 2001). There are a wide range of A_{2A} receptor antagonists that have become available in the last decade, including KF 17837 (Shimada et al., 1992), CSC (Jacobson et al., 1993), SCH 58261 (Baraldi et al., 1996), ZM 241385 (Poucher et al., 1995) and KW 6002 (Kanda et al., 1998a). The development of the selective and potent pharmacological tools along with the molecular knowledge about the A_{2A} receptor has enabled insight into the distribution and function of this receptor in the CNS. Outside of the brain, A_{2A} receptors are expressed heterogeneously throughout the body with the highest levels in the vasculature, heart, lungs and thymus (Stehle et al., 1992a). In the CNS, the A_{2A} receptor distribution is heterogeneous. The A_{2A} receptor was initially described in tissue samples from the striatum (Huang et al., 1972). Thus, it is not surprising that striatal regions such as the caudate putamen, nucleus accumbens and olfactory tubercle demonstrate the highest levels of [^{3}H]CGS 21680 binding. Lower levels of binding have been demonstrated in the cortex and hippocampus. The expression of A_{2A} mRNA supports the previous reports of high levels of the A_{2A} receptor in the striatum with lower levels in the hippocampus and cortex. The function of the A_{2A} receptor correlates with its regional distribution, predominantly the striatal and vasculature localization. A_{2A} receptor knockout mice have

been reported to suffer from anxious behavior, high blood pressure and decreased exploratory and pain responses (Ledent et al., 1997). Although another A_{2A} receptor knockout mouse does not possess these abnormalities (Chen et al., 2000; Chen et al., 1999), the previous report may document the role of the A_{2A} receptor in the control of mood, which is supported by the physiological effects of caffeine. A recent area of intense research is the co-localization of the adenosine A_{2A} receptor and dopamine D_2 receptor in the striatum (Johansson et al., 1997a; Johansson et al., 1997b), which may be important in the progression and treatment of Parkinson's Disease (PD). In numerous studies of PD in primate models, A_{2A} receptor antagonists have been reported to decrease PD symptoms and progression (Kanda et al., 1998a; Kanda et al., 1998b). The A_{2A} receptors in the cerebrovasculature are important in the regulation of vessel diameter and CNS blood flow (Meno et al., 1991). Activation of these A_{2A} receptors is believed to play a role in vascular headaches such as those induced by caffeine withdrawal (Daval et al., 1996). The A_{2A} receptors have also been implicated in the regulation of respiration, and may play a role in ischemic and convulsion-related neuronal damage (Ongini et al., 1997). Thus, despite the limited distribution of the A_{2A} receptor in the CNS, these receptors are important in numerous physiological functions.

2.2.3 Adenosine A_{2B} Receptors

The A_{2B} receptor was initially distinguished from the A_{2A} receptor by differing sensitivities to adenosine analogues inducing intracellular cAMP accumulation (Huang et al., 1972). Similar to the A_{2A} receptor, the A_{2B} receptor conventionally couples to G_S G-proteins, which activate AC and increase intracellular cAMP levels. These receptors have also been reported to couple to PLC (Pilitsis and Kimelberg, 1998), ERK and MAP

kinase pathways (Feoktistov et al., 1999) via cAMP-independent mechanisms. The A_{2B} receptor was cloned from human and rat tissues in 1992 (Jacobson et al., 1995; Pierce et al., 1992; Rivkees and Reppert, 1992; Stehle et al., 1992b). The cDNA for the rat A_{2B} receptor produces a protein of 332 AAs, which has a MW of 36.4 kDa (Palmer and Stiles, 1995). The human A_{2B} receptor has been localized to chromosome 17p12 (Jacobson et al., 1995). The cloning of the A_{2B} receptor was an important development in the purinergic field as the pharmacological tools for this receptor have lagged behind that of the other P_1 receptors. To date no selective A_{2B} receptor agonists or antagonists have been reported. A_{2B} receptor-mediated responses have been demonstrated pharmacologically using adenosine or the non-selective agonist NECA in the presence of selective A_1 and A_{2A} antagonists. There has been little progress in the development of selective and potent A_{2B} agonists (de Zwart et al., 1998). A recent report has demonstrated analogues of the non-selective antagonist CGS 15943 that have high potency as antagonists at the A_{2B} receptor (Kim et al., 1998; Klotz, 2000), however these are also potent inhibitors of the A_3 receptor. Without these pharmacological tools, the distribution and function of the A_{2B} receptor has been based primarily on molecular studies. Outside of the CNS, A_{2B} receptor mRNA is ubiquitous with high levels in the gastrointestinal tract and lower levels in the lungs. Within the brain, the A_{2B} receptor distribution appears to be ubiquitous but in low levels (Dixon et al., 1996; Sebastiao and Ribeiro, 1996), possibly correlating to its localization on astrocytes (Pilitsis and Kimelberg, 1998). Due to its high K_m for adenosine, it has been widely proposed that the A_{2B} receptor function primarily during pathophysiological conditions when adenosine

levels are elevated. To gain a better understanding of the function of the A_{2B} receptor, there must be development of potent and selective pharmacological tools.

2.2.4 Adenosine A_3 Receptors

Unlike the other three subtypes of adenosine receptors, the A_3 receptor was not anticipated, due to the lack of adenosine-mediated physiological and pharmacological data. The A_3 receptor was initially identified from a rat testis cDNA by RT-PCR using degenerate oligonucleotide primers designed from conserved regions of G-protein coupled receptors (Meyerhof et al., 1991). The PCR products had the highest homology with adenosine A_1 and A_{2A} receptors (>40% amino acid identity). In 1992, Zhou and co-researchers identified an A_3 receptor from a rat striatal cDNA library (Zhou et al., 1992). This designation was based on specific binding of $[^{125}I]$APNEA in CHO-transfected cells and inhibition of forskolin-induced cAMP formation. The A_3 receptor was subsequently cloned from human tissues (Atkinson et al., 1997; Salvatore et al., 1993). The cDNA for the cloned A_3 receptor produces a protein of 320 AAs that has a MW of 36.6 kDa (Palmer and Stiles, 1995), which is similar in size to the A_1 and A_{2B} receptors. The human A_3 receptor has been localized to chromosome 1p13.3 (Atkinson et al., 1997). Similar to the A_1 receptor, the A_3 receptor couples to G_i/G_0 G-proteins. Activated A_3 receptors have been reported to decrease AC activity as well as activate PLC (Abbracchio et al., 1995). During the past five years, there has been a tremendous advance in the pharmacological tools available for the A_3 receptor. Adenosine *per se* has been reported to have a wide range of K_m values for the A_3 receptor between 30 nM (Li et al., 1999) and >1 μM. The higher values may reflect physiological inactivation of adenosine (Li et

al., 1999). Many of the initial 'selective' A_3 agonists such as IB-MECA have low selectivity between the A_1 and A_3 receptors (Klotz, 2000). Newer compounds such as AB-MECA (Li et al., 1999) and Cl-IB-MECA (Klotz, 2000) have been reported to have greater selectivity for the A_3 receptor in both rat and human systems. There have also been a number of selective A_3 receptor antagonists reported including MRS 1191 and MRS 1460 (Jacobson, 1998). An interesting species difference has been reported for the sensitivity of the A_3 receptor to methylxanthine antagonists. While most A_3 receptors including rat, mouse and rabbit are insensitive to methylxanthines, human A_3 receptors are more sensitive to methylxanthine antagonism (Klotz et al., 1998; Linden, 1994). The molecular and pharmacological developments have enabled a better understanding of the distribution and function of the A_3 receptor, however this knowledge is still limited. Outside of the CNS in rat, the A_3 receptor is abundant in the testis, lung, kidney and heart while in humans the highest levels of expression are in the liver and lung (Salvatore et al., 1993; von Lubitz, 1997). In the CNS, the A_3 receptor has relatively low expression, ~10-30 fold lower than A_1 receptors in the cortex or the A_{2A} receptors in the striatum (von Lubitz, 1997). In fact, the existence of the A_3 receptor in brain has been questioned because the radioligands that have been used to demonstrate A_3 receptors are not selective (Rivkees et al., 2000). Although A_3 receptor mRNA has been demonstrated in sheep and rat brains (Linden, 1994; von Lubitz, 1997), no studies have reported regional differences in mRNA levels. Binding assays have indicated expression of A_3 receptors in the cortex, cerebellum and striatum of rat brains (von Lubitz, 1997), however the validity of these results may be in doubt (Rivkees et al., 2000). As with the A_{2B} receptor, there is limited knowledge of the function of the A_3 especially in the CNS. Outside of the brain,

this receptor is most widely recognized as a regulator of immune function (Linden, 1994), as seen with an A_3 receptor knockout mouse (Salvatore et al., 2000). In the CNS, A_3 receptor-mediated effects have been predominantly documented in astrocytes and microglia cells (Abbracchio et al., 1997; von Lubitz, 1997). These effects may be important during ischemia or seizures, as A_3 receptors are less sensitive to physiological levels of endogenous adenosine.

2.2.5 Limitations of Adenosine Agonists/Antagonists Therapeutically

Although in vitro and in vivo models may indicate possible therapeutic value of adenosine agonists and antagonists, the CNS potential of these compounds is limited by two major impediments; BBB permeability and peripheral adenosine receptor-mediated effects. The former is a limitation of most compounds that are directed against CNS disorders. Many of the *in vivo* studies demonstrating protective properties against ischemic or convulsant animal models administer the adenosine receptor agonists using i.c.v. injections, a route with little therapeutic value. The second and more important limitation is the peripheral side effects. Adenosine A_1 receptor agonists have been reported to produce decreased heart rate, blood pressure and body temperature while A_{2A} receptor agonists produce decreased blood pressure (Guieu et al., 1996; Von Lubitz, 1999). The side effects profile of adenosine receptor antagonists appears to be less problematic, as evidenced by the success of KW-6002 in numerous long term Parkinsonian models (Kanda et al., 1998a; Kanda et al., 1998b). However, the majority of potential clinical uses are based on adenosine receptor activation. A so-called 'centrally active' adenosine A_1 receptor agonist, ADAC, has been reported to be neuroprotective

against ischemic damage without having deleterious peripheral side effects (Von Lubitz et al., 1996). Although this indicates some potential for clinical usefulness, the more recent trend in adenosinergic-based research is in the development of regulators of endogenous adenosine levels (REAL) (Geiger et al., 1997) agents or adenosine regulating agents (ARAs) (Mullane and Bullough, 1995). Unlike direct adenosine receptor activation with agonists, these agents attempt to make use of endogenous adenosine. As these agents have been proposed to increase adenosine in a "site" and "event" specific manner, there may be a lower incidence of systemic side effects. These agents either directly interact with the nucleoside transporters, which allow adenosine to permeate the cell membrane, or one of the key enzymes involved in adenosine metabolism. Although these agents are thought to be selective at their site of action, an overall understanding of the adenosinergic system is required to have insight into why and how these agents produce their effects. In the subsequent sections, this thesis will describe the metabolic pathways, nucleoside transporters and potential release mechanisms involved in the overall regulation of extracellular adenosine levels.

3.0 Regulation of Extracellular Adenosine in the CNS

As stated previously (see section 1.1), adenosine has been known to have CNS activity since approximately 1970. In the 30 years since these early reports, adenosine has been reported to be involved in many physiological and pathophysiological CNS functions. However, little is conclusively known regarding the regulation of endogenous adenosine in the extracellular compartment of the CNS. Extracellular adenosine levels are regulated by a number of enzymatic pathways, transport systems and cell types in the

CNS. Although much is known about the individual enzymes and transporters (see fig 2), the global scheme of adenosine regulation in the CNS is not clear. Questions arise regarding the cellular source of adenosine (neuron vs. astrocyte or other cell type) and pathway involved in extracellular accumulation (adenosine release per se vs. nucleotide release and extracellular metabolism). It has been proposed that under physiological conditions extracellular adenosine arises from both adenosine release and adenosine derived from extracellular adenine nucleotides (Geiger et al., 1997). Regardless of the source, *in vivo* estimates of CNS extracellular adenosine levels range from 20nM to 2 μM with microdialysis, cranial window or cortical cup techniques (Ballarin et al., 1991; Chen et al., 1992; Hagberg et al., 1987; Meno et al., 1991; Phillis et al., 1987; Van Wylen et al., 1986). It is conventionally believed that during pathophysiological conditions, extracellular adenosine arises from released adenosine due to decreased cellular ATP pools (Von Lubitz, 1999). However, some reports have indicated a role of extracellular adenine nucleotides as the source of adenosine during these conditions (Koos et al., 1997). As these pathways have not been directly shown, one must use indirect evidence to gain a better understanding of the processes involved. This thesis will focus on the role of extracellular adenine nucleotides, nucleoside transporters, purine anabolic and catabolic enzymes and the blood-brain barrier in regulating interstitial adenosine levels in the CNS.

3.1 Extracellular Adenine Nucleotides

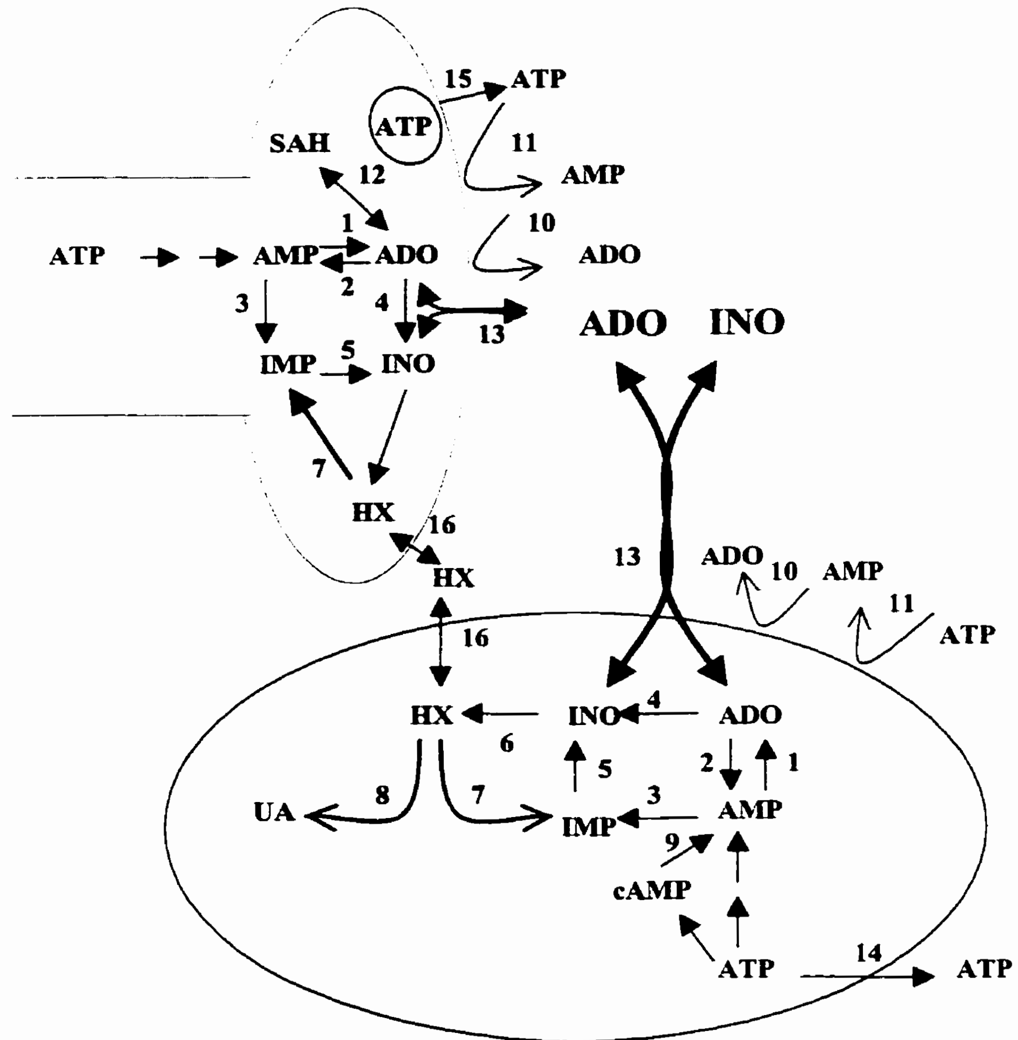
The release of adenine nucleotides such as ATP, ADP and cAMP has been documented from numerous CNS and non-CNS cell types. As charged compounds are

membrane impermeable, it is not surprising that extracellular enzymatic pathways exist to metabolize the nucleotides to a membrane permeable form, which is adenosine. Thus, adenine nucleotides released from cells are a major potential source of extracellular adenosine.

3.1.1 Release of Adenine Nucleotides

Adenine nucleotides are released from different cell types and via different mechanisms in the CNS. ATP is the most widely documented adenine nucleotide released in the CNS. It is released from neurons, astrocytes and microvasculature endothelial cells under physiological and pathophysiological conditions. Neuronal release is predominantly vesicular as inhibitors of synaptic Ca^{2+} channels or vesicular proteins will decrease the majority of ATP release from neurons (Fields and Stevens, 2000; Zimmermann, 1997). Astrocytic and endothelial ATP release occurs via a poorly characterized mechanism. Increased intracellular Ca^{2+}, increased cell volume and membrane perturbations have been reported to induce non-vesicular ATP release from these cells. Gap junctional hemichannels (Cotrina et al., 2000) and ABC transporters (Zimmermann, 1996) have been hypothesized to be involved in this form of ATP release. It has been reported that astrocytic release of ATP is important in astrocyte-astrocyte and astrocyte-neuron communication (Cotrina et al., 1998a; Guthrie et al., 1999). As most CNS cells contain P_2 receptors and are capable of ATP release, extracellular ATP is intricately involved in CNS function. In addition to ATP, other nucleotides have been documented to be released into the extracellular environment, and therefore may be potential sources of adenosine. cAMP release has been reported from both neurons and astrocytes when AC activity is high (Rosenberg et al., 1994; Rosenberg and Li, 1996).

Figure 2: Basic Overview of the Adenosinergic System



A basic two cell model for the regulation of adenosine and other purine nucleotides and nucleosides. The system is further complicated by the asymmetrical distribution of the enzymes and transporters between the cells. ADO-adenosine, INO-inosine, HX-hypoxanthine, UA-uric acid, SAH-S-adenosyl homocysteine, 1-AMP-selective 5'nucleotidase, 2-adenosine kinase, 3-AMP deaminase, 4-adenosine deaminase, 5-IMP-selective 5'nucleotidase, 6-purine nucleoside phosphorylase, 7- hypoxanthine-guanine phosphoribosyl transferase, 8-xanthine oxidase, 9-phosphodiesterases, 10-ecto-5'nucleotidase, 11-ecto-ATPases, 12- S-adenosylhomocysteine hydrolase, 13-nucleoside transporters, 14-non-vesicular ATP release, 15-vesicular ATP release,16-nucleobase transporters

This has been documented in cortical, striatal and hippocampal preparations. The release of cAMP has been proposed to occur by a furosemide sensitive mechanism, indicating a role for Cl^- channels. There have also been reports of AC acting as the cAMP transporter (Krupinski et al., 1989). ADP and AMP have also been reported to be released from cells, however the mechanism and physiological significance of these processes are unknown. In addition to conventional adenine nucleotides, diadenosine polyphosphates and NAD^+ are released and metabolized in the extracellular milieu to adenosine (Zimmermann, 1996). Therefore, there are numerous adenine nucleotides that can potentially be metabolized to adenosine in the interstitial environment of the CNS.

3.1.2 Metabolism of Adenine Nucleotides and Nucleosides

Adenine nucleotides, because of their charged phosphate groups, are impermeable to the plasma membrane, in the absence of facilitated mechanisms. When released into the extracellular environment, adenine nucleotides must be metabolized to a membrane-permeable metabolite, which is conventionally believed to be adenosine. The metabolic pathways involved in extracellular adenine nucleotide metabolism have been well characterized in the CNS.

3.1.2.1 Ecto-5'Nucleotidase

Ecto-5'-nucleotidase (e-N, EC 3.1.3.5; a.k.a. CD 73) is the best characterized of the ecto-purinergic enzymes (Zimmermann, 1992; Zimmermann, 1996). It is responsible for the extracellular dephosphorylation of AMP to adenosine and free phosphate. Thus, it is the direct source of extracellular adenosine formed from extracellular adenine

nucleotide. This enzyme has been well characterized and can be easily distinguished from the cytoplasmic 5'NTases c-N-I and c-N-II (see section 3.5) by molecular and kinetic properties. A glycosyl phosphatidylinositol (GPI) at the C-terminus of e-N anchors the protein to the plasma membrane, in an extracellular facing orientation. Cleavage of the GPI-anchor via PLC has been reported to produce a soluble form of e-N, designated e-Ns. This soluble protein has been reported to represent approximately 30% of the total 5'NTase activity in the CNS. e-N-s has been reported in the cytoplasm, in vesicles and in synaptic spaces. The molecular and biochemical properties of e-N and e-N-s indicate that these are the same proteins.

The primary sequence of the e-N subunit was identified in 1990 (Misumi et al., 1990a; Misumi et al., 1990b). This subunit has 574-577 AA, with a MW of 62-74 kDa. As e-N is an $\alpha\alpha$-dimer of these subunits, the native protein has a molecular weight of approximately 125-150 kDa (Zimmermann, 1992). The cloned enzyme was similar in size to e-N isolated from various species. The biochemical properties of e-N are very similar between neural and peripheral tissues as well as between species. Although values vary from preparation to preparation, the K_m of e-N is between 1-50 μM for both AMP and IMP (Zimmermann, 1992). This affinity is much higher than for either c-N-I or c-N-II. A number of endogenous compounds are involved in the regulation of e-N activity. Zinc is required for activity. Unlike the cytosolic 5' NTases, micromolar levels of ATP and ADP potently inhibit e-N activity. Thus, during conditions where extracellular ATP and/or ADP are elevated, extracellular adenosine levels may not rise concurrently, as e-N activity will be decreased. Free phosphate does not appear to have any effect on e-N activity, which is different from most purinergic enzymes. In addition,

oxidative stress may decrease the activity of e-N (Domanska-Janik and Bourre, 1990). Alpha, beta-methyleneadenosine 5'-diphosphate (AMPCP) has been used to pharmacologically inhibit e-N to gain a better understanding of the role of e-N. AMPCP is not an inhibitor of c-N-I or c-N-II. Interestingly, methylxanthines such as caffeine or theophylline inhibit e-N at concentrations used to antagonize P_1 purinergic receptors (Fredholm et al., 1978; Heyliger et al., 1981). Thus, care must be used when interpreting results with these compounds when endogenous adenosine is thought to play a role. The biochemical properties of e-N indicate that it is important for extracellular adenosine production as the affinity for AMP is in the physiological range.

The distribution of e-N in the nervous system is well characterized. Early immunocytochemical techniques in rat brain detected expression of e-N predominantly on non-neuronal cells such as astrocytes, oligodendrocytes and myelinated fibers (Kreutzberg et al., 1978; Snyder et al., 1983). *In vitro* preparations have indicated that e-N is expressed in both neuronal and astrocytic cultures (Meghji et al., 1989; Snyder et al., 1983; Stefanovic et al., 1976). As e-N has not been documented in neurons *in vivo* except in hippocampal neurons (Zimmermann, 1996; Zimmermann, 2000), this may point to the importance of e-N to cell survival in culture, possibly via scavenging of released purine nucleotides. In addition to the differences in cellular distributions, there is heterogeneity between brain regions, with the highest activity seen in the hippocampus and cerebellum with lower signals in the cortex, basal ganglia and superior colliculus in rat brain (Zimmermann, 1996; Zimmermann, 2000). Further information is required on the distribution of e-N in species other than rat, especially the cellular and regional

distribution of e-N in human brains. This will help to determine whether e-N is important in extracellular adenosine formation in certain cells/areas in the brain.

3.1.2.2 Ecto-ATPase

Extracellular ATP is dephosphorylated to ADP or AMP by ecto-ATPases. There have been at least 4 different types of ecto-ATPases reported in the CNS. Little information has been reported regarding the specific enzymes in the CNS, although ecto-ATPase activity is present in neurons, astrocytes and endothelial cells. The most well characterized enzyme is ecto-ATP-diphosphohydrolase (ATPdase, EC 3.6.1.5; a.k.a. ecto-apyrase or CD 39), which metabolizes tri- and diphosphates. A number of ecto-ATPdase isoforms have been cloned and the distribution of ecto-NTPdase 1 appears to be limited to microglia and the brain vasculature (Braun et al., 2000). The activity of ecto-NTPdases has been reported in neurons and astrocytes, indicating other isoforms of ecto-NTPDase. The activity of this class of enzyme is tightly regulated by glycosylation (Smith and Kirley, 1999), phosphorylation and reactive oxygen species (ROS). The importance of ecto-NTPdase may be seen in reports of its deficiency in humans with temporal lobe epilepsy and mice with audiogenic seizures (Chadwick and Frischauf, 1998; Wang et al., 1997). Specific ecto-ATPase (EC 3.6.1.3; ATP $\rightarrow$ ADP + P_i) (Kegel et al., 1997), ecto-ADPase (EC 3.6.1.6; ADP $\rightarrow$ AMP + P_i), extracellular adenylate kinase (EC 2.7.4.3; ATP + AMP $\rightarrow$ 2 ADP) (Nagy et al., 1989; Terrian et al., 1989) and alkaline phosphatase (EC 3.1.3.1) (Ogata et al., 1988) have also been reported in the CNS. Further research is needed to determine the specific role of each enzyme in the metabolism of

extracellular ATP and whether there are cellular and regional differences in the activity of each enzyme.

3.1.2.3 Ecto-Adenosine Deaminase

ADA_1 is conventionally seen as an intracellular enzyme, which metabolizes adenosine with a K_m of 40-50 μM (see section 3.4). The existence of ecto-ADA was initially described from fibroblasts as ADA_1 from these cells was sensitive to detergent treatments in a similar manner to ecto-5'nucleotidase. The ecto-ADA_1 has been reported in blood and endothelial cells since the original documentation (Franco et al., 1998). The majority of studies investigating the physiological significance of ecto-ADA has been performed in immune cells, where ecto-ADA binds CD26 to maintain its extracellular orientation. In the immune system, it has been hypothesized to play a role in cell signalling and immune system development (Franco et al., 1998). The role of ecto-ADA in the CNS has been poorly defined. It has been hypothesized to be involved in CNS development and neuronal activity, likely through regulation of adenosine levels (Franco et al., 1997). From a pharmacological point of view, ecto-ADA could be important not only in regulating extracellular adenosine levels but also in regulating the affinity of the adenosine A_1 receptor for adenosine. *In vitro* studies have demonstrated that exogenous ADA_1 can bind to the adenosine A_1 receptor and induce a change in the proportion of receptors in a high affinity state (Ciruela et al., 1996; Saura et al., 1996; Saura et al., 1998). Therefore, ecto-ADA may act as an important facilitator of rapid and sensitive adenosine A_1 receptor activation as it increases the affinity of the receptor and will degrade adenosine. However, the role and distribution of ecto-ADA in the CNS must be

further elucidated before the impact of this ecto-enzyme on extracellular adenosine levels is known.

3.1.2.4 Other enzymes

Although ecto-5'nucleotidase and ecto-ATPases are the most important enzymes involved in extracellular adenosine formation, other extracellular enzymes are important in the degradation of adenine nucleotides other than ATP, and therefore may be involved in adenosine production. Extracellular phosphodiesterases (ecto-PDE) are involved in the production of extracellular adenosine from cAMP released from cells. Ecto-PDE was initially discovered in liver cells (Smoake et al., 1981) and has since been discovered in numerous tissues (Goding et al., 1998). Although little information exists regarding the role of ecto-PDE in the CNS, its activity has been described in rat and human astrocytes (Rosenberg et al., 1994; Rosenberg and Li, 1995; Rosenberg and Li, 1996) as well as neuroblastoma cells (Orlov and Maksimova, 1999). Other enzymes such as extracellular Ap_nAses, which metabolize diadenosine polyphosphates, ecto-NAD-glycohydrolase and ecto-alkaline phosphatase may play a role in extracellular adenosine production from nucleotides, however there is limited knowledge about the function and distribution of these enzymes in the CNS (Zimmermann, 1996; Zimmermann, 2000).

3.2 Nucleoside Transporters

Nucleoside transporters are membrane proteins that allow the movement of purine and pyrimidine nucleosides across the plasma membrane. The primary function of these transporters is for the salvage of nucleosides for metabolic processes such as cellular

replication and transcription. This is a required mechanism for cells lacking *de novo* purine and pyrimidine pathways. These transporters also function in the regulation of adenosine, which is biologically active in the extracellular environment. Pharmacologically, these transporters are important in the cellular permeability of numerous chemotherapeutic compounds. This thesis will concentrate primarily on the role of nucleoside transporters in maintenance of intra- and extra-cellular adenosine levels.

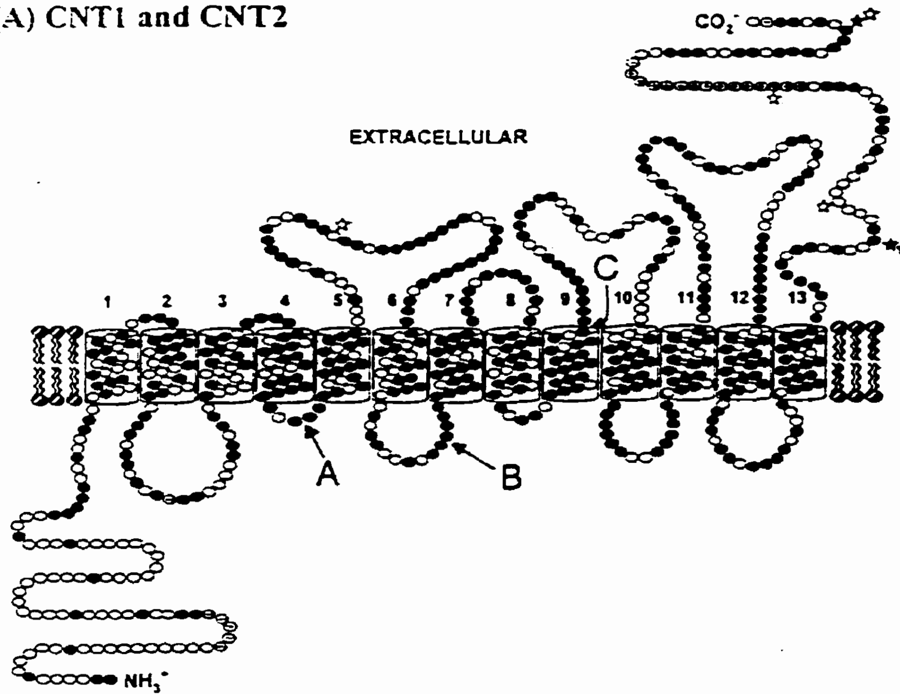
In 1963, Kubler and Bret-Schneider were the first group to report the cellular transport of purine and pyrimidine nucleosides across red blood cell (RBC) plasma membranes (Kubler and Bretschneider, 1963). During the past 38 years, extensive research has led to a better understanding of nucleoside transport processes (Cass et al., 1998).

3.2.1 Equilibrative Nucleoside Transporters

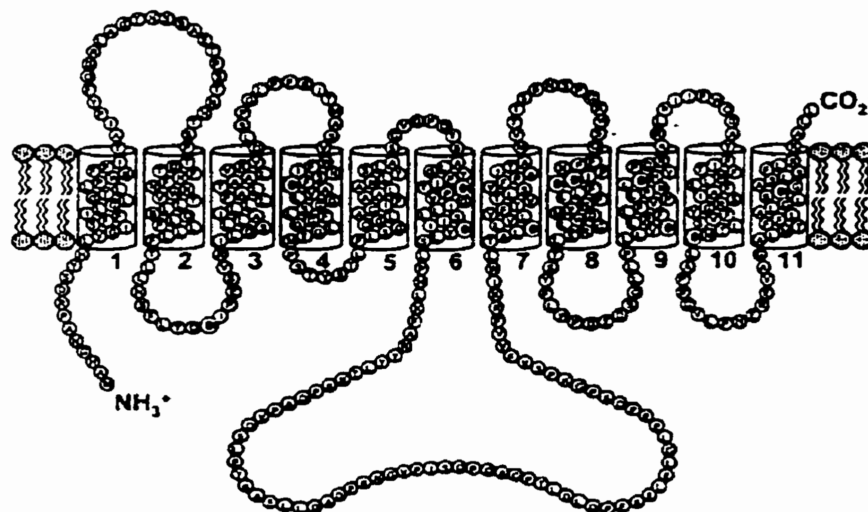
The equilibrative nucleoside transporters are integral-membrane proteins that allow the facilitative, or equilibrative, bi-directional flux of adenosine and other nucleosides across the plasma membrane. Initial studies in erythrocytes detailed the bi-directional movement of nucleoside (Kubler and Bretschneider, 1963), that was later found to be potently and selectively inhibited by the nucleoside analog nitrobenzylmercaptopurine riboside (NBMPR) (Pickard et al., 1973; Pickard and Paterson, 1972). Studies later emerged that documented equilibrative nucleoside transport that was not potently inhibited by NBMPR (Jarvis and Young, 1986; Plagemann and Wohlhueter, 1984). Based

Figure 3: Schematic Diagram of Nucleoside Transporters

(A) CNT1 and CNT2



(B) ENT1 AND ENT2



Diagrams taken from Yao et. al.(Yao et al., 2001) and Loewen et. al.(Loewen et al., 1999).

on the sensitivity to NBMPR inhibition, the equilibrative nucleoside transporters were designated equilibrative sensitive (*es*) and equilibrative insensitive (*ei*).

3.2.1.1 *es* Nucleoside Transporter

The *es* transporter is the best characterized of the nucleoside transporters.

3.2.1.1.1 Molecular Biology and Purification

The *es* transporter was initially isolated using detergent and ion exchange chromatography to purify the protein from human and pig erythrocytes. The purified protein, when reconstituted in phospholiposomes, enabled transport of uridine, binding of [^{3}H]NBMPR and had an apparent MW of 64 kDa (Jarvis, 1987). Immunoaffinity chromatography from human RBC produced a protein of 55 kDa on SDS-PAGE gels. The *es* transporter has also been purified from rat, guinea pig and rabbit sources. The differences in the apparent size of the isolated proteins are due to differences in the level of glycosylation as endo-glycosidase treatment produces a protein of 47-51 kDa (Griffith and Jarvis, 1996). The species differences and function of the glycosylation of the transporters is unknown. The *es* transporter was cloned in 1997 from rat, mouse and human tissues (Griffiths et al., 1997a; Yao et al., 1997). The clones, regarded as ENT1 (equilibrative nucleoside transporter) demonstrate that there are differences in glycosylation sites between the different species. The human clone is 456 AA encoding a protein of 50.2 kDa (Griffiths et al., 1997a) while the rat clone is 457 AA producing a protein of 50 kDa (Yao et al., 1997). The cloning of the *es* transporter enabled further

investigation of the kinetics and molecular determination of the distribution of the transporter.

3.2.1.1.2 Endogenous Activity

The endogenous activity of the *es* transporter is determined by the permeant concentrations, the kinetics of the transporter and the distribution of the transporter. As stated earlier, a wide range of purine and pyrimidine nucleosides are transported by the *es* transporter. The affinity of each of these nucleosides for the *es* transporter is species dependent. Influx studies (see section 4.1.1) report that the *es* transporter has a K_m of 20-50 μM for adenosine. As the *es* transporter is an equilibrative transporter, the kinetics of nucleoside release has also been investigated (see section 4.1.2). The release of [^{3}H]uridine and [^{3}H]formycin B (FB) have similar kinetics to influx in cells containing *es* transporters.

The distribution of the *es* transporter has been investigated with activity studies, [^{3}H]NBMPR binding, northern blots, in situ hybridization, RT-PCR and immunohistochemistry. Specific [^{3}H]NBMPR binding in rat brain has demonstrated high levels of the *es* transporter in the striatum, cortex, superior colliculus, substantia nigra, hypothalamus, choroid plexus and thalamic structures while very low levels are seen in the hippocampus, cerebellum and white matter (Anderson et al., 1999b; Geiger and Nagy, 1990). Studies have indicated a good correlation between [^{3}H]NBMPR binding and the distribution of ADA_1 activity in rat brain (Deckert et al., 1988a; Deckert et al., 1988b; Geiger and Nagy, 1990). Molecular studies have indicated widespread distribution of ENT1 mRNA transcript in rat and human brain (Anderson et al., 1999b). In addition to the regional distribution of the *es* transporter in the CNS, there have been reports of

cellular differences. *In vitro* studies have demonstrated *es* transport in neurons, astrocytes and endothelial cells. In cells derived from chick embryos, neurons were reported to have higher affinity for adenosine transport than astrocytes while the astrocytes have a greater capacity for uptake (Thampy and Barnes, 1983a; Thampy and Barnes, 1983b). Although much work involving *es* transport in the CNS has been produced, more studies are needed to determine further cellular and regional differences in *es* transport and potential co-localization with purinergic receptors and enzymes.

3.2.1.1.3 Pharmacological Regulation

The nomenclature of the *es* transporter is based on the potent and selective inhibition of the transport system by NBMPR. This compound was initially used to differentiate between *es* and *ei* transporter subtypes. NBMPR inhibits *es* transport of adenosine with a K_i of 0.1-1.0 nM, which is similar among species. The inhibition involves tight but reversible binding of NBMPR to a single non-cooperative site on the es transporter (Geiger and Nagy, 1990) thought to be present in transmembrane domain 4 (Yao et al., 2001). Inhibition of nucleoside transport through the *es* transporter by NBMPR closely correlates to [^{3}H]NBMPR binding. Inhibition of nucleoside influx by NBMPR occurs in a competitive manner while efflux inhibition occurs via a non-competitive mechanism. This is consistent with an NBMPR binding site on the extracellular surface of the *es* transporter.

Although NBMPR is the most potent and selective inhibitor of the *es* transporter, a vast array of non-nucleoside compounds has been reported to inhibit this transporter. The vasodilators dipyridamole (DPR) and dilazep (DZP) have been widely used as

inhibitors of nucleoside transport. DPR is a potent inhibitor of *es* nucleoside transport in human, rabbit and guinea pig tissues with a K_i of 1-10 nM. However, in rat and mouse tissues, it is not a potent inhibitor of this transporter with K_i values of greater than 1 μM. DZP is also not selective at inhibiting the *es* transporter but it is a relatively potent inhibitor of this transporter in all species except rat. Lidoflazine and its analogues draflazine, soluflazine and mioflazine have been reported to be potent inhibitors of human and rabbit *es* transport but not in rat or mouse cells (Griffith and Jarvis, 1996). The interest in these compounds is based on the high oral bioavailibility compared to DPR or DZP. The xanthine derivative propentofylline has been reported to inhibit *es* transport at concentrations around >1 μM (Ohkubo et al., 1991; Parkinson et al., 1993), which are similar to the concentrations at which propentofylline inhibits adenosine A_1 receptors (Borgland et al., 1998).

Due to the role of the *es* transporter in regulating adenosine levels, nucleoside transport inhibitors have been proposed as therapeutic agents in a number of CNS conditions. Outside of the CNS, nucleoside transport inhibitors have been used therapeutically for anti-platelet aggregation and adjuncts in chemotherapy. In the CNS, a clinical role has not been attained but many uses have been investigated at a basic level. NBMPR, DPR and propentofylline have been investigated for neuroprotective properties during conditions of cerebral ischemia. NBMPR-P, which is a water-soluble pro-drug form of NBMPR, has been demonstrated to protect against ischemic injury in rats when given by intracerbrovasculature (ICV) or intraperitoneal injections (i.p.) (Parkinson et al., 2000; Parkinson et al., 2001). When NBMPR (i.p.) was given in gerbil forebrain ischemia, no significant effect was seen upon neuronal damage (DeLeo et al., 1988a),

likely due to low solubility and blood-brain barrier (BBB) permeability (Anderson et al., 1996a). Propentofylline has been demonstrated to decrease ishemic damage in rat and gerbil models (Andine et al., 1990; Dux et al., 1990; Gidday et al., 1995; Haag et al., 2000; Johnson et al., 1998; Matsumoto et al., 1996; Park and Rudolphi, 1994). Although propentofylline is not selective for inhibition of the *es* transporter, an adenosine-mediated pathway is likely responsible for the decreased neuronal damage as there is increased CNS adenosine levels (Andine et al., 1990; Dux et al., 1990), increased blood flow (Turcani and Tureani, 2001) and was sensitive to theophylline inhibition (DeLeo et al., 1988b; Fern et al., 1994). DPR, because of its low solubility, poor oral bioavailibility and BBB permeability (German et al., 1989; Sollevi et al., 1984), has not been widely tested for its neuroprotective properties. However, it has been reported to increase blood flow to ischemic brain regions and increase post ischemic adenosine levels (Park and Gidday, 1990; Phillis et al., 1989). Also, it has been reported to decrease the incidence of strokes clinically, possibly due to its effects on CNS adenosine levels (Picano and Abbracchio, 1998). In addition to cerebral ischemia, a number of nucleoside transport inhibitors have been investigated as anti-convulsants. DZP has been reported to decrease kainic acid- and bicuculline methiodide-induced seizures in rats, by an adenosine receptor sensitive mechanism (Zhang et al., 1990; Zhang et al., 1993). DPR has been reported to decrease bicuculline methiodide-, hypoxic- and penicillin-induced seizures (Eldridge et al., 1989; Thorat and Kulkarni, 1990a; Thorat and Kulkarni, 1990b). Interestingly, many clinically used anti-convulsant agents such as midazolam and diazepam are inhibitors of *es* nucleoside transport, although not at clinically relevant doses (Phillis, 1984; Seubert et al., 2000; Ujfalusi et al., 1999). The effects of caffeine on wakefulness exemplify the role

of adenosine and sleep regulation. Nucleoside transport inhibitors may potentially be useful alternatives to conventional sleep inducing agents such as benzodiazepines or barbiturates. The nucleoside transport inhibitors NBMPR and mioflazine have been reported to promote sleep in cats and dogs, respectively (Porkka-Heiskanen et al., 1997; Wauquier et al., 1987). Therefore, nucleoside transport inhibitors have potential for a number of CNS related conditions. More selective and potent inhibitors of the *es* transporter that are orally active, water soluble and BBB permeable are needed to further investigate the clinical usefulness of these compounds.

In addition to the compounds that were designed/discovered to be potent inhibitors of the *es* transporter, a number of commonly used compounds have been reported to interact with the *es* transporter. As stated above benzodiazepines have been reported to inhibit *es* transport in the micromolar range (Phillis, 1984; Seubert et al., 2000). Calcium channel inhibitors such as verapamil, nimodipine, and nitrendine have been reported to inhibit NBMPR sensitive nucleoside transport (Deckert and Gleiter, 1994; Hammond et al., 1985; Plagemann and Woffendin, 1987). Antidepressants such as desipramine and fluoxetine are weak inhibitors of *es* nucleoside transport (Parkinson, unpublished data). Ethanol has been reported to inhibit *es* transport by a cAMP-dependent mechanism (Coe et al., 1996; Krauss et al., 1993). The adenosine A_1 receptor agonist CHA and the adenosine kinase inhibitor ITU have been reported to inhibit the *es* transporter (Borgland and Parkinson, 1998; Geiger et al., 1985; Parkinson and Geiger, 1996). Thus, the possible role of *es* transporter-mediated effects must be considered when adenosinergic pathway might be involved with these compounds.

3.2.1.2 *ei* Nucleoside Transporter

Following the initial studies demonstrating nucleoside influx that was sensitive to inhibition by nanomolar levels of NBMPR, numerous studies were published documenting inhibition of nucleoside transport by NBMPR only at concentrations greater than 1 μM. In comparison to the *es* transporter, little is known about the function, distribution and molecular characteristics of the *ei* transporter as selective inhibitors and radiolabeled markers are not available.

3.2.1.2.1 Molecular Biology and Purification

Until the cloning of the *ei* transporter in 1997 from human and rat tissues (Crawford et al., 1998; Griffiths et al., 1997b), the molecular nature of the transporter was not characterized. Only one report had demonstrated isolation and functional reconstitution of the *ei* transporter (Hammond, 1994). Until the cloning of the *ei* transporter, it was unclear whether the *ei* and *es* transporters were just post-translational modifications of the same gene product or two different proteins derived from two different genes (Plagemann et al., 1988). The cloning of the *ei* transporter demonstrated that these two transporters had separate genes. The ENT2 cDNA encodes for a 456 AA protein (MW 50.2 kDa) from both rat and human tissues, which is very similar to the ENT1. Although the size of the proteins is similar, there is only 50% identity between the ENT1 and ENT2 AA sequence in either human or rat transporters. Similar to ENT1, the ENT2 transporter has putative glycosylation sites on the N-terminus between transmembrane domains 1 and 2. The cloning of the *ei* transporter should allow for an

understanding of the function and distribution of the transporter, which until now has been minimal.

3.2.1.2.2 Endogenous Activity

Based on the kinetics of the *ei* transporter, it is understandable that many researchers believed the *es* and *ei* transporters were derived from the same gene. The *ei* transporter, similar to the *es* transporter, has both purine and pyrimidine nucleosides as permeants, with similar K_m values to the *es* transporter for each compound. Adenosine has been reported to have a K_m of 30-70 μM while uridine has a K_m of 70-280 μM (Griffith and Jarvis, 1996). These values are similar to those reported for the *es* transporter. However, guanosine has been reported to be transported with a K_m 10 fold higher by the *ei* transporter than the *es* transporter (Hammond, 1992; Ward et al., 2000). Therefore the *ei* transporter may be more specific for adenosine than guanosine transport compared to the *es* transporter.

The CNS distribution of the *ei* transporter is very poorly characterized. *Ei* transporter activity has been demonstrated in numerous CNS preparations including synaptosomes and human astrocytes (Gu et al., 1996; Lee and Jarvis, 1988). There is a species difference in CNS *es* and *ei* transport between rabbit, where 65% of transport is *es*, and guinea pig and rat where the majority is *ei* transport (Jones and Hammond, 1995). Although *ei* transport has been demonstrated, there is no conclusive data demonstrating regional or cellular differences in CNS *ei* activity. [^{3}H]DPR in the presence of NBMPR was proposed as a way of detecting the *ei* transporter, similar to the use of [^{3}H]NBMPR and the *es* transporter (Jones and Hammond, 1992). However, this method does not

produce reliable results due to high non-specific binding of $[^3H]$DPR (Griffith and Jarvis, 1996). RT-PCR, northern blots and in situ hybridization demonstrated mRNA transcript for the ENT2 transporter ubiquitously in human and rat brain (Anderson et al., 1999a). The important next step is to perform immunohistochemistry with antibodies for the ENT2 transporter to determine if there are regional and cellular differences in transporter expression.

3.2.1.2.3 Pharmacological Regulation

As stated above, there are no available selective inhibitors of the *ei* transporter. Although described as NBMPR insensitive, the more appropriate description would be poor or low sensitivity as micromolar concentrations of NBMPR can inhibit *ei* transport. DPR, DZP and propentofylline have also been shown to inhibit *ei* transport, but normally at higher concentrations than required for the *es* transporter. The most selective of the conventional transport inhibitors at inhibiting *ei* transport is the lidoflazine analogue, soluflazine. Soluflazine has been reported to be 8 fold more potent at inhibiting *ei* transport than *es* (Hammond, 1991). The further development of selective and potent *ei* transport inhibitors will enable further understanding of the role of this transporter in the CNS.

3.2.2 Na^+-dependent Nucleoside Transporters

Na^+-dependent nucleoside transport was initially described in rabbit choroid plexus (Spector, 1980). These studies documented intracellular accumulation of nucleosides greater than 10 fold the extracellular concentration (Plagemann et al., 1988).

The Na^+ dependent transport could be inhibited by dinitrophenol (DNP) and iodoacetate (IAA) and was unaffected by NBMPR or DPR. Other studies soon began to emerge documenting Na^+-dependent transport in kidney, intestinal, liver and epithelial cells obtained from different species (Plagemann et al., 1988). It became apparent that Na^+-dependent nucleoside transport was widely expressed and that there were numerous forms of this type of transport mechanism. In fact, six different Na^+-dependent nucleoside transporters have been described that differ in permeant selectivity, Na^+/nucleoside stoichiometry, and sensitivity to NBMPR inhibition (Cass et al., 1998). Although numerous transporters have been described, little is known about the function and distribution of each transporter.

3.2.2.1 CIF Nucleoside Transporters

The N1 or *cif* (*c*oncentrative, *i*nsensitive to NBMPR, *f*ormycin B selective) transporter is a Na^+-dependent transporter which transports predominantly purine nucleosides such as adenosine, guanosine and formycin B as well as the pyrimidine nucleoside uridine. This is the only Na^+-dependent transporter characterized to date that has adenosine as a preferred permeant.

3.2.2.1.1 Molecular Biology and Purification

Isolation of the *cif* transporter was initially described from rat intestinal and liver samples using poly(A)$^+$mRNA injected into *Xenopus* oocytes (Che et al., 1995). The *cif* transporter was cloned in 1995 and initially regarded as SPNT (sodium dependent purine nucleoside transporter) (Che et al., 1995), which is now more commonly known as CNT2

(concentrative nucleoside transporter) (Yao et al., 1996). The rat and human CNT2 clones encode a protein of 659 or 658 AAs, respectively, with a MW of ~72kDa. Initial hydropathy plots indicated that the transporter contained 14 transmembrane domains while more recent studies indicate 13 transmembrane domains (Cass et al., 1998). The protein has numerous putative regulatory sites including 3 protein kinase A (PKA) and 6 protein kinase C (PKC) phosphorylation sites and 5 potential glycosylation sites (Che et al., 1995). The CNT2 transporters show no significant homology to either ENT1 or ENT2 but there is 64% identity to the CNT1 transporter AA sequence (see section 3.2.2.2). The cloning of the CNT2/cif transporter has enabled research into the function and distribution of the transporter.

3.2.2.1.2 Endogenous Activity

The *cif* transporter is selective for the transport of purines, with the exception of uridine. *In vitro* studies using native and recombinant *cif*/CNT2 transporters have demonstrated that the affinity of the transporter for adenosine and uridine is 1-14 μM and 9-40 μM, respectively (Cass et al., 1998; Griffith and Jarvis, 1996). The transport is unaffected by the equilibrative transport inhibitors NBMPR or DPR nor is it altered by pyrimidine nucleosides such as cytidine or thymidine. The Na^+ gradient drives the accumulation or concentration of adenosine or other purine nucleosides into cells, even during conditions where intracellular levels are higher than extracellular levels. Similar to Na^+-dependent glutamate transporters, the *cif* transporter has been demonstrated to release its permeants when the Na^+ gradient is removed (Borgland and Parkinson, 1997). Thus, this transporter, may function not only in removing adenosine from the

extracellular space, but may also be involved in the cellular release of adenosine under pathophysiological conditions where Na^+ gradients are disturbed.

The absence of radiolabeled inhibitors and antibodies to the *cif* transporter has limited the progress of knowledge about the distribution and function of this transporter. Transporter activity and mRNA expression have demonstrated widespread distribution of the *cif* transporter. Outside the CNS, *cif* activity has been reported in numerous tissues in the body, primarily in liver, kidney and intestine (Griffith and Jarvis, 1996). In the rat CNS, synaptosomes, neurons and astrocytes have been reported to possess 30-90% of their total nucleoside transport as Na^+-dependent. The distribution of rCNT2 mRNA has been demonstrated in most regions of the rat brain with RT-PCR, northern blots and *in situ* hybridization (Anderson et al., 1996b). Although the *cif* transporter has been demonstrated in the CNS, very little is known about the role of the transporter in the brain.

3.2.2.1.3 Pharmacological Regulation

Similar to all reported nucleoside transporters except the *es* and N5 transporters, selective inhibitors of *cif* nucleoside transporters have not been reported. The transporter is unaffected by the conventional *es* inhibitors NBMPR, DPR or DZP. Gemcitabine, a chemotherapeutic nucleoside analogue, has been reported to inhibit *cif*-mediated nucleoside transport but not *es*, *ei* or *cit* (see 3.2.2.2) (Mackey et al., 1998; Mackey et al., 1999). The method used to inhibit *cif* and other Na^+-dependent nucleoside transporters for *in vitro* studies is the disruption of the Na^+ gradient by replacing extracellular Na^+ with lithium, choline or N-methylglucamine, inhibiting Na^+/H^+ or Na^+/K^+ exchangers or depleting cellular ATP levels (Plagemann et al., 1988). However, each of these methods

has non-selective effects, which may affect the interpretation of the results. Along with antibodies to the *cif* transporter, selective and potent inhibitors are required to obtain a better understanding of the function, role and distribution of this transporter.

3.2.2.2 Other Concentrative Nucleoside Transporters

In addition to the *cif* transporter, five other Na^+-dependent nucleoside transporters have been reported, two of which have been cloned. The best characterized of these transporters is the N2 or *cit* (concentrative, insensitive to NBMPR, thymidine selective), which is pyrimidine-selective except for permeation of adenosine. Human and rat *cit* transporters have been cloned and are regarded as CNT1. The CNT1 clones produce a protein of 648 AAs (~71 kDa) (Huang et al., 1994). The native and cloned transporter has a K_m for adenosine and uridine of 15-26 and 7-31 μM, respectively (Cass et al., 1998). Although adenosine has a high affinity for *cit*, the V_{max} for adenosine permeation is low, indicating relatively poor permeability. The activity of *cit* has been demonstrated primarily in the kidney and intestines (Griffith and Jarvis, 1996) while in rat brain rCNT1 mRNA expression has been demonstrated widely (Anderson et al., 1996b).

The N3 or *cib* (concentrative, insensitive to NBMPR, broadly selective) transporter has been reported to have both purines and pyrimidines as permeants. The *cib* transporter was recently cloned from rat and human tissues and was designated CNT3 (Ritzel et al., 2001). A clone with *cib*-like activity (called SNST1) has been reported but it does not appear to have similar kinetics or distribution to the *cib* transporter, which has been reported in leukemic blast cells, choroid plexus and intestine (Cass et al., 1998). The recent cloning of this transporter should allow for a better understanding of its distribution and function.

The other three reported Na^+-dependent nucleoside transporters are poorly characterized. The N4 transporter, which was isolated from renal brush border membranes, has been reported to have *cit*-like characteristics except for the fact that it is inhibited by guanosine (Griffith and Jarvis, 1996). The N5 transporter was isolated from human leukemic cells and appears to be guanosine selective and sensitive to NBMPR inhibition (Griffith and Jarvis, 1996). The N6 transporter has similar properties to the N5 transporter except that it allows permeation of adenosine analogues as well (Griffith and Jarvis, 1996). Therefore, if SNST1 is included, there are up to seven Na^+-dependent transporters that have been reported, with little known about the role each of these transporters play in extracellular adenosine regulation.

The nucleoside transporters are an integral component involved in regulation of extracellular adenosine levels as these proteins can allow release and cellular uptake of adenosine. The primary role of the nucleoside transporters in regulation of extracellular adenosine derived from extracellular adenine nucleotides is to sequester adenosine from the interstitial spaces. The role of these transporters in release of adenosine per se is dependent upon the intracellular metabolic pathways regulating adenosine anabolism and catabolism.

3.3 Adenosine Kinase

Adenosine kinase (EC 2.7.1.20, ATP:adenosine 5'phosphotransferase) is a ubiquitous enzyme in mammalian tissues. It is responsible for the phosphorylation of adenosine and related nucleosides and analogues to 5'monophosphate nucleotides. Although ATP is typically considered the phosphate donor, a wide range of nucleoside triphosphates can

act as the donor. In general, purine triphosphates are better phosphate donors than pyrimidine triphosphates, with GTP, dGTP, dATP and ITP being the best alternate phosphate donors (Schnebli et al., 1967). In addition, divalent cations are required for AK activity, with Mg^{2+} or Co^{2+} as the most widely accepted cofactors (Chang et al., 1980; Schnebli et al., 1967).

3.3.1 Purification and Molecular Biology

The activity of this enzyme was initially discovered in yeast (Kornberg and Pricer, 1951) and subsequently in liver and kidney extracts (Caputto, 1951). Mammalian AK was initially isolated from rat, human, mouse and rabbit tissues using ion-exchange chromatography or gel filtration and affinity chromatographic techniques. The isolated protein from the different species is a monomeric protein with a molecular weight of 38-56 kDa (Kowaluk et al., 1998). The AK gene has been reported to reside on chromosome 10 in humans and chromosome 14 in mice (Kowaluk et al., 1998). AK has been isolated from almost every human tissue (Andres and Fox, 1979). AK activity has been reported to have similar levels in liver, kidney, pancreas and brain from human autopsy specimens (Snyder and Lukey, 1982). The highest level of AK activity in rat brain appears to be in the olfactory bulb and cerebellum with the lowest activity present in the septum and posterior hypothalamus (Geiger et al., 1997).

In 1996, AK research was enhanced markedly when three laboratories independently cloned human and rat AK (McNally et al., 1997; Singh et al., 1996; Spychala et al., 1996). Two human AK cDNA clones were isolated by McNally *et al*; hAK-short and hAK-long (McNally et al., 1997). The hAK-short was nearly identical to the clones

Table 2: Endogenous Regulators of Purine Metabolism

Regulator	AK	ADA_1	e-N	c-N-I	c-N-II	AMPDA	PNP
P_i	↑ activity by ↓ substrate inhibition	N.E.	N.E.	↓ activity by 30% at [ ] > 20mM	↓	↓	↑
optimal pH	6.2-6.8	7.0-7.4	7.0-8.0	7.0-8.0; inactive below 6.8	6.0-7.0	6-7	5-7.5
Mg^{2+}	↓	↑	N.E. requires Zn^{2+}	required for activity; ↑ activity at [ ] > 10mM	required for activity↑ activity at [ ] > 10mM	↓	N.D.
ATP	required; but ↓ activity when it exceeds Mg^{2+}	N.E.	↓ activity at [ ] > 10 μM	N.E.	↑ activity, ↓K_m for IMP and AMP to μM	N.D.	N.D.
ADP	↓	N.E.	↓ activity at [ ] > 10 μM	↑ activity, ↓K_m for AMP to μM	↑ activity, ↓K_m for IMP to μM	N.D.	N.D.
AMP	↓	N.E.	N.D.	↑ activity due to increased substrate	low affinity substrate	N.D.	N.D.
Ap_nAs	↓	N.E.	N.D.	N.D.	↑ activity, ↓K_m for IMP to μM	↑	N.D.
Substrate	↓ ADO >10 μM, and ATP, Mg^{2+}	N.E.	N.D.	N.D.	N.D.	N.D.	↓ by high INO
Product	see above for ADP and AMP	↓ by INO and HX	N.D.	N.D.	N.D.	N.D.	N.D.
Others	N.D.	↓ by UTP, dGTP	N.D.	N.D.	↑ 2,3-BPG	↓2,3-BPG, GTP, ↑ PKC	↓ by PRPP

A general overview of the effects of different endogenous compounds on the different purinergic enzymatic pathways. N.E.-no effect, N.D.- not determined, ↑ increased enzyme activity, ↓ decreased enzyme activity. For references please see specific sections.

produced by Spychala *et al* and Singh *et al* (Singh et al., 1996; Spychala et al., 1996). There was no apparent difference in the kinetics or regulation between the two hAK clones, indicating probable alternative splicing of a single transcriptional product (Spychala et al., 1996). The cloned hAK has 345 (hAK-short) or 361 (hAK-long) AAs with a corresponding protein size 38.7 or 40.5 kDa, which corresponds to the size estimated from purified AK. The short and long clones of AK are believed to be a result of post-transcriptional or post-translational modifications (McNally et al., 1997). The cloning of AK has demonstrated that AK is not related to other nucleoside kinases due to its preference for 2'-nucleosides and the lack of a classical N-terminal P-loop motif, which functions in ATP or GTP binding. The current hypothesis is that AK is derived from microbial sugar kinases, which also lack the N-terminal P-loop and have high sequence homology with AK (Singh et al., 1996; Spychala et al., 1996). Thus, the cloning of AK has given researchers the ability to have a better understanding of AK at the molecular level especially with pharmacological interactions and endogenous regulation.

3.3.2 Endogenous Activity

The kinetic mechanism by which adenosine kinase phosphorylates adenosine is uncertain. Two potential mechanisms have been proposed. An ordered Bi-Bi reaction, in which $MgATP^{2-}$ and adenosine bind to AK and the subsequent release of AMP and MgADP, is the more documented model. A ping-pong mechanism, with the production of a phosporolenzyme, has been proposed but no reports of the phosphorylated AK intermediate have been documented. The possibility of AK isozymes has been proposed

but there is little data to support this hypothesis (Kowaluk et al., 1998; McNally et al., 1997).

AK is a cytosolic enzyme that is regulated by numerous endogenous mechanisms (see fig 2). AK, as opposed to ADA (see section 3.3), is regarded as a high affinity, low capacity enzyme. The affinity of mammalian AK for adenosine has been reported to range from 0.2 – 20 μM (Chang et al., 1980) with a V_{max} ranging from 0.3 μmol/min/mg prot (Chang et al., 1980) – 2.2 μmol/min/mg prot (Miller and Adamczyk, 1979). The optimal activity of AK has been reported to occur between 1-10 times the K_m of adenosine. Above these concentrations, adenosine produces substrate inhibition of AK activity. The biphasic effect of adenosine on AK activity is believed to be due to high and low affinity binding sites on AK for adenosine. The high affinity site is the catalytic site while the low affinity site is believed to be an inhibitory (regulatory) site (Geiger et al., 1997), which may also be the ATP catalytic site. A large range of K_m, K_i and V_{max} values have been reported for AK activity, likely due to the complex regulation of AK by a number of endogenous factors, principally free Mg^{2+}, ATP, pH and P_i.

Each of these is important in regulating AK activity, but pH is the most important factor in determining the effects of Mg^{2+} and ATP upon AK activity. The optimal pH for AK activity has been reported to be between 5.5-7.2. This large range is likely due to contamination of isolated AK with endogenous Mg^{2+} (Palella et al., 1980). A better estimate of the optimal pH is between 6.2-6.8. This is due to two factors. First, the true substrate for AK is $MgATP^{2-}$, rather than ATP. As the pK of the terminal phosphate group of ATP is 6.5, an increased proportion of ATP will be found as ATP^{4-} as the pH drops from physiological pH. Free Mg^{2+} can then complex with ATP^{4-} to form $MgATP^{2-}$,

the actual substrate for AK. In addition, it has been reported that decreasing the pH from 7.4 to 6.2 decreases adenosine's K_m and increases its K_i for AK (Maj et al., 2000). At pH between 5-6, AK activity is present and does not appear to be subject to inhibition by free Mg^{2+}, ATP or adenosine. AK activity decreases appreciably as the pH increases above 7.5. As cellular pH decreases, AK activity should increase due to increased levels of $MgATP^{2-}$ and increased affinity for adenosine.

The role of free Mg^{2+} in regulating AK activity, as described above, is pH dependent and linked to ATP levels. When Mg^{2+} levels exceed available ATP^{4-}, free Mg^{2+} will result, which will potently inhibit AK. This may occur during conditions where ATP levels are depleted such as hypoxia/ischemia or metabolic poisoning. The ideal ratio of Mg^{2+}/ATP levels are approximately 0.5-1. If the ratio exceeds one, free Mg^{2+} will inhibit AK activity. The role of ATP is similar to Mg^{2+}. ATP or another purine nucleoside triphosphate is required for AK activity. However, if ATP, in the dissociated form ATP^{4-}, is not complexed with Mg^{2+}, AK activity will be decreased. The inhibition of AK by free ATP is less pronounced than with free Mg^{2+} and will occur at supraphysiological concentrations greater than 10-15 mM.

In addition to pH, Mg^{2+} and ATP, AK is regulated by other common endogenous compounds. Inorganic phosphate, which is normally found intracellularly below 1 mM, has been reported to decrease adenosine's K_m and increase its K_i for AK and increase the V_{max} when P_i concentrations exceed 5mM (Maj et al., 2000). In addition to substrate inhibition, product inhibition by AMP and ADP also occurs. AMP produces competitive (K_i = 140 μM) and non-competitive (K_i = 500 μM) inhibition of AK activity while ADP produces non-competitive inhibition (K_i = 11-500 μM) (Mimouni et al., 1994; Palella et

al., 1980). The adenosine polyphosphates, Ap_4A and Ap_5A, have been reported to inhibit AK activity, at concentrations between 30-75 nM (Delaney et al., 1997; Rotllan and Miras Portugal, 1985; Schnebli et al., 1967). The glycolytic intermediate 2,3-bisphosphoglycerate, which also regulates other purinergic enzymes, has been reported to stimulate AK activity.

Thus, AK activity is complex and highly regulated, likely a reflection of the important role of adenosine in cellular function. Based on conventional views, AK is important in regulating adenosine levels during physiological conditions as basal cellular adenosine levels have been estimated to be close to the K_m for AK. This has led to the hypothesis that inhibition of AK will lead to elevation of adenosine during physiological conditions but only when adenosine levels are below the K_i values of adenosine for AK. However, when adenosine levels are elevated, inhibition of AK with exogenous compounds has been hypothesized to have minimal effects on adenosine levels, as AK should be inhibited by substrate inhibition. An interesting phenomenon of unknown significance is the dynamic flux between AMP and adenosine that is thought to involve AK and AMP-preferring 5'nucleotidase. In some cells, it has been reported that AMP is constantly being dephosphorylated to adenosine, which is then rephosphorylated to AMP in a futile cycle (Bontemps et al., 1983). The importance of this cycle is unclear but it may be important in second to second regulation of adenosine, as AK activity is highly regulated by a multitude of endogenous factors. The role of each of these regulatory pathways in a more complex system must be determined. An important point to remember with all the AK data that was cited above is that this work was performed on

isolated enzymes. Further research is needed to investigate the kinetics and regulation of AK *in vitro* and *in vivo*.

3.3.3 Pharmacological Regulation

Based on the therapeutic potential of adenosine and the critical role of AK in regulating adenosine levels, it is not surprising that a vast amount of research has gone into developing AK inhibitors. Two early reports screened an array of purine and pyrimidine nucleosides, which led to the discovery of the two most widely used AK inhibitors, 5'iodotubericidin (Henderson et al., 1972) and 5'amino-5'-deoxyadenosine (Miller et al., 1979). More recent research has investigated AK inhibitors that have high potency similar to ITU or NH_2dAdo, but are not metabolized by AK or other enzymes and have increased cellular permeability and bioavailibility (Kowaluk et al., 1998). Researchers at Metabasis Therapeutics (previously Gensia-Sicor) and Abbott Laboratories have been and are currently developing analogues of ITU and NH_2dAdo that have shown *in vitro* and *in vivo* efficacy against AK. An important point to remember with the AK inhibitors that have been developed is that these compounds are nucleoside analogues. Therefore, they may require similar transport mechanisms as adenosine and endogenous nucleosides for their cell permeability. AK inhibitors have been tested for potential therapeutic value in a number of CNS conditions. The rationale for using these compounds is site- and event-specific elevation of CNS adenosine levels, which should bypass the majority of side effects seen with using adenosine *per se* or adenosine receptor agonists. Site- and event-specific elevation of adenosine refers to the ability of these compounds to elevate adenosine levels in areas of the body where

adenosine is being produced during specific conditions such as hypoxia or ischemia, but not in areas unaffected by the stimulus. AK inhibitors have been widely documented to produce analgesia *in vivo*, whether administered peripherally or spinally. AK inhibitors such as ITU and 5'deoxy-ITU have proven effective in numerous pain paradigms of acute nociceptive pain, chemically induced pain and neuropathic pain (Kowaluk and Jarvis, 2000; Kowaluk et al., 1998). The effects of these AK inhibitors are blocked by adenosine receptor antagonists and have been mimicked clinically using intrathecal adenosine administration. AK inhibitors have been reported to be effective anti-inflammatory agents in *in vivo* models of inflammation (Kowaluk and Jarvis, 2000; Kowaluk et al., 1998). The anti-inflammatory effects are peripherally mediated. Adenosine has been widely reported to be an endogenous neuroprotective compound for seizure-induced and ischemic damage. Thus, it is not surprising that AK inhibitors have been tested for anticonvulsant and anti-ischemic properties. Bicuculline metiodide-, PTZ- and maximal electroshock-induced seizures are effectively reduced by the administration of AK inhibitors (Ugarkar et al., 2000a; Ugarkar et al., 2000b; Wiesner et al., 1999; Zhang et al., 1993). In each of these studies, centrally acting adenosine receptor antagonists were able to block the effects of the AK inhibitors, indicating elevation of endogenous adenosine levels. Although AK has been conventionally viewed as important in regulating adenosine during basal conditions and less so during conditions of increased adenosine production, AK inhibitors have been reported as effective experimental treatments for cerebral ischemia. Adenosine levels increase during hypoxic/ischemic conditions *in vitro* and *in vivo*, due to increased ATP catabolism and/or decreased ATP anabolism. Based on the biphasic relationship of adenosine levels and

AK activity, one would hypothesize that AK inhibitors would be ineffective at elevating adenosine levels during ischemic conditions, due to substrate inhibition of AK. However, AK inhibitors have been reported to decrease neuronal infarct size and neurological deficits in numerous cerebral ischemia models (Miller et al., 1996; Tatlisumak et al., 1998). These effects may be species and model dependent (Phillis and Smith-Barbour, 1993). Thus, further research is needed to determine the potential therapeutic value of AK inhibitors for cerebral ischemia. The use of AK inhibitors as analgesic, anti-inflammatory, anti-convulsants and anti-ischemic agents is continuing at both the basic and clinical levels. The first phase 1 clinical trial with an AK inhibitor is currently underway, and may provide insight into the feasibility and usefulness of these compounds clinically.

3.4 Adenosine Deaminase

Adenosine deaminase (ADA; adenosine aminohydrolase, EC 3.5.4.4) catalyses the irreversible deamination of adenosine and deoxyadenosine producing ammonia and inosine or deoxyinosine, respectively. There are three principle forms of ADA; double stranded RNA ADA, tRNA ADA and free nucleoside ADA. For the purposes of this thesis, only the free nucleoside ADA will be discussed. The importance of ADA in humans is exemplified by conditions in which ADA activity is altered. In 1972, it was documented that near to complete loss of ADA activity is the cause of severe combined immunodeficiency (SCID) in 25% of cases (Giblett et al., 1972). Overproduction of ADA produces hemolytic anemia (Fox, 1981). These syndromes demonstrate the importance of purine metabolism in man.

ADA activity has been reported to be ubiquitous in mammalian tissues; however, there is a great degree of variability in the amount of activity in various tissues. The thymus, spleen and GI tract have been reported to have the highest activities while much lower levels are found in kidney, lung and CNS tissues (Van der Weyden and Kelley, 1976). ADA activity has been reported to be present in all areas of the brain with the highest activities found in the hypothalamus and olfactory bulb while the lowest are in the hippocampus and striatum (Geiger and Nagy, 1986). There appears to be a difference in the cellular localization of ADA activity in the brain as astrocytes have ~10 fold higher ADA activity than neurons (Ceballos et al., 1994). Thus, the control of endogenous adenosine may be dependent upon the cellular and regional differences in ADA.

3.4.1 Purification and Molecular Biology

György and Röthler, who reported the production of ammonia and inosine from adenosine, initially discovered ADA activity in 1927. Soon after the specificity of ADA for adenosine and purine nucleosides was described (Schmidt, 1932). Early studies demonstrated multiple forms of ADA when ion exchange chromatography, starch gel electrophoresis or gel filtration were used to purify ADA. Three ADA isoforms have been described; ADA_{1A}, ADA_{1C} and ADA_2. ADA_{1C} is the enzyme that has best been characterized and is involved in the development of SCID. ADA_{1C} is a monomeric protein with a MW of 30-47 kDa (Van der Weyden and Kelley, 1976) while ADA_{1A} has a MW of 230-440 kDa and is formed by a dimer of ADA_{1C} complexed with an ADA binding protein. Although unclear at present, several roles have been proposed for the ADA binding protein including regulator of ADA_{1C} activity, ADA_{1C} cell surface receptor,

and anchor for ADA_{1C} in the extracellular space (Schrader et al., 1990). As there are no differences in the kinetics between ADA_{1A} and ADA_{1C}, it is unlikely that the ADA binding protein is involved directly in regulation of ADA activity. For this thesis, ADA_1 will reflect ADA_{1A} and ADA_{1C}. Unlike ADA_1, ADA_2 has been poorly characterized. It is a separate gene product that encodes for a protein that has a MW of 100-110 kDa. Although ADA_{1C} makes up >95% of the body's total ADA activity, ADA_2 is the predominant ADA in serum and plasma. Interestingly, ADA_2 levels are significantly increased in HIV-infected individuals, a phenomenon of unknown importance (Franco et al., 1998).

The understanding of ADA's role in physiological and pathophysiological situations was enhanced by the cloning of ADA_1 from human and rodent tissues in the mid 1980s (Adrian et al., 1984; Daddona et al., 1984; Hunt and Hoffee, 1983; Orkin et al., 1983; Wiginton et al., 1983). The cloned ADA_1 is ~ 41kDa made of 363 amino acids that has a high degree of sequence conservation between species (Franco et al., 1998). The gene has been localized to the long arm of chromosome 20 in humans, precisely at band 20q13.11. The ADA_1 promoter sequence lacks a conventional TATA or CAAT box, possibly indicating a 'housekeeping' role for ADA_1 in cellular activities (Valerio et al., 1985). The three-dimensional structure of ADA_1 was discovered in 1991 (Wilson and Quiocho, 1993; Wilson et al., 1991). This molecular information has helped ADA_1 and purine research and allowed the human gene therapy trials with recombinant human ADA for SCID patients.

3.4.2 Endogenous activity

As indicated in section 3.2.2.2, ADA_1 is a low, affinity high capacity enzyme. The K_m of ADA_1 for adenosine has been reported in the range of 17 – 100 μM in human and rat tissues with the majority of studies indicating 40 – 50 μM. The V_{max} of ADA_1 is 2-177 times higher than that of AK (Geiger and Nagy, 1990). Based on the kinetic properties, it has long been hypothesized that if AK and ADA_1 are present in one cell, AK will be more important in adenosine metabolism during physiological conditions because of its higher affinity for adenosine. Under pathophysiological conditions, ADA_1 would predominantly metabolize adenosine due to the substrate inhibition of AK and the adenosine concentrations that approach ADA_1's K_m for adenosine. This hypothesis has led to the development of ADA_1 inhibitors to maintain adenosine levels in conditions where endogenous adenosine levels are elevated.

In contrast to AK activity, ADA_1 activity does not appear to be highly regulated by endogenous factors. Despite extensive studies, few endogenous regulators have been reported (Arch and Newsholme, 1978). The optimum pH for the ADA_1 isoform is 7.0 – 7.4 while ADA_2 is approximately 5.5 (Van der Weyden and Kelley, 1976). ADA_1 is subject to product inhibition by inosine (K_i 60-153 μM) (Centelles et al., 1988; Fox and Kelley, 1978) as well as the purines hypoxanthine (K_i = 410 μM) and guanosine (K_i = 190 μM) (Centelles et al., 1988). The lack of endogenous regulation may also point to a role of ADA_1 as a "housekeeping" gene in mammalian cells.

3.4.3 Pharmacological Regulation

The pharmacological knowledge about ADA_1 has been widely investigated due not only to the over- and underproduction of ADA_1 in genetic conditions but also ADA's importance in the metabolism of chemotherapeutic antiparasitic, antitumor and antiviral agents (Agarwal et al., 1978; Cohen, 1985). Two of the most widely used ADA_1 inhibitors are deoxycoformycin (DCF; Pentostatin®) and erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA). DCF is a non-competitive, tight binding, almost irreversible transition state inhibitor of ADA_1, with a K_i in the picomolar range (Klohs and Kraker, 1992). Attempts have been made to develop stereoisomers of DCF, however alterations to the molecule have decreased ADA_1 inhibition (Montgomery et al., 1985; Schramm and Baker, 1985). EHNA is a competitive, weakly binding inhibitor of ADA_1 (Skolnick et al., 1978) which has a K_i in the low nanomolar range. Unlike DCF, analogues of EHNA have been developed which increase the affinity and alter the enzyme binding properties (Antonini et al., 1984; Barankiewicz et al., 1997; Harriman et al., 1992). DCF has been used clinically to treat hairy cell leukemia and has been hypothesized to be an effective treatment for other cancers (Klohs and Kraker, 1992). For CNS disorders, neither DCF nor EHNA are used clinically but *in vivo* studies have demonstrated possible clinical utility for strokes or seizures. DCF has been reported to decrease neuronal damage in rats during hypoxia/ischemia via increased adenosine production (Gidday et al., 1995; Lin and Phillis, 1992; Phillis and O'Regan, 1989). Similar results have been reported for EHNA with *in vitro* but not *in vivo* studies (Barankiewicz et al., 1997). Administration of DCF has also been reported to protect against bicuculline-methiodide induced seizures (Zhang et al., 1993). Unlike AK inhibitors, DCF or EHNA have not been reported to be

effective anti-inflammatory or anti-nociceptive agents *in vivo* (Geiger et al., 1997). The development of reversible, potent and selective ADA_1 inhibitors (Barankiewicz et al., 1997) that are BBB permeable (Mendelson et al., 1983) may have a future as ischemic or convulsant therapies.

3.5 Cytosolic 5'Nucleotidase

5'nucleotidase (EC 3.1.3.5) catalyzes the hydrolysis of a phosphate group from the ribose and deoxyribose moieties of nucleotides. The activity of this enzyme was first documented approximately 65 years ago (Reis, 1934). Three isoforms of this enzyme have been cloned; c-N-I and c-N-II (cytosolic 5'nucleotidase I and II; see section 3.4.1 and 3.4.2) and e-N (extracellular 5'nucleotidase; see section 3.1.2.1). In addition, there have been numerous reports of soluble cytosolic forms of e-N, regarded as e-N-s, which is found intracellularly. Each of these isoforms predominantly metabolizes nucleoside 5'monophosphates. In addition, these enzymes are also capable of metabolizing nucleotides and complex nucleotides such as NAD with varying affinities and capacities. The cytosolic 5'NTases, c-N-I and c-N-II, are thought to be important for the metabolism of intracellular AMP and IMP, respectively.

3.5.1 5'AMP-preferring 5'nucleotidase

5'AMP-preferring 5'nucleotidase (c-N-I; EC 3.1.3.5) dephosphorylates AMP to adenosine and free phosphate. c-N-I preferentially metabolizes AMP over IMP or GMP, with K_m values for AMP between 2-8 mM. Although c-N-I is conventionally regarded as the most important enzyme in production of adenosine, little is known about the enzyme,

its biochemical properties or its distribution. Until recently, c-N-I has only been reported in vertebrate cardiac tissue. It is hypothesized that when ATP levels are depleted, adenosine levels rise intracellularly through increased metabolism of AMP via c-N-I, which can then be released from cells. There is evidence to support increased extracellular adenosine during ATP-depleted conditions but until recently the role of c-N-I was elusive.

3.5.1.1 Molecular Biology and Purification

A 5'NTase that preferentially metabolized AMP over IMP was originally described in pigeon hearts (Gibson and Drummond, 1972). It was purified from pigeon heart by Newby in 1988 (Newby, 1988) and subsequently from rabbit, canine, rodent and human hearts. The native protein, when purified using $(NH_4)_2SO_4$ precipitation, phosphocellulose chromatography and high performance gel filtration, is approximately 150 kDa (Skladanowski and Newby, 1990). When the isolated protein is run on SDS-PAGE, 40 kDa bands are obtained, indicating the native protein is a tetramer. In 1999, c-N-I was cloned from pigeon heart (Sala-Newby et al., 1999) and recently cloned from human heart (Hunsucker et al., 2000). The cloned e-N-I had an open reading frame that codes for 358 AAs yielding a protein of 39.5 kDa. The cloned c-N-I has no sequence homology to the previously published sequences of e-N or c-N-II. Northern blot analysis of pigeon and human tissues demonstrated high mRNA expression of c-N-I in the oxidative cardiac and skeletal muscle tissues, and lower levels in total brain. No mRNA was detected in kidney, blood vessel or liver samples. The cloning of c-N-I should enable further insight into the role of c-N-I in adenosine regulation.

3.5.1.2 Endogenous Activity

Based on the lack of molecular similarity, it is not surprising that the kinetic properties of c-N-I are different than e-N or c-N-II. ADP (at concentrations >10 μM) is a potent activator of c-N-I activity by increasing the affinity for AMP and increasing the V_{max}. The increased affinity for AMP is very important as AMP can be metabolized at concentrations less than 1 mM. ATP has no significant effect on c-N-I activity. Free Mg^{2+} is an absolute requirement for c-N-I activity. Magnesium is capable of increasing e-N-I activity when Mg^{2+} levels are >1 mM and ADP level are <25 μM (Yamazaki et al., 1991) but when Mg^{2+} levels are >10 mM it is inhibitory (Skladanowski and Newby, 1990). Although consensus does not exist, the majority of studies indicate that the optimum pH is between 6.8-7.8 (Darvish and Metting, 1993; Plagemann et al., 1988; Skladanowski and Newby, 1990; Yamazaki et al., 1991). Below pH of 6.8, there is a total loss of activity when the isolated protein is assayed.

Identification of the role of c-N-I in endogenous adenosine production is hampered by the fact that it has only been isolated from cardiac tissue. The lack of enzyme purification from tissues other than the heart does not support the conventional view that c-N-I is responsible for intracellular adenosine production during ATP-depleting conditions. However, the transfection of cells with c-N-I directly demonstrates adenosine release mediated by c-N-I during ATP-depletion (Sala-Newby et al., 2000). The hypothesis that c-N-I is important for hypoxic increases in adenosine production are supported by the increases in AMP, ADP and Mg^{2+} during ATP-depleting conditions. This should increase the activity of c-N-I based on the properties of the isolated enzyme. As inhibition of e-N does not decrease, while nucleoside transport inhibitors will

decrease, extracellular adenosine release during hypoxic-like conditions, evidence supports an intracellular mechanism of adenosine production. The lack of selective, cell permeable inhibitors of c-N-I limits the conclusions that can be made regarding the role of c-N-I in adenosine production. In addition, the lack of knowledge of c-N-I in non-cardiac tissues raises further questions about the role of c-N-I. The recent cloning of c-N-I should enable researchers to gain a better understanding of its role in CNS adenosine regulation.

3.5.1.3 Pharmacological Regulation

As stated above, there are no available potent, selective and cell permeable c-N-I inhibitors. Researchers at GlaxoWellcome have reported an inhibitor based on 5-ethynyl-2',3'-dideoxyuridine (Garvey and Prus, 1999) but this compound has not been made available to basic researchers (Garvey, personal communication). Attempts have been made to use high concentrations of dideoxythymdine or pentoxifylline (Bolling et al., 1992) to inhibit c-N-I but the selectivity of these compounds is a major concern. 5'-deoxy-5'-isobutylthioadenosine (IBTA) has been used to inhibit c-N-I but it is also an inhibitor of c-N-II (Skladanowski and Newby, 1990). To gain a better understanding of c-N-I's role in adenosine production, pharmacological inhibitors need to be developed.

3.5.2 5'IMP-preferring 5'nucleotidase

IMP-preferring 5'NTase (c-N-II) metabolizes IMP or GMP preferentially to AMP, to produce inosine or guanosine. The phosphate removed from the IMP or GMP can either be released as free phosphate or can be added to a nucleoside such as inosine

or guanosine. The definition of c-N-II as a 5'nucleotidase/phosphotransferase is based on this property. Although c-N-II preferentially metabolizes IMP, it is capable of metabolizing AMP at low millimolar concentrations, levels that are similar to c-N-I. Thus, there is a potential role for c-N-II in the production of adenosine.

3.5.2.1 Molecular Biology and Purification

c-N-II has been purified from a number of species using gel filtration. The native enzyme has an apparent molecular mass of 200-265 kDa (Itoh et al., 1992; Itoh and Yamada, 1990; Pinto et al., 1987). Similar to c-N-I, SDS-PAGE produces subunits of 51-70 kDa, thereby predicting a homo-tetramer formation. Enzyme activity has been reported to be ubiquitous, however with varying levels of activity between tissues. The enzyme has the highest level in tissues having high levels of RNA and DNA turnover. The highest levels are seen in the testis, spleen and lymphocytes while skeletal muscle and erythrocytes have very low levels. In the brain, c-N-II has been demonstrated (Marques et al., 1998; Torrecilla et al., 2001), however there have been no reports of regional differences in activity. c-N-II has been cloned from human, chicken and bovine tissues (Allegrini et al., 1997; Oka et al., 1994). The human clone encodes for 561 AAs, which produces a protein of 65 kDa. There is a high degree of sequence similarity between the three clones. Northern blot analysis of human tissues supports the activity studies that indicate ubiquitous but varying levels of expression (Allegrini et al., 1997). The cloning of c-N-II has enabled researchers the opportunity to better understand c-N-II's role in purine homeostasis.

3.5.2.2 Endogenous Activity

Under optimal conditions, c-N-II has apparent K_m values of 0.1-0.6 and 1-15 mM for IMP and AMP, respectively (Zimmermann, 1992). Unlike e-N or c-N-I, c-N-II activity is increased by millimolar concentrations of ATP and ADP, by increasing the affinity for its substrates and increasing the enzyme's maximal capacity. This includes increased affinity of c-N-II for AMP. Thus, under the ideal conditions, c-N-II may be involved in adenosine production. As with c-N-I, Mg^{2+} is required for enzyme activity. In addition to ATP, ADP and Mg^{2+}, c-N-II activity is determined by a number of endogenous regulators. When free phosphate levels increase above 5 mM, the affinity of c-N-II for IMP and AMP decreases. Similar to AK, free phosphate and ATP have opposing effects on the enzyme activity, indicating a possible regulatory mechanism for determining pathways of purine metabolism. The glycolytic intermediate 2,3-BPG (Bontemps et al., 1989; Pesi et al., 1996; Tozzi et al., 1991) and diadenosine polyphosphates (Marques et al., 1998; Pinto et al., 1986) are potent stimulators of c-N-II activity and may have additive effects with ATP or ADP. The effect of pH on c-N-II is seen on two levels. The pH spectrum of c-N-II nucleotidase activity is maximal between 6-7 while the phosphotransferase activity of c-N-II is maximal at 7.4. The phosphotransferase activity of c-N-II was initially reported in 1982, while attempting to determine the apparent inhibitory properties of inosine on c-N-II activity (Worku and Newby, 1982). It has since been widely documented that c-N-II has phosphotransferase activity (Baiocchi et al., 1996; Pesi et al., 1994; Tozzi et al., 1991), which is the predominant activity during physiological conditions (Pesi et al., 1994). A

phosphorylated c-N-II has been isolated that is capable of either releasing free phosphate or phosphorylating a phosphate group to inosine, guanosine and poorly to adenosine.

At present evidence does not support a major role of c-N-II in the regulation of adenosine levels based on *in vitro* experiments. However, c-N-II can not be overlooked as it can metabolize AMP and adenosine, it is subject to endogenous regulation and the role of c-N-I has been poorly characterized.

3.5.2.3 Pharmacological Regulation

Similar to c-N-I, there are no potent and selective inhibitors for c-N-II currently available. IBTA has been reported to be a selective inhibitor of c-N-II but it also inhibits c-N-I (Meghji et al., 1993; Skladanowski et al., 1989). Through the use of c-N-I and c-N-II transfected cells (Sala-Newby et al., 2000), it has been reported that c-N-II is not involved in adenosine production during certain paradigms. However, the role of endogenous c-N-II *in vivo* will not be known until selective and potent inhibitors of this enzyme are available.

3.6 Other Intracellular Metabolic Pathways

Although the majority of research indicates that AK, ADA and 5'nucleotidases are the most important regulators of adenosine levels in the CNS, a number of other enzymatic pathways may also be important in the overall regulation of adenosine levels. S-adenosyl homocysteine hydrolase (EC 3.3.1.1, SAHH) is an enzyme capable of the reversible cleavage of s-adenosyl homocysteine into adenosine and homocysteine. Although SAHH appears to be important in the regulation of adenosine levels in the

heart, liver and kidneys, SAHH has been reported to have a limited role in the regulation of CNS adenosine levels. Despite the wide distribution of SAHH in the CNS, low intracellular homocysteine levels limit metabolism of adenosine via SAHH. Adenosine does not appear to be produced from s-adenosylhomocysteine during conditions that stimulate adenosine production. Although little evidence exists to support a major role of SAHH in the regulation of CNS adenosine, it could potentially be important in adenosine metabolism when CNS homocysteine levels are elevated, such as during alcoholism (Bleich et al., 2000a; Bleich et al., 2000b),vitamin B_{12} deficiency (Penix, 1998) or patients suffering from homocystinuria or at high risk of vascular disease and stroke (Lipton et al., 1997).

AMP deaminase (AMPDA, EC 3.5.4.6; a.k.a. adenylate deaminase) catalyzes the deamination of AMP producing IMP and ammonia. Although not directly involved in adenosine production, AMPDA is important in determining the levels of 5'AMP that are present in cells. When c-N-I and AMPDA are present in the same cell, the kinetic properties and endogenous regulators of each enzyme will determine whether adenosine or IMP will be predominantly formed from AMP. It has been reported that inhibition of AMPDA can lead to enhanced cellular adenine nucleotide levels and release of adenosine during hypoxic situations (Bookser et al., 2000a; Bookser et al., 2000b). Therefore, AMPDA is an important enzyme in the overall determination of adenosine levels, especially when AMP levels are elevated.

Purine nucleoside phosphorylase (PNP, EC) reversibly catalyzes the phosphorolysis of inosine to its free base hypoxanthine and ribose-1-phosphate (Morris and Montgomery, 1998). The importance of this enzyme to adenosine levels is that it

metabolizes inosine, which acts both as an inhibitor of ADA_1 and will be a competitive inhibitor of equilibrative adenosine transport. As inosine is predominantly metabolized by PNP, PNP activity allows production of hypoxanthine, which can be salvaged into IMP via HGPRT. As PNP has been reported predominantly in astrocytes (Castellano et al., 1990; Van Reempts et al., 1988), this may indicate a role for the regulation of adenosine levels in astrocytic cells. Although SAHH, AMPDA and PNP have not been shown to directly regulate CNS adenosine levels, it is probable that these enzymes are important in the global regulation of brain adenosine.

3.7 Adenosine Transport Across the Blood-Brain Barrier

The function of the BBB is to restrict the movement of compounds into and out of the brain parenchyma. This is maintained by tight cellular junctions between the endothelial cells of the BBB and the selective transporter systems these cells express. During physiological conditions, the integrity of the BBB maintains control over the flux of metabolites, ions and other compounds from the blood into the brain interstitial spaces and vice versa. However, during pathophysiological conditions such as ischemia and reperfusion, the permeability of the BBB has been reported to increase dramatically (Abbott, 2000; del Zoppo and Hallenbeck, 2000). Although not widely investigated, the BBB may play a role in regulating interstitial adenosine levels. This may be important in the rapid decrease in extracellular adenosine levels during reperfusion as there are high levels of adenosine in the parenchyma, the BBB integrity is decreased and blood flow is re-initiated. The BBB may also play a role in regulating basal adenosine levels as the

nucleoside transport inhibitor DPR, which does not cross the BBB, has been hypothesized to increase interstitial adenosine levels.

4.0 Investigating the Regulation of Extracellular Adenosine

All of the enzymes and transporters described above detail the levels of complexity in regulation of adenosine levels. In order to gain a better understanding of how each of these pathways is involved, *in vitro* assays are often used. These involve basic monocultures and mixed cell cultures as well as *in situ* tissue slice models. These models allow, with the use of selective enzyme and transport inhibitors, the dissection of the role of each pathway in regulating extracellular adenosine levels. Two basic types of studies are conducted; influx and efflux studies. As the source and mechanism of extracellular adenosine in the CNS is poorly characterized, these studies using cell culture models provide an initial starting point for understanding how adenosine is produced in physiological and pathophysiological conditions in the brain.

4.1 Adenosine uptake

A precise definition of adenosine "uptake" is the net result of inward transport across the plasma membrane followed by intracellular metabolism. In contrast, transport or influx studies represent the influx of substrate in its unmetabolized form across the plasma membrane by a specific transport or facilitated mechanism. This distinction has not always been recognized, thus, many publications purport to characterize adenosine transport but in fact describe adenosine uptake.

With respect to adenosine, distinguishing between transport and uptake is difficult because of the high affinity of AK. For cells that express equilibrative transporters as

their only nuceloside transporter, three techniques have been used to study transport *per se*. Initial rates of transport are often used to measure [^{3}H]adenosine influx at short time intervals ($t<15s$). This method limits the role of intracellular metabolism and, thus, the kinetics more closely represent those of the transporter for adenosine. The limitation of this technique is intracellular metabolism may still be extensive in short time periods and the signal to noise ratio may be low if transporter expression or activity is low. The second method involves [^{3}H]adenosine uptake in the presence of inhibitors of AK and ADA_1 to limit intracellular metabolism. The concern with these studies is that AK and ADA_1 inhibitors may directly interact with nucleoside transporters. The final method involves the use of poorly metabolized nucleoside transporter permeants such as [^{3}H]uridine or [^{3}H]formycin B (FB). This typically removes the contribution of intracellular metabolism but it does not address the questions regarding the kinetics of adenosine transport *per se*.

Uptake studies have their uses, for example to explore intracellular metabolism of adenosine. These studies can give information into whether transport or intracellular metabolism is the rate-limiting factor and whether AK or ADA_1 is more important in intracellular metabolism of adenosine in specific cells or during specific cell treatments.

4.2 Adenosine Release

Although not as precisely defined, release is the opposite of uptake and, thus, encompasses both adenosine formation and transport across plasma membranes. As discussed previously, adenosine release can be by two distinct pathways, release of

adenine nucleotides that are subsequently metabolized to adenosine extracellularly or intracellular formation of adenosine followed by its transporter-mediated cellular efflux.

Adenosine derived from ATP or other nucleotides released into the extracellular environment has been reported using numerous protocols including hypo-osmotic shock (Roman et al., 1999; Wang et al., 1996), physical stimulation of cells, glutamate receptor activation (Queiroz et al., 1997), and increased intracellular cAMP. Cell culture models may document the release of adenine nucleotides but this does not demonstrate the site of adenosine production as not all cells have the ability to metabolize extracellular nucleotides to adenosine.

Adenosine release has been investigated using three principle methods. The first involves utilization of techniques used for influx studies (Fernandez-Rivera-Rio and Gonzalez-Garcia, 1985). Cells can be loaded with [^{3}H]adenosine in the presence of AK and ADA_1 inhibitors. The cells are then washed and placed into buffer not containing tritium in order to initiate zero-trans efflux. The benefit of these studies is that the kinetics of adenosine release via the nucleoside transporter is examined. However, similar to influx studies, these studies are limited by potential interaction of the enzyme inhibitors with the transporters. The poorly metabolized nucleoside [^{3}H]FB can be used for release studies but this provides limited information about adenosine release (Gu et al., 1995). The second method involves receptor-mediated or activity-mediated adenosine release. Activation of NMDA (Craig et al., 1994; Craig and White, 1993; White, 1996), non-NMDA (Craig and White, 1993; White, 1996), opioid (Phillis et al., 1980), and serotonin receptors has been reported to stimulate adenosine release from *in vitro* neuron and astrocyte models. In addition, depolarization of cells with electrical (Lloyd et al.,

1993; Maire et al., 1984) or high K^+ stimulation (Philibert and Dutton, 1989) has been shown to elevate extracellular adenosine levels. The mechanism by which adenosine levels are elevated by receptor- or activity-mediated effects has been poorly characterized. The final protocol involves stimulation of adenosine release by ATP depletion. Due to the proposed correlation between decreased cellular ATP levels during cerebral or cardiac ischemia and increased adenosine levels, the use of ischemic-like conditions in *in vitro* assays has been widely used. These studies often involve loading cells with [^{3}H]adenosine or [^{3}H]adenine to produce an intracellular pool of radiolabeled adenine nucleotides. Both of these techniques produce >90 % of total intracellular tritium present as adenine nucleotide. It has been well documented that the use of tritium loaded cells to measure [^{3}H]adenosine and [^{3}H]purine release by TLC and scintillation spectroscopy produces similar results to non-radiolabeled experiments assessed by HPLC. Loading of cells with [^{3}H]adenine is preferred to [^{3}H]adenosine in order to avoid any autoregulatory effects of adenosine receptor activation. However, this relies on [^{3}H]adenine influx through nucleobase transporters, which are poorly characterized. The conditions that are often used to deplete cellular ATP are hypoxia-hypoglycemia, or inhibition of glycolysis and/or oxidative phosphorylation. As hypoxic-ischemic conditions such as a stroke are a clinical condition of interest for elevation of endogenous adenosine levels, hypoxic cell culture models have been used to investigate regulation of adenosine levels *in vitro*. In buffer lacking glucose and depleted of O_2 (bubbled with N_2), adenosine release has been reported from a number of cardiac cell culture systems (Decking et al., 1997a; He et al., 1992; Meghji et al., 1985; Newby et al., 1983) and brain slice models (Doolette, 1997; Fowler, 1993; Fredholm et al., 1984; Wallman-Johansson

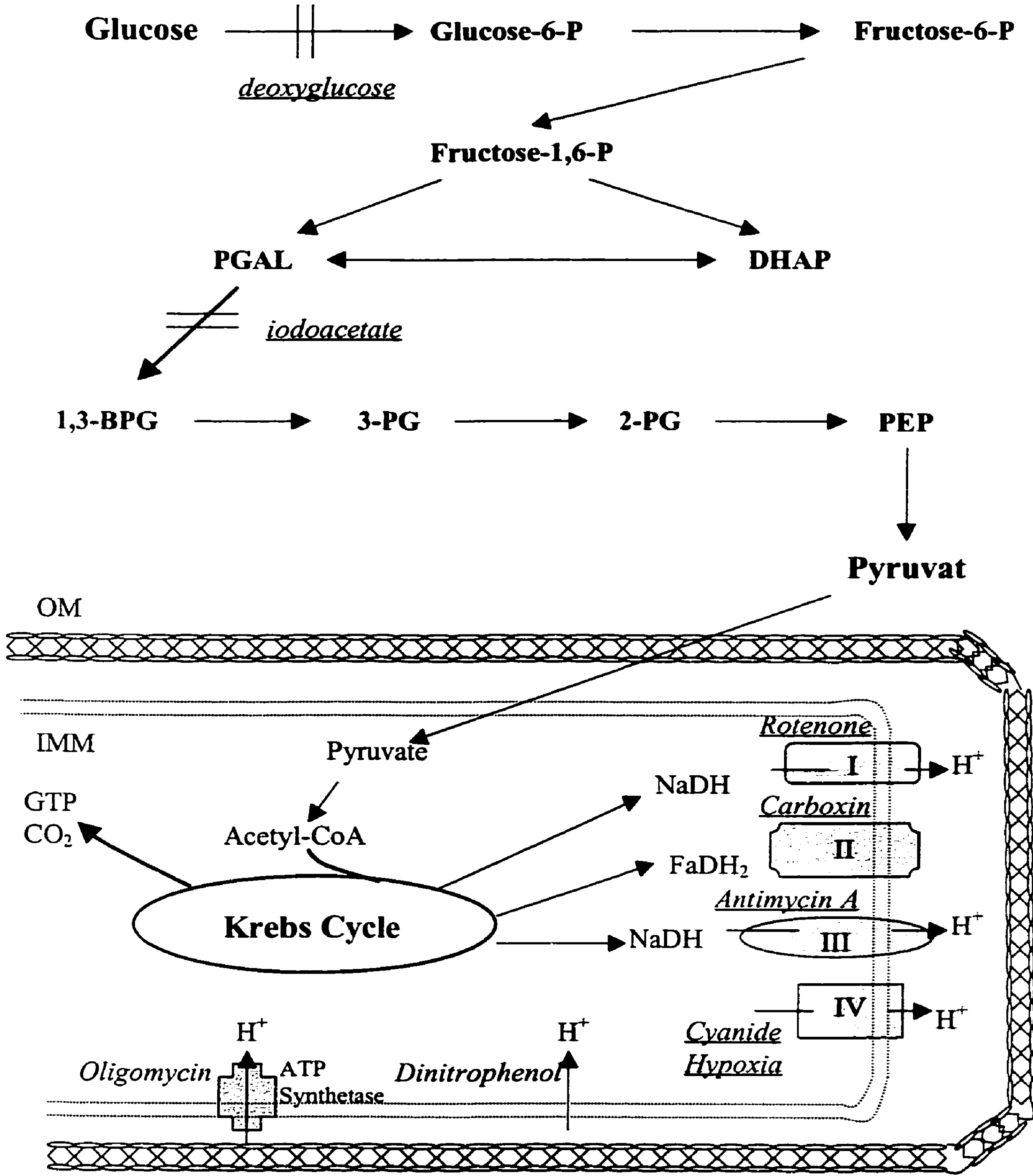
and Fredholm, 1994). Adenosine release has also been documented from hypoxia-treated neuronal (Lobner and Choi, 1994; Lynch et al., 1998; Meghji et al., 1989) and astrocytic cultures (Ciccarelli et al., 1999; Meghji et al., 1989), although the mechanism by which astrocytes release adenosine remains in question. The problem with hypoxia-treated cells with regards to extracellular adenosine release *in vitro* is the length of time required for ATP levels to decrease (>1hr), which, when compared to the short half-life of adenosine, makes it hard to know minute-to-minute changes in extracellular adenosine levels. Thus, researchers have often used conditions that decrease cellular ATP levels more rapidly. This is done with inhibitors of glycolysis or oxidative phosphorylation. As not every cell type responds to each stimulus similarly, there is not a gold standard way of depleting ATP.

Cells that rely on glycolysis for energy charge are susceptible to glycolytic inhibitors. Iodoacetate (IAA) inhibits glyceraldehyde-3-phosphate dehydrogenase, a critical step in the glycolytic pathway (Sabri and Ochs, 1971) (see fig 4). Deoxyglucose (DOG) inhibits glycolysis by competing with glucose metabolism by hexokinase. Although capable of decreasing ATP, care must be taken when these compounds are used for investigating [^{3}H]purine release as IAA and DOG decrease cellular levels of 1,3-BPG (Zoref-Shani et al., 1988) and P_i (Chen and Gueron, 1996; Chen et al., 1996), respectively, which are regulators of intracellular purine metabolism (see table 2).

Inhibition of oxidative phosphorylation can be done with a number of compounds that interact with the electron transport chain (see fig 4). Dinitrophenol (DNP) uncouples the electron transport chain by removing the H^+ gradient across the inner mitochondrial membrane (IMM). Rotenone decreases the proton gradient by decreasing the NADH

hydrogenase activity in complex I of the electron transport chain. Carboxin blocks complex II by inhibiting $FADH_2$ reduction and stops electron transport to complex III. Antimycin A inhibits complex III and inhibits cytochrome C acceptance of electrons. Cyanide and azide bind to reduced cytochrome C at complex IV, thereby inhibiting electron transfer to oxygen. Cyanide treatment has been widely used as "chemical hypoxia" (Goldberg and Choi, 1993; Goldberg et al., 1987) as its effects are believed to be the closest to hypoxia based on the site of action in the electron transport chain and its interactions with heme proteins (Bunn and Poyton, 1996; Chandel and Schumacker, 2000; Semenza, 1999). Although each of these mitochondrial toxins should deplete cellular ATP levels and increase purine release, differences have been reported in how cells respond to the different inhibitors with regards to ATP depletion and adenosine release (Doolette, 1997).

Figure 4: Mechanisms of ATP depletion



IMM-inner mitochondrial membrane; OMM-outer mitochondrial membrane; BPG- bisphosphoglycerate; PG- phosphoglycerate; PEP- phosphoenolpyruvate

5.0 Hypotheses

The purpose of this thesis work was to gain a better understanding of how extracellular adenosine levels are regulated by different nucleoside transporters and metabolic enzymes *in vitro*.

1. Recent *in vivo* studies have demonstrated that i.p. administration of NBMPR during cerebral ischemia is able to decrease neuronal damage (Parkinson, unpiblished data). As NBMPR poorly crosses the BBB (Anderson et al., 1996a), the site of action would appear to be on the lumenal side of the BBB. In addition, i.p. administration of DPR, which does not cross the BBB (Sollevi et al., 1983), increases adenosine levels in rat brain during hypoxia/reperfusion (Phillis et al., 1989). Thus, I investigated the effects of nucleoside transport inhibition on adenosine permeation of a physiological BBB model.

   Hypothesis: Administration of DPR to the lumenal compartment of a dynamic *in vitro* blood brain barrier model will inhibit bidirectional adenosine permeation of this system.

2. Activation of G-protein coupled serotonin, glutamate and norepinephrine receptors has been demonstrated to have an autoregulatory effect on the levels of serotonin, glutamate and norepinephrine, respectively. Activation of adenosine receptors has been reported to increase adenosine uptake in bovine adrenal chromaffin cells (Delicado et al., 1990). I was interested in determining if similar effects are seen with adenosine release and whether transporters or metabolic enzymes may be involved.

Hypothesis: Activation of adenosine receptors in DDT_1 MF-2 hamster smooth muscle cells during ATP-depleting conditions will decrease the total release of adenosine.

3. Adenosine kinase inhibitors such as ITU or NH_2dAdo are adenosine analogues that competitively inhibit AK activity. It has been previously reported that these compounds are potent inhibitors of isolated AK while AK inhibition in whole cells is less conclusive. Based on the structural similarity between the AK inhibitors and adenosine, I investigated whether the expression of different nucleoside transporters would affect the ability of ITU or NH_2dAdo to inhibit AK in whole cell preparations.

   Hypothesis: Expression of rENT1 nucleoside transporters but not rCNT2 will produce inhibition of adenosine uptake by adenosine kinase inhibitors in rat C6 glioma cells.

4. The release of adenosine from cells expressing the *es* nucleoside transporter has been demonstrated in various cell types during ATP-depleting conditions. The role of the *ei* transporter in adenosine release has not been demonstrated during similar conditions. The rat C6 glioma cell line possesses predominantly *ei* nucleoside transport and I was interested in the mechanisms of adenosine release from these cells.

   Hypothesis: ATP-depletion with sodium cyanide and iodoacetate will induce the release of adenosine from rat C6 glioma cells through *ei* nucleoside transporters.

5. From the previous study, the question arose: was the lack of adenosine release due to the cell type used (i.e. tumor cell line, incapable of adenine nucleotide release) or do "normal" cells respond in a similar manner? I was interested in determining which

purines are released from primary neurons and astrocytes exposed to ATP-depleting conditions.

Hypothesis: Hypoxia, inhibition of glycolysis or inhibition of oxidative phosphorylation will produce the release of adenosine from rat primary cortical neurons and the release of inosine and hypoxanthine from rat primary cortical astrocytes.

These projects will be addressed in the five subsequent chapters with a description of the background/rationale, materials/methods, results and relevant discussion for each of the projects. Overall conclusions of the thesis will be discussed in relation to the purinergic field in Chapter 7.

Chapter 2: Adenosine permeation of a dynamic *in vitro* blood-brain barrier inhibited by dipyridamole.

Published in Brain Research (Sinclair et al., 2001)

Abstract

Adenosine is an inhibitory neuromodulator in the central nervous system and has been reported to have neuroprotective properties. Using a dynamic *in vitro* blood-brain barrier, we tested the hypothesis that inhibition of adenosine transporters on the lumenal side of the blood-brain barrier may decrease the loss of adenosine from the brain. Our results indicate that lumenal administration of dipyridamole, a nucleoside transport inhibitor, can inhibit adenosine permeation from the extracapillary space into the lumen.

Adenosine is an endogenous neuromodulator that is involved in the regulation of numerous physiological and pathophysiological CNS events via the activation of membrane-bound adenosine receptors (Von Lubitz, 1999). The regulation of extracellular adenosine levels in the brain is complex, involving multiple adenosine-generating and adenosine-metabolizing enzymes as well as nucleoside transporters, which are responsible for flux of adenosine across cellular membranes (Geiger et al., 1997). Under experimental hypoxia/ischemia in rats, adenosine levels in brain rise rapidly then quickly decrease during reperfusion (Parkinson et al., 2000). Peripheral administration of dipyridamole (DPR), a nucleoside transport inhibitor, slowed the decrease in brain adenosine levels during reperfusion (Phillis et al., 1989). As DPR does not appear to cross the blood-brain barrier (BBB) (Sollevi et al., 1983), its ability to increase reperfusion adenosine levels may be mediated by inhibition of adenosine permeation of the BBB (Phillis et al., 1989). In addition, DPR, when prescribed prophylactically for stroke prevention, has been proposed to produce neuroprotection by elevation of brain adenosine levels via effects at the BBB (Picano and Abbracchio, 1998). In order to test the hypothesis that peripheral administration of DPR may inhibit adenosine movement from the brain into the blood, we have used a dynamic *in vitro* BBB (DIV-BBB) model.

The DIV-BBB was cultured with bovine aortic endothelial cells (BAEC) (obtained from Dr. H. Sage at the University of Washington) on the lumenal surface and rat C6 glioma cells (from ATCC in Rockville, MD) in the extracapillary space (ECS) as previously described (Stanness et al., 1996; Stanness et al., 1997). Cells were grown in a hollow fiber culturing system using CELLMAX Quad system and cartridge, which were purchased from Spectrum Laboratories (Rancho Dominguez, CA). Dulbecco's modified

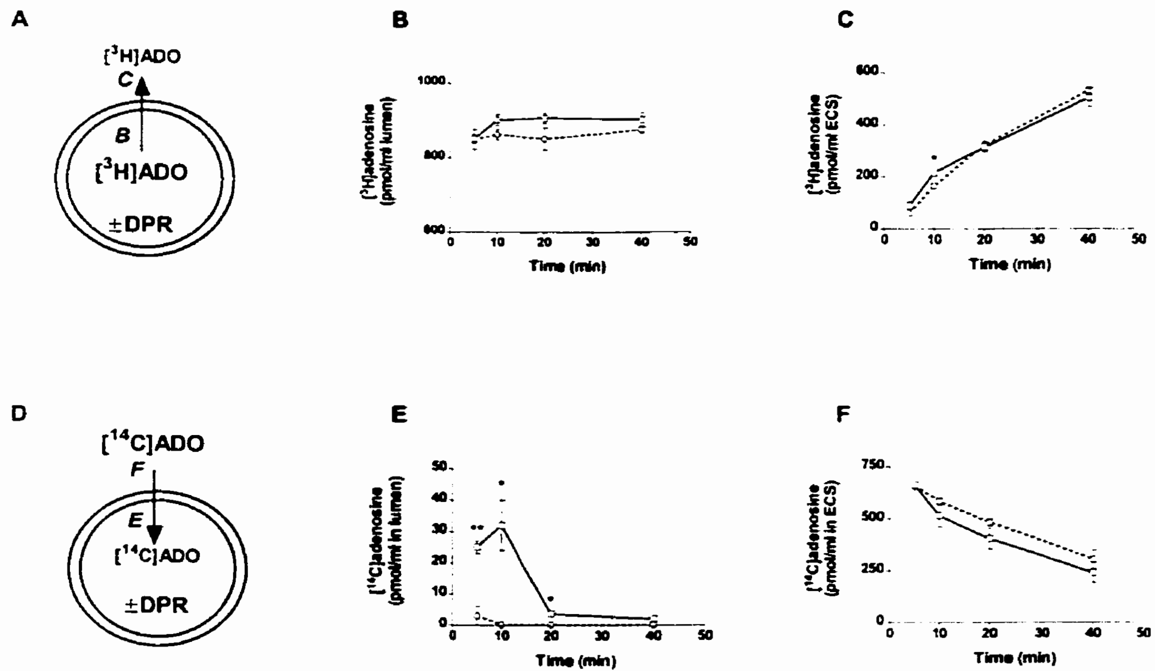
Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Life Technologies (Burlington, Ontario). Development of barrier properties in the DIV-BBB was assessed by measuring glucose consumption of the co-culture system and permeation of [^{3}H]polyethylene glycol (PEG) and [^{14}C]sucrose (Stanness et al., 1996; Stanness et al., 1997). Consumption of glucose from the culture media was assessed during the first month of culture to ensure BAEC and C6 cell growth and the results (data not shown) were similar to those previously reported (Stanness et al., 1996; Stanness et al., 1997). Barrier function of the DIV-BBB was investigated by administration of a bolus of [^{3}H]PEG and [^{14}C]sucrose into the lumen then media samples were removed from the lumen and ECS over 40 min. These experiments were performed in the absence of cells, in the presence of BAEC alone or co-culture of BAEC and C6 cells. Permeability was calculated using the formula

$$p = \frac{k * [x]_{ECS,final} - [x]_{ECS,\, initial}}{\int_{0\rightarrow t} [x]_{lumen} - \int_{0\rightarrow t} [x]_{ECS}}$$

where k is a constant for normalizing efflux rate for a lumenal surface area of 70 cm^2 and a lumen/ECS volume ratio of 50; $[x]_{lumen}$ and $[x]_{ECS}$ are the lumen and ECS concentrations of the radiolabeled permeant (Stanness et al., 1996; Stanness et al., 1997). [^{14}C]Sucrose permeation of the DIV-BBB was similar to that previously reported (Stanness et al., 1996; Stanness et al., 1997). In addition, [^{3}H]PEG has similar permeation properties as [^{14}C]sucrose. In the absence of cells or in the presence of BAEC alone, there was no barrier to the movement of either [^{3}H]PEG or [^{14}C]sucrose. In the presence of BAEC and C6 cells, the permeability of [^{3}H]PEG and [^{14}C]sucrose ranged from 1×10^{-6} – 1×10^{-5} cm/sec over a 40 min period (n=3).

Adenosine permeation of the DIV-BBB was investigated by addition of 10 μM [^{3}H]adenosine to the lumen (fig. 5a) and 10 μM [^{14}C]adenosine to the ECS (fig. 5d). All radiochemicals were purchased from NEN Life Sciences (Mississauga, Ontario). Adenosine was purchased from the Sigma Chemical Co. (St. Louis, MO). Samples were taken from the lumenal and ECS compartments over 40 min. We investigated [^{14}C] and [^{3}H]adenosine permeation of the DIV-BBB in the presence or absence of the nucleoside transport inhibitor dipyridamole (DPR) (10 μM) administered to the lumenal compartment. DPR was purchased from Research Biochemicals International (Natlick, MA). The amount of [^{3}H]adenosine in the lumen remained constant over time (fig 5b). Although endothelial cells are able to take up adenosine, loss of [^{3}H]adenosine was not detected, likely due to the large extracellular volume (70 ml) circulating through the lumen. The amount of [^{3}H]adenosine in the lumen was unaffected by the addition of DPR to the lumen (fig. 5b). There was a steady increase in the transfer of [^{3}H]adenosine from the lumen to the ECS (fig 5c) and this movement was only modestly affected by lumenal DPR administration. In contrast, permeation of [^{14}C]adenosine from the ECS into the lumen was low and was further inhibited by lumenal administration of DPR (fig 5e). Decreases in ECS [^{14}C]adenosine over time were reduced by lumenal administration of DPR; however, this effect of DPR did not reach statistical significance (fig 5f). Depletion of ECS [^{14}C]adenosine is likely due to a combination of uptake by C6 cells and efflux across the *in vitro* BBB. The small amounts of [^{14}C]adenosine measured in the lumen,

Figure 5



Effect of lumenal administration of dipyridamole on [^{3}H]adenosine or [^{14}C]adenosine permeation of the DIV-BBB. 10 μM [^{3}H]adenosine was placed into the lumen and 10 μM [^{14}C]adenosine was placed into the extracapillary space (ECS) at time zero and the lumen and ECS were sampled for radioactivity as indicated schematically in panels A and D. Experiments were run in the presence (circles) or absence (squares) of dipyridamole (10 μM) in the lumen of the capillary vessels. Samples were taken from the lumen (B, E) and the ECS (C, F) at 5, 10, 20 and 40 minutes after the addition of [^{3}H]adenosine and [^{14}C]adenosine. Statistical analyses using student's unpaired t-tests were performed using Graph Pad Prism. Data are expressed as mean ± SEM. (*$p<0.05$, **$p<0.01$; n=3)

even in the absence of DPR, result from its dilution by the constant flow of media through the lumen.

The permeation of the *in vitro* BBB by DPR was evaluated using fluorescence detection with excitation and emission wavelengths of 355 nm and 535 nm, respectively. DPR was perfused through the system for up to 2 hrs then samples from the lumen and ECS compartments were analyzed and compared to a standard curve. We found that DPR in the ECS was undetectable (n = 3) to 0.7 μM (n = 1) whereas concentrations of 5 – 10 μM were measured in the lumen (n = 4).

This study demonstrates that lumenal administration of DPR is able to inhibit adenosine permeation of a DIV-BBB system from the ECS to the lumen but not from the lumen to the ECS. As DPR appears to permeate the DIV-BBB poorly, the site of action is likely on the lumenal side of the DIV-BBB. The mechanism involved in the asymmetrical inhibition of adenosine permeation of the DIV-BBB by DPR administration is unknown at present. We propose that different nucleoside transporters are present on the lumenal and ablumenal surfaces of endothelial cells in the DIV-BBB. Four nucleoside transporter subtypes have been characterized and cloned. Two equilibrative (Na^+-independent) transporters, ENT1 and ENT2, accept adenosine and other nucleosides as permeants. DPR inhibits both ENT1 and ENT2 with inhibition constants ranging from 100 nM to 5 μM (Griffith and Jarvis, 1996). Two concentrative (Na^+-dependent) transporters, which function as symporters, have been described and adenosine is a permeant for CNT2, but not CNT1. Neither of the concentrative transporters is inhibited by DPR at concentrations less than 100 μM. Using conventional cell culture techniques, BAEC possess ENT1 as the predominant nucleoside transport mechanism (Sinclair and

Parkinson, unpublished data). Our results suggest that differentiation of BAEC in the DIV-BBB co-culture system may induce the expression of another nucleoside transport mechanism, one that is unaffected by DPR administration. Induced expression of CNT2 in the BAEC may explain the asymmetrical effects of DPR. There have been few reports of the nucleoside transporters present in the BBB *in vivo*. Rat BBB has a specific transport mechanism permeated by adenosine, inosine, guanosine and uridine but not cytidine and thymidine (Cornford and Oldendorf, 1975), a profile similar to rat CNT2 (Cass et al., 1998). Studies in the choroid plexus have indicated that both concentrative and equilibrative nucleoside transport mechanisms are present with concentrative transporters present on the lumenal side of the endothelial cell and equilibrative transporters present on either side (Spector, 1982). Although differentiation induced changes in BAEC nucleoside transporters has not been demonstrated, the expression of concentrative nucleoside transporters has been reported in other cell types undergoing differentiation (Kichenin et al., 2000; Soler et al., 1998). Thus, induction of BBB endothelial cell phenotype in BAEC may lead to the lumenal expression of concentrative nucleoside transporters as reported for cells *in vivo*. Co-expression of CNT2 and ENT1 on the lumenal surface of BAEC would enable influx of [^{3}H]adenosine through either transporter and efflux selectively via ENT1. The presence of DPR would inhibit ENT1, resulting in [^{3}H]adenosine influx but not efflux. Once antibodies or sequence information is available for bovine CNT2, further studies to examine the induction and localization of Na^+-dependent nucleoside transport processes in the BAEC of the DIV-BBB can be performed.

DPR has been used clinically for decades as an anti-platelet drug for the decreased risk of stroke development. DPR has been reported to increase plasma adenosine levels in man (Edlund et al., 1987; German et al., 1989; Sollevi et al., 1984) without affecting the CSF adenosine levels (Sollevi et al., 1983). Although the effects of DPR on adenosine levels in brain interstitium have not been investigated, it has been proposed that DPR-mediated elevations in brain adenosine levels may play a role in the prevention of stroke events in individuals taking oral DPR (Picano and Abbracchio, 1998). In rats, it has been reported that DPR is capable of slowing the decrease in adenosine levels in the brain following hypoxia/ischemia. While severe ischemia is likely to increase BBB permeability to adenosine and dipyridamole, in milder insults dipyridamole may block adenosine efflux across the BBB and preserve brain adenosine levels.

Chapter 3: Stimulation of Nucleoside Efflux and Inhibition of Adenosine Kinase by A_1 Adenosine Receptor Activation.

Published in Biochemical Pharmacology (Sinclair et al., 2000a)

Abstract

Adenosine is produced intracellularly during conditions of metabolic stress and is an endogenous agonist for four subtypes of G-protein linked receptors. Nucleoside transporters are membrane-bound carrier proteins that transfer adenosine, and other nucleosides, across biological membranes. We examined whether adenosine receptor activation could modulate transporter-mediated adenosine efflux from metabolically stressed cells. DDT_1 MF-2 smooth muscle cells were incubated with 10 μM [^{3}H]adenine to label adenine nucleotide pools. Metabolic stress with the glycolytic inhibitor iodoacetic acid (IAA, 5 mM) increased tritium release by 63% ($P<0.01$), relative to cells treated with buffer alone. The IAA-induced increase was blocked by the nucleoside transport inhibitor nitrobenzylthioinosine (NBMPR, 1 μM), indicating that the increased tritium release was primarily a purine nucleoside. High performance liquid chromatography verified this to be [^{3}H]adenosine. The adenosine A_1 receptor selective agonist N^6-cyclohexyladenosine (CHA, 300 nM) increased the release of [^{3}H]purine nucleoside induced by IAA treatment by 39% ($P<0.05$). This increase was blocked by the A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 10 μM). Treatment of cells with uridine triphosphate (UTP, 100 μM), histamine (100 μM), or phorbol-12-myristate-13-acetate (PMA, 10 μM) also increased [^{3}H]purine nucleoside release. The protein kinase C (PKC) inhibitor chelerythrine chloride (500 nM) inhibited the increase in [^{3}H]purine nucleoside efflux induced by CHA or PMA treatment. The adenosine kinase activity of cells treated with CHA or PMA was found to be significantly decreased compared to buffer-treated cells. These data indicate that adenosine A_1 receptor activation can increase nucleoside efflux from metabolically stressed DDT_1 MF-2 cells

by a PKC-dependent inhibition of adenosine kinase activity.

Adenosine, through the stimulation of membrane bound receptors, has many biological functions; it is an inhibitory neuromodulator, a potent vasodilator, an inhibitor of lipolysis, and an anti-inflammatory agent (Griffith and Jarvis, 1996). There are four subtypes of adenosine receptors; A_1 and A_3 activate G-proteins of the G_i/G_o family to inhibit adenylyl cyclase activity, inhibit Ca^{2+} influx, enhance K^+ efflux and/or alter phospholipase C (PLC) activity while A_{2A} and A_{2B} couple to G_s proteins to stimulate adenylyl cyclase activity. Under normoxic conditions, adenosine levels are maintained at low basal concentrations by three metabolic enzymes; S-adenosylhomocysteine hydrolase, adenosine deaminase and adenosine kinase. Hypoxia or ischemia increases intracellular adenosine levels due to faster rates of ATP hydrolysis than synthesis.

Nucleoside transporters are carrier proteins that transfer nucleosides, including adenosine, across plasma membranes. Two subtypes of equilibrative nucleoside transporters, termed *es* and *ei* (Vijayalakshmi and Belt, 1988), can transport adenosine in either direction according to its concentration gradient. Na^+-dependent nucleoside transporters are symporters that couple the movement of nucleosides to the inward movement of Na^+. However, when trans-membrane Na^+ gradients are disrupted, Na^+-dependent nucleoside transporters can also mediate cellular release of nucleosides (Borgland and Parkinson, 1997).

A number of physiological systems have been reported to have autoregulatory feedback mechanisms; for example, stimulation of presynaptic α_2-adrenoceptors inhibits noradrenaline release. Evidence for positive or negative autoregulation of release has also been reported for the neurotransmitters glutamate (Liu and Moghaddam, 1995; Patel and Croucher, 1997), serotonin (Gebauer et al., 1993) and GABA (Kamermans and

Werblin, 1992). We hypothesized that an autocrine mechanism exists through which adenosine receptor activation can modulate adenosine release. To investigate this, we used DDT_1 MF-2 smooth muscle cells that have adenosine A_1 and A_2 receptors as well as *es* nucleoside transporters (Parkinson et al., 1996; Ramkumar et al., 1991).

Materials and Methods

Materials. [^{3}H]Adenine was purchased from NEN Life Sciences (Mississauga, Ontario). Phorbol-12-myristate-13-acetate (PMA), adenine, adenosine, uridine triphosphate (UTP), histamine and iodoacetic acid (IAA) were purchased from the Sigma Chemical Co. (St. Louis, MO). CGS 21680, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 5'-N-ethylcarboxamidoadenosine (NECA), N^6-cyclohexyladenosine (CHA) and nitrobenzylthioinosine (nitrobenzylmercaptopurine riboside, NBMPR) were purchased from Research Biochemicals International (Natlick, MA). Chelerythrine chloride was purchased from Calbiochem (LaJolla, CA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco BRL (Burlington, Ontario).

Cell Culture. DDT_1 MF-2 smooth muscle cells were obtained from the American Type Culture Collection and cultured as previously described (Parkinson et al., 1996). Cells were harvested, washed twice (100 x *g*, 5 min) and resuspended in physiological buffer (in mM; NaCl, 118; HEPES, 25; KCl, 4.9; K_2HPO_4, 1.4; $MgCl_2$, 1.2; $CaCl_2$, 1; glucose, 11; to pH 7.4 with NaOH) to a concentration of 1×10^6 cells/ml. At the end of each experiment, cell viability was evaluated by trypan blue exclusion staining and found to be greater than 90%.

Measurement of [^{3}H]Purine Release. DDT_1 MF-2 smooth muscle cells (1×10^6 cells/ml) were incubated with 10μM [^{3}H]adenine for 30 min at 37°C. At the end of the 30 min loading period, 500μl aliquots of cell solutions were centrifuged for 5s (16, 000 x *g*) and the supernatants were aspirated. Release was initiated by resuspending the cell pellets in 500 μl of buffer (37°C) containing drug additions as indicated below. Release was terminated after 10 min by layering aliquots (400 μl) of each cell mixture over 200 μl of oil (85% silicone oil: 15% mineral oil) and centrifuging at 16,000 x *g* for 30 s. Supernatants (250 μl) were added to 5 ml of scintillation cocktail and assayed for radioactivity.

The effects of glycolytic inhibition on tritium release were determined through replacement of glucose in the buffer with 5 mM IAA. The selective inhibitor of the *es* subtype of equilibrative nucleoside transporters, NBMPR (1 μM), was used to block efflux of [^{3}H]purine nucleosides through *es* transporters.

To determine the effects of adenosine receptor activation or blockade on IAA-induced release of tritiated nucleosides, the A_1 adenosine receptor antagonist DPCPX (1 μM), the A_1 and A_2 nonselective agonist NECA (1 μM), the A_{2A} selective agonist CGS 21680 (1 μM) or the A_1 selective agonist CHA (300 nM) were placed into the resuspension buffer along with 5 mM IAA. DPCPX (10 μM) in combination with CHA (300 nM) was also tested. These concentrations of agonists and antagonists ensured maximal activation or blockade of the respective receptors without directly inhibiting *es* transporter function.* Activation of the histamine H_1 receptor with histamine (100 μM) or the purinergic P2Y receptor with UTP (100 μM) was investigated in the presence or

absence of CHA. PMA (10 μM) and chelerythrine chloride (500 nM), a stimulator and an inhibitor of protein kinase C (PKC), respectively, were tested separately, together and in combination with CHA for effects on [^{3}H]purine release.

HPLC Analysis of Adenosine. The release of adenosine *per se* from cells treated with IAA was determined by high performance liquid chromatography (HPLC). Cells were pretreated with adenine (10 μM) and release assays were performed as described above. Supernatants were analyzed for adenosine using a slight modification of the method described by Delaney and Geiger (Delaney and Geiger, 1996). Briefly, equal volumes of supernatant, 0.3M $ZnSO_4$ and 0.3M $BaOH_2$ were added sequentially, vortexed, and centrifuged for 4 min at 16,000 x *g*. Supernatant (150 μl) was derivitized by adding 25 μl 5% chloracetaldehyde and incubating for 1hr at 80°C. Samples were injected into a μBondapak C_{18} column (3.9 x 150 mM) using a mobile phase of 0.01 M KH_2PO_4 with 12% methanol (pH 5) and run isocratically at 1.5 ml/min. The excitation wavelength was set at 275 nm and the emission wavelength was 407 nm.

Adenosine Kinase Activity. Cells were harvested, washed twice (100 x *g*, 5 min) and resuspended at a concentration of 1 x 10^6 cells/ml in physiological buffer alone or containing CGS 21680 (1 μM), CHA (300 nM), DPCPX (1 μM), or CHA + DPCPX at 37°C for 10 minutes. The PKC inhibitor chelerythrine chloride (500 nM) was also tested in the presence or absence of PMA (10 μM) or CHA. For co-incubations, chelerythrine chloride or DPCPX was added 2 min prior to CHA or PMA. As IAA (5 mM) was found to have no significant effect on adenosine kinase activity (data not shown), IAA was not included in the assay. Adenosine kinase activity was determined as previously described

---

* Parkinson et. al., unpublished work.

(Parkinson and Geiger, 1996). Briefly, cells were harvested, homogenized in ice-cold 50 mM Tris-HCl (pH 7.4), then centrifuged at 38,000 X *g* (1hr, 4°C). Assay reaction mixtures (100 μl) contained 50 mM Tris-HCl (pH 7.4), 0.1% (w/v) bovine serum albumin, 500 nM EHNA, 50% (v/v) glycerol, 1.6 mM $MgCl_2$, 50 mM 2-mercaptoethanol, 50 mM KCl, 1.2 mM ATP, 2 μM (0.25 μCi) [^{3}H]adenosine and 2 μg of cytosolic protein. After incubation at 37°C for 5 min, reactions were terminated by heating to 90°C. Reaction products (20 μl) were spotted, in triplicate, on DE81 ion exchange filters, dried, and washed sequentially with 1 mM NH_4COOH, distilled deionized water and 100% ethanol. HCl (0.25 ml, 0.2 M) and KCl (0.25 ml, 0.8M) were then added to the filters to elute [^{3}H]adenine nucleotides, and the tritium content was determined by scintillation spectroscopy (Lin et al., 1988).

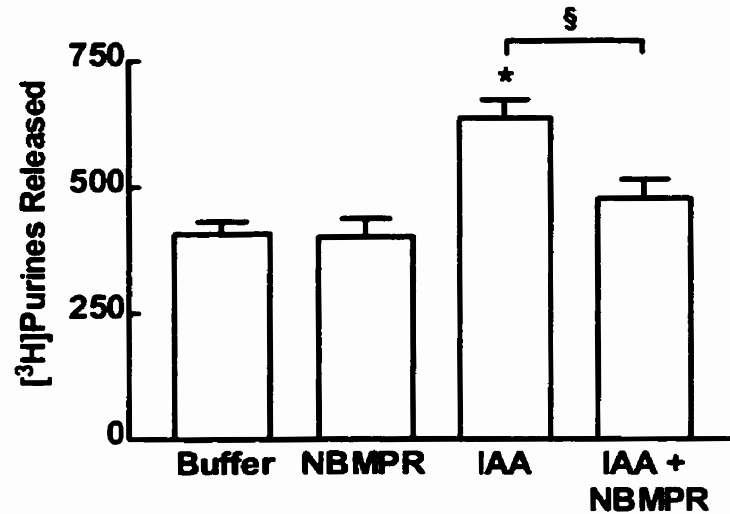
Data Analysis. Tritium release measurements were performed in triplicate and each experiment was performed at least three times. All values are expressed as means ± S.E. and statistical significance was determined by ANOVA followed by Bonferroni's post hoc test. Statistical analyses were performed using the software package GraphPad PRISM Version 2.0.

Results

DDT_1 MF-2 smooth muscle cells, loaded with [^{3}H]adenine, were subjected to glycolytic inhibition with IAA (5 mM) (fig. 6). IAA increased tritium efflux by 63% ($P<0.01$) over release into buffer alone. The *es* transport inhibitor NBMPR (1 μM) was used to determine whether the increase in tritium release was due to increased efflux of [^{3}H]purine nucleosides. NBMPR significantly blocked the IAA-induced increase ($P<0.05$). As both adenosine and its metabolite inosine are permeants of *es* nucleoside transporters, we treated cells with adenine and then measured, by HPLC, adenosine release induced by buffer alone or by IAA (table 3). Release into buffer alone showed undetectable levels of adenosine in the extracellular fluid; however, IAA-treatment produced extracellular adenosine concentrations of 75 ± 5 nM. By scintillation spectroscopy, [^{3}H]purines were detected in the supernatants of cells treated with buffer alone; however, following treatment with IAA, released [^{3}H]purines increased by 142 ± 25 nM (Table 3). Thus, about half of the increase in tritium efflux seen following treatment of cells with IAA was due to release of [^{3}H]adenosine.

The effect of adenosine receptor stimulation or inhibition was determined by resuspending the [^{3}H]adenine loaded cells into buffer containing IAA with or without the A_1 selective agonist CHA, the nonselective agonist NECA, the A_{2A} agonist CGS 21680 or the A_1 antagonist DPCPX (fig. 7A). Of the compounds tested, only CHA significantly changed release; it produced a 39% increase ($P<0.05$) over IAA alone. None of the agonists or the antagonist had any significant effects on tritium release in the absence of IAA (data not shown). To indicate if this increase by CHA was mediated by the A_1 receptor, the experiments were repeated in the presence of DPCPX (fig. 7B).

Figure 6



Effect of glycolytic inhibition on release of [^{3}H]purines from DDT$_1$ MF-2 cells loaded with [^{3}H]adenine. Cells were loaded for 30 min with 10 μM [^{3}H]adenine then pelleted and resuspended in buffer or buffer containing 5 mM iodoacetate (IAA) for 10 min in the absence or presence of the nucleoside transport inhibitor NBMPR (1 μM). Values represent means ± S.E. of pmoles/10^6 cells. Statistical significance was determined by ANOVA followed by Bonferroni's post hoc test (§ $P<0.05$ between IAA and IAA+NBMPR, * $P<0.01$ between buffer and IAA, n=3).

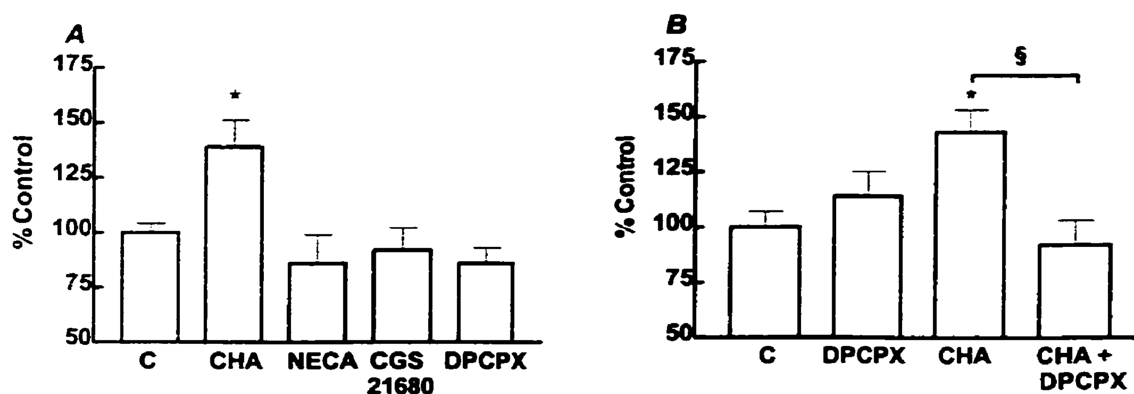
TABLE 3

IAA-induced release of adenosine or total purines from DDT_1 MF-2 cells.

Treatment	adenosine	$[^3H]$purines
Buffer	ND (5)	201 ± 8 nM (21)
IAA	72 ± 4 nM (4)	329 ± 24 nM (21)

Cells were preincubated with 10 μM adenine or 10 μM $[^3H]$adenine for 30 min at 37°C. Cells were then pelleted and resuspended in buffer alone or in 5 mM IAA. After 10 min, cells were pelleted through oil and supernatants were analyzed by HPLC for adenosine content or by scintillation spectroscopy for $[^3H]$purine content. Data are mean concentrations ± S.E. (n). ND-not detected; IAA-iodoacetate.

Figure 7



Effects of adenosine receptor agonists and antagonists on IAA-stimulated efflux of [^{3}H]purines from DDT$_1$ MF-2 cells loaded with [^{3}H]adenine. **A.** Cells were loaded for 30 min with 10 μM [^{3}H]adenine then pelleted and resuspended into buffer containing IAA alone (C; control) or with the A_1 agonist CHA (300 nM), the nonselective agonist NECA (1 μM), the A_{2A} agonist CGS 21680 (1 μM) or the A_1 antagonist DPCPX (1 μM). **B.** Cells were resuspended into buffer containing IAA alone (C; control) or with DPCPX (10 μM), CHA (300 nM) or DPCPX and CHA. Data for [^{3}H]purines released during 10 min (37°C) are means ± S.E. expressed as percentage of purines evoked by IAA treatment. In panel A, control was 120 ± 20 pmoles/ 10^6 cells while in panel B it was 118 ± 29 pmoles/ 10^6 cells. Statistical significance was determined using ANOVA and Bonferroni's post hoc test was used to compare treatments (§ $P<0.05$ between CHA and CHA+DPCPX, * $P<0.05$ between C and CHA, $n \geq 4$).

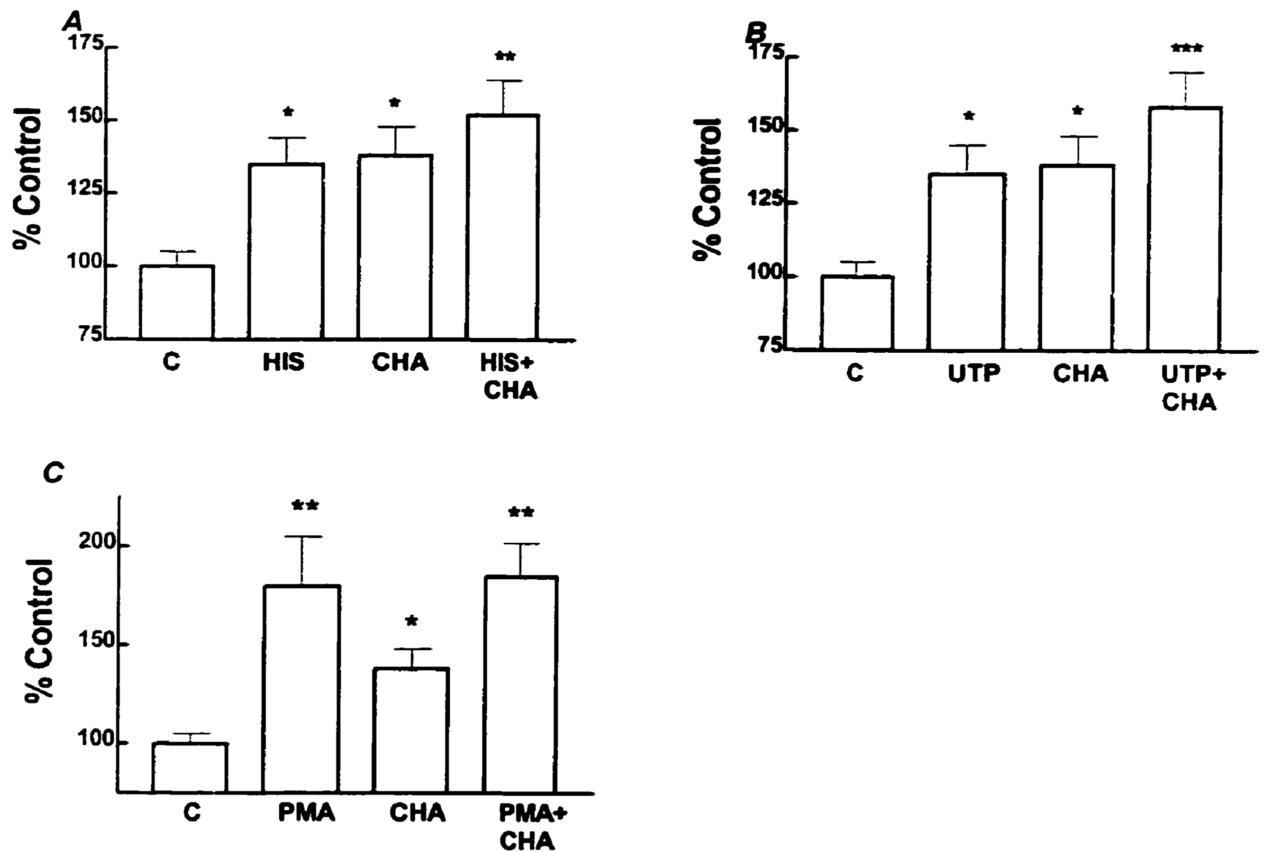
DPCPX completely inhibited the CHA-mediated increase in [^{3}H]purine nucleoside efflux ($P<0.05$). As A_1 adenosine receptor stimulation is known to inhibit adenylyl cyclase activity, we investigated the role of cAMP in altering purine nucleoside release. Forskolin (10 μM) as well as CGS 21680 (1 μM), both of which will activate adenylyl cyclase in these cells (Borgland and Parkinson, 1998)* failed to alter IAA-induced nucleoside release (data not shown; fig. 7A). Thus, we found no evidence that cAMP formation affects adenosine efflux.

As A_1 receptors in DDT_1 MF-2 cells are known to activate PLC directly and to enhance the effects of other PLC activators (Dickenson and Hill, 1994; Gerwins and Fredholm, 1995b; Schachter and Wolfe, 1992), we examined the role of PLC and PKC activation on IAA stimulation of [^{3}H]purine release. Both nucleotide P2Y (Gerwins and Fredholm, 1995a) and histamine H_1 (Dickenson and Hill, 1994) receptor stimulation have been shown to increase PLC activity in these cells. We investigated whether UTP (100 μM) or histamine (100 μM) could enhance IAA-stimulated efflux of [^{3}H]purines and found that each compound caused a 35% increase relative to that of IAA ($P<0.05$) (fig. 8A and fig. 8B). When UTP or histamine was combined with CHA, there appeared to be an additive effect and release was increased by 58% and 52%, respectively ($P<0.01$), relative to IAA alone. The role of PKC in these increases in [^{3}H]purine release was investigated with the phorbol ester PMA or chelerythrine chloride to activate or inhibit PKC, respectively. PMA (10 μM) caused a significant increase in tritium efflux over that of IAA alone ($P<0.05$); however, unlike UTP or histamine there was no additional effect

---

* Parkinson et. al., unpublished work.

Figure 8

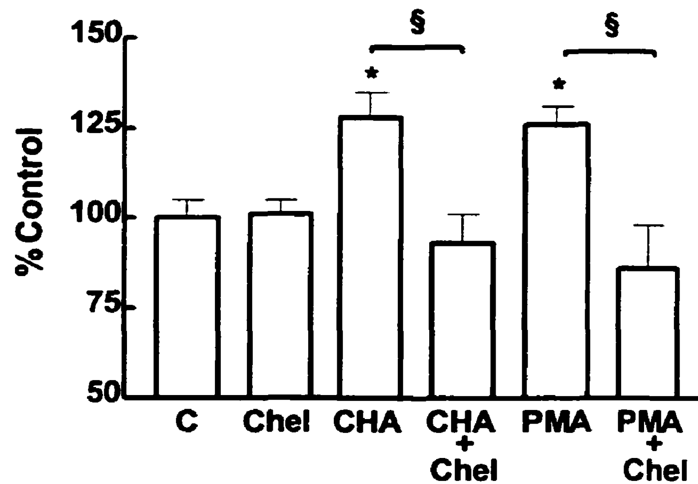


The effects of PLC and PKC activation on purine efflux induced by IAA with or without CHA. Cells were loaded for 30 min with 10 μM [^{3}H]adenine then pelleted and resuspended into buffer containing IAA alone (C; control) or with histamine (100 μM; A), UTP (100 μM; **B**) or PMA (10 μM; **C**) in the presence or absence of CHA (300 nM) at 37°C for 10 min. Values represent means ± S.E. expressed as percentages of [^{3}H]purine nucleosides evoked by IAA-treatment. In panels A and B, control was 121 ± 23 pmoles/ 10^6 cells while in panel C it was 65 ± 19 pmoles/ 10^6 cells. Statistical significance was determined using ANOVA and Bonferroni's post hoc test was used to compare treatments (* $P<0.05$, **$P<0.01$ relative to C; $n \geq 5$).

with CHA (fig. 8C). A concentration of chelerythrine chloride (500 nM) that did not modify basal efflux completely inhibited both CHA- and PMA-mediated increases in [^{3}H]purine release ($P<0.05$) (fig. 9).

Previous work in our laboratory showed that CHA at concentrations of less than 5 μM did not directly affect nucleoside transporter function in DDT_1 MF-2 (Borgland and Parkinson, 1998). We examined whether the increased [^{3}H]purine release induced by PMA or CHA was associated with inhibition of one of the adenosine metabolizing pathways. The activity of the enzyme adenosine kinase, which phosphorylates adenosine to AMP, was found to be significantly inhibited by adenosine A_1 receptor activation by CHA but not by activation of A_2 receptors with CGS 21680 (fig. 10A). These effects were receptor mediated, as the A_1 receptor antagonist DPCPX was able to block the CHA-mediated effects on adenosine kinase (fig. 10B). Activation of PKC with the phorbol ester PMA was able to mimic the effects of CHA on adenosine kinase activity and chelerythrine chloride was able to attenuate the inhibitory effects of CHA and PMA (Table 4).

Figure 9



The effects of PKC inhibition on CHA or PMA mediated increases in [^{3}H]purine nucleoside efflux. Cells were loaded for 30 min with 10 μM [^{3}H]adenine then pelleted and resuspended into buffer containing IAA alone (C; control) or with PMA (10 μM) or CHA (300 nM) or the PKC inhibitor chelerythrine chloride (Chel; 500 nM) alone or in combination with PMA or CHA at 37°C for 10 min. Data are expressed as percentage of [^{3}H]purine nucleosides evoked by IAA-treatment; bars represent means ± S.E. Control was 191 ± 29 pmoles/ 10^6 cells. Statistical significance was determined using ANOVA and Bonferroni's post hoc test was used to compare treatments (* $P<0.05$ relative to C; §$P<0.05$ for CHA±Chel or PMA±Chel, $n \geq 4$).

TABLE 4

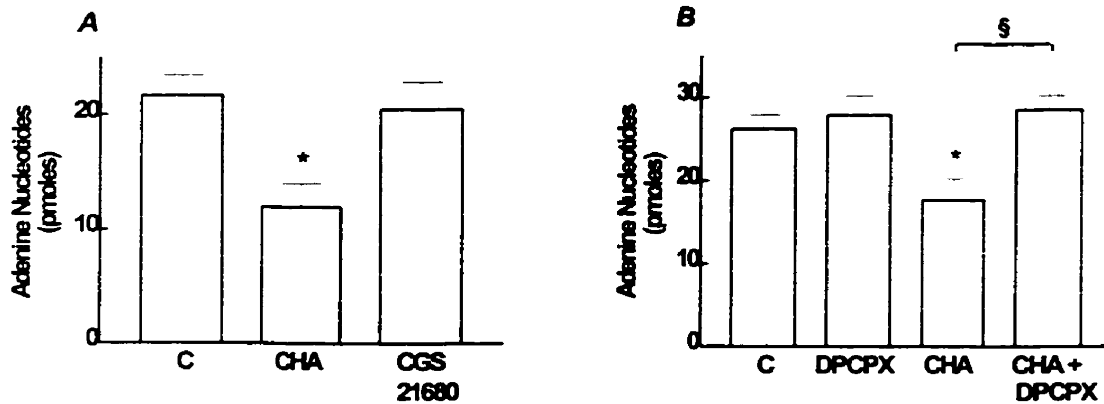
Effects of CHA and PMA on adenosine kinase activity.

Treatment	Adenine Nucleotides (pmoles)	% Control
Buffer	26.2 ± 1.7 (8)	100
CHA	18.3 ± 1.7 (7)*	67 ± 4.0 *,§
PMA	14.9 ± 1.5 (5)**,§§	61 ± 4.3 **,§§
Chel	24.9 ± 1.9 (6)	85 ± 4.5
CHA + Chel	25.0 ± 2.1 (5)	85 ± 2.5
PMA + Chel	27.7 ± 1.4 (3)	97 ± 6.0

Cells were stimulated with CHA (300 nM) or PMA (10 μM) alone or with chelerythrine chloride (Chel; 500 nM) at 37°C for 10 min then lysed and adenosine kinase activity was measured as described in the text. Values are means ± S.E.(n). Adenosine kinase activity is expressed as picomoles [^{3}H]adenine nucleotides produced/2 μg protein/5 min or as % control of adenosine kinase activity relative to buffer-treated cells.

* $P<0.05$, $^{**}P<0.01$ relative to buffer-treatment; $^{\S}P<0.05$, $^{\S\S}P<0.01$ relative to Chel treated-cells. ANOVA followed by Bonferroni's post hoc test

Figure 10



The effects of adenosine receptor agonist or antagonist treatment of DDT_1 MF-2 cells on cytosolic adenosine kinase activity. **A.** Cells were stimulated for 10 min with buffer (C; control), CHA (300 nM) or CGS 21680 (1 μM) at 37°C then lysed and adenosine kinase activity was determined as described in the text. **B.** Cells were treated with buffer, CHA (300 nM), DPCPX (1 μM) or CHA + DPCPX for 10 min at 37°C. Cytosolic protein was isolated and adenosine kinase activity was determined. Data for adenine nucleotides are mean ± S.E. expressed as picomoles adenine nucleotides/ 2 μg cytosolic protein/5min. Statistical significance was determined using ANOVA and Bonferroni's post hoc test was used to compare treatments (§ $P<0.05$ between CHA and CHA+DPCPX, * $P<0.05$ between C and CHA, $n \geq 4$).

Discussion

The main findings of this study were that adenosine A_1 receptor activation can increase the efflux of [^{3}H]purines from DDT$_1$ MF-2 cells during glycolytic inhibition. Stimulation of PLC by histamine H_1 and P2Y nucleotide receptor activation as well as direct PKC activation with PMA can mimic this increase in efflux. Adenosine kinase assays showed that CHA or PMA treatment inhibited adenosine metabolism, an effect that could elevate intracellular adenosine levels and enhance adenosine efflux.

Depleting cellular ATP by blocking glycolysis or oxidative phosphorylation is an effective way of enhancing intracellular adenosine production and release (Bukoski and Sparks, 1986; Zoref-Shani et al., 1988). We found that the glycolytic inhibitor IAA was effective at stimulating the release of [^{3}H]purines during a 10 min release period. NBMPR blocked this increase, indicating that IAA treatment induced [^{3}H]purine nucleoside release from cells via *es* nucleoside transporters. Release of [^{3}H]purines was also detected following treatment of cells with buffer alone; as this release was not affected by NBMPR and adenosine levels were undetectable by HPLC, this basal release could reflect [^{3}H]adenine or its degradation products.

HPLC analysis showed that adenosine accounts for about 50% of the IAA-evoked release of purine nucleosides. Inosine is another *es* transporter permeant that may be released under these conditions. Previously, IAA treatment was reported to cause release of similar amounts of adenosine and inosine from neural (Rego et al., 1997) and cardiac tissue (Jennings et al., 1989). Inosine can be formed from adenosine by adenosine deaminase or from dephosphorylation of inosine monophosphate following deamination of adenosine monophosphate. Inosine production may have physiological

importance as a recent study has reported that inosine can stimulate A_3 receptors in mast cells (Jin et al., 1997).

In this study cAMP-dependent mechanisms did not appear to modulate adenosine release. Both the adenylyl cyclase activator forskolin and the A_{2A} agonist CGS 21680 can stimulate cAMP production in these cells (Borgland and Parkinson, 1998)* but did not affect the release of [^{3}H]purine nucleoside. Thus, *es* transporters in DDT_1 MF-2 cells do not appear to be regulated by cAMP-dependent mechanisms although such regulation may occur in other cell types (Coe et al., 1996; Delicado et al., 1990; Sen et al., 1990).

Stimulation of adenosine A_1, histamine H_1, and nucleotide P2Y receptors enhanced IAA-induced [^{3}H]purine nucleoside release from DDT_1 MF-2 cells. Stimulation of these receptors leads to activation of PLC and PKC (Gerwins and Fredholm, 1995a; Gerwins and Fredholm, 1995b; Schachter and Wolfe, 1992) and A_1 receptor activation can enhance PLC activity induced by other PLC-coupled receptors in these cells (Gerwins and Fredholm, 1995b; Schachter et al., 1992; Schachter and Wolfe, 1992). The phorbol ester PMA also enhanced [^{3}H]purine nucleoside release. The PKC inhibitor chelerythrine chloride inhibited both the CHA- and the PMA-induced increases in nucleoside efflux, supporting the role of PKC in this efflux. In contrast to our data, it was reported previously that PKC activation decreased adenosine uptake and decreased the number of functional transporters in bovine adrenal chromaffin cells (Delicado et al., 1991) although not in bovine endothelial cells (Sen et al., 1996).

Adenosine kinase is a high affinity enzyme that has a K_m value for adenosine of 0.2 - 0.5 μM and is subject to substrate inhibition (Fisher and Newsholme, 1984; Lin et

* Parkinson et. al., unpublished work.

al., 1988). Inhibition of adenosine kinase can elevate endogenous adenosine levels (Golembiowska et al., 1996; White, 1996) and has been reported to have neuroprotective (Tatlisumak et al., 1998) anti-inflammatory (Cronstein et al., 1995), and anti-nociceptive effects (Keil and DeLander, 1996; Sawynok et al., 1998). Treatment of these cells with CHA inhibited the activity of adenosine kinase while the A_{2A} agonist CGS 21680 did not affect adenosine kinase activity. Previously, CHA was reported to inhibit adenosine kinase activity directly with a K_i value of 220 μM (Lin et al., 1988); however, the concentration of CHA used in the present study was almost three orders of magnitude lower (300 nM). Inhibition of adenosine kinase by CHA was attenuated by the A_1 selective antagonist DPCPX or the PKC inhibitor chelerythrine chloride, indicating an A_1 receptor-mediated and PKC-dependent mechanism. The finding that PKC activation with PMA or CHA can inhibit adenosine kinase activity is novel. DDT_1 MF-2 cells have been reported to contain PKC-α, PKC-ε and PKC-ζ (Assender et al., 1994). As PKC-α or PKC-ε are activated by PMA, one or both of these isoforms may cause direct or indirect inhibition of adenosine kinase activity. Phosphorylation of adenosine kinase by PKC activation is a potential mechanism as the adenosine kinase sequence has numerous PKC phosphorylation consensus sites (McNally et al., 1997; Singh et al., 1996; Spychala et al., 1996). Although increased intracellular cAMP levels stimulated by CGS 21680 administration does not directly modulate adenosine kinase activity, it is possible that cAMP may modulate the PKC-mediated inhibition of AK in these cells (Schachter et al., 1992). Whether inhibition of adenosine kinase activity by PKC-dependent mechanisms underlies PMA- or carbachol-induced potentiation of NMDA-evoked adenosine release from cortical slices (Semba and White, 1997; Wang and White, 1998) has yet to be

determined. It is tempting to speculate that inhibition of adenosine kinase may provide a way for local adenosine levels to reach sufficiently high concentrations to activate receptors with relatively low affinity for adenosine, such as the A_{2B} or A_3 subtypes.

In summary, we have shown that adenosine A_1 receptor activation can inhibit adenosine kinase activity by a PKC-dependent pathway. In DDT_1 MF-2 cells, this led to enhanced cellular release of purine nucleosides. This may indicate a mechanism by which adenosine can potentiate adenosine levels under conditions of metabolic stress.

Chapter 4: Nucleoside Transporter Subtype Expression: Effects on Potency of Adenosine Kinase Inhibitors.

Submitted to British Journal of Pharmacology

Abstract

Adenosine kinase (AK) inhibitors, by blocking one pathway of adenosine metabolism, can enhance adenosine levels and potentiate adenosine receptor activation. As the AK inhibitors 5'iodotubercidin (ITU) and 5'amino-5'-deoxyadenosine (NH_2dAdo) are nucleoside analogues, we hypothesized that the expression of nucleoside transporter subtypes can affect the potency of these inhibitors in intact cells. Three nucleoside transporter subtypes that mediate adenosine permeation of rat cells have been characterized and cloned: equilibrative transporters rENT1 and rENT2 and concentrative transporter rCNT2. For the present study, rat C6 glioma cells, which express rENT2 nucleoside transporters, were stably transfected with rENT1 (rENT1-C6 cells) or rCNT2 (rCNT2-C6 cells) nucleoside transporters. As expected, ITU and NH_2dAdo inhibited AK isolated from C6 cells, and IC_{50} values of 4 nM and 1.8 μM, respectively, were obtained. We tested the effects of ITU and NH_2dAdo on [^{3}H]adenosine uptake and conversion to [^{3}H]adenine nucleotides in the three cell types. NH_2dAdo did not show any cell type selectivity. In contrast, ITU showed significant inhibition of [^{3}H]adenosine uptake and [^{3}H]adenine nucleotide formation at concentrations $\leq$ 100 nM in rENT1-C6 cells, while concentrations $\geq$ 3 μM were required for C6 or rCNT2-C6 cells. Nitrobenzylthioinosine (100 nM), a selective inhibitor of rENT1, abolished the effects of nanomolar concentrations of ITU in rENT1-C6 cells. This study demonstrates that ITU is a potent inhibitor of isolated AK, but its effects in whole cells are dependent upon nucleoside transporter subtype expression. Thus, cellular and tissue differences in expression of nucleoside transporter subtypes may affect the pharmacological actions of some AK inhibitors.

Adenosine kinase (AK) inhibitors have been reported to have therapeutic potential in the areas of inflammation, analgesia, anti-convulsion and cerebral ischemia (Kowaluk et al., 1998). These properties are believed to be due to elevation of endogenous adenosine levels and activation of adenosine receptors (Britton et al., 1999). As AK is an intracellular enzyme, only cell permeable inhibitors will exhibit pharmacological effects. Many AK inhibitors are purine nucleoside analogues (Henderson et al., 1972; Miller et al., 1979), so we hypothesized that the cellular permeability of these compounds may require nucleoside transporters.

Transmembrane fluxes of purine and pyrimidine nucleosides, including adenosine, occur via nucleoside transporters. These transporters are broadly categorized into two classes: concentrative and equilibrative. Concentrative nucleoside transporters, of which six subtypes have been characterized, are Na^+-dependent and couple influx of adenosine or other nucleosides to influx of Na^+ (Cass et al., 1998; Geiger et al., 1997). Three concentrative nucleoside transporters have been cloned from human and rodent tissues (Che et al., 1995; Huang et al., 1994; Ritzel et al., 2001): CNT1 (concentrative nucleoside transporter 1; previously N2/cit) is selective for pyrimidine permeants, CNT2 (N1/cif) is selective for purine nucleosides and uridine, and CNT3 (N3/cib) has purine and pyrimidine nucleosides as permeants. Two equilibrative nucleoside transporter subtypes have been characterized and cloned (Crawford et al., 1998; Griffiths et al., 1997b; Yao et al., 1997). Both transport purine and pyrimidine nucleosides across plasma membranes in a direction dictated by their concentration gradients. The equilibrative transporters are two unique gene products and are functionally differentiated based on their sensitivity to nitrobenzylthioinosine (NBMPR). ENT1 (equilibrative

nucleoside transporter 1; cloned *es* transporter) is sensitive to low nanomolar concentrations of NBMPR while ENT2 (*ei* transporter) is relatively insensitive to NBMPR, with IC_{50} values >1 μM (Griffith and Jarvis, 1996). Several studies have investigated the effects of ITU on nucleoside transport processes. At up to 10 μM ITU, some reports have indicated no effect on nucleoside transport processes (Lloyd and Fredholm, 1995) while others have demonstrated inhibition of transport (Davies and Cook, 1995; Henderson et al., 1972; Wu et al., 1984). We have previously reported that ITU, at concentrations of 4-15 μM, can inhibit both ENT1 nucleoside transport and ligand binding to ENT1 (Parkinson and Geiger, 1996).

The objectives of this study were to determine if the expression of nucleoside transporter subtypes affects the potency of the AK inhibitors ITU or NH_2dAdo to inhibit adenosine uptake and metabolism in rat C6 glioma cells. Our results indicate that potency of ITU, but not NH_2dAdo, was influenced by nucleoside transporter subtype expression.

Materials and Methods

Materials

PCR primers, low and high glucose Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Moloney murine leukemia virus (MMLV) reverse transcriptase, oligo $(dT)_{12\text{-}18}$, random primers DNA labeling kits, LIPOFECTIN® reagent, neomycin (G418), EcoRV, Not I and Sac II were purchased from Life Technologies (Burlington, Ontario). The SNAP RNA isolation kit and pcDNA 3.1 (-) mammalian expression vector were purchased from Invitrogen (Carlsbad, CA). T4 DNA polymerase, T4 DNA ligase and Wizard® DNA clean-up system were purchased from Promega. Ready To Go™ PCR beads and Apa I were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Epicurian Coli® XL1-Blue MRF' Kan supercompetent cells were purchased from Stratagene (La Jolla, CA). $[^3H]$Adenosine, $[^3H]$uridine and $[^3H]$NBMPR were purchased from NEN Life Sciences (Mississauga, Ontario). Iodotubericidin (ITU) was purchased from Alberta Nucleoside Therapeutics (Edmonton, Alberta). Erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA), dipyridamole (DPR), nitrobenzylthioinosine (nitrobenzylmercaptopurine riboside, NBMPR) and nitrobenzylthioguanosine were purchased from Research Biochemicals International (Natlick, MA). All other compounds were purchased from the Sigma Chemical Co. (St. Louis, MO).

Transfection of C6 Glioma Cells with rENT1, rCNT2 or vector

The nucleoside transporter subtypes rENT1 and rCNT2 were originally cloned in pGEM-T vectors (Griffiths et al., 1997a; Yao et al., 1996). The rENT1 cDNA insert was

excised from the pGEM-T vector using Sac II and Not I (simultaneously at 37° C for 1hr) and then treated with T4 DNA polymerase to produce blunt ends. The rENT1 insert was ligated into the EcoRV restriction site of pcDNA 3.1(-). The rCNT2 was excised with Apa I and Not I (simultaneously at 37° C for 1hr) and then inserted into the Apa I and Not I sites in pcDNA 3.1(-). pcDNA 3.1 (-) with and without inserts was amplified in Epicurian Coli® XL1-Blue MRF' Kan supercompetent cells, isolated and purified using Wizard® DNA clean-up system.

Rat C6 glioma cells were transfected with pcDNA 3.1(-) containing no insert, rENT1 or rCNT2 using LIPOFECTIN® reagent and the manufacturer's protocol. Stably transfected C6 cells were selected using 800 μg G418/ ml of culture media. Single clones were isolated and cultured in the presence of 400 μg/ml G418.

RT-PCR Analysis

RT-PCR analysis was performed as previously described (Sinclair et al., 2000b). Total RNA was isolated from rat C6 glioma cells using the SNAP RNA isolation kit and treated with DNase I. cDNA synthesis was performed at 37°C for 60 min with a total reaction volume of 60 μl consisting of 300 ng oligo$(dT)_{12-18}$ primer, 5 μg total RNA, 3 mM dNTPs, 6.7 μM dithiothreitol (DTT), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$ and 3.3 Units MMLV reverse transcriptase. Control reactions were performed by omitting reverse transcriptase.

For PCR, control and reverse transcriptase-treated solutions (2 μl) were amplified using Ready To Go™ PCR beads. The amplification consisted of 30 cycles of: 30 s at 94°C, 30 s at 56°C and 1 min at 72°C. A final 10 min 72°C elongation step followed and

samples were held at –9°C then analyzed by electrophoresis on a 1.0% agarose gel. DNA bands were viewed and photographed under UV light following ethidium bromide staining.

rENT1 was amplified with the 5' primer 5'-CACCATGACAACCAGTCACCAG -3' and the 3' primer 5'-TGAAGGCACCTGGTTTCTGTC -3' to produce a 1.76-kb product. rENT2 was amplified using the 5' primer 5'-TTACCCAACCTGCACCCTCTC -3' and the 3' primer 5'-GTAGCCACATTGCATATGGTGA -3' to produce a 1.67-kb product. rCNT2 was amplified using the 5' primer 5'-AACCTCCACTTCCTGCTTGTCA-3' and the 3' primer 5'-CTTCACTCCCTCCTTGCTCTTG-3' to produce a 1.43-kb product. The presence of mRNA for glyceraldehyde-3'-phosphate dehydrogenase (GAPDH), a ubiquitous housekeeping gene, was used as a loading control, and was detected using the 5' primer 5'-GCTGGGGCTCACCTGAAGGG-3' and the 3' primer 5'-GGATGACCTTGCCCACAGCC-3' to amplify a 343-bp DNA product (bases 346 to 688) from the rat GAPDH cDNA.

Nucleoside Uptake Assays

Rat C6 glioma cells were cultured in 24-well plates until confluent as previously described (Sinclair et al., 2000b). Cells were washed twice in physiological buffer (in mM; NaCl, 118; HEPES, 25; KCl, 4.9; K_2HPO_4, 1.4; $MgCl_2$, 1.2; $CaCl_2$, 1; glucose, 11; to pH 7.4 with NaOH) or buffer in which NaCl was replaced with N-methylglucamine (NMG). Cells were incubated with [^{3}H]adenosine (1 μM) or [^{3}H]uridine (1 μM) in 250 μl of Na^+ or NMG^+ buffer for times ranging from 0-300 sec. Nucleoside transporter

subtypes were determined by inhibition of [^{3}H]uridine uptake by 100nM NBMPR (rENT1), 10μM NBMPR (rENT1 and rENT2) or NMG^+ buffer (rCNT2). To examine the effect of AK inhibitors, cells were exposed to graded concentrations of ITU (1 nM – 30 μM) or NH_2dAdo (300 nM – 30 μM) 15 min prior to and during the uptake assays. To terminate uptake, the extracellular solutions were aspirated and the cells were rapidly washed twice with ice-cold buffer. Cellular protein was dissolved by incubating cells overnight with NaOH (1M; 500 μl) at 37°C. Separate aliquots of the dissolved cells were used for protein determination, using the Bradford assay, and for liquid scintillation spectroscopy. Uptake values were determined from the radioactivity in the dissolved cells and are expressed as pmol/mg cellular protein using the specific activity of the uptake assay buffer.

Adenosine Kinase Assays

Activity of isolated AK was assessed as previously described (Sinclair et al., 2000a). Briefly, cells were homogenized in ice-cold 50 mM Tris-HCl (pH 7.4), then centrifuged at 38,000 X *g* (1hr, 4°C). Assay reaction mixtures (100 μl) contained 50 mM Tris-HCl (pH 7.4), 0.1% (w/v) bovine serum albumin, 500 nM EHNA, 50% (v/v) glycerol, 1.6 mM $MgCl_2$, 50 mM 2-mercaptoethanol, 50 mM KCl, 1.2 mM ATP, 2 μM (0.25 μCi) [^{3}H]adenosine and 2 μg of cytosolic protein in the presence or absence of ITU (1 nM –1 μM) or NH_2dAdo (1 nM –10 μM). After incubation at 37°C for 5 min, reactions were terminated by heating to 90°C. Reaction products (20 μl) were spotted, in triplicate, on DE81 ion exchange filters, dried, and washed sequentially with 1 mM NH_4COOH, distilled deionized water and 100% ethanol. HCl (0.25 ml, 0.2 M) and KCl

(0.25 ml, 0.8M) were then added to the filters to elute [^{3}H]adenine nucleotides, and the tritium content was determined by scintillation spectroscopy.

Inhibition of AK activity in intact cells was investigated in C6 cells as previously described, with minor modifications (Rosenberg et al., 2000; Wiesner et al., 1999). Cells were cultured and treated as described for nucleoside uptake assays. Following a five min [^{3}H]adenosine (1 μM) uptake interval, cells were washed with ice-cold buffer and dissolved in 250 μl of 2% trichloroacetic acid (TCA). 100 μl of the cell extract was taken for scintillation spectroscopy and 50 μl was placed onto DE-81 sephadex filters to determine the percentage of the total uptake that was [^{3}H]adenine nucleotides. The ion-exchange filters were then washed as described above.

[^{3}H]NBMPR binding assays

Cells were washed with Na^+ buffer then incubated (22 °C) with 0.1 – 5 nM [^{3}H]NBMPR in the absence or presence of 1 μM nitrobenzylthioguanosine. After a 1 hr incubation interval, cells were washed twice with ice-cold Na^+ buffer then dissolved with NaOH. Samples were analyzed for both tritium and protein content.

Data Analysis

Each experiment was performed at least three times in duplicate or triplicate, unless otherwise stated. All values are expressed as means ± S.E. and statistical significance was determined by ANOVA followed by Tukey's post hoc test. Statistical analyses were performed using the software package GraphPad PRISM Version 3.0.

Results

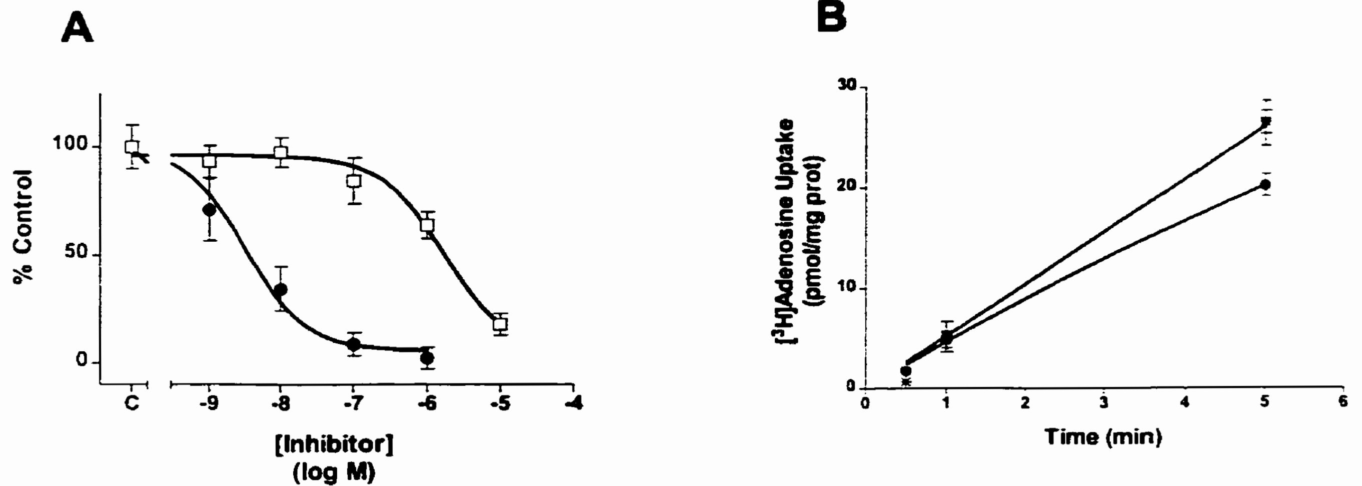
AK assays were performed to determine the potencies of ITU and NH_2dAdo for inhibition of rat C6 glioma cell AK activity (fig 11A). ITU inhibited AK by 98% at 1 μM and had an IC_{50} value of 4 nM. NH_2dAdo produced 82% inhibition at 10 μM and had an IC_{50} value of 1.8 μM. Rat C6 glioma cells contain predominantly (>95%) rENT2-mediated nucleoside transport and 1 μM [^{3}H]adenosine uptake is linear over time (fig 11B) (Sinclair et al., 2000b). In the absence of intracellular metabolism, [^{3}H]adenosine transport via rENT2 would be predicted to saturate quickly, thus, the apparent linearity of uptake indicates extensive metabolism of [^{3}H]adenosine. Indeed, after 5 min uptake intervals, approximately 90% of intracellular tritium was associated with adenine nucleotides. Thus, we investigated maximally inhibitory concentrations of ITU (1 μM) and NH_2dAdo (10 μM) on [^{3}H]adenosine uptake. Neither ITU nor NH_2dAdo had a significant effect on 1 μM [^{3}H]adenosine accumulation over 5 min (fig 11b). These results indicate that ITU and NH_2dAdo are ineffective in live C6 cells and led us to hypothesize that nucleoside transporter subtype expression can affect cell permeability and, thus, efficacy of AK inhibitors.

To investigate this hypothesis, we stably transfected the C6 glioma cells with pcDNA 3.1 (-) containing no insert (vector-C6), rENT1 cDNA sequence (rENT1-C6) or rCNT2 cDNA sequence (rCNT2-C6). To demonstrate effective transfection, we performed 1 μM [^{3}H]uridine uptake, RT-PCR analysis and [^{3}H]NBMPR binding. The accumulation of [^{3}H]uridine in rENT1-C6 and rCNT2-C6 cells was increased by 45 ± 17 % and 47 ± 20 %, respectively, compared to wt-C6 cells. The uptake of 1 μM [^{3}H]uridine uptake in wt-C6 (fig 12a) or vector-C6 cells (data not shown) was not significantly

affected by 100 nM NBMPR or NMG^+ buffer while 10 μM DPR inhibited [^{3}H]uridine uptake by 100 ± 5 %. As we have previously reported (Sinclair et al., 2000b), this indicates predominantly rENT2-mediated nucleoside transport in rat C6 glioma cells. In the rENT1-C6 cells, 100 nM NBMPR inhibited [^{3}H]uridine uptake by 32 ± 10 %. RT-PCR analysis indicates mRNA transcript for rENT1 in the ENT1-C6 cells but not in the other three cell types (fig 12d). In addition, specific [^{3}H]NBMPR binding was detected in rENT1-C6 cells (K_D = 0.18 ± 0.03 nM and B_{MAX} = 298 ± 10 fmol/mg protein, n = 4) but not in wt-C6 cells (n = 4). This indicates successful transfection of rENT1 into these cells. Although rCNT2 is a purine selective nucleoside transporter, uridine is transported by rCNT2 (Ritzel et al., 1998). In the rCNT2-C6 cells, [^{3}H]uridine uptake was inhibited by 37 ± 11 % when NMG^+ buffer replaced Na^+ buffer (fig 12c). RT-PCR (fig 2d) demonstrated that rCNT2 mRNA transcript is present in rCNT2-C6 cells but not in the other three cell types. These data indicate successful transfection of C6 cells with the rCNT2 nucleoside transporter.

The interaction of ITU or NH_2dAdo with the nucleoside transporters was investigated in the C6 cells in two ways: 1 μM [^{3}H]uridine uptake to investigate transporter-mediated effects and 1 μM [^{3}H]adenosine uptake to investigate AK-mediated and transporter-mediated effects. Previous reports have demonstrated direct interaction of ITU with nucleoside transporters at concentrations greater than 1 μM (Davies and Cook, 1995; Henderson et al., 1972; Parkinson and Geiger, 1996; Wu et al., 1984). To investigate the hypothesis that ITU and NH_2dAdo inhibit nucleoside transport directly, we measured uptake of 1 μM [^{3}H]uridine into wt-C6, rENT1-C6 and rCNT2-C6 cells.

Figure 11



Effect of ITU or NH_2dAdo on AK activity or 1 µM [^{3}H]adenosine uptake in C6 cells. A. Cytosolic protein from C6 cells was isolated and AK activity was determined in the presence of ITU (•; 1 nM- 1 µM) or NH_2dAdo (□; 1 nM- 10 µM). Data are expressed as %control where control represents 28.7 ± 5.5 pmol adenine nucleotides/ 2 µg cytosolic protein/5min. **B**. Adenosine accumulation was measured by incubating C6 cells with 1 µM [^{3}H]adenosine for 5-300 s in the presence of buffer (✳), 1 µM ITU (•) or 10 µM NH_2dAdo (□). Accumulation is expressed as pmol/mg cellular protein. Symbols represent means and error bars represent SEM. Experiments were performed at least two times in triplicate.

Figure 12

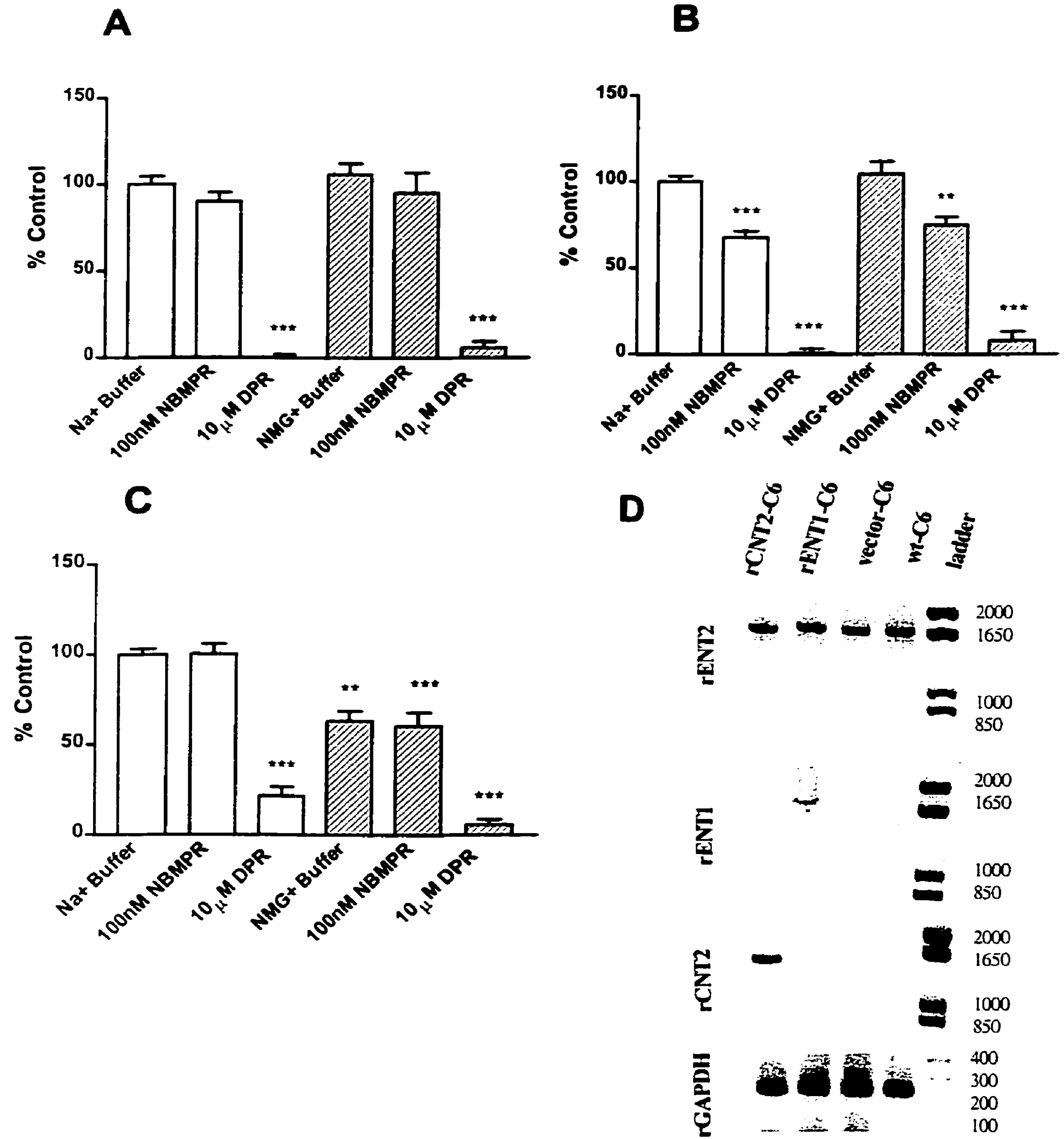


Figure 12:continued

Determination of nucleoside transporter subtypes in wt-C6, rENT1-C6, rCNT2-C6 and vector-C6 rat glioma cells by inhibition of nucleoside accumulation and RT-PCR. [^{3}H]Uridine accumulation was measured by incubating the wt-C6 (**A**), rENT1-C6 (**B**) or rCNT2-C6 (**C**) with 1 μM [^{3}H]uridine in Na^+ (open bars) or NMG^+ (shaded bars) buffer in the presence or absence of 100 nM NBMPR or 10 μM DPR for 5 min. RT-PCR analysis of total RNA from wt-C6, vector-C6, rENT1-C6 or rCNT2-C6 cells was performed for detection of rENT1 (1.76 kb product), rENT2 (1.67 kb product), rCNT2 (1.43 kb product) or rGAPDH (343 bp product) transcript (**D**). Data in panels A-C are expressed as % control where control is 4.0 ± 0.7, 5.3 ± 0.8 and 5.0 ± 1.2 pmol [^{3}H]uridine/mg prot/ 5min in A, B and C, respectively. Symbols represent means and error bars represent SEM. Experiments were performed at least three times in duplicate.

ITU inhibited [^{3}H]uridine uptake with an apparent IC_{50} value of 2.7-5.6 μM, with no statistically significant differences among the three cell types (fig 13a). NH_2dAdo had similar effects on [^{3}H]uridine uptake to ITU; IC_{50} values of ≥ 10 μM were obtained (fig 13b).

[^{3}H]Adenosine uptake was performed using relatively long assays, 5 min, because AK activity accounts for most of the intracellular accumulation that occurs during this time period. In wt-C6 and rCNT2-C6 cells, inhibition of [^{3}H]adenosine uptake by ITU was evident only with concentrations ≥ 3 μM and only 50-55% inhibition was detected with 30 μM (fig 14a). However, in rENT1-C6 cells, ITU produced biphasic inhibition of [^{3}H]adenosine uptake. The first component was observed with ITU at concentrations of 1 – 300 nM and produced 50% inhibition of [^{3}H]adenosine uptake with an IC_{50} value of 4.6 nM (fig 14a). This IC_{50} value is very close to that obtained for inhibition of isolated AK by ITU (fig 11a). At concentrations greater than 300 nM ITU, [^{3}H]adenosine uptake was decreased further, with a maximum of 76% inhibition observed with 30 μM (fig. 14A). Pre-treatment (5 min) of ENT1-C6 cells with NBMPR (100 nM), completely blocked the effects of nanomolar concentrations of ITU (fig 14a) indicating that rENT1 was responsible for these effects. The inhibition of [^{3}H]adenosine uptake by NH_2dAdo was similar in all three cell types (fig 14b). At 30 μM NH_2dAdo, [^{3}H]adenosine uptake was inhibited by 40-60%.

Whole cell adenosine kinase assays were performed to demonstrate that inhibition of [^{3}H]adenosine uptake by ITU or NH_2dAdo correlated to decreased [^{3}H]adenine nucleotide incorporation. In wt-C6 and ENT1-C6 cells, approximately 90% of [^{3}H]adenosine accumulated by cells was metabolized to [^{3}H]adenine nucleotide (fig 14c).

10nM ITU significantly decreased the adenine nucleotide incorporation in ENT1-C6 cells but not wt-C6 cells. 1μM ITU significantly decreased [^{3}H]adenine nucleotide formation in both the wt-C6 and ENT1-C6 cells but a significantly greater inhibition was seen in the ENT1-C6 cells. No inhibition of adenine nucleotide incorporation was seen with NH_2dAdo at concentrations < 30 μM (data not shown). This demonstrates that inhibition of [^{3}H]adenosine uptake correlated to decreased incorporation into [^{3}H]adenine nucleotides.

Figure 13

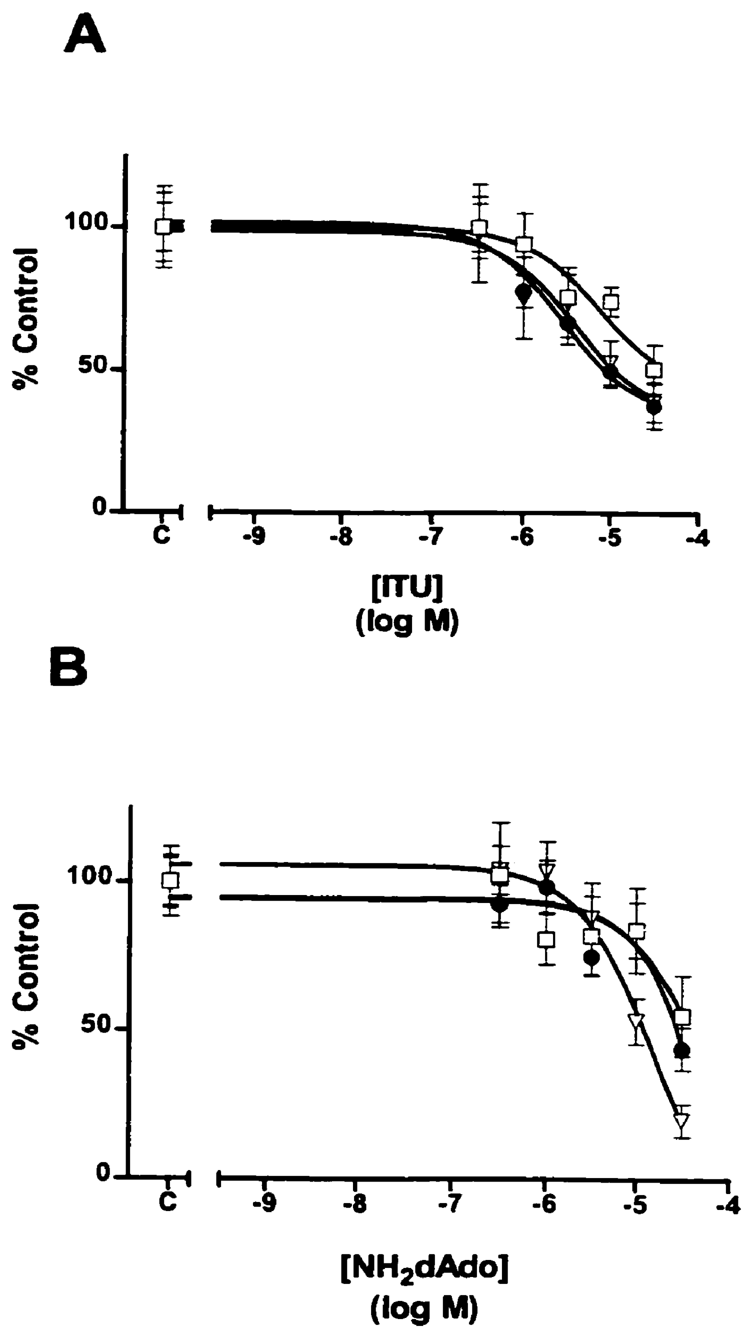


Figure 13: continued

Effect of ITU or NH_2dAdo on [^{3}H]uridine uptake in wt-C6, rENT1-C6 and rCNT2-C6 cells. [^{3}H]Uridine accumulation was measured by incubating the wt-C6 (□), rENT1-C6 (•) or rCNT2-C6 (∇) with 1 μM [^{3}H]uridine for 5 min in the presence of ITU (1nM – 30 μM) (**A**) or NH_2dAdo (300 nM – 30 μM) (**B**). Data are expressed as % control. Control represents 5.5 ± 0.9 (□), 9 ± 2.2 (•) or 7.1 ± 1.8 (∇) pmol [^{3}H]uridine/mg cellular protein/5min in A or 4.2 ± 0.7 (□), 4.9 ± 1.0 (•) or 8.2 ± 1.5 (∇) pmol [^{3}H]uridine/mg cellular protein/5min in B. Symbols represent means and error bars represent SEM. Experiments were performed at least three times in duplicate.

Figure 14

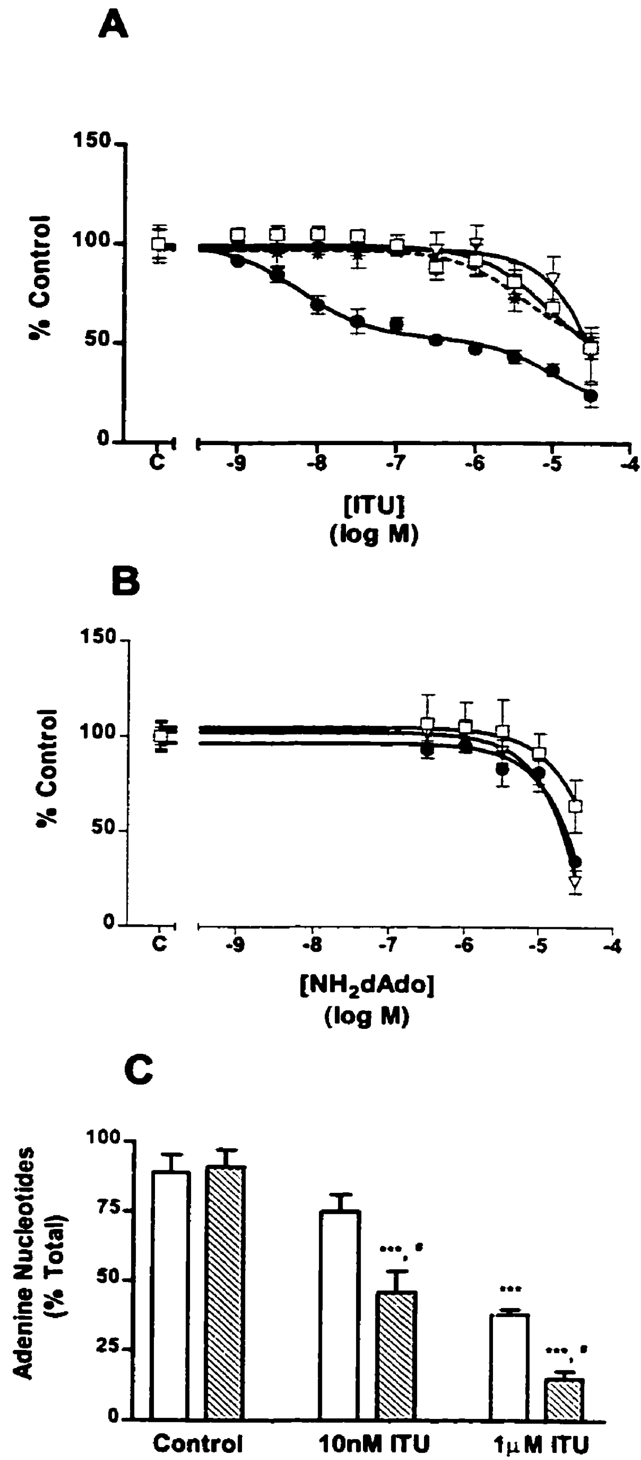


Figure 14: continued

Effect of ITU or NH_2dAdo on [^{3}H]adenosine uptake in wt-C6, rENT1-C6 and rCNT2-C6 cells. A. [^{3}H]Adenosine accumulation was measured by incubating wt-C6 (□), rENT1-C6 (•) or rCNT2-C6 (∇) cells, or rENT1-C6 cells preincubated with 100 nM NBMPR(*, dashed line), with 1 μM [^{3}H]adenosine for 5 min in the presence of ITU (1nM – 30 μM). **B.** [^{3}H]Adenosine accumulation was measured by incubating the wt-C6 (□), rENT1-C6 (•) or rCNT2-C6 (∇) with 1 μM [^{3}H]adenosine for 5 min in the presence of NH_2dAdo (300 nM – 30 μM). **C.** Incorporation of [^{3}H]adenosine into [^{3}H]adenine nucleotides was measured using whole cell AK assays in wt-C6 (open bars) and rENT1-C6 (hatched bars) cells in the presence of buffer, 10nM ITU or 1μM ITU. Data are expressed as % control (A and B) or % total (C). Control represents 29 ± 9 (□), 37 ± 5 (•) or 44 ± 6 (∇) pmol [^{3}H]adenosine/mg cellular protein/5min in A or 30 ± 8 (□), 35 ± 7 (•) or 56 ± 6 (∇) pmol [^{3}H]adenosine/mg cellular protein/5min in B. In C, total represents 115 ± 14 (wt-C6) or 139 ± 26 (rENT1-C6) pmol/mg cellular protein/5min. Symbols represent means and error bars represent SEM. Experiments were performed at least three times in duplicate. Statistical significance was determined between experimental groups by ANOVA followed by Tukey's post hoc test (*** $p<0.001$ from control; # $p<0.05$ from wt-C6 cell at the same concentration).

Discussion

AK inhibitors, by blocking adenosine phosphorylation to AMP, can elevate adenosine levels and potentiate adenosine receptor activation (Kowaluk and Jarvis, 2000; Kowaluk et al., 1998). In this report, we demonstrated that expression of the nucleoside transporter rENT1 increased ITU-mediated inhibition of [^{3}H]adenosine uptake compared to cells expressing rENT2 or rCNT2 nucleoside transporters. In contrast, NH_2dAdo had similar potency for inhibition of [^{3}H]adenosine uptake in rat C6 glioma cells expressing different subtypes of nucleoside transporters. This study demonstrates that the effects of ITU, and potentially other AK inhibitors, are influenced by nucleoside transporter subtype expression.

Adenosine uptake is the result of transport across the plasma membrane followed by intracellular metabolism. Under physiological conditions, adenosine metabolism is primarily to AMP by AK. Adenosine transport *per se* can be measured with rapid [^{3}H]adenosine uptake intervals (<15s), while longer uptake intervals usually result in intracellular metabolic trapping of adenosine in the form of adenine nucleotides. AK inhibitors, when used during longer accumulation intervals, would be expected to significantly decrease cellular accumulation of [^{3}H]adenosine by decreasing its metabolism to [^{3}H]adenine nucleotides. Surprisingly, neither ITU nor NH_2dAdo inhibited the uptake of [^{3}H]adenosine into C6 glioma cells during 5 min intervals. ITU inhibited isolated AK from C6 cells with an IC_{50} value of 4 nM, which is similar to previously reported values (Jarvis et al., 2000; Wiesner et al., 1999). NH_2dAdo inhibited isolated AK with an IC_{50} value of 1.8 μM, which is 5-100 fold higher than previous reports (9.2 – 173 nM) (Jarvis et al., 2000; Wiesner et al., 1999). This may indicate cell type or species

differences in the affinity of NH_2dAdo for AK. It is clear, however, that NH_2dAdo has poor cell penetrability and low potency for AK inhibition in C6 glioma cells (figs 11b and 13b, (Jarvis et al., 2000)). ITU has been previously documented to have high cell penetrability, which produces similar inhibitory profiles with whole cell and isolated AK (Jarvis et al., 2000; Wiesner et al., 1999). In our experiments, ITU appeared to permeate wt-C6 cells poorly as ITU did not decrease [^{3}H]adenosine accumulation unless concentrations were 1000-fold higher than the IC_{50} value for isolated AK (compare fig 11a and 14a). The difference between the previous studies and our study appears to be the nucleoside transporter subtypes present in the cell types used. The rat C6 glioma cells contain predominantly rENT2 nucleoside transporters while the previous studies used human neuroblastoma cells (Jarvis et al., 2000) and bovine endothelial cells (Wiesner et al., 1999), which contain ENT1 nucleoside transporters (Jones et al., 1994), unpublished results, Sinclair and Parkinson).

With rENT1 or rCNT2 transfected C6 cells we tested whether nucleoside transporter subtype expression affected the potency and efficacy of ITU or NH_2dAdo. As AK inhibitors have been previously demonstrated to inhibit nucleoside transport directly (Davies and Cook, 1995; Henderson et al., 1972; Parkinson and Geiger, 1996; Wu et al., 1984), we investigated the effects of ITU and NH_2dAdo on the uptake of 1μM [^{3}H]uridine, a nucleoside that is transported by rENT1, rENT2 and rCNT2 but is not metabolized by AK. ITU and NH_2dAdo inhibited [^{3}H]uridine uptake with IC_{50} values of approximately 10 μM, indicating direct interaction of both AK inhibitors with the nucleoside transporters at micromolar concentrations. ITU and NH_2dAdo have been used to inhibit AK in many studies at concentrations of 10-50 μM. Our data suggest that, at

these concentrations, some of the observed effects may be due to inhibition of nucleoside transporters.

[^{3}H]Adenosine uptake during 5 min allowed us to investigate AK- and transporter-mediated effects of ITU and NH_2dAdo. While ITU had similar effects in wt-C6 and rCNT2-C6 cells, it was much more potent in rENT1-C6 at decreasing [^{3}H]adenosine accumulation. NBMPR, a potent and selective inhibitor of rENT1, inhibited the effects of nanomolar concentrations of ITU in the rENT1-C6 cells. This finding indicates that transfection with rENT1 nucleoside transporters facilitated the cellular permeation of ITU into C6 glioma cells. In contrast, transfection of the C6 cells with either rENT1 or rCNT2 did not affect the potency of NH_2dAdo. As NH_2dAdo inhibited AK and nucleoside transport at similar concentrations, it is not possible to gain a true understanding of the role of each nucleoside transporter subtype in NH_2dAdo-mediated effects.

AK inhibitors have been studied for their effects on heart rate, blood pressure, inflammation, pain, stroke and seizure activity (for review see (Kowaluk et al., 1998) or (Kowaluk and Jarvis, 2000)). The reported benefit of using AK inhibitors relative to adenosine receptor agonists is their proposed site- and event-specific properties, which produce decreased systemic effects such as alterations in heart rate and blood pressure. Our results demonstrate a mechanism through which AK inhibitors such as ITU can have cell or tissue selective sites of action based on nucleoside transporter subtype distribution and expression. As other AK inhibitors are in late pre-clinical and early clinical development (Kowaluk and Jarvis, 2000), it is important to determine the role of the different nucleoside transporters in the effects mediated by these compounds.

Chapter 5: Purine uptake and Release in Rat C6 Glioma Cells: Nucleoside transport and Purine Metabolism under ATP Depleting conditions

Published in the Journal of Neurochemistry (Sinclair et al., 2000b)

Abstract

Adenosine, through activation of membrane bound receptors, has been reported to have neuroprotective properties during strokes or seizures. The role of astrocytes in regulating brain interstitial adenosine levels has not been clearly defined. We have determined the nucleoside transporters present in rat C6 glioma cells. RT-PCR analysis, [^{3}H]nucleoside uptake experiments and [^{3}H]nitrobenzylthioinosine (NBMPR) binding assays indicated that the primary functional nucleoside transporter in C6 cells was rENT2, an equilibrative nucleoside transporter that is relatively insensitive to inhibition by NBMPR. [^{3}H]Formycin B, a poorly metabolized nucleoside analogue, was used to investigate nucleoside release processes and rENT2 transporters mediated [^{3}H]formycin B release from these cells. Adenosine release was investigated by first loading cells with [^{3}H]adenine to label adenine nucleotide pools. Tritium release was initiated by inhibiting glycolytic and oxidative ATP generation and, thus, depleting ATP levels. Our results indicate that during ATP depleting conditions, AMP catabolism progressed via the reactions AMP → IMP → inosine → hypoxanthine, which accounted for >90% of the evoked-tritium release. Suprisingly, adenosine was not released during ATP depleting conditions. However, inhibition of AMP deaminase/adenosine deaminase or purine nucleoside phosphorylase during ATP depletion produced release of adenosine or inosine, respectively, via the rENT2 transporter. This indicates that C6 glioma cells possess primarily rENT2 nucleoside transporters that function in adenosine uptake but intracellular metabolism prevents the release of adenosine from these cells even during ATP-depleting conditions.

Adenosine is an endogenous nucleoside that is present in brain at concentrations of 40 – 460 nM (Ballarin et al., 1991) during physiological conditions and increases up to 100-fold during ATP-depleting conditions, such as ischemia (Parkinson et al., 2000). Adenosine interacts with a family of four receptor subtypes and activation of A_1 receptors, in particular, is associated with neuroprotection during ischemia (Rudolphi et al., 1992; Schubert et al., 1994; Von Lubitz, 1999). The use of adenosine *per se* or adenosine receptor agonists as neuroprotective agents is limited by systemic side effects such as decreased heart rate and blood pressure (Von Lubitz, 1999). Inhibitors of cellular adenosine uptake or adenosine metabolism have been proposed as site- and event-specific agents for increasing adenosine levels in the brain interstitium (Geiger et al., 1997) and such compounds are predicted to have fewer systemic side effects than receptor agonists. However, the role of specific cell types for adenosine uptake, release and metabolism in brain are poorly characterized.

Adenosine is formed primarily by dephosphorylation of ATP via intermediate formation of ADP and AMP. Extracellular adenosine has been reported to originate either from cellular release of adenosine *per se* or from extracellular metabolism of adenine nucleotides (Fredholm, 1997). During ischemia, release of adenosine *per se* is thought to be the quantitatively more important source of extracellular adenosine (Whittingham, 1990). Cellular release of adenosine occurs via nucleoside transporters, which are membrane proteins that mediate transmembrane fluxes of purine and pyrimidine nucleosides, including adenosine. These transporters are broadly categorized into two classes: concentrative and equilibrative. Concentrative nucleoside transporters, of which six subtypes have been characterized, are Na^+-dependent and couple influx of adenosine

or other nucleosides to influx of Na^+ (Cass et al., 1998; Geiger et al., 1997). Two equilibrative nucleoside transporter subtypes have been characterized and cloned. Both transport purine and pyrimidine nucleosides across plasma membranes in a direction dictated by their concentration gradients. The equilibrative transporters are two unique gene products and are functionally differentiated based on their sensitivity to nitrobenzylthioinosine (NBMPR). ENT1 (equilibrative nucleoside transporter 1; cloned *es* transporter) is sensitive to low nanomolar concentrations of NBMPR while ENT2 (*ei* transporter) is relatively insensitive to NBMPR, with IC_{50} values >1 μM (Griffith and Jarvis, 1996). Both ENT1 and ENT2 have been reported to have broad distribution in rat brain, including both neurons and astrocytes (Anderson et al., 1999a; Anderson et al., 1999b). Many studies have demonstrated that ENT1 can mediate the cellular release of adenosine (Gu et al., 1995; Sinclair et al., 2000a); however, similar investigations of ENT2 are limited.

The objectives of this study in rat C6 glioma cells were to determine the nucleoside transporters present in these cells, to investigate the efflux of purines and to characterize purine catabolic pathways during ATP-depleting conditions. Astrocytes have been reported to have a greater capacity for adenosine uptake than neurons (Bender and Hertz, 1986); therefore, astrocytes may be an important regulator of adenosine levels during ischemia-like conditions. Rat C6 glioma cells were chosen for these experiments because they exhibit low levels of adenine nucleotide release (Cotrina et al., 1998b), a potential nucleoside transporter-independent source of extracellular adenosine. Our results demonstrate 1) that C6 glioma cells express ENT2, which are capable of nucleoside uptake and release, and 2) during ATP depletion, ATP metabolism occurs via

an IMP pathway resulting in formation and release of hypoxanthine, rather than adenosine.

Materials and Methods

Materials

PCR primers, low and high glucose Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Moloney murine leukemia virus (MMLV) reverse transcriptase, oligo $(dT)_{12-18}$ and random primers DNA labeling kits were purchased from Life Technologies (Burlington, Ontario). The SNAP RNA isolation kit was purchased from Invitrogen (Carlsbad, CA). Ready To Go™ PCR beads were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). 2-Amino-1,5-dihydro-7-(3-pyridinylmethyl)-4H-pyrrolo[3,2,-d]pyridin-4-one (BCX-34; peldesine) was a generous gift from Dr. Phillip Morris of Biocryst Pharmaceuticals (Birmingham, Alabama). $[^3H]$Adenosine, $[^3H]$formycin B, $[^3H]$adenine and $[^3H]$NBMPR were purchased from NEN Life Sciences (Mississauga, Ontario). Silica Gel GF TLC plates were purchased from Fisher Scientific (Whitby, Ontario). Erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA), dipyridamole (DPR), nitrobenzylthioinosine (nitrobenzyl-mercaptopurine riboside, NBMPR) and nitrobenzylthioguanosine were purchased from Research Biochemicals International (Natlick, MA). All other compounds were purchased from the Sigma Chemical Co. (St. Louis, MO).

RT-PCR Analysis

Total RNA was isolated from rat C6 glioma cells using the SNAP RNA isolation kit and treated with DNase I. cDNA synthesis was performed at 37°C for 60 min with a total reaction volume of 60 μl consisting of 300 ng oligo$(dT)_{12\text{-}18}$ primer, 5 μg total RNA, 3 mM dNTPs, 6.7 μM dithiothreitol (DTT), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$ and 3.3 Units MMLV reverse transcriptase. Control reactions were performed by omitting reverse transcriptase.

For PCR, control and reverse transcriptase-treated solutions (2 μl) were amplified using Ready To Go™ PCR beads. The amplification consisted of 30 cycles of: 30 s at 94°C, 30 s at 56°C and 1 min at 72°C. A final 10 min 72°C elongation step followed and samples were held at –9°C then analyzed by electrophoresis on a 1.0% agarose gel. DNA bands were viewed and photographed under UV light following ethidium bromide staining.

rENT1 was amplified with the 5' primer 5'-CACCATGACAACCAGTCACCAG -3' and the 3' primer 5'-TGAAGGCACCTGGTTTCTGTC -3' to produce a 1.76-kb product. rENT2 was amplified using the 5' primer 5'-TTACCCAACCTGCACCCTCTC -3' and the 3' primer 5'-GTAGCCACATTGCATATGGTGA -3' to produce a 1.67-kb product (Yao et al., 1997). The presence of mRNA for glyceraldehyde-3'-phosphate dehydrogenase (GAPDH), a ubiquitous housekeeping gene, was used as a loading control, and was detected using the 5' primer 5'-GCTGGGGCTCACCTGAAGGG-3' and the 3' primer 5'-GGATGACCTTGCCCACAGCC-3' to amplify a 343-bp DNA product (bases 346 to 688) from the rat GAPDH cDNA.

Nucleoside Uptake Assays

Rat C6 glioma cells were cultured as previously described (Stanness et al., 1997) in 24-well plates until confluent. Cells were washed twice in physiological buffer (in mM; NaCl, 118; HEPES, 25; KCl, 4.9; K_2HPO_4, 1.4; $MgCl_2$, 1.2; $CaCl_2$, 1; glucose, 11; to pH 7.4 with NaOH) or buffer in which NaCl was replaced with N-methylglucamine (NMG). Cells were incubated with [^{3}H]adenosine (1 μM) or [^{3}H]formycin B (10 μM) in 250 μl of Na^+ or NMG^+ buffer for times ranging from 0-300 sec. To examine the effect of nucleoside transport inhibitors, cells were exposed to graded concentrations of NBMPR or DPR (1 nM – 30 μM) prior to and during the uptake assays. To examine the effect of ATP depletion, cells were treated with 5 mM IAA and 1 mM NaCN in glucose-free buffer for 10 min prior to [^{3}H]adenosine uptake. For kinetic analysis of [^{3}H]adenosine uptake, cells were incubated with graded concentrations of [^{3}H]adenosine (300 nM – 30 μM) for 1 min. To terminate uptake, the extracellular solutions were aspirated and the cells were rapidly washed twice with ice-cold Na^+ or NMG^+ buffer. Cellular protein was dissolved by incubating cells overnight with NaOH (1M; 500 μl) at 37°C. Separate aliquots of the dissolved cells were used for protein determination, using the Bradford assay, and for liquid scintillation spectroscopy. Uptake values were determined from the radioactivity in the dissolved cells and are expressed as pmol/mg cellular protein using the specific activity of the uptake buffer.

Nucleoside Release Assays

Release assays were performed with confluent C6 cells cultured in 24-well plates. Cells were washed twice in Na^+ buffer then incubated with [^{3}H]adenine (10 μM) or [^{3}H]formycin B (10 μM) at 22°C. This inwardly-directed concentration gradient resulted

in tritium accumulation within the cells. After 30 min, cells were washed twice with buffer to remove extracellular tritium. Cellular release of [^{3}H]formycin B was initiated by incubating the cells in fresh buffer, thus providing an outwardly-directed concentration gradient. As [^{3}H]adenine is rapidly metabolized to [^{3}H]adenine nucleotides by these cells, cells were treated with glucose-free buffer alone or with the glycolytic inhibitor IAA (5 mM) and the oxidative phosphorylation inhibitor NaCN (1 mM) to stimulate metabolism of [^{3}H]adenine nucleotides and release of the [^{3}H]purine metabolites. Trypan blue exclusion staining indicated no significant difference in viability ($\geq$ 90%) between buffer treated and IAA/NaCN treated cells (data not shown). The effects of the nucleoside transport inhibitors DPR (1 nM – 30 μM) or NBMPR (1 nM – 30 μM) on [^{3}H]purine release were tested. In addition, the contribution of specific enzymes to the metabolism and release of [^{3}H]purines was examined using 1 μM EHNA to inhibit adenosine deaminase, 100 μM EHNA to inhibit both adenosine deaminase and AMP deaminase (Fishbein et al., 1981), and 10 μM BCX-34 to inhibit purine nucleoside phosphorylase (PNP) (Jurkowitz et al., 1998). In some experiments cells were treated with IAA and NaCN then washed and returned to Na^+ buffer to mimic a return to normoxic conditions. [^{3}H]Purine release into the extracellular media was quantified by liquid scintillation spectroscopy and thin layer chromatography. Cells were dissolved in NaOH and analyzed for protein content. Release data are expressed as pmol/mg cellular protein using the specific activity of the loading buffer.

Thin Layer Chromatography

The method of Schrader and Gerlach (Schrader and Gerlach, 1976) was used to identify the [^{3}H]purines released from C6 cells. Briefly, n-butanol, ethyl acetate, methanol and ammonium hydroxide (7:4:3:4) were mixed, placed in a TLC tank and allowed to equilibrate for 90 min. Extracellular media (20 μl) obtained from the nucleoside release assays was spotted onto Silica Gel GF plates with 5 μl of cold carrier. Cold carrier consisted of 15 mM each of adenosine, inosine, hypoxanthine, adenine and AMP, 7.5 mM uric acid and 6.5 mM xanthine. Plates were run for 3 hours, and samples migrated in the order AMP/uric acid, inosine, xanthine, hypoxanthine, adenosine and adenine. Spots were outlined under ultraviolet light, scraped, transferred to scintillation vials and 500 μl of 0.2 M HCl was added to each tube. After 1 hr, scintillation fluid (5 ml) was added and radioactivity was determined using scintillation spectrometry.

Adenosine deaminase and AMP deaminase assays

Adenosine deaminase and AMP deaminase assays were performed as previously described (Martinek, 1963; Padua et al., 1990). Briefly, C6 cells were homogenized in 0.2M phosphate buffer (pH 7.4), and homogenate protein was adjusted to 0.5 mg/ml. 50 μL of homogenate was combined with 50 μL of 0.2M phosphate buffer containing either 20mM AMP (for AMP deaminase) or 1mM adenosine (for adenosine deaminase) in the presence or absence of 0.1-500 μM EHNA for 60 min at 37°C. The reactions were terminated with 500 μL of phenol color reagent consisting of 1% (wt/vol) phenol and 0.005% (wt/vol) sodium nitroferricyanide in water. Maximal color from the reaction developed in 15min at 37°C upon the addition of 500 μL of a solution containing 0.125 M NaOH and 0.042% sodium hypochlorite. The amount of activity for either enzyme

corresponded stoichiometrically (1:1) with the production of ammonia. Standard calibration curves for ammonia production were calculated using ammonium sulfate. AMP deaminase or adenosine deaminase activity was determined measuring absorbance at 650nM with a Molecular Devices Emax precision microplate reader.

[^{3}H]NBMPR binding assays

Cells were washed with Na^+ buffer then incubated (22 °C) with 0.1 – 5 nM [^{3}H]NBMPR in the absence or presence of 1 μM nitrobenzylthioguanosine. After a 1 hr incubation interval, cells were washed twice with ice-cold Na^+ buffer then dissolved with NaOH. Samples were analyzed for both tritium and protein content.

Data Analysis

Each experiment was performed at least three times in duplicate or triplicate, unless otherwise stated. All values are expressed as means ± S.E. and statistical significance was determined by ANOVA followed by Bonferroni's post hoc test. Statistical analyses were performed using the software package GraphPad PRISM Version 2.0.

Results

[^{3}H]Nucleoside uptake

To identify the nucleoside transporter subtypes present in rat C6 glioma cells we performed [^{3}H]adenosine and [^{3}H]formycin B uptake experiments. [^{3}H]Adenosine accumulation in C6 cells was linear for time intervals up to 5 min (figure 15A, 15B and 15C). This could indicate either concentrative transport or equilibrative transport followed by intracellular metabolism of [^{3}H]adenosine to [^{3}H]adenine nucleotides (figure 19; reaction 7). To distinguish between these possibilities, we first used the poorly metabolizable nucleoside transporter permeant [^{3}H]formycin B. The accumulation of [^{3}H]formycin B was saturable indicating accumulation via an equilibrative transporter. [^{3}H]Formycin B accumulation in the C6 cells had a $t_{½}$ of 60 ± 9 s and maximal accumulation of 56 ± 3 pmol/mg protein. Second, the presence of concentrative Na^+-dependent transport was investigated by replacing NaCl in the buffer with NMG^+. At intervals up to 5 min, [^{3}H]adenosine accumulation was not significantly affected by Na^+ substitution (figure 15B). Third, depletion of intracellular ATP is another method of abolishing concentrative nucleoside transport (Ohkubo et al., 1991; Thampy and Barnes, 1983a); however, treatment of C6 cells with 5 mM IAA and 1 mM NaCN had no significant effect on [^{3}H]adenosine uptake (figure 15C). Thus, no evidence for concentrative, Na^+-dependent nucleoside transport was found in these cells. Concentration-dependent accumulation of adenosine (0.3-30 μM) in 1 min had a K_m value of 12 ± 1 μM and a V_{max} value of 157 ± 23 pmol/mg protein/min (figure 15D). To determine which equilibrative nucleoside transporter subtypes are present in C6 cells, we performed RT-PCR using rENT1 or rENT2 sequence-specific primers, assayed

inhibition of [^{3}H]adenosine or [^{3}H]formycin B accumulation by DPR or NBMPR, and tested for specific binding of [^{3}H]NBMPR. RT-PCR analysis of C6 cell total RNA indicated that rENT2 was the predominant nucleoside transporter as a strong band was seen at 1.67 kb using rENT2 primers while a very weak band was seen at 1.76 kb using rENT1 primers (figure 16A). NBMPR inhibited the accumulation of [^{3}H]adenosine (1 μM; 1 min) with an IC_{50} value of 1.05 ± 0.25 μM while DPR had an IC_{50} value of 119 ± 17 nM (figure 16B). Similar results were seen for the inhibition of [^{3}H]formycin B accumulation with IC_{50} values for NBMPR and DPR of 5.5 ± 0.75 μM and 152 ± 42 nM, respectively (figure 16C). Specific binding of [^{3}H]NBMPR was not detected in C6 cells (data not shown). Thus, RT-PCR analysis, IC_{50} values for NBMPR of ≥ 1 μM and the lack of specific binding sites for [^{3}H]NBMPR indicate that rENT2 is the predominant nucleoside transporter in C6 cells.

[^{3}H]Purine release

ENT1 transporters have been reported to mediate both accumulation and release of nucleosides (Gu et al., 1996; Sinclair et al., 2000a) but similar data is not available for ENT2. Therefore, we examined the cellular release of [^{3}H]formycin B from C6 glioma cells. A hyperbolic release profile was observed (figure 17A) and [^{3}H]formycin B release in 1 min was blocked by DPR and NBMPR with IC_{50} values of 370 ± 12 nM and 3.3 ± 0.4 μM, respectively (figure 17B).

To determine if rENT2 transporters can mediate [^{3}H]adenosine release from C6 cells, we used ATP depleting conditions, which have been used previously to elevate

Figure 15

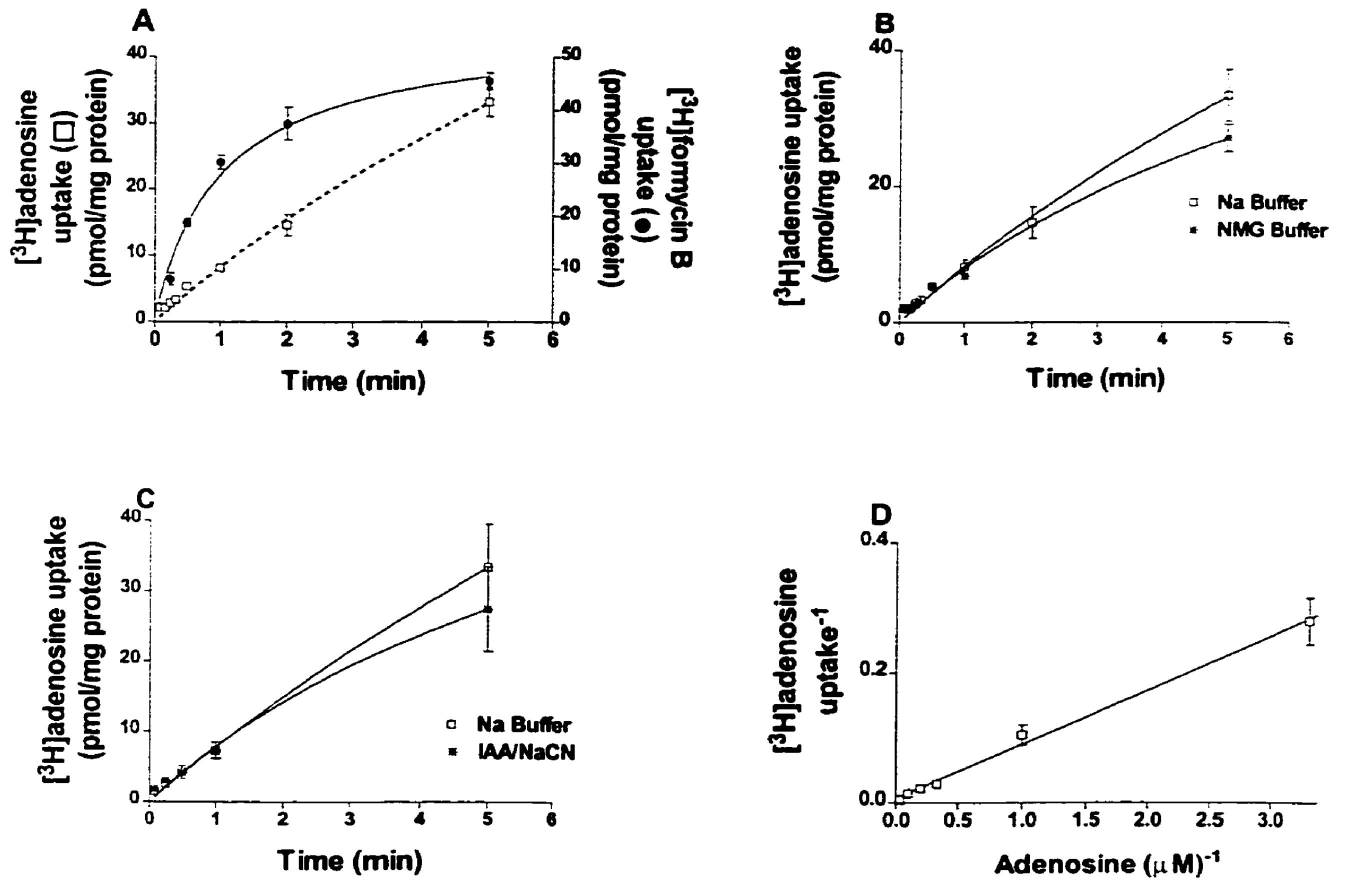


Figure 15: continued

Accumulation of 1 μM [^{3}H]adenosine or 10 μM [^{3}H]formycin B in C6 cells.

Nucleoside accumulation was measured by incubating C6 cells with 1 μM [^{3}H]adenosine or 10 μM [^{3}H]formycin B for 5-300 s (A). Na^+-dependent transport was investigated in C6 cells by replacing NaCl with NMG^+ as described in the text (B). Cells were treated for 10 min with 5 mM IAA and 1 mM NaCN to deplete the cells of ATP and accumulation of 1 μM [^{3}H]adenosine was examined (C). Concentration-dependent accumulation of [^{3}H]adenosine (0.3-30 μM) during 1 min time intervals was analyzed by double reciprocal analysis (D). Accumulation is expressed as pmol/mg cellular protein. Symbols represent means and error bars represent SEM. Experiments were performed at least three times in duplicate.

intracellular adenosine levels and to initiate adenosine release (Daval et al., 1980; Reyes et al., 1995). To radiolabel adenine nucleotides without activating adenosine receptors, we incubated C6 cells with 10 μM [^{3}H]adenine for 30 min. Intracellular [^{3}H]adenine nucleotides accounted for approximately 95% of the total tritium within the cells (n=2). Cells were then treated for 10 min with 5 mM IAA and 1 mM NaCN to block glycolytic and oxidative ATP generation. This resulted in net catabolism of [^{3}H]adenine nucleotides (Ogata et al., 1995). Previously, we used this protocol to stimulate [^{3}H]adenosine release from DDT_1 MF-2 smooth muscle cells (Sinclair et al., 2000a). This protocol increased the release of [^{3}H]purines from C6 cells; however, NBMPR and DPR had no inhibitory effects on this release (figure 18A). TLC analysis of the extracellular medium of IAA/NaCN-stimulated cells indicated that this treatment protocol resulted primarily in the cellular release of [^{3}H]hypoxanthine and not [^{3}H]adenosine (table 5; experiment 2A). HPLC analysis confirmed that the release of endogenous purines matched the release of [^{3}H]purines (data not shown).

A recent report using primary cultures of rat astrocytes demonstrated that hypoxia/hypoglycemia induced the release of hypoxanthine; however, returning the cells to normoxic/normoglycemic buffer induced cellular release of both hypoxanthine and adenosine (Ciccarelli et al., 1999). We investigated this phenomenon in C6 cells and found a 465% increase in release of [^{3}H]purines from cells exposed first to IAA/NaCN and then to buffer relative to cells exposed to buffer alone (figure 18B). Cells that were only exposed to IAA/NaCN exhibited an intermediate level of purine release, 110 ± 25 %

Figure 16

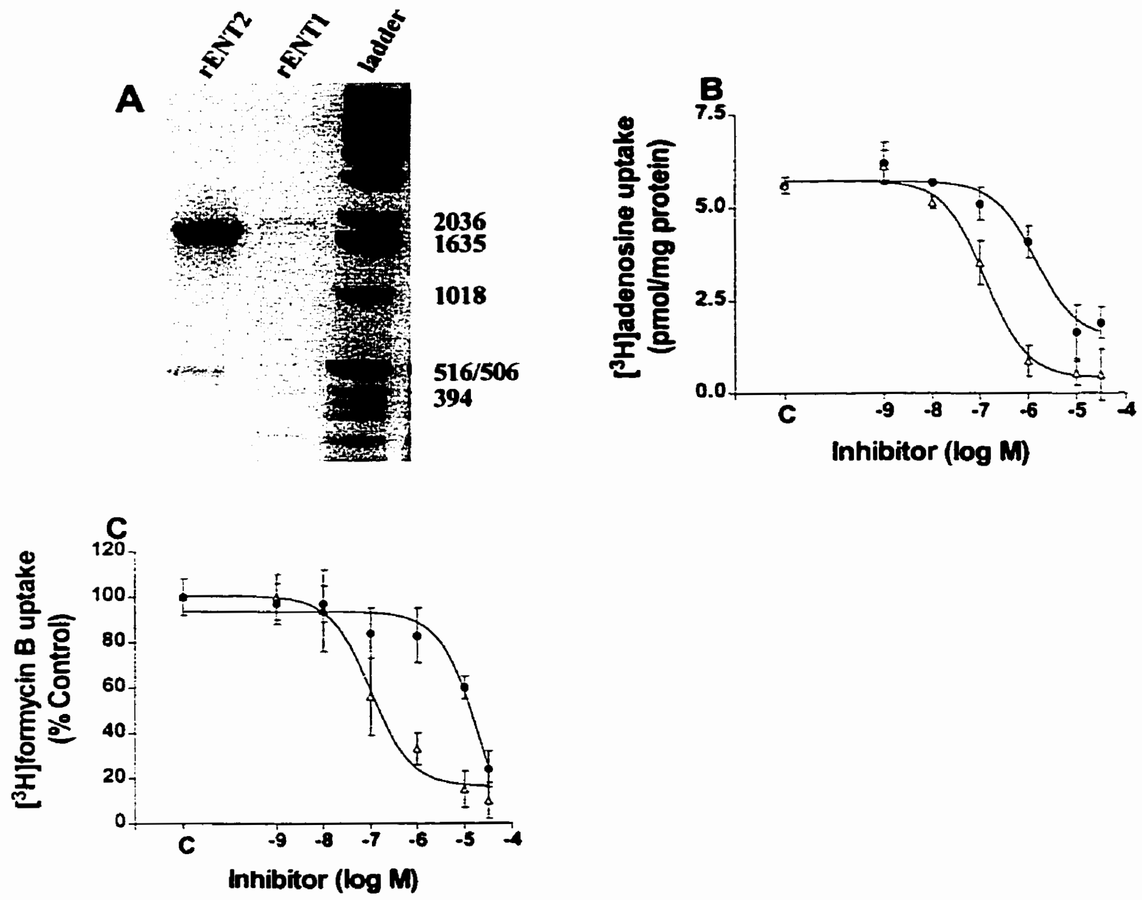
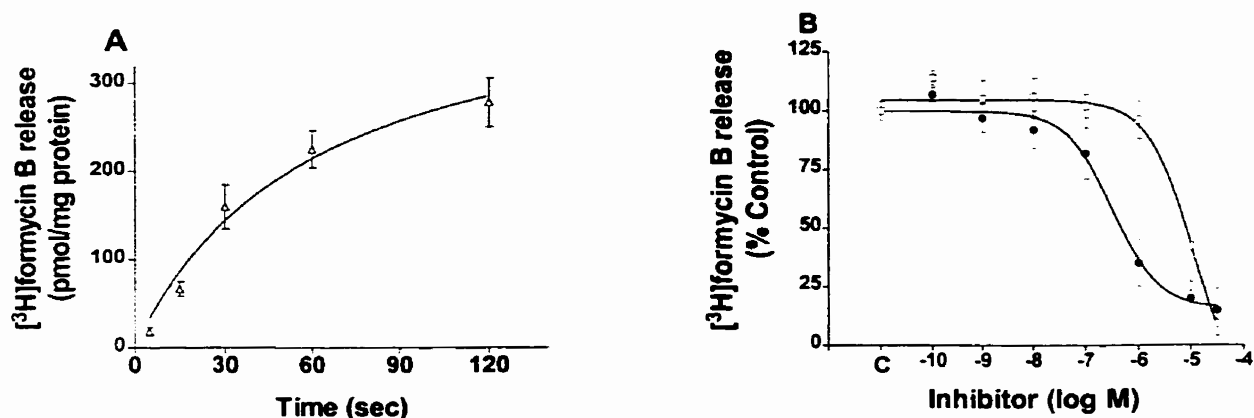


Figure 16: continued

Determination of equilibrative nucleoside transporter subtypes in C6 rat glioma cells by RT-PCR and inhibition of nucleoside accumulation. RT-PCR analysis of total RNA for presence of rENT1 (1.76 kb product) or rENT2 (1.67 kb product) transcript was performed as described in the text (A). [^{3}H]Nucleoside accumulation was measured by incubating the C6 cells with 1 μM [^{3}H]adenosine (B) or 10 μM [^{3}H]formycin B (C) for 1 min in the presence or absence of 0.1 nM- 10 μM concentrations of NBMPR (closed circle) or DPR (open triangles). DPR and NBMPR inhibited [^{3}H]adenosine accumulation with IC_{50} values of 119 ± 17 nM and 1.1 ± 0.25 μM, respectively, and inhibited [^{3}H]formycin B accumulation with IC_{50} values of 152 ± 42 nM and 5.5 ± 0.75 μM, respectively. Data in B and C are expressed as pmol/mg cellular protein. Symbols represent means and error bars represent SEM. Experiments were performed at least three times in duplicate.

Figure 17



[^{3}H]Formycin B release from C6 cells. Cells were loaded for 30 min with 10 μM [^{3}H]formycin B, rinsed and placed into buffer for 5-120s (A) or in buffer containing graded concentrations (0.1 nM – 10 μM) of NBMPR or DPR for 1 min (B). [^{3}H]Formycin B release was inhibited by DPR (closed circles) and NBMPR (open squares) with IC_{50} values of 370 ± 12 nM and 3.3 ± 0.4 μM, respectively. Data in (A) are expressed as pmol [^{3}H]formycin B released/ mg cellular protein. Data in (B) are expressed as a percentage of release into buffer alone (control, C); control values were 118 ± 28 pmol/min/mg protein. Symbols represent means and error bars represent SEM. Experiments were performed at least three times in duplicate.

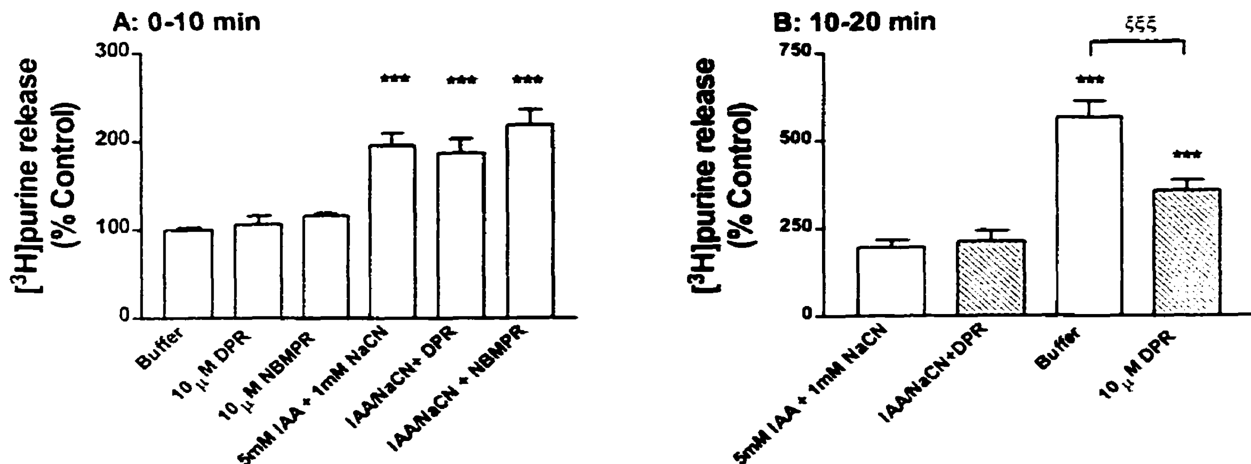
increase over buffer treated cells. While DPR had no significant effect on purine release induced by IAA/NaCN treatment, release evoked by removal of IAA/NaCN was inhibited by approximately 50% (figure 18B). TLC analysis indicated that the increase in tritium release after removal of IAA/NaCN was due to [^{3}H]inosine (table 5; experiment 2B); thus, in contrast to the previous study (Ciccarelli et al., 1999), we found no evidence for release of [^{3}H]adenosine during or following IAA/NaCN treatment.

We investigated further the lack of [^{3}H]adenosine release from IAA/NaCN-stimulated C6 cells by examining the roles of adenosine deaminase, AMP deaminase and PNP in the production and release of [^{3}H]purines. The role of adenosine deaminase was investigated with 1 μM EHNA, which inhibited adenosine deaminase in these cells by ~90% (n=2). EHNA (1 μM) alone or in combination with 10 μM DPR did not attenuate either the increase in [^{3}H]purine release during IAA/NaCN treatment or following return to Na^+ buffer (n = 4 , data not shown). This indicates that [^{3}H]hypoxanthine production was not via intermediary formation of [^{3}H]adenosine, but that catabolism occurred by the pathway AMP → IMP → inosine → hypoxanthine (shown in dark arrows in figure 19). The role of AMP deaminase was investigated using 100 μM EHNA, which inhibited AMP deaminase by ~90% (n=2) as well as adenosine deaminase. EHNA (100 μM) did not significantly affect IAA/NaCN-induced [^{3}H]purine release, suggesting that AMP was metabolized by AMP-preferring 5'nucleotidase to adenosine (figure 19; reaction 6) which exited the cells via rENT2 nucleoside transporters. Following removal of IAA/NaCN, 100μM EHNA inhibited release by 75% (table 6; experiment 3B), indicating reduced adenosine formation and release during these conditions. Thus, with AMP deaminase inhibited and ATP-depleting conditions removed, AMP kinase (figure 19; reaction 8)

activity may have resumed in addition to AMP-preferring 5'nucleotidase activity. EHNA (100 μM) in conjunction with 10 μM DPR was able to inhibit the increase in [^{3}H]purine release induced during IAA/NaCN treatment and following return to Na^+ buffer by 78% and 96%, respectively (table 6; experiments 4A and 4B). The effect of DPR was greater in the presence of IAA/NaCN (table 6; compare experiments 3A and 4A) than following their removal (table 6; compare experiments 3B and 4B). This provides further evidence that, in the presence of 100 μM EHNA to inhibit AMP deaminase, a greater amount of adenosine was produced in the presence than following removal of IAA/NaCN.

PNP consumes inorganic phosphate to convert inosine to hypoxanthine and ribose-1-phosphate. The role of PNP in [^{3}H]purine release was investigated with the cell permeable PNP inhibitor BCX-34 (10 μM) (Jurkowitz et al., 1998; Litsky et al., 1999; Morris and Montgomery, 1998). Similar to 100 μM EHNA, BCX-34 (10 μM) did not significantly affect the IAA/NaCN-mediated release of [^{3}H]purines (table 6; experiment 5A) but caused a 35% inhibition of the release that occurred following removal of IAA/NaCN (table 6; experiment 5B). Thus, with PNP inhibited and ATP-depleting conditions removed, inosine release was decreased possibly due to product inhibition of IMP-preferring 5'nucleotidase (Worku and Newby, 1982). In combination with DPR, BCX-34 inhibited the increase in [^{3}H]purine release both during IAA/NaCN treatment and following return to Na^+ buffer by 78% and 91%, respectively (table 6; experiments 6A and 6B). Thus, the experiments during and following ATP-depleting conditions indicate that [^{3}H]inosine and [^{3}H]hypoxanthine were produced primarily by an AMP deaminase-dependent pathway not an adenosine deaminase-dependent pathway (figure 19; reactions 1 and 2).

Figure 18



Effect of nucleoside transport inhibitors on purines released from C6 cells during or following treatment with IAA and NaCN. (A) Cells were loaded for 30 min with 10 μM [^{3}H]adenine, rinsed and resuspended into buffer or 5 mM IAA and 1 mM NaCN in the presence or absence of 10 μM DPR or 10 μM NBMPR for 10 min (0-10 min interval). Supernatants were removed and analyzed for tritium content. (B) Cells treated with IAA/NaCN (open bars) or IAA/NaCN plus DPR (hatched bars) for the first 10 min interval were washed and placed into buffer or IAA/NaCN in the absence or presence of 10 μM DPR as indicated for another 10 min (10-20 min interval). Supernatants were again removed and analyzed for tritium content. Data are means ± SEM and are expressed as percentages of control values (buffer-treated cells) for the 0-10 or 10-20 min intervals. Control values were 177 ± 22 pmol/mg protein for 0-10 min and 87 ± 19 pmol/mg protein for 10-20 min. Statistical significance was determined by ANOVA followed by Bonferroni's post hoc test (*** $p<0.001$ from respective control value; ξξξ $p<0.001$ from buffer). Experiments were performed at least 4X in duplicate.

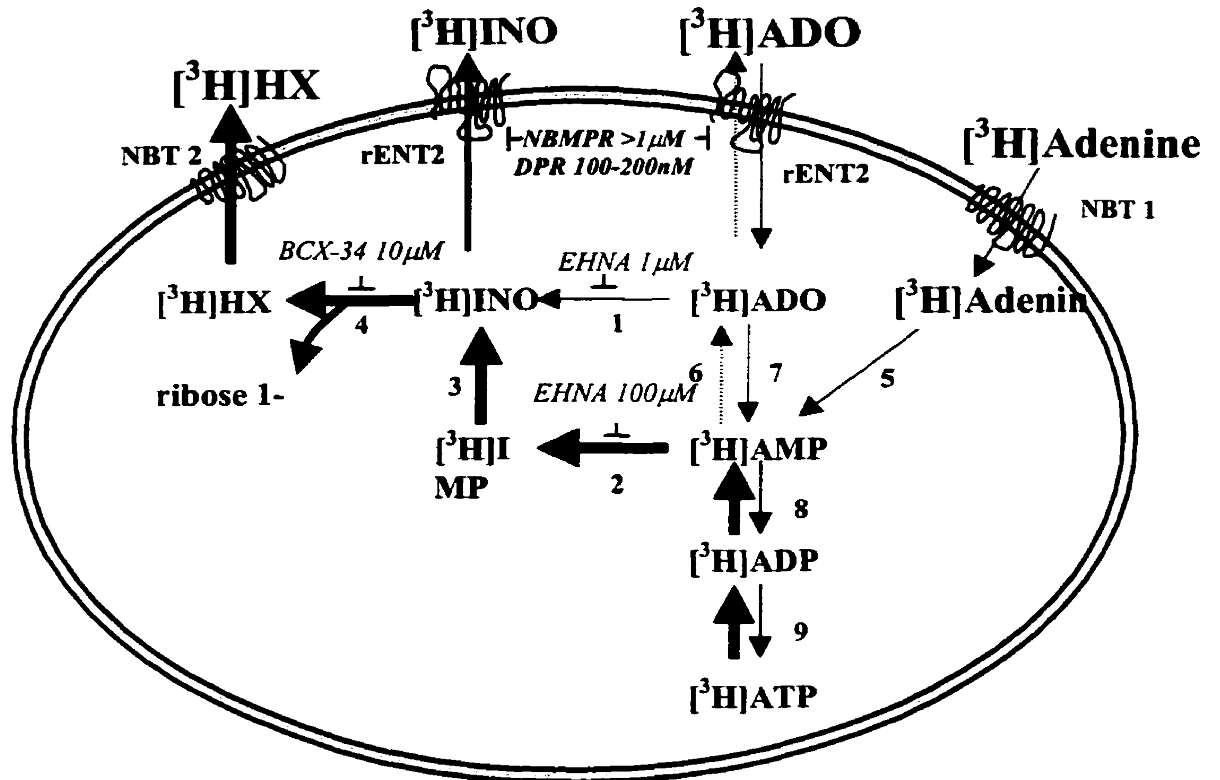
Table 5

TLC analysis of purines released (pmol/mg protein) from rat C6 glioma cells

Expt	Treatment	Inosine	Hypoxanthine	Adenosine	Adenine
1	A) Buffer	21 ± 6	28 ± 7	8 ± 3	23 ± 7
	B) Buffer	19 ± 6	20 ± 9	16 ± 6	11 ± 4
2	A) IAA/NaCN	52 ± 5 *	185 ± 19 ***	4 ± 2	39 ± 8
	B) Buffer	129 ± 12 ***	168 ± 14 ***	11 ± 4	13 ± 4
3	A) IAA/NaCN + DPR	39 ± 10	191 ± 23 ***	12 ± 5	41 ± 8
	B) DPR	25 ± 7 ξξξ	148 ± 16 ***	9 ± 2	9 ± 4

Cells were loaded with 10 μM [^{3}H]adenine for 30 min then rinsed and incubated with buffer (experiment 1A), 5 mM IAA and 1 mM NaCN (experiment 2A) or IAA, NaCN and 10 μM DPR (experiment 3A). After 10 min, solutions were removed and analyzed by TLC for purine content. Cells in experimental experiments 1B and 2B were washed and placed into buffer; cells in experiment 3B were washed and incubated with 10 μM DPR. After 10 min solutions were again removed and analyzed for purine content. TLC analysis was performed as described in the text. AMP/uric acid and xanthine were below detectable levels. Data are expressed as pmol purine released/mg cellular protein. Statistical significance was determined between experimental groups during the same time interval by ANOVA followed by Bonferroni's post hoc test (* $p<0.05$, *** $p<0.001$ from experiment 1; ξξξ $p<0.001$ from experiment 2; n=3)

Figure 19



Schematic diagram of IAA/NaCN-induced [^{3}H]purine pathways in rat C6 glioma cells. Bold arrows represent the pathways of purine metabolism during 5 mM IAA and 1 mM NaCN treatment. Dashed arrows represent pathways that are only detected in the presence of 100 µM EHNA. Narrow arrows represent metabolism of either [^{3}H]adenosine or [^{3}H]adenine during loading phase. ADO- adenosine; INO- inosine; HX- hypoxanthine; rENT2- rat equilibrative nucleoside transporter 2; NBT 1- adenine preferring nucleobase transporter; NBT 2- hypoxanthine preferring nucleobase transporter; 1 - adenosine deaminase; 2 - AMP deaminase; 3 - IMP preferring 5'nucleotidase; 4 - purine nucleoside phosphorylase; 5 - adenine phosphoribosyltransferase; 6 - AMP preferring 5'nucleotidase; 7 – adenosine kinase; 8 – AMP kinase; 9 – ADP kinase.

Table 6

Effect of inhibition of adenosine deaminase and AMP deaminase or purine nucleoside phosphorylase on IAA/NaCN-evoked [^{3}H]purine release (pmol/mg protein or % control).

Expt	Treatment	[^{3}H]purines	% Control
1	A) IAA/NaCN	193 ± 20	100 ± 4
	B) Buffer	299 ± 16	100 ± 4
2	A) IAA/ NaCN + DPR	174 ± 17	98 ± 7
	B) DPR	164 ± 11 ***	55 ± 4 ***
3	A) IAA/NaCN + EHNA	174 ± 24	75 ± 10
	B) EHNA	77 ± 15 ***	25 ± 5 ***
4	A) IAA/NaCN + EHNA + DPR	54 ± 17 ***, ξξξ	22 ± 6 ***, ξξξ
	B) EHNA + DPR	15 ± 9 ***	4 ± 2 ***, ξξξ
5	A) IAA/NaCN + BCX-34	111 ± 13	76 ± 8
	B) BCX-34	183 ± 13 ***	65 ± 4 ***
6	A) IAA/NaCN + BCX-34 + DPR	33 ± 11 ***, δδδ	22 ± 7 ***, δδδ
	B) BCX-34 + DPR	26 ± 5 ***, δδδ	9 ± 2 ***,δδδ

Table 6: continued

Cells were loaded for 30 min with 10 μM [^{3}H]adenine, rinsed and incubated with buffer containing 5 mM IAA and 1 mM NaCN (experiment 1A) or in the added presence of 10 μM DPR (experiment 2A), 100 μM EHNA (experiment 3A), DPR and EHNA (experiment 4A), 10 μM BCX-34 (experiment 5A) or DPR and BCX-34 (experiment 6A). After 10 min, solutions were removed and analyzed for [^{3}H]purines using scintillation spectroscopy. Cells were washed and incubated with buffer (experiment 1B), DPR (experiment 2B), EHNA (experiment 3B), EHNA + DPR (experiment 4B), BCX-34 (experiment 5B) or BCX-34 + DPR (experiment 6B). Note the absence of IAA and NaCN in the second incubation interval. After 10 min solutions were again removed and analyzed for [^{3}H]purines. [^{3}H]Purines are expressed as pmol purine released/mg cellular protein. Statistical significance was determined between groups during the same time interval by ANOVA followed by Bonferroni's post hoc test (*** $p<0.001$ from experiment 1; ξξξ $p<0.001$ from experiment 3; δδδ $p<0.001$ from experiment 5; n = 10 for experiments 1 and 2, n = 4 for experiments 3 and 4, n = 6 for experiments 5 and 6).

Discussion

A main finding of this study was that rat C6 glioma cells express nucleoside transporters primarily of the ENT2-subtype. While these transporters are bidirectional, as indicated by both uptake and release of the purine nucleoside [^{3}H]formycin B, ATP-depleting conditions did not induce cellular release of adenosine. Our evidence indicates that AMP was metabolized to IMP via AMP deaminase, IMP was dephosphorylated to inosine by IMP-preferring 5'nucleotidase and inosine was phosphorolyzed to produce hypoxanthine and ribose-1-phosphate. Hypoxanthine and lesser quantities of inosine were released from the C6 cells.

C6 cells exhibited a linear rate of accumulation of adenosine for time intervals up to 5 min. However, as removal of Na^+ or depletion of intracellular ATP had no effect on adenosine accumulation, the apparent concentration of adenosine within C6 cells was likely due to intracellular metabolism and preservation of an inwardly-directed concentration gradient for adenosine rather than the presence of Na^+-dependent nucleoside transporters. Previously, Na^+-dependent nucleoside transport has been reported in primary cultures of rat cerebellar and spinal cord astrocytes (Hosli and Hosli, 1988) but not in cortical or hippocampal astrocytes from human, rat or chick brain (Gu et al., 1996; Ohkubo et al., 1991; Thampy and Barnes, 1983a).

Kinetic constants for adenosine transport have been reported for astrocytes from a variety of species and brain regions. Our data indicate a K_m value of 12 μM and a V_{max} value of 156 pmol/min/mg protein. Previously, K_m values of 3.4 – 12 μM and V_{max} values of 150 – 360 pmol/min/mg protein were reported for primary cultures from human, mouse and chick (Bender and Hertz, 1986; Gu et al., 1996; Hertz, 1978; Matz

and Hertz, 1990; Thampy and Barnes, 1983a). Thus, adenosine transport kinetics appear similar among C6 cells and astrocytes from several species.

Rat brain is known to express high levels of rENT2; most studies report finding 40 – 60 % rENT2, <10% Na^+-dependent transport and the remainder rENT1 (Geiger et al., 1988; Johnston and Geiger, 1990; Lee and Jarvis, 1988). This study demonstrates that C6 cells have mRNA transcript primarily for rENT2. The RT-PCR data was supported by data from functional assays showing that NBMPR inhibited accumulation of adenosine and formycin B with IC_{50} values of ≥ 1 μM. Therefore, C6 cells express almost exclusively rENT2, making these cells a useful model for examining the function and regulation of these transporters.

DPR is less useful than NBMPR to distinguish ENT1 from ENT2 because it exhibits species differences in potency. DPR inhibits hENT1 at nanomolar concentrations but micromolar concentrations are usually required to inhibit rENT1 (Griffiths et al., 1997a; Yao et al., 1997). Typically, both DPR and NBMPR have micromolar affinity for h- and rENT2 (Crawford et al., 1998; Griffiths et al., 1997b; Yao et al., 1997) although recently DPR was reported to inhibit hENT2 in transfected cells (Ward et al., 2000) and rENT2 in rat synaptosomes (Sweeney et al., 1993) with IC_{50} values between 150-360 nM. In the present study, DPR exhibited higher affinity for inhibition of rENT2 in C6 cells than for recombinant rENT2 expressed in *Xenopus* oocytes (Yao et al., 1997). The reason for the high affinity of DPR for rENT2 in C6 cells is not known but is currently under investigation. It may indicate multiple isoforms of this nucleoside transporter. The values for NBMPR inhibition of rENT2 reported here are similar to those seen in recombinant systems and cell culture models (Griffith and Jarvis,

1996; Yao et al., 1997). DPR and NBMPR inhibited adenosine uptake into primary mouse cortical astrocytes with similar IC_{50} values (DPR 100-390 nM; NBMPR 1.64-5.33 μM) (Bender and Hertz, 1986; Bender and Hertz, 1987) to those reported here for C6 cells. Thus, nucleoside transporters in mouse cortical astrocytes may closely resemble the ENT2 found in rat C6 cells. In contrast, human fetal astrocytes appear to express both hENT1 and hENT2 transporter activity and exhibit biphasic inhibition curves for DPR and NBMPR (Gu et al., 1996).

Directional symmetry of equilibrative nucleoside transporters has been reported in a number of tissues containing ENT1 (Gu et al., 1996; Sinclair et al., 2000a). Prior to assessing the role of ENT2 transporters in mediating the cellular release of adenosine during ATP-depleting conditions, we tested for directional symmetry of [^{3}H]formycin B transport. Both uptake and release of [^{3}H]formycin B were evident and were inhibited similarly by NBMPR or DPR. These data indicate a potential role of ENT2 in cellular release of adenosine. However, under ATP-depleting conditions with IAA and NaCN the C6 cells did not release adenosine but instead released hypoxanthine and, to a lesser extent, inosine. Interestingly, adenine (1-100 μM) was unable to inhibit the release of hypoxanthine and hypoxanthine (10-300 μM) was unable to inhibit the uptake of [^{3}H]adenine (10 μM) in C6 cells (data not shown). This suggests the presence of at least two nucleobase transporters in these cells (figure 19; NBT 1 and 2). The nucleobase transporter involved in adenine transport appears similar to that described in human erythrocytes and LLC-PK_1 cells (Griffith and Jarvis, 1996). Hypoxanthine release may occur via an equilibrative nucleobase transporter similar to that reported in human placenta (Barros, 1994) or possibly by reversal of Na^+ dependent nucleobase transport as

has been reported for reversal of Na^+ dependent nucleoside transport during conditions that perturb the Na^+ gradient (Borgland and Parkinson, 1997).

The pathways involved in IAA/NaCN-induced [^{3}H]hypoxanthine release from rat C6 glioma cells are detailed in figure 19: AMP → IMP → inosine → hypoxanthine. Our results are in contrast to previous reports of various stimuli inducing adenosine release from primary rat astrocytes (Ballerini et al., 1995; Caciagli et al., 1989; Ciccarelli et al., 1994; Ciccarelli et al., 1999). However, these reports do not differentiate between adenosine release *per se* and adenine nucleotide release followed by extracellular metabolism to adenosine. As primary cultures of astrocytes can release ATP (Guthrie et al., 1999), and as the nucleoside transport inhibitor propentofylline had no effect on adenosine release (Caciagli et al., 1999), it is possible that the primary cultures of astrocytes released ATP, which was metabolized extracellularly to adenosine. In contrast, C6 cells have been reported to be incapable of ATP release (Cotrina et al., 1998b).

Treatment of chick glia with deoxyglucose and oligomycin (Meghji et al., 1989) increased release of adenosine, inosine and, in particular, hypoxanthine. In our experiments, we did not see increased adenosine release from C6 cells treated with IAA and NaCN (table 1) or deoxyglucose and dinitrophenol (data not shown). The differences between our results and those with chick glia might be due to differences in nucleoside transporter subtypes or metabolic enzymes expressed in these two cell types. Regardless, data from several studies indicate that hypoxanthine is the major constituent of purine release from astrocytes during ATP-depleting conditions (Ciccarelli et al., 1999; Meghji et al., 1989).

Purine metabolism may differ between C6 cells and primary astrocytes. While the removal of hypoxic stimulus induced adenosine release in astrocytes (Ciccarelli et al., 1999), this was not observed with C6 cells following removal of IAA/NaCN. There was, however, a large increase in inosine release, which was effectively inhibited by DPR. As 1μM EHNA did not decrease inosine release, it is unlikely that the difference in results was due to higher adenosine deaminase activity in C6 cells compared to primary astrocytes. As has been described for T lymphocytes (Barankiewicz et al., 1990), adenosine release from C6 cells may be precluded by high levels of AMP deaminase (figure 19; reaction 2) and/or adenosine kinase (figure 19; reaction 7) or low levels of AMP 5'nucleotidase (figure 19; reaction 6). Simultaneous inhibition of AMP deaminase and adenosine deaminase, using 100 μM EHNA, induced release that was sensitive to inhibition by DPR, indicating that adenosine was formed and released when these enzymes were inhibited.

Removal of IAA/NaCN might be expected to enhance purine nucleotide salvage and decrease purine release; in fact, the opposite was observed both in this study and a previous report (Ciccarelli et al., 1999). One possible explanation for the increase in purine release may be that purine salvage enzymes can consume nucleotides and, thus, may simultaneously promote both salvage and release of purines. For example, conversion of ribose-1-phosphate to phosphoribosyl pyrophosphate (PRPP) by ribose-phosphate pyrophosphokinase requires ATP as a pyrophosphate donor. The resulting AMP may enter the pathways illustrated in figure 19. Alternatively, reperfusion following simulated hypoxia with metabolic inhibitors can lead to free radical production (Myers et al., 1995; Quaife et al., 1991) that may alter purine metabolism (Spragg et al.,

1985) and induce release of inosine and hypoxanthine. The mechanism for the enhanced inosine formation following IAA/NaCN removal is unclear. One possibility is that PNP activity may be decreased due to high PRPP or inosine or low P_i concentrations within the cells (Ropp and Traut, 1991).

PNP-mediated production of hypoxanthine may be a protective mechanism during ATP-depleting conditions. Inosine and adenosine have been reported to increase the survival of glial cells (Haun et al., 1996; Jurkowitz et al., 1998) and co-cultures of neurons and astrocytes (Litsky et al., 1999) during ATP-depleting conditions; this protection was reduced by inhibition of PNP. PNP activity may maintain the adenine nucleotide pool via production of ribose-1-phosphate, which can enter into glycolysis, and hypoxanthine, which can be converted into IMP via hypoxanthine-guanine phosphoribosyl transferase. Additionally, release of hypoxanthine by astrocytes may facilitate adenine nucleotide salvage by neurons.

In summary, our results demonstrate that C6 glioma cells do not release adenosine during ATP-depleting events. Unlike primary cultures of astrocytes, C6 cells do not release ATP; thus, our data imply that release of ATP and its extracellular metabolism to adenosine is the primary route of adenosine release from astrocytes. C6 cells metabolized ATP without forming adenosine and were able to accumulate adenosine during ATP-depleting conditions. From these findings, we propose that during ATP-depleting conditions astrocytes may salvage extracellular adenosine derived from ATP released from astrocytes or neurons or from adenosine *per se* released from neurons. Hypoxanthine released from astrocytes during and following an ischemic insult may be important for adenine nucleotide salvage by neurons.

Chapter 6: Release of [^{3}H]purines from rat primary cortical neurons and astrocytes during treatment with hypoxia, iodoacetate or sodium cyanide.

manuscript in preparation for Neuropharmacology.

Abstract

In brain, the levels of adenosine increase up to 100-fold during cerebral ischemia; however, the role of specific cell types, enzymatic pathways and membrane transport processes in regulating intra- and extracellular concentrations of adenosine is poorly characterized. Rat primary cortical neurons and astrocytes were cultured for 6-13 and 12-21 days, respectively, prior to incubation with [^{3}H]adenine for 30 min to radiolabel intracellular ATP. Cells were then treated with glucose-free buffer, hypoxia/hypoglycemia, 500 μM iodoacetate (IAA) or 100 μM sodium cyanide (NaCN) for 1 hour to stimulate metabolism of ATP and release of [^{3}H]purines. The nucleoside transport inhibitor dipyridamole (DPR) (10 μM), the adenosine kinase inhibitor iodotubercidin (ITU) (1 μM), the adenosine deaminase inhibitor EHNA (1 μM) and the purine nucleoside phosphorylase inhibitor BCX-34 (10 μM) were tested to investigate the contribution of specific enzymes and transporters in the metabolism and release of purines from each cell type. Our results indicate that (a) hypoxia stimulated the release of [^{3}H]purines from neurons but not from astrocytes, (b) neurons released greater amounts of [^{3}H]purines during inhibition of glycolysis compared to inhibition of oxidative phosphorylation while astrocytes released similar amounts with both stimuli, (c) hypoxia, NaCN and IAA treatments induced the release of similar amounts of [^{3}H]inosine and [^{3}H]hypoxanthine from neurons and ~2-4 fold more [^{3}H]hypoxanthine than [^{3}H]inosine from astrocytes, (d) hypoxia, NaCN and IAA produced significant increases in [^{3}H]adenosine release from neurons in the presence of EHNA or EHNA and ITU, (e) adenosine release from astrocytes was not enhanced by hypoxia, NaCN and IAA unless EHNA and ITU were present. These data suggest that, in these experimental conditions,

adenosine was formed by an intracellular pathway in neurons and then released via a nucleoside transporter. In contrast, adenine nucleotide release and extracellular metabolism to adenosine appeared to predominate in astrocytes. However, in both cell types, adenosine release was far lower than inosine and hypoxanthine release.

Introduction

Endogenous adenosine plays the role of a neuroprotective compound during strokes and seizures (Rudolphi et al., 1992; Von Lubitz, 1999). The protective nature of adenosine has been reported to be due to activation of membrane-bound adenosine receptors. Activation of adenosine A_1 receptors decreases excitatory amino acid release and cellular Ca^{2+} influx and increases K^+ influx while A_{2A} receptors increase blood flow and growth factor production (Von Lubitz, 1999). Although the role of endogenous adenosine has been widely investigated, less research has been done to document the mechanism of extracellular adenosine formation. Basal levels of adenosine in brain have been reported to be between 20 nM- 2 μM, depending upon the region of brain tested and the detection method used (Ballarin et al., 1991; Chen et al., 1992; Hagberg et al., 1987; Meno et al., 1991; Phillis et al., 1987; Van Wylen et al., 1986). These levels may increase up to 100 fold during forebrain ischemia (Parkinson et al., 2000). Extracellular adenosine may be derived from cellular release of adenosine *per se* or metabolism of extracellular adenine nucleotides. Both pathways have been reported to be important for basal adenosine levels (Fredholm, 1997) while release of adenosine *per se* has been proposed as the prominent source during ischemic or stimulated conditions (Whittingham, 1990). In addition, the cellular source of adenosine has been poorly defined. Adenosine release has been documented in slice and *in vivo* models, however whether the adenosine is primarily from neurons or astrocytes has not been determined.

Intracellular metabolism and nucleoside transporters tightly regulate release of adenosine. Adenosine kinase (AK) phosphorylates adenosine with a K_m of 0.5-2 μM (Chang et al., 1980; Kowaluk and Jarvis, 2000) while adenosine deaminase (ADA)

removes an amino group from adenosine with a K_m of 20 - 50 μM (Geiger and Nagy, 1990). AK has been proposed to be more important in regulating adenosine levels during basal conditions while ADA becoming more important during stimulated, or ATP-depleting conditions (Geiger et al., 1997). Nucleoside transporters are important for cellular release and uptake of extracellular adenosine. There are two broad classes of nucleoside transporters: Na^+-dependent and -independent (Griffith and Jarvis, 1996). The Na^+-dependent transporters couple influx of adenosine or other nucleosides to the Na^+ gradient; thus, these transporters are important primarily for influx (Cass et al., 1998). The equilibrative transporters allow adenosine to permeate cell membranes based on its concentration gradient, thus playing a potential role for both uptake and release (Gu et al., 1996; Sinclair et al., 2000a). If extracellular adenosine is released as adenosine *per se*, the pathway of metabolism is $ATP_i \rightarrow ADP_i \rightarrow AMP_i \rightarrow ADO_i \rightarrow ADO_e$. In this scenario, nucleoside transport inhibitors would be expected to decrease extracellular adenosine while AK and ADA inhibitors would likely increase release. If extracellular adenosine is produced via metabolism of released adenine nucleotides, the production of adenosine would be expected to include $ATP_e \rightarrow ADP_e \rightarrow AMP_e \rightarrow ADO_e \rightarrow ADO_i$. In this situation, nucleoside transport inhibitors would likely increase extracellular adenosine while AK and ADA inhibitors may have minimal effects on adenosine levels.

The objective of this study was to investigate the release of purines from primary rat cortical neurons and astrocytes during basal, hypoxic and ATP-depleting conditions. Previously, we have demonstrated that rat C6 glioma cells release hypoxanthine but not adenosine during ATP-depleting conditions (Sinclair et al., 2000b). We wished to determine whether primary astrocytes would respond in a similar manner and how this

would compare to primary neurons. Our results demonstrate that (a) neurons are more sensitive to hypoxia than astrocytes, (b) neurons release primarily inosine and hypoxanthine while astrocytes release predominantly AMP and hypoxanthine (c) neither cell type releases large quantities of adenosine levels unless adenosine metabolizing enzymes are inhibited.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium F12 (DMEM-F12), fetal bovine serum (FBS), Neurobasal™ medium and B-27 supplement were purchased from Life Technologies (Burlington, Ontario). 2-Amino-1,5-dihydro-7-(3-pyridinylmethyl)-4H-pyrrolo[3,2,-d]pyridin-4-one (BCX-34; peldesine) was a generous gift from Dr. Phillip Morris of Biocryst Pharmaceuticals (Birmingham, Alabama). Iodotubericidin (ITU) was purchased from Alberta Nucleoside Therapeutics (Edmonton, Alberta). [^{3}H]Adenosine, and [^{3}H]adenine were purchased from NEN Life Sciences (Mississauga, Ontario). Silica Gel GF TLC plates were purchased from Fisher Scientific (Whitby, Ontario). Erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA), and dipyridamole (DPR) were purchased from Research Biochemicals International (Natlick, MA). All other compounds were purchased from the Sigma Chemical Co. (St. Louis, MO).

Cell Culture

All experimental procedures were performed in adherence to the guidelines of the Canadian Council on Animal Care (CCAC) and were approved by the University of Manitoba Animal Protocol Management and Review Committee.

Rat primary cortical neurons and astrocytes were isolated as previously described, with minor modifications. Briefly, time-mated E19 rats were anesthetized with ether, laparotomy was performed and fetuses were collected in sterile petri dishes. An incision was made at the base of the neck of the fetus then continued up until the cortex was exposed. The cerebral hemispheres were dissected and pooled in 3 ml of Hank's solution. The meninges, hippocampus and basal structures were dissected away from the cortex.

The cortical tissue was placed in a sterile 15 ml conical tube and the media was aspirated off and replaced with fresh Ca^{2+} and Mg^{2+} free Hank's solution. The tissue was cut up, triturated numerous times and centrifuged for 10 min. The supernatant was aspirated and the pellet was resuspended in DMEM-F12 with 10% FCS and 1% penicillin/streptomycin/amphotericin. At this point, the isolated cells were split with some for astrocytic culture and some for neuronal culturing. For the astrocytes, the cells were plated in 10 ml of media in T-175 flasks pre-coated with poly-d-lysine (20 μg/ml). After 5-7 days in culture, the flasks were shaken vigorously at 250 rpm for ~12 hours to remove neurons, type II astrocytes, microglia and oligodendrocytes. The media was aspirated and the cells were trypsinized (3 ml; 25 μg/ml trypsin) for 10 min at 37°C. Fresh media was added to inactivate the trypsin, cells were dissociated with glass blown pipettes and split 1:1 with a final volume of 10 ml in each T-175 flask. Once the plates reached confluence, the cells were trypsinized and plated into 12 well plates, which were coated with poly-L-lysine. For the neuronal culture, cells were plated in 12 well plates that were coated with poly-L-lysine. The cells were left in DMEM-F12 for 4-6 hrs in the incubator. After this time, the media was aspirated and Neurobasal™ media with B27 supplement was added to each well. Astrocytes were used between days 12-21 *in vitro* while neurons were used days 6-13 *in vitro*.

Nucleoside Release Assays

Release assays were performed with primary neurons or astrocytes cultured in 12-well plates. Cells were washed twice in Na^+ buffer then incubated with $[^3H]$adenine (1

μM) at 22°C. The inwardly-directed concentration gradient resulted in tritium accumulation within the cells. After 30 min, cells were washed twice with buffer to remove extracellular tritium. The [^{3}H]adenine that accumulated in the astrocytes and neurons was rapidly metabolized to [^{3}H]adenine nucleotides (>85%; data not shown). Cells were treated with glucose-free buffer alone, N_2-bubbled glucose-free buffer (then placed in N_2-aerated environment), the glycolytic inhibitor IAA (500 μM) or the oxidative phosphorylation inhibitor NaCN (100 μM) for 1 hr to stimulate metabolism of [^{3}H]adenine nucleotides and release of the [^{3}H]purine metabolites. Trypan blue exclusion staining and LDH release indicated no significant difference in viability between the four different treatments in either cell type (data not shown). The effects of the nucleoside transport inhibitor DPR (10 μM) on [^{3}H]purine release was tested. In addition, the contribution of specific enzymes to the metabolism and release of [^{3}H]purines was examined using 1 μM ITU to inhibit adenosine kinase, 1 μM EHNA to inhibit adenosine deaminase, and 10 μM BCX-34 to inhibit purine nucleoside phosphorylase (PNP). After 1 hr treatment, extracellular buffer was removed and analyzed for [^{3}H]purines by thin layer chromatography. Cells were dissolved in NaOH and analyzed for protein content. Release data are expressed as pmol/mg cellular protein using the specific activity of the loading buffer.

Thin Layer Chromatography

The method of Schrader and Gerlach was used to identify the [^{3}H]purines release (Schrader and Gerlach, 1976). Briefly, n-butanol, ethyl acetate, methanol and ammonium hydroxide (7:4:3:4) were mixed, placed in a TLC tank and allowed to equilibrate for 90

min. Extracellular media (15 μl) obtained from the nucleoside release assays was spotted onto Silica Gel GF plates with 5 μl of cold carrier. Cold carrier consisted of 15 mM each of adenosine, inosine, hypoxanthine, adenine and AMP, 7.5 mM uric acid and 6.5 mM xanthine. Plates were run for 3 hours, and samples migrated in the order AMP/uric acid, inosine, xanthine, hypoxanthine, adenosine and adenine. Uric acid, xanthine and adenine were low or not detected in all experiments; therefore, data obtained for these purines are not reported. Spots were outlined under ultraviolet light, scraped, transferred to scintillation vials and 500 μl of 0.2 M HCl was added to each tube. After 1 hr, scintillation fluid (5 ml) was added and radioactivity was determined using scintillation spectrometry.

Data Analysis

Each experiment was performed at least three times in duplicate or triplicate, unless otherwise stated. All values are expressed as means ± S.E. and statistical significance was determined by ANOVA followed by Bonferroni's post hoc test. Statistical analyses were performed using the software package GraphPad PRISM Version 3.0.

Results

To compare the release of [^{3}H]purines from the primary neurons and astrocytes, we stimulated the cells with four conditions; glucose-free buffer alone, hypoxic buffer, 100 μM NaCN or 500 μM IAA. The neurons released 90 ± 6 pmol/ mg prot/ hr of [^{3}H]purines into glucose-free buffer (30 % AMP, 29% inosine, 21% hypoxanthine and 9% adenosine) (fig 20a). Hypoxia significantly elevated the total [^{3}H]purine release from the neurons to 147 ± 8 pmol/mg prot/hr. There was a significant increase in the release of [^{3}H]inosine (29 ± 4 vs. 49 ± 4 pmol/mg prot/hr, $p<0.05$), and [^{3}H]hypoxanthine release (21 ± 1 vs. 41 ± 7 pmol/mg prot/hr, $p<0.05$) by hypoxic treatment compared to normoxic buffer while [^{3}H]adenosine and [^{3}H]AMP release was not significantly changed (fig 20a). Similar but larger increases in release were seen from neurons treated with NaCN or IAA and amounted to 225 ± 13 and 467 ± 21 pmol/mg prot/hr, respectively. The increased release in the presence of NaCN or IAA was due to increased release of both [^{3}H]inosine and [^{3}H]hypoxanthine. The ratio of [^{3}H]hypoxanthine: [^{3}H]inosine release was ~1:1 in each of the four conditions (fig 20a). Stimulation of the neurons with the glycolytic inhibitor IAA produced significantly higher [^{3}H]purine release from the primary neurons than did the oxidative phosphorylation inhibitor NaCN.

The release of [^{3}H]purines from the primary astrocytes treated with glucose-free buffer was 170 ± 24 pmol/ mg prot/ hr (67% AMP, 14% inosine, 6% hypoxanthine and 14% adenosine) (fig 20b). The release of [^{3}H]AMP and [^{3}H]adenosine from astrocytes was significantly higher than from neurons. Unlike neurons, astrocytes were unaffected by treatment of 1 hour in hypoxic buffer (152 ± 24 pmol/ mg prot/ hr) (fig 20b). There was no significant difference between the release of [^{3}H]purines in astrocytes stimulated

with glucose-free buffer or hypoxic buffer. NaCN and IAA significantly increased the release of [^{3}H]purines to 237 ± 22 and 299 ± 13 pmol/ mg prot/ hr, respectively (fig 20b). There was no significant difference in the total [^{3}H]purine release between NaCN and IAA treated primary astrocytes. NaCN produced a significant increase in [^{3}H]hypoxanthine release but not [^{3}H]inosine compared to buffer treated astrocytes, while IAA produced significant increases in both [^{3}H]hypoxanthine and [^{3}H]inosine release. Neither IAA nor NaCN treatment induced significant changes in [^{3}H]adenosine or [^{3}H]AMP release compared to release from astrocytes in glucose-free buffer. The ratio of [^{3}H]hypoxanthine: [^{3}H]inosine release was ~1:1, 1:1, 4:1 and 2:1 in the astrocytes treated with glucose free buffer, hypoxic buffer, NaCN and IAA, respectively (fig 20b). When deoxyglucose (10mM) or dinitrophenol (25μM) was used in place of IAA or NaCN as inhibitors of glycolysis or oxidative phosphorylation, respectively, neurons and astrocytes released similar quantities of [^{3}H]purines (data not shown).

In all four conditions in both cell types, [^{3}H]AMP release was unaffected by the enzyme inhibitors or transport blocker. In general, the enzyme inhibitors had similar effects on release in all four conditions in the two cell types. The general effects will be described in the section on release into glucose-free buffer. The specific differences seen in release into hypoxic-buffer, NaCN or IAA will be described in the subsequent sections.

Glucose-Free Buffer

The neurons released 90 ± 6 pmol/ mg prot/ hr of [^{3}H]purines into glucose-free buffer (30 % AMP, 29% inosine, 21% hypoxanthine and 9% adenosine) (fig 21a). Total

[^{3}H]purine release was significantly increased in the presence of ITU and EHNA (90 ± 6 vs. 123 ± 8 pmol/mg prot/hr, $p<0.05$), which was blocked by DPR (75 ± 9 pmol/mg prot/hr, $p<0.001$) (fig 21a). DPR significantly decreased the release of [^{3}H]inosine from cells treated with buffer, ITU and/or EHNA or BCX-34 (fig 21a; $p<0.001$). Compared to buffer treated neurons, EHNA significantly decreased the release of [^{3}H]inosine (29 ± 4 vs. 14 ± 2 pmol/mg prot/hr, $p<0.05$). BCX-34 significantly increased the release of [^{3}H]inosine (61 ± 7 pmol/mg prot/hr; $p<0.01$) and decreased the release of [^{3}H]hypoxanthine (21 ± 1 vs. 0 ± 1 pmol/mg prot/hr; $p<0.05$). [^{3}H]Hypoxanthine release was also significantly increased by treatment of the cells with ITU and DPR (21 ± 1 vs. 39 ± 3 pmol/mg prot/hr; $p<0.05$). The release of [^{3}H]adenosine was significantly increased from buffer treated cells by the combination of ITU+EHNA (9 ± 1 vs. 45 ± 4 pmol/mg prot/hr; $p<0.001$), which was significantly decreased by addition of DPR (14 ± 3 pmol/mg prot/hr; $p<0.001$). The effects of ITU and EHNA on [^{3}H]adenosine release together were significantly greater than either compound alone.

The release of [^{3}H]purines from primary astrocytes treated with glucose-free buffer was 170 ± 24 pmol/ mg prot/ hr (67% AMP, 14% inosine, 6% hypoxanthine and 14% adenosine) (fig 20b). Total [^{3}H]purine release from primary astrocytes was not significantly affected by DPR, ITU, EHNA or BCX-34, however, the pattern of release was different. As with the neurons, ITU had no significant effect on [^{3}H]inosine, [^{3}H]adenosine or [^{3}H]hypoxanthine release. EHNA significantly decreased [^{3}H]inosine release (26 ± 8 vs 1 ± 1 pmol/mg prot/hr, $p<0.01$) but did not significantly affect [^{3}H]adenosine release. BCX-34 significantly increased [^{3}H]inosine release ($p<0.05$) and decreased [^{3}H]hypoxanthine (12 ± 5 vs. 2 ± 1 pmol/mg prot/hr, $p<0.05$) release compared

to glucose-free buffer. Similar to the primary neurons, DPR significantly decreased the release of [^{3}H]inosine from primary astrocytes treated with glucose-free buffer (26 ± 8 vs. 10 ± 2 pmol/mg prot/hr, $p < 0.05$), ITU (17 ± 3 vs. 8 ± 2 pmol/mg prot/hr, $p<0.05$) or BCX-34 (36 ± 10 vs. 10 ± 2, $p<0.001$). Unlike in neurons where DPR tended to decrease [^{3}H]adenosine release, DPR did not affect [^{3}H]adenosine release in astrocytes. ITU + EHNA significantly increased [^{3}H]adenosine release compared to buffer alone (24 ± 6 vs. 70 ± 8 pmol/mg prot/hr, $p<0.001$) but this was not significantly decreased by DPR (59 ± 7 pmol/mg prot/hr) (fig 21b).

Hypoxic Glucose-Free Buffer

The total [^{3}H]purine release from hypoxic-buffer treated neurons was significantly inhibited by DPR in all groups except the neurons treated with ITU (fig 22a). The [^{3}H]purine release from hypoxic-buffer treated neurons (fig 22a) in the presence of ITU, EHNA, BCX-34 and DPR was similar to the buffer treated neurons (compare fig 22a and fig 21a). As with neurons in glucose-free buffer, DPR significantly decreased the release of [^{3}H]inosine from cells treated with buffer, ITU and/or EHNA or BCX-34 (fig 22a; $p<0.001$). One major difference between neurons treated with hypoxia compared to neurons in glucose free buffer was seen in the fact that EHNA alone significantly increased the release of [^{3}H]adenosine during hypoxic treatment (15 ± 1 vs. 29 ± 2 pmol/mg prot/hr, $p<0.001$), which was significantly decreased by DPR (10 ± 2 pmol/mg prot/hr, $p<0.01$; fig 22a). The [^{3}H]adenosine release from hypoxic neurons treated with EHNA (29 ± 2 pmol/mg prot/hr; fig 22a) was significantly higher ($p<0.05$) than release from neurons treated with EHNA in normoxic buffer (16 ± 2 pmol/mg prot/hr; fig 21a).

Figure 20

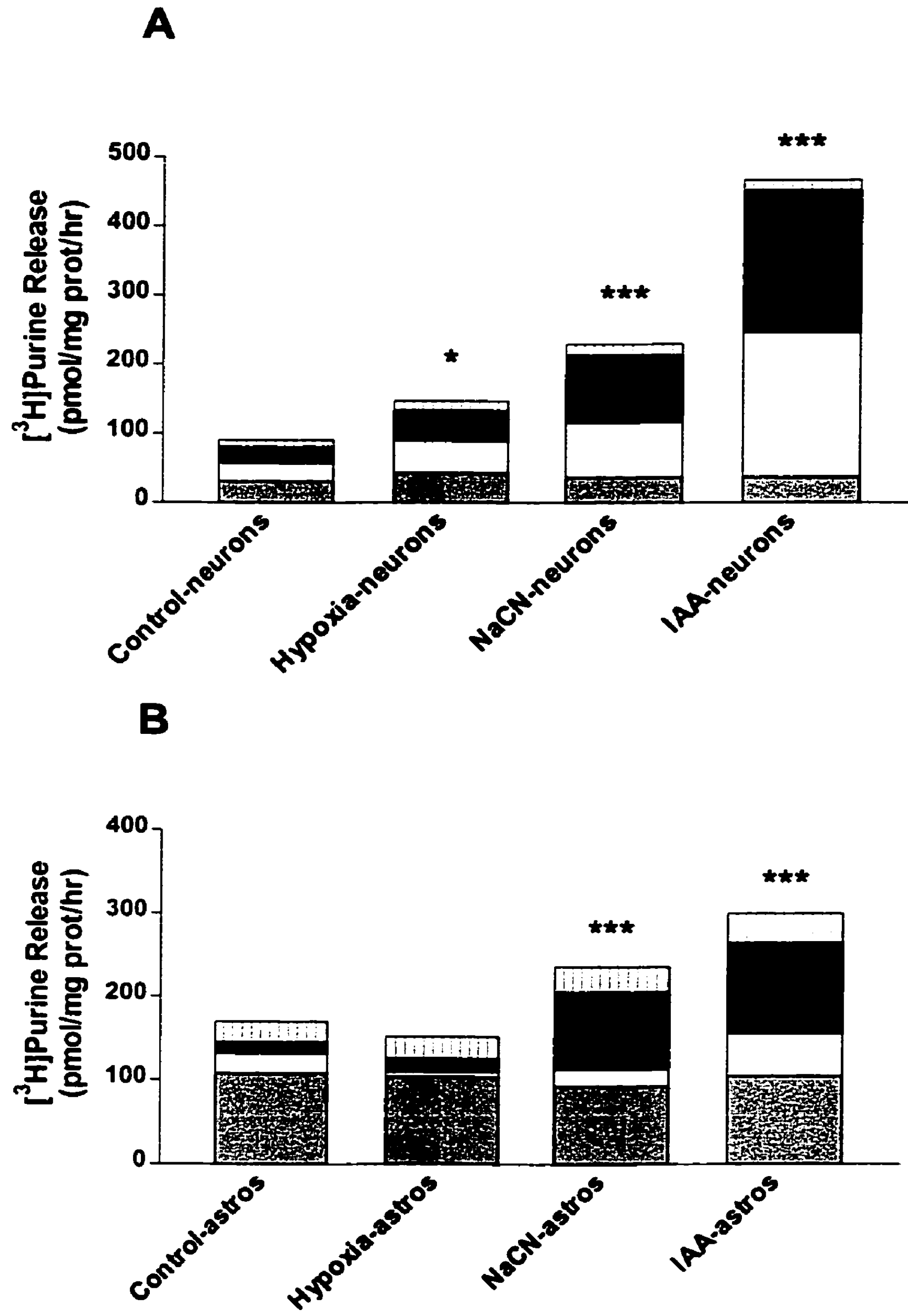
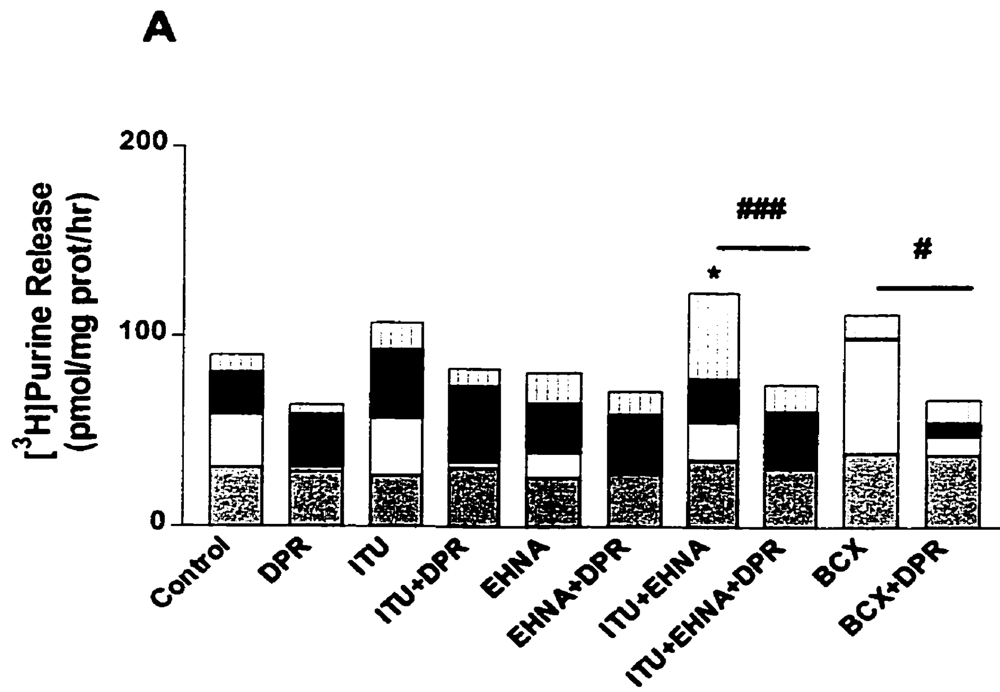


Figure 20 continued:

Effect of glucose-free buffer, hypoxic-buffer, 100 μM NaCN or 500 μM IAA upon [^{3}H]purine release from rat primary cortical neurons (A) or astrocytes (B). Cells were loaded for 30 min with 1 μM [^{3}H]adenine, rinsed and placed into appropriate buffer for 1 hour at 22°C. Buffer was removed and analyzed by TLC for [^{3}H]purines. Data are expressed as total [^{3}H]purines released which is the sum of [^{3}H]AMP (gray bars), [^{3}H]inosine (white bars), [^{3}H]hypoxanthine (black bars) and [^{3}H]adenosine (hatched bars). Statistical significance was determined by ANOVA followed by Bonferroni's post hoc test (* $p<0.05$, *** $p<0.001$ from respective from respective glucose-free buffer group).

Figure 21



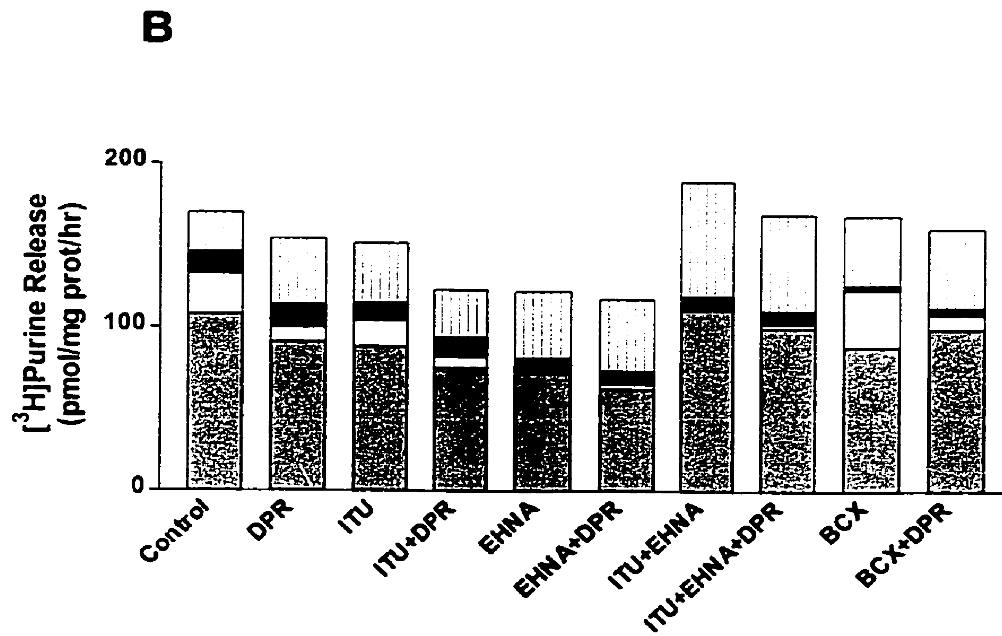


Figure 21:continued

Effects of ITU, EHNA, BCX-34 and DPR on the release of [^{3}H]purines from glucose-free buffer treated rat primary cortical neurons (A) or astrocytes (B). Cells were loaded for 30 min with 1 μM [^{3}H]adenine, rinsed and placed into glucose free-buffer in the presence or absence of 10 μM DPR, 1 μM ITU ± DPR, 1 μM EHNA ± DPR or 10 μM BCX-34 ± DPR for 1 hour at 22°C. Buffer was removed and analyzed by TLC for [^{3}H]purines. Data are expressed as total [^{3}H]purines released which is the sum of [^{3}H]AMP (gray bars), [^{3}H]inosine (white bars), [^{3}H]hypoxanthine (black bars) and [^{3}H]adenosine (hatched bars). Statistical significance was determined for total [^{3}H]purine release by ANOVA followed by Bonferroni's post hoc test (* $p<0.05$ from respective glucose-free buffer group; $^{\#}$ $p<0.05$, $^{\#\#\#}$ $p<0.001$ from the respective DPR treated groups). Statistical significance of inhibitors on [^{3}H]AMP, [^{3}H]inosine, [^{3}H]hypoxanthine and [^{3}H]adenosine release are provided in the text.

As detailed in fig 20, hypoxia significantly elevated the total [^{3}H]purine release from the neurons but not astrocytes (fig 22 a, b). Therefore, the release from the astrocytes during treatment with hypoxic buffer looks nearly identical to release in glucose-free buffer (compare fig 22b to fig 21b).

100 μM NaCN in Glucose-Free Buffer

Primary neurons treated with 100 μM NaCN for 1 hr released [^{3}H]purines similar to hypoxic-buffer treated neurons (fig 23a). The total [^{3}H]purine release was 225 ± 13 pmol/mg prot/hr (19 % AMP, 39 % inosine, 35 % hypoxanthine and 7% adenosine) and was significantly inhibited by DPR in all the groups (fig 23a). This inhibition of total release was predominantly due to inhibition of [^{3}H]inosine release. DPR significantly inhibited the release of [^{3}H]inosine when in NaCN alone or in the presence of ITU and/or EHNA or BCX-34. DPR significantly decreased [^{3}H]adenosine release in the presence of ITU (20 ± 2 vs. 9 ± 1 pmol/mg prot/hr, $p<0.05$), EHNA (42 ± 3 vs. 16 ± 2 pmol/mg prot/hr, $p<0.001$) and ITU + EHNA (67 ± 4 vs. 15 ± 2 pmol/mg prot/hr, $p<0.001$). While BCX-34 significantly increased [^{3}H]inosine release (82 ± 11 vs. 176 ± 15 pmol/mg prot/hr, $p<0.001$; fig 23a) and decreased [^{3}H]hypoxanthine release (94 ± 10 vs. 10 ± 5 pmol/mg prot/hr, $p<0.001$), BCX-34 + DPR did not significantly change [^{3}H]hypoxanthine release (64 ± 5 pmol/mg prot/hr) relative to NaCN alone. Similar to hypoxia treated neurons, EHNA alone significantly increased adenosine release from NaCN-treated neurons (18 ± 2 vs. 42 ± 3 pmol/mg prot/hr, $p<0.001$; fig 23a). The combination of ITU and EHNA significantly increased [^{3}H]adenosine release over ITU or EHNA alone ($p<0.001$). The release of [^{3}H]adenosine from NaCN treated neurons in

the presence of EHNA or EHNA and ITU (42 ± 3 or 67 ± 4 pmol/mg prot/hr, respectively fig 23a) was significantly greater than release from neurons treated with EHNA or EHNA and ITU in glucose-free buffer (16 ± 2 or 45 ± 4 pmol/mg prot/hr, respectively; fig 21a; $p<0.01$).

In primary astrocytes, none of the drug combinations significantly affected the total release of [^{3}H]purines from NaCN-treated cells (fig 23b). Although there was not a significant change in total release, the release of specific [^{3}H]purines was altered. BCX-34 was the only compound that significantly increased [^{3}H]inosine release (22 ± 6 vs. 188 ± 31 pmol/mg prot/hr, $p<0.001$; fig 23b), which was blocked by DPR (23 ± 7pmol/mg/prot/hr). The combination of ITU + DPR significantly increased [^{3}H]hypoxanthine release (91 ± 21 vs. 225 ± 35 pmol/mg prot/hr, $p<0.001$; fig 23b) while BCX-34 significantly decreased [^{3}H]hypoxanthine release (23 ± 7 pmol/mg/prot/hr, $p<0.05$). Unlike the primary neurons, EHNA alone did not significantly alter [^{3}H]adenosine release from NaCN-treated astrocytes (30 ± 6 vs. 58 ± 9 pmol/mg/prot/hr, $p>0.05$). [^{3}H]Adenosine release from NaCN-treated astrocytes was significantly increased by the combination of ITU and EHNA (30 ± 6 vs. 118 ± 18 pmol/mg/prot/hr, $p>0.001$) and this was significantly greater than release from glucose-free buffer treated astrocytes with ITU and EHNA (118 ± 18 vs. 70 ± 8 pmol/mg prot/hr, $p<0.001$; fig 21b).

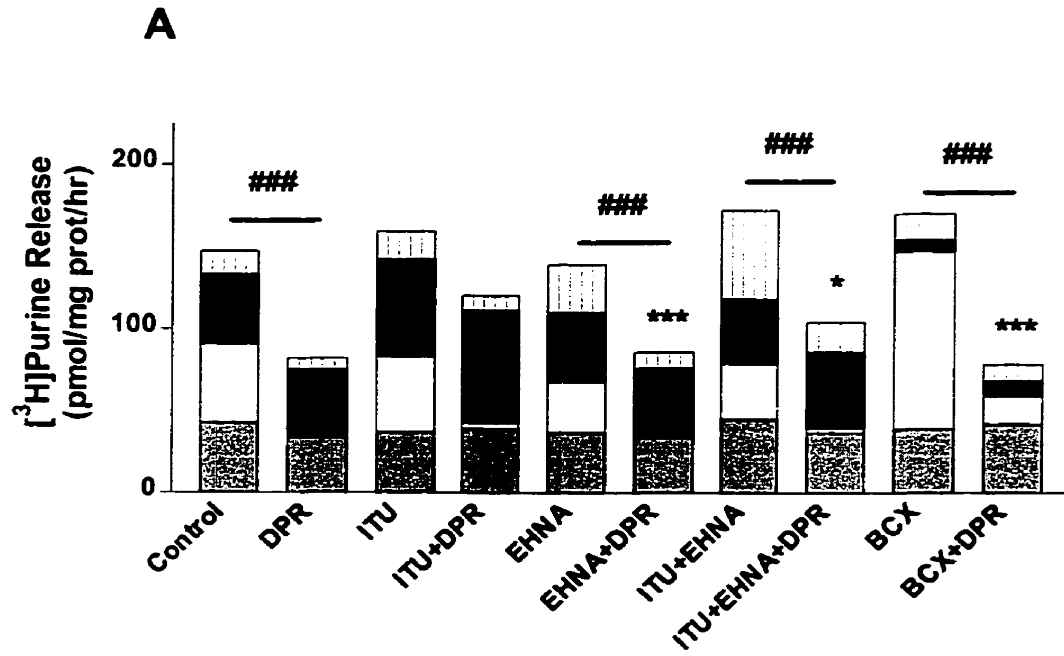
500 μM IAA in Glucose-Free Buffer

The total [^{3}H]purine release from neurons treated with 500 μM IAA for 1 hr was significantly decreased by DPR in each of the five combinations (fig 24a, $p<0.001$). The decrease in total [^{3}H]purine release was predominantly due to the significant inhibition of

[^{3}H]inosine release by DPR in each of the groups. Compared to IAA alone, BCX-34 significantly increased [^{3}H]inosine release (211 ± 16 vs. 322 ± 24 pmol/mg prot/hr, $p<0.001$; fig 24a) and significantly decreased [^{3}H]hypoxanthine release (203 ± 20 vs. 19 ± 3 pmol/mg prot/hr, $p<0.001$). Similar to the hypoxia and NaCN treated neurons, EHNA alone significantly increased [^{3}H]adenosine release (16 ± 1 vs. 41 ± 5 pmol/mg prot/hr, $p<0.001$; fig 24a), which was inhibited by DPR (13 ± 3 pmol/mg prot/hr, $P<0.001$). As with each of the hypoxic buffer and NaCN treatments, the neuronal release of [^{3}H]adenosine into 500 μM IAA containing EHNA or EHNA and ITU (41 ± 5 or 70 ± 8 pmol/mg prot/hr, respectively; fig 24a) was significantly greater than from neurons treated with EHNA or EHNA and ITU in glucose-free buffer (16 ± 2 or 45 ± 4 pmol/mg prot/hr, respectively; fig 21a).

In primary astrocytes, only one combination of inhibitors significantly affected the total release of [^{3}H]purines from NaCN-treated cells (fig 24b). BCX-34 + DPR significantly decreased the total [^{3}H]purine release. This was due the inhibition of PNP by BCX-34 which led to a significant increase of [^{3}H]inosine release and significant decrease of [^{3}H]hypoxanthine release compared to 500 μM IAA alone. As with all other treatments (figs 21b-23b), DPR significantly decreased [^{3}H]inosine release from all groups. The release of [^{3}H]inosine, [^{3}H]hypoxanthine and [^{3}H]adenosine into 500 μM IAA (fig 24b) was very similar to the release from astrocytes treated with 100 μM NaCN (fig 23b).

Figure 22



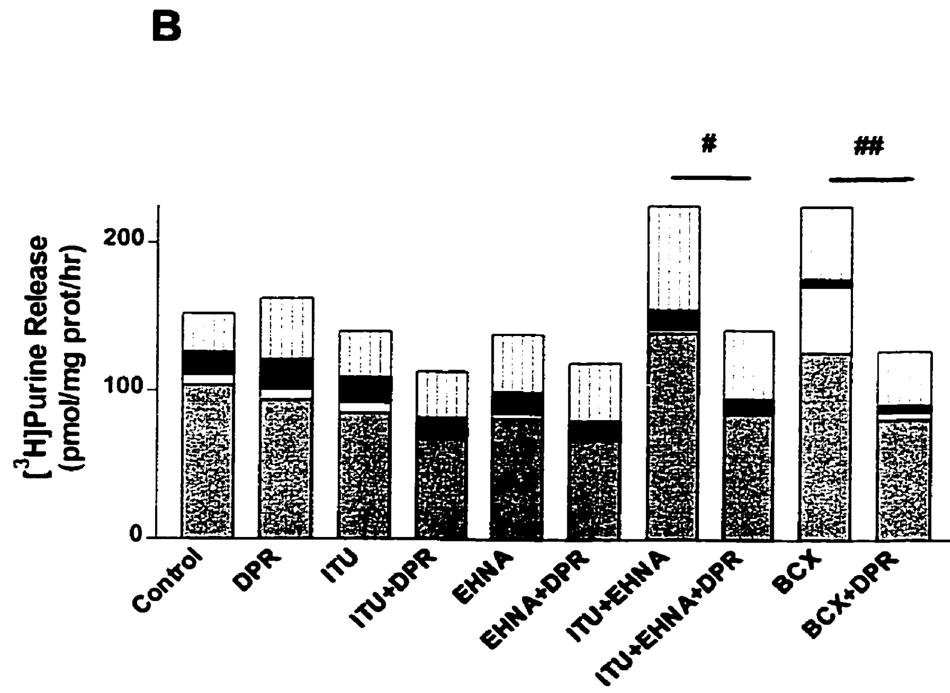


Figure 22:continued

Effects of ITU, EHNA, BCX-34 and DPR on the release of [^{3}H]purines from hypoxic buffer treated rat primary cortical neurons (A) or astrocytes (B). Cells were loaded for 30 min with 1 μM [^{3}H]adenine, rinsed and placed into hypoxic-buffer in the presence or absence of 10 μM DPR, 1 μM ITU ± DPR, 1 μM EHNA ± DPR or 10 μM BCX-34 ± DPR for 1 hour at 22°C. Buffer was removed and analyzed by TLC for [^{3}H]purines. Data are expressed as total [^{3}H]purines released which is the sum of [^{3}H]AMP (gray bars), [^{3}H]inosine (white bars), [^{3}H]hypoxanthine (black bars) and [^{3}H]adenosine (hatched bars). Statistical significance was determined for total [^{3}H]purine release by ANOVA followed by Bonferroni's post hoc test (* $p<0.05$, *** $p<0.001$ from respective from respective glucose-free buffer group; $^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.01$, $^{\#\#\#}$ $p<0.001$ from the respective DPR treated groups). Statistical significance of inhibitors on [^{3}H]AMP, [^{3}H]inosine, [^{3}H]hypoxanthine and [^{3}H]adenosine release are provided in the text.

Figure 23

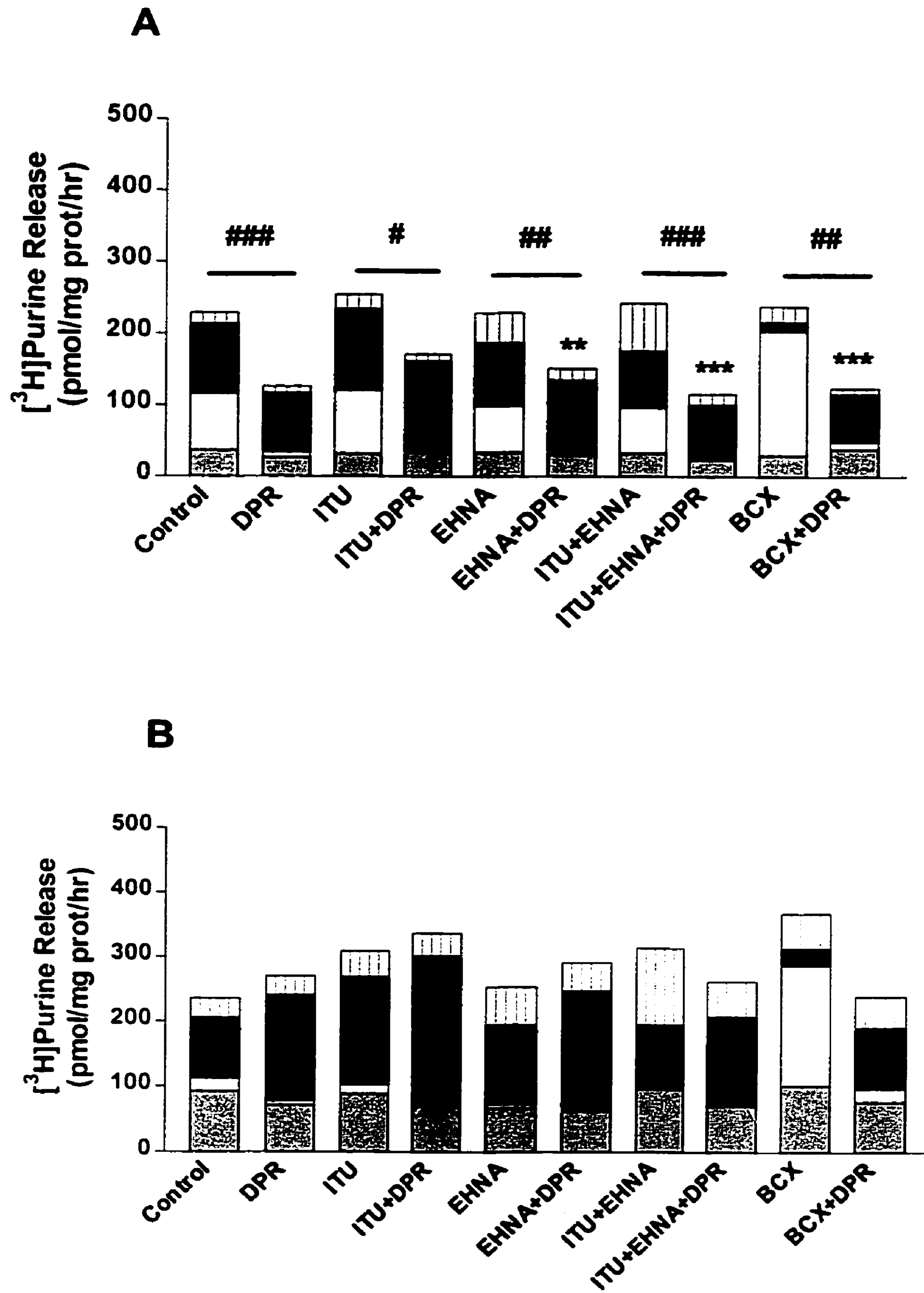
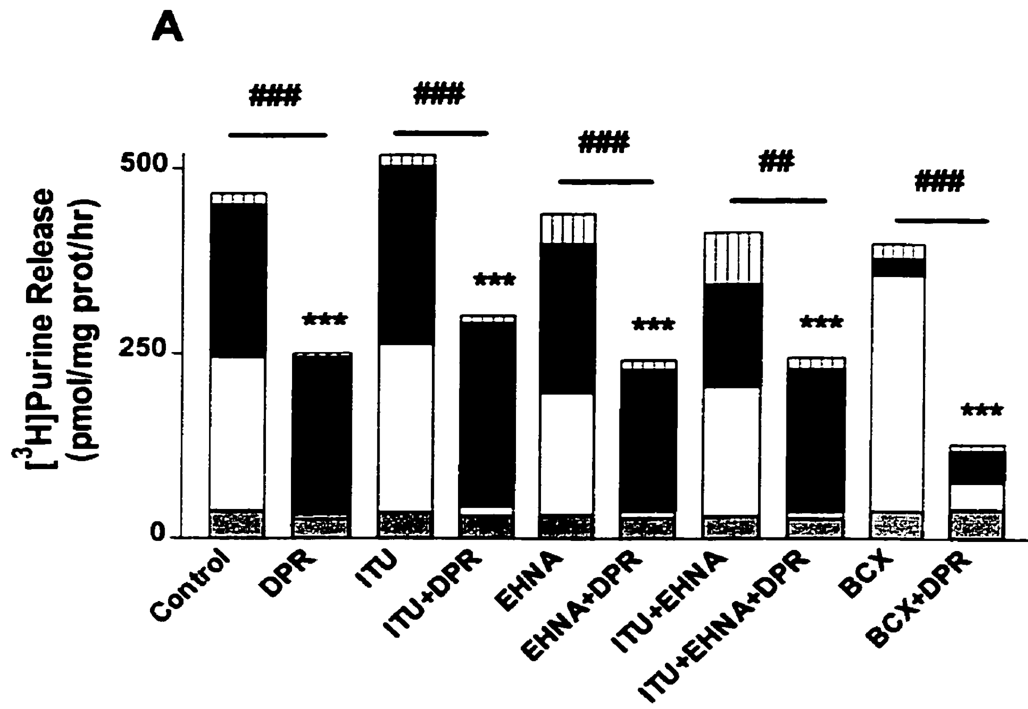


Figure 23:continued

Effects of ITU, EHNA, BCX-34 and DPR on the release of [^{3}H]purines from 100 μM NaCN treated rat primary cortical neurons (A) or astrocytes (B). Cells were loaded for 30 min with 1 μM [^{3}H]adenine, rinsed and placed into 100 μM NaCN in the presence or absence of 10 μM DPR, 1 μM ITU ± DPR, 1 μM EHNA ± DPR or 10 μM BCX-34 ± DPR for 1 hour at 22°C. Buffer was removed and analyzed by TLC for [^{3}H]purines. Data are expressed as total [^{3}H]purines released which is the sum of [^{3}H]AMP (gray bars), [^{3}H]inosine (white bars), [^{3}H]hypoxanthine (black bars) and [^{3}H]adenosine (hatched bars). Statistical significance was determined for total [^{3}H]purine release by ANOVA followed by Bonferroni's post hoc test (** $p<0.01$, *** $p<0.001$ from respective from respective glucose-free buffer group; $^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.01$, $^{\#\#\#}$ $p<0.001$ from the respective DPR treated groups). Statistical significance of inhibitors on [^{3}H]AMP, [^{3}H]inosine, [^{3}H]hypoxanthine and [^{3}H]adenosine release are provided in the text.

Figure 24



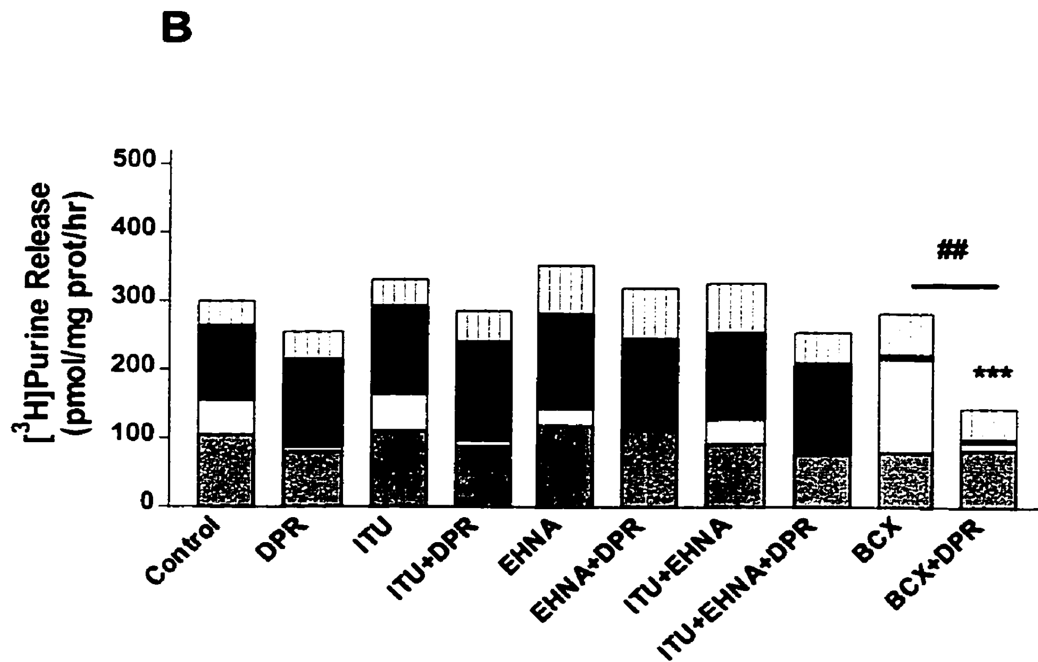


Figure 24:continued

Effects of ITU, EHNA, BCX-34 and DPR on the release of [^{3}H]purines from 500 μM IAA treated rat primary cortical neurons (A) or astrocytes (B). Cells were loaded for 30 min with 1 μM [^{3}H]adenine, rinsed and placed into 500 μM IAA in the presence or absence of 10 μM DPR, 1 μM ITU ± DPR, 1 μM EHNA ± DPR or 10 μM BCX-34 ± DPR for 1 hour at 22°C. Buffer was removed and analyzed by TLC for [^{3}H]purines. Data are expressed as total [^{3}H]purines released which is the sum of [^{3}H]AMP (gray bars), [^{3}H]inosine (white bars), [^{3}H]hypoxanthine (black bars) and [^{3}H]adenosine (hatched bars). Statistical significance was determined for total [^{3}H]purine release by ANOVA followed by Bonferroni's post hoc test (*** $p<0.001$ from respective from respective glucose-free buffer group; ## $p<0.01$, ### $p<0.001$ from the respective DPR treated groups). Statistical significance of inhibitors on [^{3}H]AMP, [^{3}H]inosine, [^{3}H]hypoxanthine and [^{3}H]adenosine release are provided in the text.

Discussion:

The primary findings of this study are that (a) hypoxia stimulated the release of [^{3}H]purines from neurons but not astrocytes, (b) neurons released greater amounts of [^{3}H]purines during inhibition of glycolysis compared to inhibition of oxidative phosphorylation while astrocytes released similar amounts with both stimuli, (c) hypoxia, NaCN and IAA treatments induced the release of similar amounts of [^{3}H]inosine and [^{3}H]hypoxanthine from neurons and ~2-4 fold more [^{3}H]hypoxanthine than [^{3}H]inosine from astrocytes, (d) hypoxia, NaCN and IAA conditions produced significant increases in [^{3}H]adenosine release from neurons in the presence of EHNA or EHNA and ITU, (e) adenosine release from astrocytes was not enhanced by hypoxia, NaCN and IAA unless EHNA and ITU were present.

Total [^{3}H]Purine Release

There were significantly greater amounts of [^{3}H]purines released from primary astrocytes than neurons during basal conditions in glucose free-buffer (fig 1). This was due to higher levels of [^{3}H]AMP released by astrocytes. Hypoxia significantly increased the overall release of [^{3}H]purines from neurons but not astrocytes. It has previously been reported that neurons are more dependent upon oxidative phosphorylation than astrocytes for maintenance of ATP levels (Ames, 2000). In addition, neurons are more susceptible to hypoxia-induced cell death than are astrocytes (Litsky et al., 1999), which correlates to depletion of cellular ATP levels. Thus, extracellular purines that appear in brain during conditions of low O_2 availability are likely from neurons. If ATP levels are maintained better by astrocytes during hypoxic conditions, these cells may be a site for salvage of

purines released by the neurons. When IAA or NaCN was used to deplete ATP, neurons and astrocytes significantly increased the release of [^{3}H]purines. Neurons showed a greater release of [^{3}H]purines stimulated by hypoxia, NaCN or IAA than astrocytes. These results indicate that both cells are susceptible to ATP depletion by inhibition of glycolysis or oxidative phosphorylation but the neurons are more sensitive to inhibition of these pathways.

[^{3}H]AMP Release

The release of [^{3}H]AMP did not significantly differ in either cell type among the four conditions. However, there was significantly more [^{3}H]AMP release from astrocytes than neurons. Although in our TLC protocol AMP and UA migrate together, UA was not detected (data not shown); this supports previous reports demonstrating a lack of xanthine oxidase in neuronal and astrocytic cells (Ceballos and Rubio, 1995; Zoref-Shani et al., 1995). The [^{3}H]AMP release from the astrocytes was not seen from rat C6 glioma cells in similar conditions (Sinclair et al., 2000b), which have been reported to be incapable of ATP release (Cotrina et al., 1998a; Cotrina et al., 1998b). Release of adenine nucleotides from astrocytes is an important potential source of adenosine from the astrocytes as astrocytes have been reported to have all the ecto-enzymes required for extracellular adenosine production (Zimmermann, 1996; Zimmermann, 2000).

[^{3}H]Inosine Release

The amount of [^{3}H]inosine released by neurons and astrocytes was similar under basal conditions. However, neurons released significantly larger amounts of [^{3}H]inosine

than astrocytes during hypoxia, NaCN or IAA treatment. The reason for the higher [^{3}H]inosine release from neurons than astrocytes may be due to higher activity of PNP in astrocytes (Van Reempts et al., 1988) or higher activity of ADA or IMP preferring 5'nucleotidase in neurons. In both cell types, DPR inhibited the release of [^{3}H]inosine, supports intracellular inosine formation. In addition, this demonstrates the bi-directional DPR sensitivity of the nucleoside transporters in these cells as influx of nucleosides is also blocked by DPR (Bender and Hertz, 1986). As EHNA did not significantly decrease the release of [^{3}H]inosine from astrocytes during any of the conditions, this suggests that the principal route of inosine formation is through AMP deaminase followed by 5'nucleotidase (AMP→IMP→inosine) rather than 5'nucleotidase then ADA activity (AMP→adenosine→inosine); as previously reported for rat C6 glioma cells (Sinclair et al., 2000b). However, in the neurons, EHNA significantly decreased [^{3}H]inosine release during basal and hypoxic conditions, indicating that ADA contributes to inosine formation in neurons.

[^{3}H]Hypoxanthine Release

The basal, hypoxic and NaCN-stimulated release of [^{3}H]hypoxanthine were similar between neurons and astrocytes. There was a significantly greater amount of [^{3}H]hypoxanthine released from IAA treated neurons than from astrocytes, which corresponds to a larger amount of total [^{3}H]purines released. BCX-34 decreased [^{3}H]hypoxanthine release from both cell types in each of the four conditions. This supports an intracellular site of PNP activity and hypoxanthine production. Although the release of [^{3}H]hypoxanthine from astrocytes was expected, it is surprising that the

neurons released large quantities of [^{3}H]hypoxanthine, as PNP has been reported to be an astrocytic enzyme (Castellano et al., 1990; Van Reempts et al., 1988). As cultured neurons have been reported to exhibit PNP activity (Zoref-Shani et al., 1995), its expression may be induced by culture conditions. This increased expression suggests that PNP and hypoxanthine may be important for nucleotide maintenance and possibly for cell survival (Litsky et al., 1999).

[^{3}H]Adenosine Release

In CNS, adenosine is thought to be the most pharmacologically active of the adenine nucleotide metabolites reported in this study. Under basal conditions, significantly more [^{3}H]adenosine was released from astrocytes than from neurons (24 ± 6 vs. 9 ± 1 pmol/mg prot/hr). While DPR decreased [^{3}H]adenosine release from neurons, it produced a small but not significant increase in astrocytic [^{3}H]adenosine release. As large quantities of [^{3}H]AMP were found in the extracellular environment of astrocytes, it is possible that the basal [^{3}H]adenosine release was due to extracellular metabolism of [^{3}H]AMP, especially since astrocytes have the required ecto-enzymes (Zimmermann, 1992; Zimmermann, 1996). In contrast, the inhibition by DPR indicates that neuronal [^{3}H]adenosine release occurred largely as adenosine *per se*. Surprisingly, hypoxia, NaCN and IAA did not produce any significant changes in [^{3}H]adenosine release from either cell type. Using ATP-depleting conditions, two previous studies have demonstrated increased adenosine release from primary rat cortical astrocytes and chick neuronal and astrocyte models, respectively (Ciccarelli et al., 1999; Meghji et al., 1989). The difference between our results and the previous reports may be due to differences in

temperature, stimuli, species or regional source of cells. When deoxyglucose or dinitrophenol were used instead of IAA or NaCN as inhibitors of glycolysis or oxidative phosphorylation, similar levels and patterns of [^{3}H]purine release were seen from neurons and astrocytes (data not shown). Thus, it is unlikely that the stimuli used in our studies explain the lack of [^{3}H]adenosine release. Temperature changes have been reported to affect extracellular adenosine levels in hippocampal slices, through changes in the activity of the *ei* (ENT2) nucleoside transporter (Dunwiddie and Diao, 2000). Temperature may not be the principal reason for the lack of [^{3}H]adenosine release in the absence of enzyme inhibitors. In the present study, the transporters in both cell types were functional in the release of nucleosides as inosine release was blocked by DPR. In addition, adenosine release from hypoxic astrocytes at 37°C was reported to occur only after the re-introduction of oxygen (Ciccarelli et al., 1999). The lack of adenosine release may suggest that adenosine was produced in large quantities but was metabolized prior to sampling. However, the conditions used were mild in duration (hypoxia) and concentration (IAA or NaCN) and unlikely to cause rapid depletion of ATP. Furthermore, ITU and EHNA led to a 5-fold increase in adenosine release as opposed 100-fold increases measured in vivo without enzyme inhibitors (Parkinson et al., 2000).

When we investigated the effects of the enzyme inhibitors on [^{3}H]adenosine release, we were surprised by the lack of effect of ITU on [^{3}H]adenosine release from either cell type in basal or stimulated conditions. Previous studies have demonstrated increased release of adenosine under basal or stimulated conditions in the presence of AK inhibitors in brain slice models (Lloyd and Fredholm, 1995; Pak et al., 1994). Similar results have not been demonstrated in cell culture models (Lynch et al., 1998). The lack

of effect of ITU on adenosine release may indicate high basal adenosine levels that are approaching the K_i of adenosine for AK or decreased AK activity due to other endogenous factors (Decking et al., 1997b; Lynch et al., 1998). The lack of effect of ITU may also indicate that primary rat neurons and astrocytes do not express the rENT1 nucleoside transporter, which facilitates entry of ITU into cells (Sinclair et al., see chapter 4). Inhibition of ADA by EHNA did not affect the basal release of [^{3}H]adenosine from either neurons or astrocytes. While EHNA alone did not affect astrocytic [^{3}H]adenosine release during hypoxia, NaCN or IAA treatment, EHNA produced a significant increase in neuronal [^{3}H]adenosine release in these three conditions. It has been reported than ADA may be more important in adenosine metabolism in neurons than astrocytes in culture (Matz and Hertz, 1989). Therefore, EHNA may be more likely to elevate adenosine release from neurons than astrocytes, as we report. The combination of ITU and EHNA significantly increased both basal and stimulated [^{3}H]adenosine release from both neurons and astrocytes. This indicates that either enzyme inhibitor alone is not as effective at elevating adenosine release because the other pathway of metabolism is available. Simultaneous inhibition of AK and ADA has been reported to have "supra-additive" effects on adenosine release from hippocampal and spinal cord slice models (Golembiowska et al., 1996; Hebb and White, 1998). This appears to be the most effective method of elevating basal and stimulated adenosine release from both neurons and astrocytes.

Our results indicate that neurons and astrocytes respond differently to glucose-free buffer, hypoxic buffer, 100 μM NaCN or 500 μM IAA with respect to the purines each cell type releases. Neurons were more sensitive to the stimulated conditions,

especially hypoxia, than astrocytes. This indicates that under comparable conditions, neurons are likely the site of purine release while astrocytes are a potential site of purine salvage. If the therapeutic goal is to elevate extracellular CNS adenosine levels *in vivo*, compounds should be developed to inhibit adenosine metabolism by neurons or adenosine accumulation by astrocytes, as these strategies could increase production or decrease removal of interstitial adenosine.

Chapter 7: General Discussion

The basis of this thesis was to investigate the mechanisms involved in regulation of extracellular adenosine levels. I used different *in vitro* cell culture models to determine the role of nucleoside transporters, purinergic enzymes and the BBB in this process. As a number of regulators of endogenous adenosine levels are in pre-clinical and early clinical development for CNS disorders, this thesis work is important in demonstrating how these different enzymes, transporters and cell types are involved in extracellular adenosine regulation.

The first research study in this thesis investigated the potential role of the BBB in the levels of extracellular CNS adenosine levels (Chapter 2). The hypothesis of this study was that **administration of DPR to the lumenal compartment of a dynamic *in vitro* blood brain barrier model would inhibit bidirectional adenosine permeation of this system**. For this project, I used a physiologically relevant *in vitro* model of the BBB, which has blood-like media flow, low permeability and cellular phenotype similar to the BBB *in vivo*. My results demonstrated that lumenal administration of the nucleoside transport inhibitor DPR, was able to inhibit completely the movement of adenosine from the interstitial or parenchymal compartment into the lumenal compartment. Surprisingly, lumenal DPR administration was unable to affect the movement of adenosine from the lumen to the interstitial compartment. As DPR poorly crossed the DIV-BBB and poorly permeates the BBB *in vivo* (Sollevi et al., 1983), the site of action appears to be on the lumenal side of the BBB. These results support the hypothesis that DPR may be able to decrease the incidence or severity of stroke in humans by decreasing the loss of adenosine from the CNS during basal conditions, thereby promoting adenosine receptor

activation and/or maintenance of ATP levels. As one of the primary therapeutic goals for regulators of endogenous adenosine levels is for conditions of hypoxia/ischemia, further studies must be performed which investigate how DPR or other transport inhibitors affect the permeation of adenosine across the BBB during these conditions. Since non-specific BBB permeability increases dramatically during ischemia/reperfusion injury (Dobbin et al., 1989; Picozzi et al., 1985; Pluta et al., 1994; Preston and Foster, 1997; Preston et al., 1993), one would hypothesize that nucleoside transport inhibitors would not affect adenosine loss across the BBB, as the movement would be non-specific paracellular transport. However, the selective and potent nucleoside transport inhibitor NBMPR, when administered i.p., has been demonstrated to decrease neuronal damage following cerebral ischemia (Parkinson et al., unpublished data), even though it poorly permeates the BBB (Anderson et al., 1996a). In addition to *in vitro* studies, *in vivo* studies should be performed to investigate the BBB permeation of adenosine and other nucleoside transporter permeants and the effects of peripherally administered nucleoside transport inhibitors during basal and ischemic conditions. As my results indicate that there is asymmetrical cellular expression of the nucleoside transporters on the endothelial cells of the BBB, it would be interesting to determine with immunohistochemistry whether similar expression patterns exist *in vivo*.

Autoregulation of extracellular serotonin, norepinephrine, glutamate and GABA levels has been reported to occur via activation of their respective receptors. Similar events have been reported with adenosine by activation of the A_{2A} receptor (Delicado et al., 1990; Dolhun and Parkinson, 1995). However, the mechanism of action was poorly demonstrated and this study only investigated uptake not release. I wanted to determine

whether autoregulatory effects were evident in DDT_1 MF-2 smooth muscle cells stimulated to release adenosine. I hypothesized that **activation of adenosine receptors in DDT_1 MF-2 hamster smooth muscle cells during ATP-depleting conditions will decrease the total release of adenosine.** This was based on previous studies that demonstrated altered *es* nucleoside transporters on the cell surface following adenosine receptor activation (Delicado et al., 1990; Dolhun and Parkinson, 1995). However, my results showed that activation of the A_1 receptor, not the A_{2A} receptor, produced a significant increase in adenosine release during ATP-depleting conditions. These effects could be mimicked by pathways that activated PKC but not by activation of adenylate cyclase or PKA. Although these results demonstrated that AK activity was decreased by PKC activation, which lead to increased adenosine release, the direct effect of PKC on AK was not shown. Future studies must be undertaken to investigate the mechanism by which PKC decreases AK activity. As there are a number of consensus PKC phosphorylation sites on AK, the hypothesis would be that direct phosphorylation of AK by PKC leads to decreased activity. As AK has been shown to be affected by ATP, Mg^{2+}, pH and P_i, the interaction between PKC and these endogenous regulators must also be investigated. The inhibition of AK activity by adenosine A_1 receptor activation or increased PKC activity adds another layer of complexity to the regulation of AK. This provides further evidence for the potential role of AK in the control of minute-to-minute adenosine levels.

Although the DDT_1MF-2 cells are a well-characterized adenosinergic model, I wished to investigate whether similar events occurred in a CNS cell culture system. In early studies to determine the nucleoside transporters expressed by C6 rat glioma cells, I

discovered that, contrary to expectations, the AK inhibitors ITU or NH_2dAdo did not decrease [^3H]adenosine accumulation. As numerous studies have demonstrated the ability of AK inhibitors to decrease adenosine accumulation in cells containing the *es* nucleoside transporter, I hypothesized that the **expression of rENT1 nucleoside transporters but not rCNT2 will produce inhibition of adenosine uptake by adenosine kinase inhibitors in rat C6 glioma cells**. Using rat C6 glioma cells that were transfected with either rENT1 or rCNT2 cDNA, I demonstrated that the rENT1 enabled ITU to decrease the accumulation of [^3H]adenosine while neither transporter enabled NH_2dAdo to have a potent effect. These studies also demonstrated that both ITU and NH_2dAdo directly inhibited the native *ei* transporters in the C6 cells. Based on the structural similarity of ITU and NH_2dAdo to other permeants of the nucleoside transporters, it is not surprising that there is direct interaction of the compounds with the *ei* transporter. Based on other studies, the ability of the rENT1 transporter to facilitate the entry of ITU but not NH_2dAdo was expected. Future studies should investigate the next generation of adenosine kinase inhibitors that are currently being developed by Abbott and Metabasis Therapeutics to determine if similar mechanisms occur. These results demonstrate a mechanism by which AK inhibitors may cause "site" selective increases in endogenous adenosine. An important extension of this work is to determine the cellular and regional distribution of the respective nucleoside transporter subtypes, in order to predict the effect of the AK inhibitors on adenosine levels.

Astrocytes have been proposed to be the principal regulator of endogenous adenosine levels in the brain (Bender and Hertz, 1986; Matz and Hertz, 1989). However, there have been few reports, which investigate this claim. I wished to determine how

astrocytes might be involved in the regulation of extracellular adenosine and how this may be affected by different stimuli. In this project, I had hypothesized that **ATP-depletion with sodium cyanide and iodoacetate will induce the release of adenosine from rat C6 glioma cells through the *ei* nucleoside transporter**. When the cells were loaded with [^{3}H]adenine and depleted of ATP, I expected the C6 cells to release large quantities of [^{3}H]adenosine, similar to the results I saw with the DDT_1 MF-2 cells. However, no significant increase in [^{3}H]adenosine release was seen. There was a large increase in the release of [^{3}H]hypoxanthine from these cells. The lack of [^{3}H]adenosine release was not due to the inability of the *ei* transporter to facilitate adenosine release as [^{3}H]FB and [^{3}H]inosine was effectively released by these cells. The release of hypoxanthine but not adenosine was due to AMPDA metabolizing AMP to IMP rather than c-N-I dephosphorylating AMP to adenosine. This 'alternate' metabolic pathway led to the production of inosine, which was then metabolized by PNP to hypoxanthine. These results are similar to the release pattern of purines seen from ATP-depleted adipocytes (Kather, 1988; Kather, 1990) and T cells but not B cells (Barankiewicz et al., 1990). These results may support the hypothesis that astrocytes are important in producing extracellular adenosine via the release of adenine nucleotides.This could not be tested as the C6 cells do not release ATP (Cotrina et al., 1998a).

From the previous study, I was concerned that the results were due to cell type or protocol dependent effects. I wished to investigate whether "real" CNS cells produced adenosine release during hypoxia *per se* compared to mimicking hypoxia with iodoacetate and cyanide. Based on my previous study, I hypothesized that **hypoxia, inhibition of glycolysis or inhibition of oxidative phosphorylation will induce the**

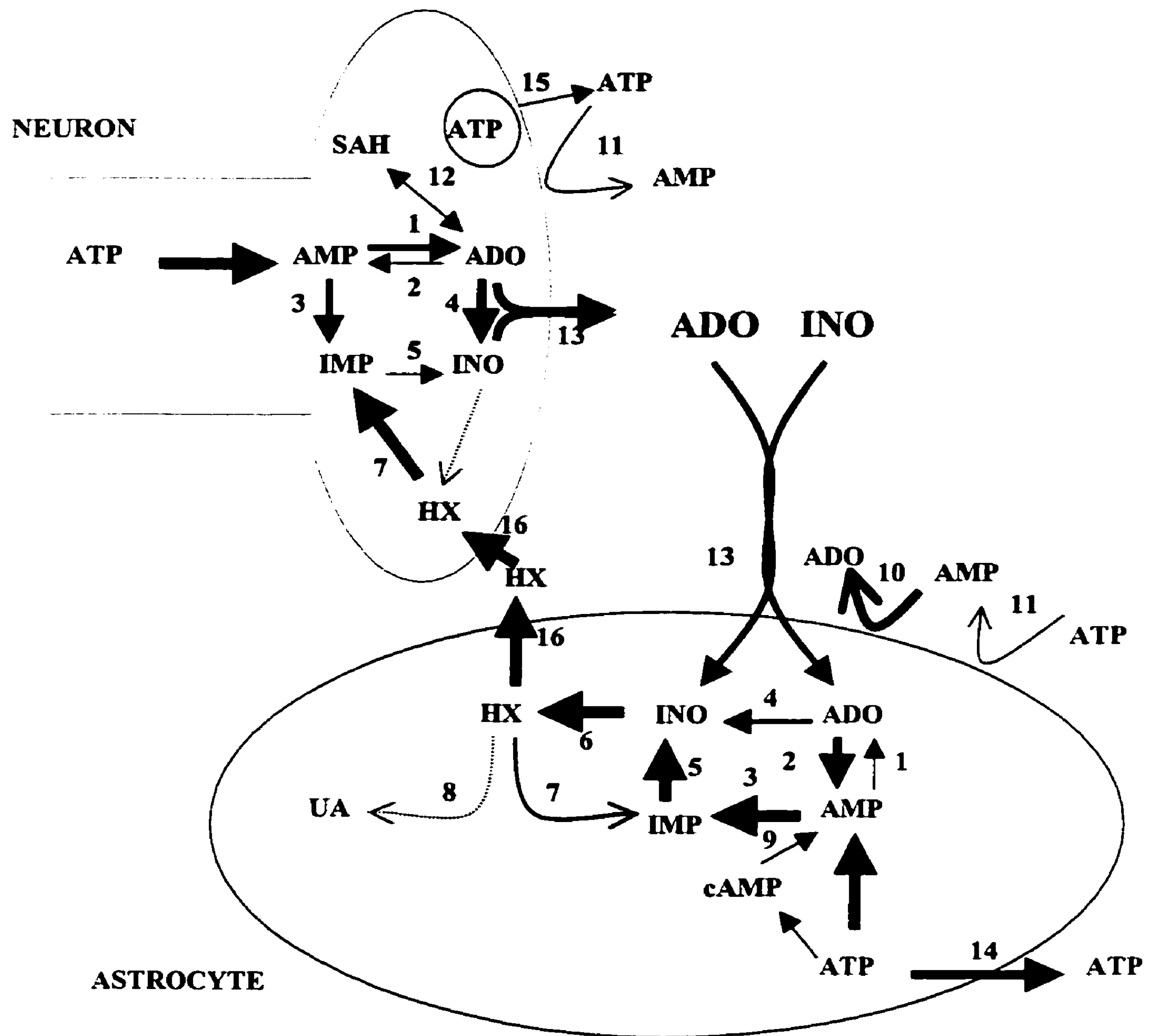
release of adenosine from rat primary cortical neurons and of inosine and hypoxanthine from rat primary cortical astrocytes. Once again, my hypothesis did not prove to be correct. Neither of these cell types released significant amounts of [^{3}H]adenosine in the absence of AK or ADA inhibitors. However, a number of interesting differences were seen between neurons and astrocytes with regard to purines released due to different stimuli. Under basal conditions, astrocytes released significantly more [^{3}H]purines than did neurons, predominantly due to large quantities of [^{3}H]AMP released by the astrocytes. This correlates with the proposed role of adenine nucleotides released from astrocytes in cell-cell signaling (Cotrina et al., 1998a; Cotrina et al., 1998b; Guthrie et al., 1999). During ATP-depeletion with hypoxic buffer, 100 μM NaCN or 500 μM IAA, neurons released significantly greater amounts of [^{3}H]purines than astrocytes. Neurons were particularly sensitive to hypoxia compared to astrocytes as neurons increased hypoxanthine and inosine release while astrocytes were not affected by this stimulus. This indicates that during hypoxia *in vivo,* neurons are likely the site of extracellular purine release while astrocytes may be a site of accumulation. In order to evaluate further the potential cycling of purines between astrocytes and neurons, future studies should evaluate purine release and accumulation between neurons and astrocytes in co-culture and physiological slice models. It is possible that mono-cultures of either cell type and cytoarchitectural effects of 2-dimensional cell culture plates may affect the mechanisms of purine regulation. In addition, future *in vitro* studies should attempt to decrease the extracellular:intracellular volume ratio, as the large extracellular volume of cell culture systems may affect the ability to gauge local purine levels. These studies along with better characterization of the cellular and regional distribution of nucleoside

transporters and purinergic enzymes will enable a better understanding of the sites of adenosine production in the CNS.

Based on the results of my final two manuscripts and the relevant literature, I would like to propose a purine "shuttle" or cycle between neurons and astrocytes (fig 25). As can be seen in fig 25, the putative cycle is complex. This hypothesis supports the notion that adenosine in the extracellular environment is derived from adenosine release *per se* predominantly from neurons and that astrocytes and neurons are both capable of releasing ATP, however, via different mechanisms (see section 3.1.1; fig 25 #14 and #15). Although ATP can be metabolized by neurons and astrocytes (fig 25 #11), the extracellular production of adenosine from adenine nucleotides occurs primarily on astrocytes as e-N has been reported to be on astrocytes but not neurons (Zimmermann, 1992) (fig 25 #10). Based on my final study, it appears that astrocytes may be more important for the release of adenine nucleotides than are neurons. It does not appear that either cell type releases appreciable quantities of adenine nucleotides during ATP depletion, indicating that this is not an important route of extracellular adenosine during these conditions.

Although neither neurons nor astrocytes released adenosine during ATP depleting conditions in the paradigms tested, adenosine release has been demonstrated in *in vivo* and slice models during similar conditions. The lack of adenosine release in the isolated cell culture systems may indicate an alteration in purine metabolism compared to *in vivo.* At present, no studies have demonstrated a differential distribution of nucleoside transporters in neurons and astrocytes. Based on molecular and transport studies (see section 3.2.1) in the mammalian CNS, one may hypothesize that both cell types possess

Figure 25: Schematic Diagram of Purine Cycle between Neurons and Astrocytes



A schematic diagram of a putative purine cycle between neurons and astrocytes. The arrows indicate the predominant direction of metabolism during ATP depleting conditions. The thick arrows indicate predominant pathways, narrow arrows indicate alternate pathways and broken arrows indicate pathways that do not appear to be present *in vivo* but have been reported *in vitro*. ADO-adenosine, INO-inosine, HX-hypoxanthine, UA-uric acid, SAH-S-adenosyl homocysteine, 1-AMP-selective 5'nucleotidase, 2-adenosine kinase, 3-AMP deaminase, 4-adenosine deaminase, 5-IMP-selective 5'nucleotidase, 6-purine nucleoside phosphorylase, 7-hypoxanthine-guanine phosphoribosyl transferase, 8-xanthine oxidase, 9-phosphodiesterases, 10-ecto-5'nucleotidase, 11-ecto-ATPases, 12- S-adenosylhomocysteine hydrolase, 13-nucleoside transporters, 14-non-vesicular ATP release, 15-vesicular ATP release,16-nucleobase transporters

equilibrative nucleoside transporters *in vivo*. Thus, it is likely neurons and astrocytes have the ability to accumulate and release adenosine and other nucleosides. The basic rationale behind this hypothesis lies not in the release of adenosine but in the asymmetric expression of purinergic enzymes. The most important difference in enzyme activity is seen in the expression of PNP in astrocytes but not in neurons *in vivo*. Although PNP has been reported in cultured neurons, astrocytic PNP expression would be expected to produce hypoxanthine rather than inosine during ATP depleting conditions, as was seen in the final two studies. Conversely, if neurons lacked PNP expression, these cells would be expected to release large quantities of inosine rather than hypoxanthine. The potential cycle depends on neuronal production of inosine and the ability of the astrocyte to metabolize inosine to hypoxanthine, which can then be released. During ATP limited conditions, inosine is primarily metabolized by PNP to hypoxanthine, as opposed to phosphotransferase by c-N-II (see section 3.5.2.2). If inosine is produced in neurons during ATP-depleting conditions, it will be released into the extracellular spaces by equilibrative nucleoside transporters. As similar increases in intracellular inosine will not be seen in astrocytes because of PNP activity, inosine released from neurons will be able to be transported into astrocytes. The inosine in astrocytes whether derived from astrocytic metabolism or accumulation from the extracellular environment will then be metabolized by PNP to hypoxanthine. The intracellular hypoxanthine can either be released into the extracellular compartment via equilibrative nucleobase transporters (fig 25 #16) or metabolized by HGPRT to IMP (fig 25 #7). Although xanthine oxidase has been reported in neurons and astrocytes *in vitro*, xanthine oxidase will not metabolize hypoxanthine in astrocytes *in vivo* as it is found in microglia and endothelial cells in the

CNS (Moriwaki et al., 1999; Terada et al., 1991). Based on this hypothesis, neurons release inosine, which is accumulate by astrocytes and metabolized to hypoxanthine that is released in the extracellular compartment. Hypoxanthine will then be accumulated into the neurons and metabolized by HGPRT to IMP. As the purine nucleotide cycle (AMP → IMP → adenyl succinate) has been reported to be important in maintaining neuronal energy charge (Van den Berghe et al., 1992), neuronal HGPRT metabolism of hypoxanthine would be important for replenishing nucleotide levels from the released inosine. This cycle would function similarly to the glutamate-glutamine cycle that has been demonstrated in astrocytes and neurons. The importance of this putative cycle to regulation of extracellular adenosine levels is unclear. This cycle also would function with adenosine release from neurons as inhibition of adenosine kinase activity during ATP depletion will limit the replenishing of adenine nucleotides from adenosine. However, there is little evidence from the literature or from my studies that would indicate that this occurs. While the hypothesis has a strong basis, a number of critical elements in addition to direct evidence is required to further support the concept. Although HGPRT activity has been demonstrated in astrocytes and neurons *in vitro*, there have been no reports of asymmetrical distribution of HGPRT activity between these cells *in vivo*. If there were greater activity of HGPRT in neurons, this would support the putative purinergic cycle. In addition, it would be very interesting to determine whether there is asymmetrical distribution of AK, ADA, c-N-I, c-N-II or AMPDA activity between astrocytes and neurons. While none of these enzymes would ultimately disprove the hypothesis, it would be strengthened if the activity of ADA, AMPDA and c-N-II were similar between the two cell types while AK and c-N-I were higher in neurons. ADA,

AMPDA and c-N-II would be integral to the production of inosine in both cells. Higher neuronal AK and c-N-I activities would indicate that adenosine is produced primarily from a neuronal source, and may be controlled on a minute-to-minute basis. While these experiments would help to elucidate this potential cycle, the only true measure of it would be to be able to follow the movement of purines between the two cells. Due to the complexity of this system, it is unlikely that this can be easily performed chemically or with radioisotopes. Thus, it may be very difficult to demonstrate the existence of this cycle for sure.

The overall importance of this thesis work is that it has provided a better understanding of how cells metabolize and transport adenosine and other purine nucleoside and nucleobases. It describes mechanisms of purine metabolism and how these can be pharmacologically regulated in order to facilitate increased levels of extracellular adenosine levels. It illustrates the complex systems involved in adenosine transport and metabolism. I am left with the opinion that designing a drug that is cell and BBB permeable, selectively active in the CNS and is reversible is a tall order (Von Lubitz, 1999). However, it is clear that adenosine has a multitude of effects and adenosine levels are affected by a wide range of drugs and stimuli.

References:

Abbott, N. J.: Inflammat y mediators and modulation of blood-brain barrier permeability. Cell Mol N urobiol **20** (2)**:** 131-47., 2000.

Abbracchio, M. P., Bran illa, R., Ceruti, S., Kim, H. O., von Lubitz, D. K., Jacobson, K. A., and Cattabeni, F.: G protein-dependent activation of phospholipase C by adenosine A3 receptors in rat brain. Mol Pharmacol **48** (6)**:** 1038-45., 1995.

Abbracchio, M. P., Ceruti, S., Brambilla, R., Franceschi, C., Malorni, W., Jacobson, K. A., von Lubitz, D. K., and Cattabeni, F.: Modulation of apoptosis by adenosine in the central nervous system: a possible role for the A3 receptor. Pathophysiological significance and therapeutic implications for neurodegenerative disorders. Ann N Y Acad Sci **825:** 11-22, 1997.

Adrian, G. S., Wiginton, D. A., and Hutton, J. J.: Structure of adenosine deaminase mRNAs from normal and adenosine deaminase-deficient human cell lines. Mol Cell Biol **4** (9)**:** 1712-7, 1984.

Agarwal, R. P., Cha, S., Crabtree, G. W., and Parks Jr., R. E.: Coformycin and Deoxycoformycin: Tight-binding inhibitors of adenosine deaminase. *In* Chemistry and Biology of Nucleosides and Nucleotides, Academic Press, 1978.

Allegrini, S., Pesi, R., Tozzi, M. G., Fiol, C. J., Johnson, R. B., and Eriksson, S.: Bovine cytosolic IMP/GMP-specific 5'-nucleotidase: cloning and expression of active enzyme in Escherichia coli. Biochem J **328** (Pt 2)**:** 483-7, 1997.

Ames, A.: CNS energy metabolism as related to function. Brain Res Brain Res Rev **34** (1-2)**:** 42-68., 2000.

Anderson, C. M., Baldwin, S. A., Young, J. D., Cass, C. E., and Parkinson, F. E.: Distribution of mRNA encoding a nitrobenzylthioinosine-insensitive nucleoside transporter (ENT2) in rat brain. Brain Res Mol Brain Res **70** (2): 293-7, 1999a.

Anderson, C. M., Sitar, D. S., and Parkinson, F. E.: Ability of nitrobenzylthioinosine to cross the blood-brain barrier in rats. Neurosci Lett **219** (3): 191-4, 1996a.

Anderson, C. M., Xiong, W., Geiger, J. D., Young, J. D., Cass, C. E., Baldwin, S. A., and Parkinson, F. E.: Distribution of equilibrative, nitrobenzylthioinosine-sensitive nucleoside transporters (ENT1) in brain. J Neurochem **73** (2): 867-73, 1999b.

Anderson, C. M., Xiong, W., Young, J. D., Cass, C. E., and Parkinson, F. E.: Demonstration of the existence of mRNAs encoding N1/cif and N2/cit sodium/nucleoside cotransporters in rat brain. Brain Res Mol Brain Res **42** (2): 358-61, 1996b.

Andine, P., Rudolphi, K. A., Fredholm, B. B., and Hagberg, H.: Effect of propentofylline (HWA 285) on extracellular purines and excitatory amino acids in CA1 of rat hippocampus during transient ischaemia. Br J Pharmacol **100** (4): 814-8., 1990.

Andres, C. M., and Fox, I. H.: Purification and properties of human placental adenosine kinase. J Biol Chem **254** (22): 11388-93, 1979.

Antonini, I., Cristalli, G., Franchetti, P., Grifantini, M., Martelli, S., Lupidi, G., and Riva, F.: Adenosine deaminase inhibitors. Synthesis of deaza analogues of erythro-9-(2-hydroxy-3-nonyl)adenine. J Med Chem **27** (3): 274-8., 1984.

Arch, J. R., and Newsholme, E. A.: The control of the metabolism and the hormonal role of adenosine. Essays Biochem **14**: 82-123, 1978.

Assender, J. W., Kontny, E., and Fredholm, B. B.: Expression of protein kinase C isoforms in smooth muscle cells in various states of differentiation. FEBS Lett **342** (1): 76-80, 1994.

Atkinson, M. R., Townsend-Nicholson, A., Nicholl, J. K., Sutherland, G. R., and Schofield, P. R.: Cloning, characterisation and chromosomal assignment of the human adenosine A3 receptor (ADORA3) gene. Neurosci Res **29** (1): 73-9., 1997.

Baiocchi, C., Pesi, R., Camici, M., Itoh, R., and Grazi Tozzi, M.: Mechanism of the reaction catalysed by cytosolic 5'-nucleotidase/phosphotransferase: formation of a phosphorylated intermediate. Biochem J **317** (Pt 3): 797-801, 1996.

Ballarin, M., Fredholm, B. B., Ambrosio, S., and Mahy, N.: Extracellular levels of adenosine and its metabolites in the striatum of awake rats: inhibition of uptake and metabolism. Acta Physiol Scand **142** (1): 97-103, 1991.

Ballerini, P., Ciccarelli, R., Di Iorio, P., Giuliani, P., and Caciagli, F.: Influence of Ca2+ channel modulators on [3H]purine release from rat cultured glial cells. Neurochem Res **20** (6): 697-704, 1995.

Baraldi, P. G., Cacciari, B., Spalluto, G., Pineda de las Infantas y Villatoro, M. J., Zocchi, C., Dionisotti, S., and Ongini, E.: Pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine derivatives: potent and selective A(2A) adenosine antagonists. J Med Chem **39** (5): 1164-71., 1996.

Barankiewicz, J., Danks, A. M., Abushanab, E., Makings, L., Wiemann, T., Wallis, R. A., Pragnacharyulu, P. V., Fox, A., and Marangos, P. J.: Regulation of adenosine concentration and cytoprotective effects of novel reversible adenosine deaminase inhibitors. J Pharmacol Exp Ther **283** (3): 1230-8, 1997.

Barankiewicz, J., Ronlov, G., Jimenez, R., and Gruber, H. E.: Selective adenosine release from human B but not T lymphoid cell line. J Biol Chem **265** (26): 15738-43, 1990.

Barros, L. F.: Hypoxanthine transport in the guinea pig and human placenta is a carrier-mediated process that does not involve nucleoside transporters. Am J Obstet Gynecol **171** (1): 111-7, 1994.

Bender, A. S., and Hertz, L.: Similarities of adenosine uptake systems in astrocytes and neurons in primary cultures. Neurochem Res **11** (11): 1507-24, 1986.

Bender, A. S., and Hertz, L.: Dissimilarities between benzodiazepine-binding sites and adenosine uptake sites in astrocytes and neurons in primary cultures. J Neurosci Res **17** (2): 154-61, 1987.

Bleich, S., Degner, D., Wiltfang, J., Maler, J. M., Niedmann, P., Cohrs, S., Mangholz, A., Porzig, J., Sprung, R., Ruther, E., and Kornhuber, J.: Elevated homocysteine levels in alcohol withdrawal. Alcohol Alcohol **35** (4): 351-4., 2000a.

Bleich, S., Spilker, K., Kurth, C., Degner, D., Quintela-Schneider, M., Javaheripour, K., Ruther, E., Kornhuber, J., and Wiltfang, J.: Oxidative stress and an altered methionine metabolism in alcoholism. Neurosci Lett **293** (3): 171-4., 2000b.

Bolling, S. F., Olszanski, D. A., Bove, E. L., and Childs, K. F.: Enhanced myocardial protection during global ischemia with 5'-nucleotidase inhibitors. J Thorac Cardiovasc Surg **103** (1): 73-7., 1992.

Bontemps, F., Van den Berghe, G., and Hers, H. G.: Evidence for a substrate cycle between AMP and adenosine in isolated hepatocytes. Proc Natl Acad Sci U S A **80** (10): 2829-33, 1983.

Bontemps, F., Vincent, M. F., Van den Bergh, F., van Waeg, G., and Van den Berghe, G.: Stimulation by glycerate 2,3-bisphosphate: a common property of cytosolic IMP-GMP 5'-nucleotidase in rat and human tissues. Biochim Biophys Acta **997** (1-2): 131-4, 1989.

Bookser, B. C., Kasibhatla, S. R., Appleman, J. R., and Erion, M. D.: AMP deaminase inhibitors. 2. Initial discovery of a non-nucleotide transition-state inhibitor series. J Med Chem **43** (8): 1495-507., 2000a.

Bookser, B. C., Kasibhatla, S. R., and Erion, M. D.: AMP deaminase inhibitors. 4. Further N3-substituted coformycin aglycon analogues: N3-alkylmalonates as ribose 5'-monophosphate mimetics. J Med Chem **43** (8): 1519-24., 2000b.

Borgland, S. L., Castanon, M., Spevak, W., and Parkinson, F. E.: Effects of propentofylline on adenosine receptor activity in Chinese hamster ovary cell lines transfected with human A1, A2A, or A2B receptors and a luciferase reporter gene. Can J Physiol Pharmacol **76** (12): 1132-8, 1998.

Borgland, S. L., and Parkinson, F. E.: Uptake and release of [3H]formycin B via sodium-dependent nucleoside transporters in mouse leukemic L1210/MA27.1 cells. J Pharmacol Exp Ther **281** (1): 347-53, 1997.

Borgland, S. L., and Parkinson, F. E.: Effect of adenosine receptor agonists on release of the nucleoside analogue [3H]formycin B from cultured smooth muscle DDT1 MF-2 cells. Eur J Pharmacol **346** (2-3): 339-44, 1998.

Braun, N., Sevigny, J., Robson, S. C., Enjyoji, K., Guckelberger, O., Hammer, K., Di Virgilio, F., and Zimmermann, H.: Assignment of ecto-nucleoside triphosphate

diphosphohydrolase-1/cd39 expression to microglia and vasculature of the brain. Eur J Neurosci **12** (12)**:** 4357-66., 2000.

Braun, S., and Levitzki, A.: Adenosine receptor permanently coupled to turkey erythrocyte adenylate cyclase. Biochemistry **18** (10)**:** 2134-8., 1979.

Britton, D. R., Mikusa, J., Lee, C. H., Jarvis, M. F., Williams, M., and Kowaluk, E. A.: Site and event specific increase of striatal adenosine release by adenosine kinase inhibition in rats. Neurosci Lett **266** (2)**:** 93-6, 1999.

Bukoski, R. D., and Sparks, H. V., Jr.: Adenosine production and release by adult rat cardiocytes. J Mol Cell Cardiol **18** (6)**:** 595-605, 1986.

Bunn, H. F., and Poyton, R. O.: Oxygen sensing and molecular adaptation to hypoxia. Physiol Rev **76** (3)**:** 839-85., 1996.

Burnstock, G.: The purinergic nerve hypothesis. Ciba Found Symp **48:** 295-314, 1977.

Caciagli, F., Ciccarelli, R., Di Iorio, P., Tacconelli, L., and Ballerini, P.: Influence of PLA2-PG system on purine release and cAMP content in dissociated primary glial cultures from rat striatum. Pharmacol Res **21** (3)**:** 271-84, 1989.

Caciagli, F., Di Iorio, P., Giuliani, G., Patricelli, P., Glasky, A., and Rathbone, M.: The hypoxanthine analog AIT-082 mimics the activity of guanosine in affecting extracellular adenosine breakdown and glutamate reuptake in rat cultured astrocytes. Society for Neuroscience Abstracts **29:** 1013, 1999.

Caputto, R. J.: The enzymatic synthesis of adenylic acid; adenosine kinase. J Biol Chem **189:** 801-814, 1951.

Cass, C. E., Young, J. D., and Baldwin, S. A.: Recent advances in the molecular biology of nucleoside transporters of mammalian cells. Biochem Cell Biol **76** (5)**:** 761-70, 1998.

Castellano, B., Gonzalez, B., Finsen, B. R., and Zimmer, J.: Histochemical demonstration of purine nucleoside phosphorylase (PNPase) in microglial and astroglial cells of adult rat brain. J Histochem Cytochem **38** (11): 1535-9, 1990.

Ceballos, G., and Rubio, R.: Coculture of astroglial and vascular endothelial cells as apposing layers enhances the transcellular transport of hypoxanthine. J Neurochem **64** (3): 991-9, 1995.

Ceballos, G., Tuttle, J. B., and Rubio, R.: Differential distribution of purine metabolizing enzymes between glia and neurons. J Neurochem **62** (3): 1144-53, 1994.

Centelles, J. J., Franco, R., and Bozal, J.: Purification and partial characterization of brain adenosine deaminase: inhibition by purine compounds and by drugs. J Neurosci Res **19** (2): 258-67, 1988.

Chadwick, B. P., and Frischauf, A. M.: The CD39-like gene family: identification of three new human members (CD39L2, CD39L3, and CD39L4), their murine homologues, and a member of the gene family from Drosophila melanogaster. Genomics **50** (3): 357-67., 1998.

Chandel, N. S., and Schumacker, P. T.: Cellular oxygen sensing by mitochondria: old questions, new insight. J Appl Physiol **88** (5): 1880-9., 2000.

Chang, C. H., Brockman, R. W., and Bennett, L. L., Jr.: Adenosine kinase from L1210 cells. Purification and some properties of the enzyme. J Biol Chem **255** (6): 2366-71, 1980.

Che, M., Ortiz, D. F., and Arias, I. M.: Primary structure and functional expression of a cDNA encoding the bile canalicular, purine-specific Na(+)-nucleoside cotransporter. J Biol Chem **270** (23): 13596-9, 1995.

Chen, J. F., Beilstein, M., Xu, Y. H., Turner, T. J., Moratalla, R., Standaert, D. G., Aloyo, V. J., Fink, J. S., and Schwarzschild, M. A.: Selective attenuation of psychostimulant-induced behavioral responses in mice lacking A(2A) adenosine receptors. Neuroscience **97** (1): 195-204, 2000.

Chen, J. F., Huang, Z., Ma, J., Zhu, J., Moratalla, R., Standaert, D., Moskowitz, M. A., Fink, J. S., and Schwarzschild, M. A.: A(2A) adenosine receptor deficiency attenuates brain injury induced by transient focal ischemia in mice. J Neurosci **19** (21): 9192-200., 1999.

Chen, W., and Gueron, M.: AMP degradation in the perfused rat heart during 2-deoxy-D-glucose perfusion and anoxia. Part II: The determination of the degradation pathways using an adenosine deaminase inhibitor. J Mol Cell Cardiol **28** (10): 2175-82, 1996.

Chen, W., Hoerter, J., and Gueron, M.: A comparison of AMP degradation in the perfused rat heart during 2- deoxy-D-glucose perfusion and anoxia. Part I: The release of adenosine and inosine. J Mol Cell Cardiol **28** (10): 2163-74, 1996.

Chen, Y., Graham, D. I., and Stone, T. W.: Release of endogenous adenosine and its metabolites by the activation of NMDA receptors in the rat hippocampus in vivo. Br J Pharmacol **106** (3): 632-8., 1992.

Chern, Y., King, K., Lai, H. L., and Lai, H. T.: Molecular cloning of a novel adenosine receptor gene from rat brain. Biochem Biophys Res Commun **185** (1): 304-9., 1992.

Ciccarelli, R., Di Iorio, P., Ballerini, P., Ambrosini, G., Giuliani, P., Tiboni, G. M., and Caciagli, F.: Effects of exogenous ATP and related analogues on the proliferation rate of dissociated primary cultures of rat astrocytes. J Neurosci Res **39** (5): 556-66, 1994.

Ciccarelli, R., Di Iorio, P., Giuliani, P., D'Alimonte, I., Ballerini, P., Caciagli, F., and Rathbone, M. P.: Rat cultured astrocytes release guanine-based purines in basal conditions and after hypoxia/hypoglycemia. Glia **25** (1): 93-8, 1999.

Ciruela, F., Saura, C., Canela, E. I., Mallol, J., Lluis, C., and Franco, R.: Adenosine deaminase affects ligand-induced signalling by interacting with cell surface adenosine receptors. FEBS Lett **380** (3): 219-23, 1996.

Coe, I. R., Dohrman, D. P., Constantinescu, A., Diamond, I., and Gordon, A. S.: Activation of cyclic AMP-dependent protein kinase reverses tolerance of a nucleoside transporter to ethanol. J Pharmacol Exp Ther **276** (2): 365-9, 1996.

Cohen, S. S.: Inhibitors of adenosine deaminase and the development of antiviral agents. Ann N Y Acad Sci **451**: 204-14, 1985.

Cornfield, L. J., Hu, S., Hurt, S. D., and Sills, M. A.: [3H]2-phenylaminoadenosine ([3H]CV 1808) labels a novel adenosine receptor in rat brain. J Pharmacol Exp Ther **263** (2): 552-61., 1992.

Cornford, E. M., and Oldendorf, W. H.: Independent blood-brain barrier transport systems for nucleic acid precursors. Biochim Biophys Acta **394** (2): 211-9, 1975.

Cotrina, M. L., Lin, J. H., Alves-Rodrigues, A., Liu, S., Li, J., Azmi-Ghadimi, H., Kang, J., Naus, C. C., and Nedergaard, M.: Connexins regulate calcium signaling by controlling ATP release. Proc Natl Acad Sci U S A **95** (26): 15735-40, 1998a.

Cotrina, M. L., Lin, J. H., Lopez-Garcia, J. C., Naus, C. C., and Nedergaard, M.: ATP-Mediated glia signaling. J Neurosci **20** (8): 2835-44, 2000.

Cotrina, M. L., Lin, J. H., and Nedergaard, M.: Cytoskeletal assembly and ATP release regulate astrocytic calcium signaling. J Neurosci **18** (21): 8794-804, 1998b.

Craig, C. G., Temple, S. D., and White, T. D.: Is cyclic AMP involved in excitatory amino acid-evoked adenosine release from rat cortical slices? Eur J Pharmacol **269** (1): 79-85, 1994.

Craig, C. G., and White, T. D.: NMDA-evoked adenosine release from rat cortex does not require the intermediate formation of nitric oxide. Neurosci Lett **158** (2): 167-9, 1993.

Crawford, C. R., Patel, D. H., Naeve, C., and Belt, J. A.: Cloning of the human equilibrative, nitrobenzylmercaptopurine riboside (NBMPR)-insensitive nucleoside transporter ei by functional expression in a transport-deficient cell line. J Biol Chem **273** (9): 5288-93, 1998.

Cronstein, B. N., Naime, D., and Firestein, G.: The antiinflammatory effects of an adenosine kinase inhibitor are mediated by adenosine. Arthritis Rheum **38** (8): 1040-5, 1995.

Daddona, P. E., Shewach, D. S., Kelley, W. N., Argos, P., Markham, A. F., and Orkin, S. H.: Human adenosine deaminase. cDNA and complete primary amino acid sequence. J Biol Chem **259** (19): 12101-6, 1984.

Daly, J. W., Padgett, W., and Seamon, K. B.: Activation of cyclic AMP-generating systems in brain membranes and slices by the diterpene forskolin: augmentation of receptor-mediated responses. J Neurochem **38** (2): 532-44., 1982.

Darvish, A., and Metting, P. J.: Purification and regulation of an AMP-specific cytosolic 5'-nucleotidase from dog heart. Am J Physiol **264** (5 Pt 2): H1528-34, 1993.

Daval, J. L., Barberis, C., and Gayet, J.: Release of [14C]adenosine derivatives from superfused synaptosome preparations. Effects of depolarizing agents and metabolic inhibitors. Brain Res **181** (1): 161-74, 1980.

Daval, J. L., Nicolas, F., and Doriat, J. F.: Adenosine physiology and pharmacology: how about A2 receptors? Pharmacol Ther **71** (3): 325-35, 1996.

Davies, L. P., and Cook, A. F.: Inhibition of adenosine kinase and adenosine uptake in guinea-pig CNS tissue by halogenated tubercidin analogues. Life Sci **56** (17): L345-9, 1995.

de Zwart, M., Link, R., von Frijtag Drabbe Kunzel, J. K., Cristalli, G., Jacobson, K. A., Townsend-Nicholson, A., and AP, I. J.: A functional screening of adenosine analogues at the adenosine A2B receptor: a search for potent agonists. Nucleosides Nucleotides **17** (6): 969-85., 1998.

Deckert, J., Bisserbe, J. C., Klein, E., and Marangos, P. J.: Adenosine uptake sites in brain: regional distribution of putative subtypes in relationship to adenosine A1-receptors. J Neurosci **8** (7): 2338-49., 1988a.

Deckert, J., and Gleiter, C. H.: Adenosine--an endogenous neuroprotective metabolite and neuromodulator. J Neural Transm Suppl **43** (21): 23-31, 1994.

Deckert, J., Morgan, P. F., and Marangos, P. J.: Adenosine uptake site heterogeneity in the mammalian CNS? Uptake inhibitors as probes and potential neuropharmaceuticals. Life Sci **42** (14): 1331-45, 1988b.

Decking, U. K., Arens, S., Schlieper, G., Schulze, K., and Schrader, J.: Dissociation between adenosine release, MVO2, and energy status in working guinea pig hearts. Am J Physiol **272** (1 Pt 2): H371-81, 1997a.

Decking, U. K., Schlieper, G., Kroll, K., and Schrader, J.: Hypoxia-induced inhibition of adenosine kinase potentiates cardiac adenosine release. Circ Res **81** (2): 154-64, 1997b.

del Zoppo, G. J., and Hallenbeck, J. M.: Advances in the vascular pathophysiology of ischemic stroke. Thromb Res **98** (3): 73-81., 2000.

Delaney, S. M., Blackburn, G. M., and Geiger, J. D.: Diadenosine polyphosphates inhibit adenosine kinase activity but decrease levels of endogenous adenosine in rat brain. Eur J Pharmacol **332** (1): 35-42, 1997.

Delaney, S. M., and Geiger, J. D.: Brain regional levels of adenosine and adenosine nucleotides in rats killed by high-energy focused microwave irradiation. J Neurosci Methods **64** (2): 151-6, 1996.

DeLeo, J., Schubert, P., and Kreutzberg, G. W.: Propentofylline (HWA 285) protects hippocampal neurons of Mongolian gerbils against ischemic damage in the presence of an adenosine antagonist. Neurosci Lett **84** (3): 307-11., 1988a.

DeLeo, J., Schubert, P., and Kreutzberg, G. W.: Protection against ischemic brain damage using propentofylline in gerbils. Stroke **19** (12): 1535-9., 1988b.

Delicado, E. G., Rodrigues, A., Sen, R. P., Sebastiao, A. M., Ribeiro, J. A., and Miras-Portugal, M. T.: Effect of 5'-(N-ethylcarboxamido)adenosine on adenosine transport in cultured chromaffin cells. J Neurochem **54** (6): 1941-6, 1990.

Delicado, E. G., Sen, R. P., and Miras-Portugal, M. T.: Effects of phorbol esters and secretagogues on nitrobenzylthioinosine binding to nucleoside transporters and nucleoside uptake in cultured chromaffin cells. Biochem J **279** (Pt 3): 651-5, 1991.

Dickenson, J. M., and Hill, S. J.: Interactions between adenosine A_1- and histamine H_1-receptors. International Journal of Biochemistry **26** (8): 959-969, 1994.

Dixon, A. K., Gubitz, A. K., Sirinathsinghji, D. J., Richardson, P. J., and Freeman, T. C.: Tissue distribution of adenosine receptor mRNAs in the rat. Br J Pharmacol **118** (6): 1461-8., 1996.

Dobbin, J., Crockard, H. A., and Ross-Russell, R.: Transient blood-brain barrier permeability following profound temporary global ischaemia: an experimental study using 14C-AIB. J Cereb Blood Flow Metab **9** (1): 71-8, 1989.

Dolhun, B. A., and Parkinson, F. E.: Effect of chronic treatment with an adenosine receptor agonist on nucleoside transport sites in DDT1 MF-2 smooth muscle cells. Proc. West. Pharmacol. Soc. **38:** 157, 1995.

Domanska-Janik, K., and Bourre, J. M.: Effect of lipid peroxidation on Na+,K(+)-ATPase, 5'-nucleotidase and CNPase in mouse brain myelin. Biochim Biophys Acta **1034** (2): 200-6., 1990.

Doolette, D. J.: Mechanism of adenosine accumulation in the hippocampal slice during energy deprivation. Neurochem Int **30** (2): 211-23, 1997.

Drury, A. N.: The physiological activity of nucleic acids and its derivatives. Physiol Rev **16:** 292-325, 1936.

Drury, A. N., and Szent-Gyorgi, A.: The physiological activity of adenine compounds with special refernce to their action upon mammalian heart. J Physiol Lond **68:** 213-237, 1929.

Dunwiddie, T. V., and Diao, L.: Regulation of extracellular adenosine in rat hippocampal slices is temperature dependent: role of adenosine transporters. Neuroscience **95** (1): 81-8, 2000.

Dux, E., Fastbom, J., Ungerstedt, U., Rudolphi, K., and Fredholm, B. B.: Protective effect of adenosine and a novel xanthine derivative propentofylline on the cell damage after bilateral carotid occlusion in the gerbil hippocampus. Brain Res **516** (2): 248-56., 1990.

Edlund, A., Siden, A., and Sollevi, A.: Evidence for an anti-aggregatory effect of adenosine at physiological concentrations and for its role in the action of dipyridamole. Thromb Res **45** (2): 183-90, 1987.

Eldridge, F. L., Paydarfar, D., Scott, S. C., and Dowell, R. T.: Role of endogenous adenosine in recurrent generalized seizures. Exp Neurol **103** (2): 179-85., 1989.

Feoktistov, I., Goldstein, A. E., and Biaggioni, I.: Role of p38 mitogen-activated protein kinase and extracellular signal-regulated protein kinase kinase in adenosine A2B receptor-mediated interleukin-8 production in human mast cells. Mol Pharmacol **55** (4): 726-34., 1999.

Fern, R., Waxman, S. G., and Ransom, B. R.: Modulation of anoxic injury in CNS white matter by adenosine and interaction between adenosine and GABA. J Neurophysiol **72** (6): 2609-16., 1994.

Fernandez-Rivera-Rio, L., and Gonzalez-Garcia, M. R.: The human erythrocyte ghost: a new experimental model for studying adenosine transport. Arch Biochem Biophys **240** (1): 246-56, 1985.

Fields, R. D., and Stevens, B.: ATP: an extracellular signaling molecule between neurons and glia. Trends Neurosci **23** (12): 625-33., 2000.

Fishbein, W. N., Davis, J. I., Winkert, J. W., and Strong, D. M.: Levels of adenosine deaminase AMP deaminase, and adenylate kinase in cultured human lymphoblast lines:

exquisite sensitivity of AMP deaminase to adenosine deaminase inhibitors. Biochem Med **26** (3): 377-86, 1981.

Fisher, M. N., and Newsholme, E. A.: Properties of rat heart adenosine kinase. Biochem J **221** (2): 521-8, 1984.

Fowler, J. C.: Purine release and inhibition of synaptic transmission during hypoxia and hypoglycemia in rat hippocampal slices. Neurosci Lett **157** (1): 83-6, 1993.

Fox, I. H.: Metabolic basis for disorders of purine nucleotide degradation. Metabolism **30** (6): 616-34, 1981.

Fox, I. H., and Kelley, W. N.: The role of adenosine and 2'-deoxyadenosine in mammalian cells. Annu Rev Biochem **47:** 655-86, 1978.

Franco, R., Casado, V., Ciruela, F., Saura, C., Mallol, J., Canela, E. I., and Lluis, C.: Cell surface adenosine deaminase: much more than an ectoenzyme. Prog Neurobiol **52** (4): 283-94, 1997.

Franco, R., Valenzuela, A., Lluis, C., and Blanco, J.: Enzymatic and extraenzymatic role of ecto-adenosine deaminase in lymphocytes. Immunol Rev **161:** 27-42, 1998.

Fredholm, B. B.: Purinoceptors in the nervous system. Pharmacol Toxicol **76** (4): 228-39, 1995.

Fredholm, B. B.: Adenosine and neuroprotection. Int Rev Neurobiol **40:** 259-80, 1997.

Fredholm, B. B., Dunwiddie, T. V., Bergman, B., and Lindstrom, K.: Levels of adenosine and adenine nucleotides in slices of rat hippocampus. Brain Res **295** (1): 127-36, 1984.

Fredholm, B. B., Hedqvist, P., and Vernet, L.: Effect of theophylline and other drugs on rabbit renal cyclic nucleotide phosphodiesterase, 5'-nucleotidase and adenosine deaminase. Biochem Pharmacol **27** (24): 2845-50, 1978.

Furlong, T. J., Pierce, K. D., Selbie, L. A., and Shine, J.: Molecular characterization of a human brain adenosine A2 receptor. Brain Res Mol Brain Res **15** (1-2): 62-6., 1992.

Garvey, E. P., and Prus, K. L.: A specific inhibitor of heart cytosolic 5'-nucleotidase I attenuates hydrolysis of adenosine 5'-monophosphate in primary rat myocytes. Arch Biochem Biophys **364** (2): 235-40., 1999.

Gebauer, A., Merger, M., and Kilbinger, H.: Modulation by 5-HT3 and 5-HT4 receptors of the release of 5- hydroxytryptamine from the guinea-pig small intestine. Naunyn Schmiedebergs Arch Pharmacol **347** (2): 137-40, 1993.

Geiger, J. D., Johnston, M. E., and Yago, V.: Pharmacological characterization of rapidly accumulated adenosine by dissociated brain cells from adult rat. J Neurochem **51** (1): 283-91, 1988.

Geiger, J. D., LaBella, F. S., and Nagy, J. I.: Characterization of nitrobenzylthioinosine binding to nucleoside transport sites selective for adenosine in rat brain. J Neurosci **5** (3): 735-40., 1985.

Geiger, J. D., and Nagy, J. I.: Distribution of adenosine deaminase activity in rat brain and spinal cord. J Neurosci **6** (9): 2707-14, 1986.

Geiger, J. D., and Nagy, J. I.: Adenosine Deaminase and [3H]Nitrobenzylthioinosine as Markers of Adenosine Metabolism and Transport in Central Purinergic Systems. *In* Adenosine and Adenosine Receptors, ed. by M. Williams, pp. 225-288, Humana Press, 1990.

Geiger, J. D., Parkinson, F. E., and Kowaluk, E. A.: Regulators of Endogenous Adenosine Levels as Therapeutic Targets. *In* Purinergic Approaches in Experimental

Therapeutics, ed. by K. A. Jacobson and M. F. Jarvis, pp. 55-84, Wiley-Liss Inc., New York, 1997.

German, D. C., Kredich, N. M., and Bjornsson, T. D.: Oral dipyridamole increases plasma adenosine levels in human beings. Clin Pharmacol Ther **45** (1): 80-4, 1989.

Gerwins, P., and Fredholm, B. B.: Activation of adenosine A1 and bradykinin receptors increases protein kinase C and phospholipase D activity in smooth muscle cells. Naunyn Schmiedebergs Arch Pharmacol **351** (2): 186-93, 1995a.

Gerwins, P., and Fredholm, B. B.: Activation of phospholipase C and phospholipase D by stimulation of adenosine A1, bradykinin or P2U receptors does not correlate well with protein kinase C activation. Naunyn Schmiedebergs Arch Pharmacol **351** (2): 194-201, 1995b.

Giblett, E. R., Anderson, J. E., Cohen, F., Pollara, B., and Meuwissen, H. J.: Adenosine-deaminase deficiency in two patients with severely impaired cellular immunity. Lancet **2** (7786): 1067-9., 1972.

Gibson, W. B., and Drummond, G. I.: Properties of 5'-nucleotidase from avian heart. Biochemistry **11** (2): 223-9., 1972.

Gidday, J. M., Fitzgibbons, J. C., Shah, A. R., Kraujalis, M. J., and Park, T. S.: Reduction in cerebral ischemic injury in the newborn rat by potentiation of endogenous adenosine. Pediatr Res **38** (3): 306-11, 1995.

Goding, J. W., Terkeltaub, R., Maurice, M., Deterre, P., Sali, A., and Belli, S. I.: Ecto-phosphodiesterase/pyrophosphatase of lymphocytes and non-lymphoid cells: structure and function of the PC-1 family. Immunol Rev **161** (1): 11-26., 1998.

Goldberg, M. P., and Choi, D. W.: Combined oxygen and glucose deprivation in cortical cell culture: calcium-dependent and calcium-independent mechanisms of neuronal injury. J Neurosci **13** (8): 3510-24., 1993.

Goldberg, M. P., Weiss, J. H., Pham, P. C., and Choi, D. W.: N-methyl-D-aspartate receptors mediate hypoxic neuronal injury in cortical culture. J Pharmacol Exp Ther **243** (2): 784-91., 1987.

Golembiowska, K., White, T. D., and Sawynok, J.: Adenosine kinase inhibitors augment release of adenosine from spinal cord slices. Eur J Pharmacol **307** (2): 157-62, 1996.

Griffith, D. A., and Jarvis, S. M.: Nucleoside and nucleobase transport systems of mammalian cells. Biochim Biophys Acta **1286** (3): 153-81, 1996.

Griffiths, M., Beaumont, N., Yao, S. Y., Sundaram, M., Boumah, C. E., Davies, A., Kwong, F. Y., Coe, I., Cass, C. E., Young, J. D., and Baldwin, S. A.: Cloning of a human nucleoside transporter implicated in the cellular uptake of adenosine and chemotherapeutic drugs [see comments]. Nat Med **3** (1): 89-93, 1997a.

Griffiths, M., Yao, S. Y., Abidi, F., Phillips, S. E., Cass, C. E., Young, J. D., and Baldwin, S. A.: Molecular cloning and characterization of a nitrobenzylthioinosine-insensitive (ei) equilibrative nucleoside transporter from human placenta. Biochem J **328** (Pt 3): 739-43, 1997b.

Gu, J. G., Foga, I. O., Parkinson, F. E., and Geiger, J. D.: Involvement of bidirectional adenosine transporters in the release of L- [3H]adenosine from rat brain synaptosomal preparations. J Neurochem **64** (5): 2105-10, 1995.

Gu, J. G., Nath, A., and Geiger, J. D.: Characterization of inhibitor-sensitive and -resistant adenosine transporters in cultured human fetal astrocytes. J Neurochem **67** (3): 972-7, 1996.

Guieu, R., Couraud, F., Pouget, J., Sampieri, F., Bechis, G., and Rochat, H.: Adenosine and the nervous system: clinical implications. Clin Neuropharmacol **19** (6): 459-74, 1996.

Guthrie, P. B., Knappenberger, J., Segal, M., Bennett, M. V. L., Charles, A. C., and Kater, S. B.: ATP released from astrocytes mediates glial calcium waves. J Neurosci **19** (2): 520-8, 1999.

Haag, P., Schneider, T., Schabitz, W., and Hacke, W.: Effect of propentofylline (HWA 285) on focal ischemia in rats: effect of treatment and posttreatment duration on infarct size. J Neurol Sci **175** (1): 52-6., 2000.

Hagberg, H., Andersson, P., Lacarewicz, J., Jacobson, I., Butcher, S., and Sandberg, M.: Extracellular adenosine, inosine, hypoxanthine, and xanthine in relation to tissue nucleotides and purines in rat striatum during transient ischemia. J Neurochem **49** (1): 227-31., 1987.

Hammond, J. R.: Comparative pharmacology of the nitrobenzylthioguanosine-sensitive and -resistant nucleoside transport mechanisms of Ehrlich ascites tumor cells. J Pharmacol Exp Ther **259** (2): 799-807, 1991.

Hammond, J. R.: Differential uptake of [3H]guanosine by nucleoside transporter subtypes in Ehrlich ascites tumour cells. Biochem J **287** (Pt 2): 431-6, 1992.

Hammond, J. R.: Functional reconstitution of pharmacologically distinct subtypes of nucleoside transporters in liposomal membranes. J Pharmacol Exp Ther **271** (2): 906-17, 1994.

Hammond, J. R., Williams, E. F., and Clanachan, A. S.: Affinity of calcium channel inhibitors, benzodiazepines, and other vasoactive compounds for the nucleoside transport system. Can J Physiol Pharmacol **63** (10): 1302-7., 1985.

Harriman, G. C., Poirot, A. F., Abushanab, E., Midgett, R. M., and Stoeckler, J. D.: Adenosine deaminase inhibitors. Synthesis and biological evaluation of C1' and nor-C1' derivatives of (+)-erythro-9-(2(S)-hydroxy-3(R)-nonyl)adenine. J Med Chem **35** (22): 4180-4., 1992.

Haun, S. E., Segeleon, J. E., Trapp, V. L., Clotz, M. A., and Horrocks, L. A.: Inosine mediates the protective effect of adenosine in rat astrocyte cultures subjected to combined glucose-oxygen deprivation. J Neurochem **67** (5): 2051-9, 1996.

He, M. X., Gorman, M. W., Romig, G. D., and Sparks, H. V., Jr.: Adenosine formation and myocardial energy status during graded hypoxia. J Mol Cell Cardiol **24** (1): 79-89, 1992.

Hebb, M. O., and White, T. D.: Co-administration of adenosine kinase and deaminase inhibitors produces supra-additive potentiation of N-methyl-D-aspartate-evoked adenosine formation in cortex. Eur J Pharmacol **344** (2-3): 121-5, 1998.

Henderson, J. F., Paterson, A. R., Caldwell, I. C., Paul, B., Chan, M. C., and Lau, K. F.: Inhibitors of nucleoside and nucleotide metabolism. Cancer Chemother Rep [2] **3** (1): 71-85, 1972.

Hertz, L.: Kinetics of adenosine uptake into astrocytes. J Neurochem **31** (1): 55-62, 1978.

Heyliger, C. E., Panagia, V., and Dhalla, N. S.: Effect of cyclic AMP phosphodiesterase inhibitors on cardiac sarcolemmal 5'-nucleotidase. J Pharmacol Exp Ther **217** (2): 489-93., 1981.

Hirano, D., Aoki, Y., Ogasawara, H., Kodama, H., Waga, I., Sakanaka, C., Shimizu, T., and Nakamura, M.: Functional coupling of adenosine A2a receptor to inhibition of the mitogen-activated protein kinase cascade in Chinese hamster ovary cells. Biochem J **316** (Pt 1)(2): 81-6., 1996.

Hosli, E., and Hosli, L.: Autoradiographic studies on the uptake of adenosine and on binding of adenosine analogues in neurons and astrocytes of cultured rat cerebellum and spinal cord. Neuroscience **24** (2): 621-8, 1988.

Huang, M., Shimizu, H., and Daly, J. W.: Accumulation of cyclic adenosine monophosphate in incubated slices of brain tissue. 2. Effects of depolarizing agents, membrane stabilizers, phosphodiesterase inhibitors, and adenosine analogs. J Med Chem **15** (5): 462-6., 1972.

Huang, Q. Q., Yao, S. Y., Ritzel, M. W., Paterson, A. R., Cass, C. E., and Young, J. D.: Cloning and functional expression of a complementary DNA encoding a mammalian nucleoside transport protein. J Biol Chem **269** (27): 17757-60, 1994.

Hunsucker, S. A., Spychala, J., and Mitchell, B. S.: Human cytosolic nucleotidase I: Charaterization and role in nucleoside analog resistance. J Biol Chem **22**: 22, 2000.

Hunt, S. W. d., and Hoffee, P. A.: Amplification of adenosine deaminase gene sequences in deoxycoformycin-resistant rat hepatoma cells. J Biol Chem **258** (21): 13185-92, 1983.

Itoh, R., Echizen, H., Higuchi, M., Oka, J., and Yamada, K.: A comparative study on tissue distribution and metabolic adaptation of IMP-GMP 5'-nucleotidase. Comp Biochem Physiol B **103** (1): 153-9., 1992.

Itoh, R., and Yamada, K.: Pig lung 5'-nucleotidase: effect of diadenosine 5',5'''-P1, P4-tetraphosphate and its related compounds. Int J Biochem **22** (3): 231-8, 1990.

Jacobson, K. A.: Adenosine A3 receptors: novel ligands and paradoxical effects. Trends Pharmacol Sci **19** (5): 184-91., 1998.

Jacobson, K. A., Gallo-Rodriguez, C., Melman, N., Fischer, B., Maillard, M., van Bergen, A., van Galen, P. J., and Karton, Y.: Structure-activity relationships of 8-styrylxanthines as A2-selective adenosine antagonists. J Med Chem **36** (10): 1333-42., 1993.

Jacobson, M. A., Johnson, R. G., Luneau, C. J., and Salvatore, C. A.: Cloning and chromosomal localization of the human A2b adenosine receptor gene (ADORA2B) and its pseudogene. Genomics **27** (2): 374-6., 1995.

Jarvis, M. F., Yu, H., Kohlhaas, K., Alexander, K., Lee, C. H., Jiang, M., Bhagwat, S. S., Williams, M., and Kowaluk, E. A.: ABT-702 (4-amino-5-(3-bromophenyl)-7-(6-morpholinopyridin-3-yl)pyrido[2, 3-d]pyrimidine), a novel orally effective adenosine kinase inhibitor with analgesic and anti-inflammatory properties: I. In vitro characterization and acute antinociceptive effects in the mouse [In Process Citation]. J Pharmacol Exp Ther **295** (3): 1156-64, 2000.

Jarvis, S. M.: Kinetic and Molecular Properties of Nucleoside Transporters in Animal Cells. *In* Topics and Perspectives in Adenosine Research, ed. by E. Gerlach and B. F. Becker, pp. 102-117, Springer-Verlag, Berlin, 1987.

Jarvis, S. M., and Young, J. D.: Nucleoside transport in rat erythrocytes: two components with differences in sensitivity to inhibition by nitrobenzylthioinosine and p-chloromercuriphenyl sulfonate. J Membr Biol **93** (1): 1-10, 1986.

Jennings, R. B., Reimer, K. A., Steenbergen, C., Jr., and Schaper, J.: Total ischemia III: Effect of inhibition of anaerobic glycolysis. J Mol Cell Cardiol **21 Suppl 1:** 37-54, 1989.

Jezer, A., Oppenheimer, B. S., and Schwartz, S. P.: The effect of adenosine on cardiac irregularities in man. Am J Heart **9:** 252-258, 1933.

Jin, X., Shepherd, R. K., Duling, B. R., and Linden, J.: Inosine binds to A3 adenosine receptors and stimulates mast cell degranulation. J Clin Invest **100** (11): 2849-57, 1997.

Johansson, B., Georgiev, V., and Fredholm, B. B.: Distribution and postnatal ontogeny of adenosine A2A receptors in rat brain: comparison with dopamine receptors. Neuroscience **80** (4): 1187-207., 1997a.

Johansson, B., Georgiev, V., and Fredholm, B. B.: Distribution and postnatal ontogeny of adenosine A2A receptors in rat brain: comparison with dopamine receptors. Neuroscience **80** (4): 1187-207, 1997b.

Johnson, M. P., McCarty, D. R., and Chmielewski, P. A.: Temporal dependent neuroprotection with propentofylline (HWA 285) in a temporary focal ischemia model. Eur J Pharmacol **346** (2-3): 151-7., 1998.

Johnston, M. E., and Geiger, J. D.: Adenosine transport systems on dissociated brain cells from mouse, guinea-pig, and rat. Neurochem Res **15** (9): 911-5, 1990.

Jones, K. W., and Hammond, J. R.: Heterogeneity of [3H]dipyridamole binding to CNS membranes: correlation with [3H]nitrobenzylthioinosine binding and [3H]uridine influx studies. J Neurochem **59** (4): 1363-71, 1992.

Jones, K. W., and Hammond, J. R.: Characterization of nucleoside transport activity in rabbit cortical synaptosomes. Can J Physiol Pharmacol **73** (12): 1733-41., 1995.

Jones, K. W., Rylett, R. J., and Hammond, J. R.: Effect of cellular differentiation on nucleoside transport in human neuroblastoma cells. Brain Res **660** (1): 104-12, 1994.

Jurkowitz, M. S., Litsky, M. L., Browning, M. J., and Hohl, C. M.: Adenosine, inosine, and guanosine protect glial cells during glucose deprivation and mitochondrial inhibition: correlation between protection and ATP preservation. J Neurochem **71** (2): 535-48, 1998.

Kamermans, M., and Werblin, F.: GABA-mediated positive autofeedback loop controls horizontal cell kinetics in tiger salamander retina. J Neurosci **12** (7): 2451-63, 1992.

Kanda, T., Jackson, M. J., Smith, L. A., Pearce, R. K., Nakamura, J., Kase, H., Kuwana, Y., and Jenner, P.: Adenosine A2A antagonist: a novel antiparkinsonian agent that does not provoke dyskinesia in parkinsonian monkeys. Ann Neurol **43** (4): 507-13, 1998a.

Kanda, T., Tashiro, T., Kuwana, Y., and Jenner, P.: Adenosine A2A receptors modify motor function in MPTP-treated common marmosets [In Process Citation]. Neuroreport **9** (12): 2857-60, 1998b.

Kather, H.: Purine accumulation in human fat cell suspensions. Evidence that human adipocytes release inosine and hypoxanthine rather than adenosine. J Biol Chem **263** (18): 8803-9, 1988.

Kather, H.: Pathways of purine metabolism in human adipocytes. Further evidence against a role of adenosine as an endogenous regulator of human fat cell function. J Biol Chem **265** (1): 96-102, 1990.

Kegel, B., Braun, N., Heine, P., Maliszewski, C. R., and Zimmermann, H.: An ecto-ATPase and an ecto-ATP diphosphohydrolase are expressed in rat brain. Neuropharmacology **36** (9): 1189-200., 1997.

Keil, G. J., 2nd, and DeLander, G. E.: Altered sensory behaviors in mice following manipulation of endogenous spinal adenosine neurotransmission. Eur J Pharmacol **312** (1): 7-14, 1996.

Kichenin, K., Pignede, G., Fudalej, F., and Seman, M.: CD3 activation induces concentrative nucleoside transport in human T lymphocytes. Eur J Immunol **30** (2): 366-70, 2000.

Kim, Y. C., de Zwart, M., Chang, L., Moro, S., von Frijtag Drabbe Kunzel, J. K., Melman, N., AP, I. J., and Jacobson, K. A.: Derivatives of the triazoloquinazoline adenosine antagonist (CGS 15943) having high potency at the human A2B and A3 receptor subtypes. J Med Chem **41** (15): 2835-45., 1998.

Klohs, W. D., and Kraker, A. J.: Pentostatin: future directions. Pharmacol Rev **44** (4): 459-77, 1992.

Klotz, K. N.: Adenosine receptors and their ligands. Naunyn Schmiedebergs Arch Pharmacol **362** (4-5): 382-91., 2000.

Klotz, K. N., Hessling, J., Hegler, J., Owman, C., Kull, B., Fredholm, B. B., and Lohse, M. J.: Comparative pharmacology of human adenosine receptor subtypes - characterization of stably transfected receptors in CHO cells. Naunyn Schmiedebergs Arch Pharmacol **357** (1): 1-9., 1998.

Koos, B. J., Kruger, L., and Murray, T. F.: Source of extracellular brain adenosine during hypoxia in fetal sheep. Brain Res **778** (2): 439-42., 1997.

Kornberg, A., and Pricer, W. E.: Enzymatic phosphorylation of adenosine and 2,6-diamopurine riboside. J Biol Chem **193:** 481-495, 1951.

Kowaluk, E., and Jarvis, M.: Therapeutic potential of adenosine kinase inhibitors. Exp. Opin. Invest. Drugs **9** (3): 551-564, 2000.

Kowaluk, E. A., Bhagwat, S. S., and Jarvis, M. F.: Adenosine kinase inhibitors. Curr Pharm Des **4** (5): 403-16, 1998.

Krauss, S. W., Ghirnikar, R. B., Diamond, I., and Gordon, A. S.: Inhibition of adenosine uptake by ethanol is specific for one class of nucleoside transporters. Mol Pharmacol **44** (5): 1021-6, 1993.

Kreutzberg, G. W., Barron, K. D., and Schubert, P.: Cytochemical localization of 5'-nucleotidase in glial plasma membranes. Brain Res **158** (2): 247-57, 1978.

Krupinski, J., Coussen, F., Bakalyar, H. A., Tang, W. J., Feinstein, P. G., Orth, K., Slaughter, C., Reed, R. R., and Gilman, A. G.: Adenylyl cyclase amino acid sequence: possible channel- or transporter- like structure. Science **244** (4912): 1558-64, 1989.

Kubler, W., and Bretschneider, H. J.: Die permeation von adenosin durch die erythrocytenmembran des hundes. Pfluger Archiv Physiol **277:** 141-149, 1963.

Le, F., Townsend-Nicholson, A., Baker, E., Sutherland, G. R., and Schofield, P. R.: Characterization and chromosomal localization of the human A2a adenosine receptor gene: ADORA2A. Biochem Biophys Res Commun **223** (2): 461-7., 1996.

Ledent, C., Vaugeois, J. M., Schiffmann, S. N., Pedrazzini, T., El Yacoubi, M., Vanderhaeghen, J. J., Costentin, J., Heath, J. K., Vassart, G., and Parmentier, M.: Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A2a receptor [see comments]. Nature **388** (6643): 674-8, 1997.

Lee, C. W., and Jarvis, S. M.: Nucleoside transport in rat cerebral-cortical synaptosomes. Evidence for two types of nucleoside transporters. Biochem J **249** (2): 557-64, 1988.

Li, A. H., Moro, S., Forsyth, N., Melman, N., Ji, X. D., and Jacobson, K. A.: Synthesis, CoMFA analysis, and receptor docking of 3,5-diacyl-2, 4-dialkylpyridine derivatives as selective A3 adenosine receptor antagonists. J Med Chem **42** (4): 706-21., 1999.

Libert, F., Parmentier, M., Lefort, A., Dinsart, C., Van Sande, J., Maenhaut, C., Simons, M. J., Dumont, J. E., and Vassart, G.: Selective amplification and cloning of four new members of the G protein-coupled receptor family. Science **244** (4904): 569-72., 1989.

Libert, F., Passage, E., Parmentier, M., Simons, M. J., Vassart, G., and Mattei, M. G.: Chromosomal mapping of A1 and A2 adenosine receptors, VIP receptor, and a new subtype of serotonin receptor. Genomics **11** (1): 225-7., 1991.

Libert, F., Van Sande, J., Lefort, A., Czernilofsky, A., Dumont, J. E., Vassart, G., Ensinger, H. A., and Mendla, K. D.: Cloning and functional characterization of a human A1 adenosine receptor. Biochem Biophys Res Commun **187** (2): 919-26., 1992.

Lin, B. B., Hurley, M. C., and Fox, I. H.: Regulation of adenosine kinase by adenosine analogs. Mol Pharmacol **34** (4): 501-5, 1988.

Lin, Y., and Phillis, J. W.: Deoxycoformycin and oxypurinol: protection against focal ischemic brain injury in the rat. Brain Res **571** (2): 272-80., 1992.

Linden, J.: Cloned adenosine A3 receptors: pharmacological properties, species differences and receptor functions. Trends Pharmacol Sci **15** (8): 298-306, 1994.

Linden, J.: Molecular approach to adenosine receptors: receptor-mediated mechanisms of tissue protection. Annu Rev Pharmacol Toxicol **41** (3): 775-87, 2001.

Lipton, S. A., Kim, W. K., Choi, Y. B., Kumar, S., D'Emilia, D. M., Rayudu, P. V., Arnelle, D. R., and Stamler, J. S.: Neurotoxicity associated with dual actions of homocysteine at the N-methyl-D-aspartate receptor. Proc Natl Acad Sci U S A **94** (11): 5923-8., 1997.

Litsky, M. L., Hohl, C. M., Lucas, J. H., and Jurkowitz, M. S.: Inosine and guanosine preserve neuronal and glial cell viability in mouse spinal cord cultures during chemical hypoxia. Brain Res **821** (2): 426-32, 1999.

Liu, J., and Moghaddam, B.: Regulation of glutamate efflux by excitatory amino acid receptors: evidence for tonic inhibitory and phasic excitatory regulation. J Pharmacol Exp Ther **274** (3): 1209-15, 1995.

Lloyd, H. G., and Fredholm, B. B.: Involvement of adenosine deaminase and adenosine kinase in regulating extracellular adenosine concentration in rat hippocampal slices. Neurochem Int **26** (4): 387-95, 1995.

Lloyd, H. G., Lindstrom, K., and Fredholm, B. B.: Intracellular formation and release of adenosine from rat hippocampal slices evoked by electrical stimulation or energy depletion. Neurochem Int **23** (2): 173-85, 1993.

Lobner, D., and Choi, D. W.: Dipyridamole increases oxygen-glucose deprivation-induced injury in cortical cell culture. Stroke **25** (10): 2085-9; discussion 2089-90., 1994.

Loewen, S. K., Ng, A. M., Yao, S. Y., Cass, C. E., Baldwin, S. A., and Young, J. D.: Identification of amino acid residues responsible for the pyrimidine and purine nucleoside specificities of human concentrative Na(+) nucleoside cotransporters hCNT1 and hCNT2. J Biol Chem **274** (35): 24475-84., 1999.

Londos, C., Cooper, D. M., and Wolff, J.: Subclasses of external adenosine receptors. Proc Natl Acad Sci U S A **77** (5): 2551-4., 1980.

Luthin, D. R., and Linden, J.: Comparison of A4 and A2a binding sites in striatum and COS cells transfected with adenosine A2a receptors. J Pharmacol Exp Ther **272** (2): 511-8., 1995.

Lynch, J. J., 3rd, Alexander, K. M., Jarvis, M. F., and Kowaluk, E. A.: Inhibition of adenosine kinase during oxygen-glucose deprivation in rat cortical neuronal cultures. Neurosci Lett **252** (3): 207-10, 1998.

Mackey, J. R., Mani, R. S., Selner, M., Mowles, D., Young, J. D., Belt, J. A., Crawford, C. R., and Cass, C. E.: Functional nucleoside transporters are required for gemcitabine influx and manifestation of toxicity in cancer cell lines. Cancer Res **58** (19): 4349-57, 1998.

Mackey, J. R., Yao, S. Y., Smith, K. M., Karpinski, E., Baldwin, S. A., Cass, C. E., and Young, J. D.: Gemcitabine transport in xenopus oocytes expressing recombinant plasma membrane mammalian nucleoside transporters. J Natl Cancer Inst **91** (21): 1876-81, 1999.

Maenhaut, C., Van Sande, J., Libert, F., Abramowicz, M., Parmentier, M., Vanderhaegen, J. J., Dumont, J. E., Vassart, G., and Schiffmann, S.: RDC8 codes for an adenosine A2 receptor with physiological constitutive activity. Biochem Biophys Res Commun **173** (3): 1169-78., 1990.

Mahan, L. C., McVittie, L. D., Smyk-Randall, E. M., Nakata, H., Monsma, F. J., Gerfen, C. R., and Sibley, D. R.: Cloning and expression of an A1 adenosine receptor from rat brain. Mol Pharmacol **40** (1): 1-7., 1991.

Maire, J. C., Medilanski, J., and Straub, R. W.: Release of adenosine, inosine and hypoxanthine from rabbit non- myelinated nerve fibres at rest and during activity. J Physiol (Lond) **357:** 67-77, 1984.

Maj, M., Singh, B., and Gupta, R. S.: The influence of inorganic phosphate on the activity of adenosine kinase. Biochim Biophys Acta **1476** (1)**:** 33-42, 2000.

Marques, A. F., Teixeira, N. A., Gambaretto, C., Sillero, A., and Sillero, M. A.: IMP-GMP 5'-nucleotidase from rat brain: activation by polyphosphates. J Neurochem **71** (3)**:** 1241-50, 1998.

Martinek, R. G.: Micromethod for estimation of serum adenosine deaminase. Clinical Chemistry **9:** 620-625, 1963.

Matsumoto, K., Sakaki, T., Kohmura, E., Hayakawa, T., and Yamada, K.: Amelioration of ischemic brain damage by the preischemic administration of propentofylline (HWA285) in a rat focal ischemia. Brain Res **723** (1-2)**:** 228-30., 1996.

Matz, H., and Hertz, L.: Adenosine metabolism in neurons and astrocytes in primary cultures. J Neurosci Res **24** (2)**:** 260-7, 1989.

Matz, H., and Hertz, L.: Effects of adenosine deaminase inhibition on active uptake and metabolism of adenosine in astrocytes in primary cultures. Brain Res **515** (1-2)**:** 168-72, 1990.

McNally, T., Helfrich, R. J., Cowart, M., Dorwin, S. A., Meuth, J. L., Idler, K. B., Klute, K. A., Simmer, R. L., Kowaluk, E. A., and Halbert, D. N.: Cloning and expression of the adenosine kinase gene from rat and human tissues. Biochem Biophys Res Commun **231** (3)**:** 645-50, 1997.

Meghji, P., Holmquist, C. A., and Newby, A. C.: Adenosine formation and release from neonatal-rat heart cells in culture. Biochem J **229** (3): 799-805, 1985.

Meghji, P., Skladanowski, A. C., Newby, A. C., Slakey, L. L., and Pearson, J. D.: Effect of 5'-deoxy-5'-isobutylthioadenosine on formation and release of adenosine from neonatal and adult rat ventricular myocytes. Biochem J **291** (Pt 3): 833-9, 1993.

Meghji, P., Tuttle, J. B., and Rubio, R.: Adenosine formation and release by embryonic chick neurons and glia in cell culture. J Neurochem **53** (6): 1852-60, 1989.

Mendelson, W. B., Kuruvilla, A., Watlington, T., Goehl, K., Paul, S. M., and Skolnick, P.: Sedative and electroencephalographic actions of erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA): relationship to inhibition of brain adenosine deaminase. Psychopharmacology **79** (2-3): 126-9, 1983.

Meno, J. R., Ngai, A. C., Ibayashi, S., and Winn, H. R.: Adenosine release and changes in pial arteriolar diameter during transient cerebral ischemia and reperfusion. J Cereb Blood Flow Metab **11** (6): 986-93., 1991.

Meyerhof, W., Muller-Brechlin, R., and Richter, D.: Molecular cloning of a novel putative G-protein coupled receptor expressed during rat spermiogenesis. FEBS Lett **284** (2): 155-60., 1991.

Miller, L. P., Jelovich, L. A., Yao, L., DaRe, J., Ugarkar, B., and Foster, A. C.: Pre- and peristroke treatment with the adenosine kinase inhibitor, 5'-deoxyiodotubercidin, significantly reduces infarct volume after temporary occlusion of the middle cerebral artery in rats. Neurosci Lett **220** (2): 73-6, 1996.

Miller, R. L., and Adamczyk, D. L.: Adenosine kinase: regulation by substrates, magnesium and pH. Adv Exp Med Biol : 145-9, 1979.

Miller, R. L., Adamczyk, D. L., Miller, W. H., Koszalka, G. W., Rideout, J. L., Beacham, L. M. d., Chao, E. Y., Haggerty, J. J., Krenitsky, T. A., and Elion, G. B.: Adenosine kinase from rabbit liver. II. Substrate and inhibitor specificity. J Biol Chem **254** (7): 2346-52, 1979.

Mimouni, M., Bontemps, F., and Van den Berghe, G.: Kinetic studies of rat liver adenosine kinase. Explanation of exchange reaction between adenosine and AMP. J Biol Chem **269** (27): 17820-5, 1994.

Misumi, Y., Ogata, S., Hirose, S., and Ikehara, Y.: Primary structure of rat liver 5'-nucleotidase deduced from the cDNA. Presence of the COOH-terminal hydrophobic domain for possible post-translational modification by glycophospholipid. J Biol Chem **265** (4): 2178-83., 1990a.

Misumi, Y., Ogata, S., Ohkubo, K., Hirose, S., and Ikehara, Y.: Primary structure of human placental 5'-nucleotidase and identification of the glycolipid anchor in the mature form. Eur J Biochem **191** (3): 563-9., 1990b.

Montgomery, J. A., Thomas, H. J., Zell, A. L., Einsphar, H. M., and Bugg, C. E.: Study on the inhibition of adenosine deaminase. J Med Chem **28** (11): 1751-3., 1985.

Moriwaki, Y., Yamamoto, T., and Higashino, K.: Enzymes involved in purine metabolism--a review of histochemical localization and functional implications. Histol Histopathol 14 (4): 1321-40, 1999.

Morris, P., and Montgomery, J.: Inhibitors of the enzyme purine nucleoside phosphorylase. Exp Opin Ther Patents **8** (3): 283-299, 1998.

Mullane, K., and Bullough, D.: Harnessing an endogenous cardioprotective mechanism: cellular sources and sites of action of adenosine. J Mol Cell Cardiol **27** (4): 1041-54, 1995.

Myers, K. M., Fiskum, G., Liu, Y., Simmens, S. J., Bredesen, D. E., and Murphy, A. N.: Bcl-2 protects neural cells from cyanide/aglycemia-induced lipid oxidation, mitochondrial injury, and loss of viability. J Neurochem **65** (6): 2432-40, 1995.

Nagy, A. K., Shuster, T. A., and Delgado-Escueta, A. V.: Rat brain synaptosomal ATP:AMP-phosphotransferase activity. J Neurochem **53** (4): 1166-72., 1989.

Newby, A. C.: Adenosine and the concept of 'retaliatory metabolites'. Trends in Biochemal Sciences **9**: 42-44, 1984.

Newby, A. C.: The pigeon heart 5'-nucleotidase responsible for ischaemia-induced adenosine formation. Biochem J **253** (1): 123-30, 1988.

Newby, A. C., Holmquist, C. A., Illingworth, J., and Pearson, J. D.: The control of adenosine concentration in polymorphonuclear leucocytes, cultured heart cells and isolated perfused heart from the rat. Biochem J **214** (2): 317-23, 1983.

Ogata, S., Hayashi, Y., Takami, N., and Ikehara, Y.: Chemical characterization of the membrane-anchoring domain of human placental alkaline phosphatase. J Biol Chem **263** (21): 10489-94., 1988.

Ogata, T., Nakamura, Y., Tsuji, K., Shibata, T., and Kataoka, K.: A possible mechanism for the hypoxia-hypoglycemia-induced release of excitatory amino acids from cultured hippocampal astrocytes. Neurochem Res **20** (6): 737-43, 1995.

Ohkubo, T., Mitsumoto, Y., and Mohri, T.: Characterization of the uptake of adenosine by cultured rat hippocampal cells and inhibition of the uptake by xanthine derivatives. Neurosci Lett **133** (2): 275-8, 1991.

Oka, J., Matsumoto, A., Hosokawa, Y., and Inoue, S.: Molecular cloning of human cytosolic purine 5'-nucleotidase. Biochem Biophys Res Commun **205** (1): 917-22, 1994.

Ongini, E., Adami, M., Ferri, C., and Bertorelli, R.: Adenosine A2A receptors and neuroprotection. Ann N Y Acad Sci **825**: 30-48, 1997.

Ongini, E., and Fredholm, B. B.: Pharmacology of adenosine A2A receptors. Trends Pharmacol Sci **17** (10): 364-72, 1996.

Orkin, S. H., Daddona, P. E., Shewach, D. S., Markham, A. F., Bruns, G. A., Goff, S. C., and Kelley, W. N.: Molecular cloning of human adenosine deaminase gene sequences. J Biol Chem **258** (21): 12753-6, 1983.

Orlov, S. N., and Maksimova, N. V.: Efflux of cyclic adenosine monophosphate from cells: mechanisms and physiological implications. Biochemistry (Mosc) **64** (2): 127-35., 1999.

Padua, R., Geiger, J. D., Dambock, S., and Nagy, J. I.: 2'-Deoxycoformycin inhibition of adenosine deaminase in rat brain: in vivo and in vitro analysis of specificity, potency, and enzyme recovery. J Neurochem **54** (4): 1169-78, 1990.

Pak, M. A., Haas, H. L., Decking, U. K., and Schrader, J.: Inhibition of adenosine kinase increases endogenous adenosine and depresses neuronal activity in hippocampal slices. Neuropharmacology **33** (9): 1049-53, 1994.

Palella, T. D., Andres, C. M., and Fox, I. H.: Human placental adenosine kinase. Kinetic mechanism and inhibition. J Biol Chem **255** (11): 5264-9, 1980.

Palmer, T. M., and Stiles, G. L.: Adenosine receptors. Neuropharmacology **34** (7): 683-94, 1995.

Park, C. K., and Rudolphi, K. A.: Antiischemic effects of propentofylline (HWA 285) against focal cerebral infarction in rats. Neurosci Lett **178** (2): 235-8., 1994.

Park, T. S., and Gidday, J. M.: Effect of dipyridamole on cerebral extracellular adenosine level in vivo. J Cereb Blood Flow Metab **10** (3): 424-7., 1990.

Parkinson, F. E., and Geiger, J. D.: Effects of iodotubercidin on adenosine kinase activity and nucleoside transport in DDT1 MF-2 smooth muscle cells. J Pharmacol Exp Ther **277** (3): 1397-401, 1996.

Parkinson, F. E., Mukherjee, K., and Geiger, J. D.: [3H]adenosine transport in DDT1 MF-2 smooth muscle cells: inhibition by metabolites of propentofylline. Eur J Pharmacol **308** (1): 97-102, 1996.

Parkinson, F. E., Paterson, A. R., Young, J. D., and Cass, C. E.: Inhibitory effects of propentofylline on [3H]adenosine influx. A study of three nucleoside transport systems. Biochem Pharmacol **46** (5): 891-6, 1993.

Parkinson, F. E., Zhang, Y. W., Shepel, P. N., Greenway, S. C., Peeling, J., and Geiger, J. D.: Effects of nitrobenzylthioinosine on neuronal injury, adenosine levels and adenosine receptor activity in rat forebrain ischemia. J. Neurochem **75** (3): 795-802, 2000.

Parkinson, F. E., Zhang, Y. W., Shepel, P. N., Greenway, S. C., Peeling, J., and Geiger, J. D.: Effects of peripheral administration of nitrobenzylthioinosine on neuronal injury and adenosine levels in rat forebrain ischemia. Brain Research **submitted**, 2001.

Patel, D. R., and Croucher, M. J.: Evidence for a role of presynaptic AMPA receptors in the control of neuronal glutamate release in the rat forebrain. Eur J Pharmacol **332** (2): 143-51, 1997.

Penix, L. P.: Ischemic strokes secondary to vitamin B12 deficiency-induced hyperhomocystinemia. Neurology **51** (2): 622-4., 1998.

Pesi, R., Baiocchi, C., Tozzi, M. G., and Camici, M.: Synergistic action of ADP and 2,3-bisphosphoglycerate on the modulation of cytosolic 5'-nucleotidase. Biochim Biophys Acta **1294** (2): 191-4, 1996.

Pesi, R., Turriani, M., Allegrini, S., Scolozzi, C., Camici, M., Ipata, P. L., and Tozzi, M. G.: The bifunctional cytosolic 5'-nucleotidase: regulation of the phosphotransferase and nucleotidase activities. Arch Biochem Biophys **312** (1): 75-80, 1994.

Peterfreund, R. A., MacCollin, M., Gusella, J., and Fink, J. S.: Characterization and expression of the human A2a adenosine receptor gene. J Neurochem **66** (1): 362-8., 1996.

Philibert, R. A., and Dutton, G. R.: Dihydropyridines modulate K+-evoked amino acid and adenosine release from cerebellar neuronal cultures. Neurosci Lett **102** (1): 97-102, 1989.

Phillis, J. W.: Adenosine's role in the central actions of the benzodiazepines. Prog Neuropsychopharmacol Biol Psychiatry **8** (4-6): 495-502, 1984.

Phillis, J. W., Jiang, Z. G., Chelack, B. J., and Wu, P. H.: Morphine enhances adenosine release from the in vivo rat cerebral cortex. Eur J Pharmacol **65** (1): 97-100, 1980.

Phillis, J. W., and O'Regan, M. H.: Deoxycoformycin antagonizes ischemia-induced neuronal degeneration. Brain Res Bull **22** (3): 537-40., 1989.

Phillis, J. W., O'Regan, M. H., and Walter, G. A.: Effects of two nucleoside transport inhibitors, dipyridamole and soluflazine, on purine release from the rat cerebral cortex. Brain Res **481** (2): 309-16, 1989.

Phillis, J. W., and Smith-Barbour, M.: The adenosine kinase inhibitor, 5-iodotubercidin, is not protective against cerebral ischemic injury in the gerbil. Life Sci **53** (6): 497-502, 1993.

Phillis, J. W., Walter, G. A., O'Regan, M. H., and Stair, R. E.: Increases in cerebral cortical perfusate adenosine and inosine concentrations during hypoxia and ischemia. J Cereb Blood Flow Metab **7** (6): 679-86., 1987.

Picano, E., and Abbracchio, M. P.: European Stroke Prevention Study-2 results: serendipitous demonstration of neuroprotection induced by endogenous adenosine accumulation? Trends Pharmacol Sci **19** (1): 14-6, 1998.

Pickard, M. A., Brown, R. R., Paul, B., and Paterson, A. R.: Binding of the nucleoside transport inhibitor 4-nitrobenzylthioinosine to erythrocyte membranes. Can J Biochem **51** (5): 666-72., 1973.

Pickard, M. A., and Paterson, A. R.: Use of 4-nitrobenzylthioinosine in the measurement of rates of nucleoside transport in human erythrocytes. Can J Biochem **50** (7): 839-40., 1972.

Picozzi, P., Todd, N. V., and Crockard, H. A.: Regional blood-brain barrier permeability changes after restoration of blood flow in postischemic gerbil brains: a quantitative study. J Cereb Blood Flow Metab **5** (1): 10-6, 1985.

Pierce, K. D., Furlong, T. J., Selbie, L. A., and Shine, J.: Molecular cloning and expression of an adenosine A2b receptor from human brain. Biochem Biophys Res Commun **187** (1): 86-93., 1992.

Pilitsis, J. G., and Kimelberg, H. K.: Adenosine receptor mediated stimulation of intracellular calcium in acutely isolated astrocytes. Brain Res **798** (1-2): 294-303., 1998.

Pinto, R. M., Canales, J., Faraldo, A., Sillero, A., and Gunther Sillero, M. A.: Cytosol 5'-nucleotidase from Artemia embryos. Purification and properties. Comp Biochem Physiol B **86** (1): 49-53, 1987.

Pinto, R. M., Canales, J., Gunther Sillero, M. A., and Sillero, A.: Diadenosine tetraphosphate activates cytosol 5'-nucleotidase. Biochem Biophys Res Commun **138** (1): 261-7, 1986.

Plagemann, P. G., and Woffendin, C.: Effects of Ca2+-channel antagonists on nucleoside and nucleobase transport in human erythrocytes and cultured mammalian cells. Biochim Biophys Acta **928** (3): 243-50., 1987.

Plagemann, P. G., and Wohlhueter, R. M.: Nucleoside transport in cultured mammalian cells. Multiple forms with different sensitivity to inhibition by nitrobenzylthioinosine or hypoxanthine. Biochim Biophys Acta **773** (1): 39-52, 1984.

Plagemann, P. G., Wohlhueter, R. M., and Woffendin, C.: Nucleoside and nucleobase transport in animal cells. Biochim Biophys Acta **947** (3): 405-43., 1988.

Pluta, R., Lossinsky, A. S., Wisniewski, H. M., and Mossakowski, M. J.: Early blood-brain barrier changes in the rat following transient complete cerebral ischemia induced by cardiac arrest. Brain Res **633** (1-2): 41-52, 1994.

Porkka-Heiskanen, T., Strecker, R. E., Thakkar, M., Bjorkum, A. A., Greene, R. W., and McCarley, R. W.: Adenosine: a mediator of the sleep-inducing effects of prolonged wakefulness. Science **276** (5316): 1265-8, 1997.

Poucher, S. M., Keddie, J. R., Singh, P., Stoggall, S. M., Caulkett, P. W., Jones, G., and Coli, M. G.: The in vitro pharmacology of ZM 241385, a potent, non-xanthine A2a selective adenosine receptor antagonist. Br J Pharmacol **115** (6): 1096-102., 1995.

Preston, E., and Foster, D. O.: Evidence for pore-like opening of the blood-brain barrier following forebrain ischemia in rats. Brain Res **761** (1): 4-10, 1997.

Preston, E., Sutherland, G., and Finsten, A.: Three openings of the blood-brain barrier produced by forebrain ischemia in the rat. Neurosci Lett **149** (1): 75-8, 1993.

Quaife, R. A., Kohmoto, O., and Barry, W. H.: Mechanisms of reoxygenation injury in cultured ventricular myocytes [see comments]. Circulation **83** (2): 566-77, 1991.

Queiroz, G., Gebicke-Haerter, P. J., Schobert, A., Starke, K., and von Kugelgen, I.: Release of ATP from cultured rat astrocytes elicited by glutamate receptor activation. Neuroscience **78** (4): 1203-8, 1997.

Ramkumar, V., Olah, M. E., Jacobson, K. A., and Stiles, G. L.: Distinct pathways of desensitization of A1- and A2-adenosine receptors in DDT1 MF-2 cells. Mol Pharmacol **40** (5): 639-47, 1991.

Rego, A. C., Santos, M. S., and Oliveira, C. R.: Adenosine triphosphate degradation products after oxidative stress and metabolic dysfunction in cultured retinal cells. J Neurochem **69** (3): 1228-35, 1997.

Reis, M. J.: Bull Soc Chim Biol **16:** 385-399, 1934.

Reppert, S. M., Weaver, D. R., Stehle, J. H., and Rivkees, S. A.: Molecular cloning and characterization of a rat A1-adenosine receptor that is widely expressed in brain and spinal cord. Mol Endocrinol **5** (8): 1037-48., 1991.

Revan, S., Montesinos, M. C., Naime, D., Landau, S., and Cronstein, B. N.: Adenosine A2 receptor occupancy regulates stimulated neutrophil function via activation of a serine/threonine protein phosphatase. J Biol Chem **271** (29): 17114-8., 1996.

Reyes, J. L., Melendez, E., Suarez, J., Rangel, M., Franco, M., and Martinez, F.: Modulation of the release of adenosine by glucose and chemical hypoxia in cultured renal cells (MDCK line). Biochem Biophys Res Commun **208** (3): 970-7, 1995.

Ritzel, M. W., Ng, A. M., Yao, S. Y., Graham, K., Loewen, S. K., Smith, K. M., Ritzel, R. G., Mowles, D. A., Carpenter, P., Chen, X. Z., Karpinski, E., Hyde, R. J., Baldwin, S. A., Cass, C. E., and Young, J. D.: Molecular identification and characterization of novel human and mouse concentrative Na{super+}-nucleoside cotransporter proteins (hCNT3 and mCNT3) broadly selective for purine and pyrimidine nucleosides (system cib). J Biol Chem **276:** 2914-2927, 2001.

Ritzel, M. W., Yao, S. Y., Ng, A. M., Mackey, J. R., Cass, C. E., and Young, J. D.: Molecular cloning, functional expression and chromosomal localization of a cDNA encoding a human Na+/nucleoside cotransporter (hCNT2) selective for purine nucleosides and uridine. Mol Membr Biol **15** (4): 203-11, 1998.

Rivkees, S. A., and Reppert, S. M.: RFL9 encodes an A2b-adenosine receptor. Mol Endocrinol **6** (10): 1598-604., 1992.

Rivkees, S. A., Thevananther, S., and Hao, H.: Are A3 adenosine receptors expressed in the brain? Neuroreport **11** (5): 1025-30., 2000.

Roman, R. M., Feranchak, A. P., Davison, A. K., Schwiebert, E. M., and Fitz, J. G.: Evidence for Gd(3+) inhibition of membrane ATP permeability and purinergic signaling. Am J Physiol **277** (6 Pt 1): G1222-30, 1999.

Ropp, P. A., and Traut, T. W.: Allosteric regulation of purine nucleoside phosphorylase. Arch Biochem Biophys **288** (2): 614-20, 1991.

Rosenberg, P. A., Knowles, R., Knowles, K. P., and Li, Y.: Beta-adrenergic receptor-mediated regulation of extracellular adenosine in cerebral cortex in culture. J Neurosci **14** (5 Pt 2): 2953-65., 1994.

Rosenberg, P. A., and Li, Y.: Adenylyl cyclase activation underlies intracellular cyclic AMP accumulation, cyclic AMP transport, and extracellular adenosine accumulation evoked by beta-adrenergic receptor stimulation in mixed cultures of neurons and astrocytes derived from rat cerebral cortex. Brain Res **692** (1-2): 227-32., 1995.

Rosenberg, P. A., and Li, Y.: Forskolin evokes extracellular adenosine accumulation in rat cortical cultures. Neurosci Lett **211** (1): 49-52., 1996.

Rosenberg, P. A., Li, Y., Le, M., and Zhang, Y.: Nitric oxide-stimulated increase in extracellular adenosine accumulation in rat forebrain neurons in culture is associated with ATP hydrolysis and inhibition of adenosine kinase activity. J Neurosci **20** (16): 6294-301, 2000.

Rotllan, P., and Miras Portugal, M. T.: Adenosine kinase from bovine adrenal medulla. Eur J Biochem **151** (2): 365-71, 1985.

Rudolphi, K. A., Schubert, P., Parkinson, F. E., and Fredholm, B. B.: Neuroprotective role of adenosine in cerebral ischaemia. Trends Pharmacol Sci **13** (12): 439-45, 1992.

Sabri, M. I., and Ochs, S.: Inhibition of glyceraldehyde-3-phosphate dehydrogenase in mammalian nerve by iodoacetic acid. J Neurochem **18** (8): 1509-14., 1971.

Sala-Newby, G. B., Freeman, N. V., Skladanowski, A. C., and Newby, A. C.: Distinct roles for recombinant cytosolic 5'-nucleotidase-I and -II in AMP and IMP catabolism in COS-7 and H9c2 rat myoblast cell lines. J Biol Chem **275** (16): 11666-71, 2000.

Sala-Newby, G. B., Skladanowski, A. C., and Newby, A. C.: The mechanism of adenosine formation in cells. Cloning of cytosolic 5'- nucleotidase-I. J Biol Chem **274** (25): 17789-93, 1999.

Salvatore, C. A., Jacobson, M. A., Taylor, H. E., Linden, J., and Johnson, R. G.: Molecular cloning and characterization of the human A3 adenosine receptor. Proc Natl Acad Sci U S A **90** (21): 10365-9., 1993.

Salvatore, C. A., Tilley, S. L., Latour, A. M., Fletcher, D. S., Koller, B. H., and Jacobson, M. A.: Disruption of the A(3) adenosine receptor gene in mice and its effect on stimulated inflammatory cells. J Biol Chem **275** (6): 4429-34., 2000.

Sattin, A., and Rall, T. W.: The effect of adenosine and adenine nucleotides on the cyclic adenosine 3', 5'-phosphate content of guinea pig cerebral cortex slices. Mol Pharmacol **6** (1): 13-23, 1970.

Saura, C., Ciruela, F., Casado, V., Canela, E. I., Mallol, J., Lluis, C., and Franco, R.: Adenosine deaminase interacts with A1 adenosine receptors in pig brain cortical membranes. J Neurochem **66** (4): 1675-82, 1996.

Saura, C. A., Mallol, J., Canela, E. I., Lluis, C., and Franco, R.: Adenosine deaminase and A1 adenosine receptors internalize together following agonist-induced receptor desensitization. J Biol Chem **273** (28): 17610-7, 1998.

Sawynok, J., Reid, A., and Poon, A.: Peripheral antinociceptive effect of an adenosine kinase inhibitor, with augmentation by an adenosine deaminase inhibitor, in the rat formalin test. Pain **74** (1): 75-81, 1998.

Schachter, J. B., Ivins, J. K., Pittman, R. N., and Wolfe, B. B.: Competitive regulation of phospholipase C responses by cAMP and calcium. Mol Pharmacol **41** (3): 577-86, 1992.

Schachter, J. B., and Wolfe, B. B.: Cyclic AMP differentiates two separate but interacting pathways of phosphoinositide hydrolysis in the DDT1-MF2 smooth muscle cell line. Mol Pharmacol **41** (3): 587-97, 1992.

Schmidt, Z.: Z. Physiol Chem **208**: 185-224, 1932.

Schnebli, H. P., Hill, D. L., and Bennett, L. L., Jr.: Purification and properties of adenosine kinase from human tumor cells of type H. Ep. No. 2. J Biol Chem **242** (9): 1997-2004, 1967.

Schrader, J., and Gerlach, E.: Compartmentation of cardiac adenine nucleotides and formation of adenosine. Pflugers Arch **367** (2): 129-35, 1976.

Schrader, W. P., West, C. A., Miczek, A. D., and Norton, E. K.: Characterization of the adenosine deaminase-adenosine deaminase complexing protein binding reaction. J Biol Chem **265** (31): 19312-8, 1990.

Schramm, V. L., and Baker, D. C.: Spontaneous epimerization of (S)-deoxycoformycin and interaction of (R)-deoxycoformycin, (S)-deoxycoformycin, and 8-ketodeoxycoformycin with adenosine deaminase. Biochemistry **24** (3): 641-6., 1985.

Schubert, P., Rudolphi, K. A., Fredholm, B. B., and Nakamura, Y.: Modulation of nerve and glial function by adenosine--role in the development of ischemic damage. Int J Biochem **26** (10-11): 1227-36, 1994.

Sebastiao, A. M., and Ribeiro, J. A.: Adenosine A2 receptor-mediated excitatory actions on the nervous system. Prog Neurobiol **48** (3): 167-89., 1996.

Semba, K., and White, T. D.: M3 muscarinic receptor-mediated enhancement of NMDA-evoked adenosine release in rat cortical slices in vitro. J Neurochem **69** (3): 1066-72, 1997.

Semenza, G. L.: Perspectives on oxygen sensing. Cell **98** (3): 281-4., 1999.

Sen, R. P., Delicado, E. G., and Miras-Portugal, M. T.: Effect of forskolin and cyclic AMP analog on adenosine transport in cultured chromaffin cells. Neurochemistry International **17** (4): 523-528, 1990.

Sen, R. P., Sobrevia, L., Delicado, E. G., Yudilevich, D., and Miras-Portugal, M. T.: Bovine adrenal endothelial cells express nucleoside transporters nonregulated by protein kinases A and C. Am J Physiol **271** (2 Pt 1): C504-10, 1996.

Seubert, C. N., Morey, T. E., Martynyuk, A. E., Cucchiara, R. F., and Dennis, D. M.: Midazolam selectively potentiates the A(2A) - but not A1- receptor--mediated effects of adenosine: role of nucleoside transport inhibition and clinical implications. Anesthesiology **92** (2): 567-77., 2000.

Sexl, V., Mancusi, G., Holler, C., Gloria-Maercker, E., Schutz, W., and Freissmuth, M.: Stimulation of the mitogen-activated protein kinase via the A2A-adenosine receptor in primary human endothelial cells. J Biol Chem **272** (9): 5792-9., 1997.

Shimada, J., Suzuki, F., Nonaka, H., Ishii, A., and Ichikawa, S.: (E)-1,3-dialkyl-7-methyl-8-(3,4,5-trimethoxystyryl)xanthines: potent and selective adenosine A2 antagonists. J Med Chem **35** (12): 2342-5., 1992.

Sinclair, C., Shepel, P., Geiger, J., and Parkinson, F.: Stimulation of Nucleoside Efflux and Inhibition of Adenosine Kinase by A1 Adenosine Receptor Activation. Biochemical Pharmacology **59** (5): 477-483, 2000a.

Sinclair, C. J., Krizanac-Bengez, L., Stanness, K. A., Janigro, D., and Parkinson, F. E.: Adenosine permeation of a dynamic in vitro blood-brain barrier inhibited by dipyridamole. Brain Res **898** (1): 122-5., 2001.

Sinclair, C. J., LaRiviere, C. G., Young, J. D., Cass, C. E., Baldwin, S. A., and Parkinson, F. E.: Purine uptake and release in rat C6 glioma cells: nucleoside transport and purine metabolism under ATP-depleting conditions. J Neurochem **75** (4): 1528-38, 2000b.

Singh, B., Hao, W., Wu, Z., Eigl, B., and Gupta, R. S.: Cloning and characterization of cDNA for adenosine kinase from mammalian (Chinese hamster, mouse, human and rat) species. High frequency mutants of Chinese hamster ovary cells involve structural alterations in the gene. Eur J Biochem **241** (2): 564-71, 1996.

Skladanowski, A. C., and Newby, A. C.: Partial purification and properties of an AMP-specific soluble 5'-nucleotidase from pigeon heart. Biochem J **268** (1): 117-22, 1990.

Skladanowski, A. C., Sala, G. B., and Newby, A. C.: Inhibition of IMP-specific cytosolic 5'-nucleotidase and adenosine formation in rat polymorphonuclear leucocytes by 5'-deoxy-5'-isobutylthio derivatives of adenosine and inosine. Biochem J **262** (1): 203-8, 1989.

Skolnick, P., Nimitkitpaisan, Y., Stalvey, L., and Daly, J. W.: Inhibition of brain adenosine deaminase by 2'-deoxycoformycin and erythro-9-(2-hydroxy-3-nonyl) adenine. J Neurochem **30** (6): 1479-82., 1978.

Smith, T. M., and Kirley, T. L.: Glycosylation is essential for functional expression of a human brain ecto-apyrase. Biochemistry **38** (5): 1509-16., 1999.

Smoake, J. A., McMahon, K. L., Wright, R. K., and Solomon, S. S.: Hormonally sensitive cyclic AMP phosphodiesterase in liver cells. An ecto-enzyme. J Biol Chem **256** (16): 8531-5., 1981.

Snyder, D. S., Zimmerman, T. R., Jr., Farooq, M., Norton, W. T., and Cammer, W.: Carbonic anhydrase, 5'-nucleotidase, and 2',3'-cyclic nucleotide-3'- phosphodiesterase activities in oligodendrocytes, astrocytes, and neurons isolated from the brains of developing rats. J Neurochem **40** (1): 120-7, 1983.

Snyder, F. F., and Lukey, T.: Kinetic considerations for the regulation of adenosine and deoxyadenosine metabolism in mouse and human tissues based on a thymocyte model. Biochim Biophys Acta **696** (3): 299-307., 1982.

Soler, C., Felipe, A., Mata, J. F., Casado, F. J., Celada, A., and Pastor-Anglada, M.: Regulation of nucleoside transport by lipopolysaccharide, phorbol esters, and tumor necrosis factor-alpha in human B-lymphocytes [In Process Citation]. J Biol Chem **273** (41): 26939-45, 1998.

Sollevi, A., Link, H., and Fredholm, B.: Dipyridamole treatment doubles the plasma adenosine level in patients with minro stroke. Thromb Hemostas **50**: 20, 1983.

Sollevi, A., Ostergren, J., Fagrell, B., and Hjemdahl, P.: Theophylline antagonizes cardiovascular responses to dipyridamole in man without affecting increases in plasma adenosine. Acta Physiol Scand **121** (2): 165-71, 1984.

Spector, R.: Thymidine transport in the central nervous system. J Neurochem **35** (5): 1092-8, 1980.

Spector, R.: Nucleoside transport in choroid plexus: mechanism and specificity. Arch Biochem Biophys **216** (2): 693-703, 1982.

Spragg, R. G., Hinshaw, D. B., Hyslop, P. A., Schraufstatter, I. U., and Cochrane, C. G.: Alterations in adenosine triphosphate and energy charge in cultured endothelial and P388D1 cells after oxidant injury. J Clin Invest **76** (4): 1471-6, 1985.

Spychala, J., Datta, N. S., Takabayashi, K., Datta, M., Fox, I. H., Gribbin, T., and Mitchell, B. S.: Cloning of human adenosine kinase cDNA: sequence similarity to microbial ribokinases and fructokinases. Proc Natl Acad Sci U S A **93** (3): 1232-7, 1996.

Stanness, K. A., Guatteo, E., and Janigro, D.: A dynamic model of the blood-brain barrier "in vitro". Neurotoxicology **17** (2): 481-96, 1996.

Stanness, K. A., Westrum, L. E., Fornaciari, E., Mascagni, P., Nelson, J. A., Stenglein, S. G., Myers, T., and Janigro, D.: Morphological and functional characterization of an in vitro blood- brain barrier model. Brain Res **771** (2): 329-42, 1997.

Stefanovic, V., Mandel, P., and Rosenberg, A.: Ecto-5'-nucleotidase of intact cultured C6 rat glioma cells. J Biol Chem **251** (13): 3900-5, 1976.

Stehle, J. H., Rivkees, S. A., Lee, J. J., Weaver, D. R., Deeds, J. D., and Reppert, S. M.: Molecular cloning and expression of the cDNA for a novel A2-adenosine receptor subtype. Mol Endocrinol **6** (3): 384-93, 1992a.

Stehle, J. H., Rivkees, S. A., Lee, J. J., Weaver, D. R., Deeds, J. D., and Reppert, S. M.: Molecular cloning and expression of the cDNA for a novel A2-adenosine receptor subtype. Mol Endocrinol **6** (3): 384-93., 1992b.

Svenningsson, P., Lindskog, M., Ledent, C., Parmentier, M., Greengard, P., Fredholm, B. B., and Fisone, G.: Regulation of the phosphorylation of the dopamine- and cAMP-

regulated phosphoprotein of 32 kDa in vivo by dopamine D1, dopamine D2, and adenosine A2A receptors. Proc Natl Acad Sci U S A **97** (4): 1856-60., 2000.

Sweeney, M. I., White, T. D., and Sawynok, J.: Morphine-evoked release of adenosine from the spinal cord occurs via a nucleoside carrier with differential sensitivity to dipyridamole and nitrobenzylthioinosine [published erratum appears in Brain Res 1993 Jul 30;618(1):179]. Brain Res **614** (1-2): 301-7, 1993.

Tatlisumak, T., Takano, K., Carano, R. A., Miller, L. P., Foster, A. C., and Fisher, M.: Delayed treatment with an adenosine kinase inhibitor, GP683, attenuates infarct size in rats with temporary middle cerebral artery occlusion. Stroke **29** (9): 1952-8, 1998.

Terada, L. S., Willingham, I. R., Rosandich, M. E., Leff, J. A., Kindt, G. W., and Repine, J. E.: Generation of superoxide anion by brain endothelial cell xanthine oxidase. J Cell Physiol **148** (2): 191-6., 1991.

Terrian, D. M., Hernandez, P. G., Rea, M. A., and Peters, R. I.: ATP release, adenosine formation, and modulation of dynorphin and glutamic acid release by adenosine analogues in rat hippocampal mossy fiber synaptosomes. J Neurochem **53** (5): 1390-9., 1989.

Thampy, K. G., and Barnes, E. M., Jr.: Adenosine transport by cultured glial cells from chick embryo brain. Arch Biochem Biophys **220** (2): 340-6, 1983a.

Thampy, K. G., and Barnes, E. M., Jr.: Adenosine transport by primary cultures of neurons from chick embryo brain. J Neurochem **40** (3): 874-9, 1983b.

Thorat, S. N., and Kulkarni, S. K.: Effect of MK-801 and its interaction with adenosinergic agents and carbamazepine against hypoxic stress-induced convulsions and death in mice. Methods Find Exp Clin Pharmacol **12** (9): 595-600., 1990a.

Thorat, S. N., and Kulkarni, S. K.: The protective effect of adenosinergic agents, Ro 5-4864 and carbamazepine against hypoxic stress-induced neurotoxicity in mice. Methods Find Exp Clin Pharmacol **12** (1): 17-22., 1990b.

Torrecilla, A., Marques, A. F., Buscalioni, R. D., Oliveira, J. M., Teixeira, N. A., Atencia, E. A., Gunther Sillero, M. A., and Sillero, A.: Metabolic fate of AMP, IMP, GMP and XMP in the cytosol of rat brain: an experimental and theoretical analysis. J Neurochem **76** (5): 1291-307., 2001.

Townsend-Nicholson, A., and Shine, J.: Molecular cloning and characterisation of a human brain A1 adenosine receptor cDNA. Brain Res Mol Brain Res **16** (3-4): 365-70., 1992.

Tozzi, M. G., Camici, M., Pesi, R., Allegrini, S., Sgarrella, F., and Ipata, P. L.: Nucleoside phosphotransferase activity of human colon carcinoma cytosolic 5'-nucleotidase. Arch Biochem Biophys **291** (2): 212-7, 1991.

Turcani, P., and Tureani, M.: Effect of propentofylline on cerebral blood flow in a gerbil focal cerebral ischemia. J Neurol Sci **183** (1): 57-60., 2001.

Ugarkar, B. G., Castellino, A. J., DaRe, J. M., Kopcho, J. J., Wiesner, J. B., Schanzer, J. M., and Erion, M. D.: Adenosine kinase inhibitors. 2. Synthesis, enzyme inhibition, and antiseizure activity of diaryltubercidin analogues. J Med Chem **43** (15): 2894-905, 2000a.

Ugarkar, B. G., DaRe, J. M., Kopcho, J. J., Browne, C. E., 3rd, Schanzer, J. M., Wiesner, J. B., and Erion, M. D.: Adenosine kinase inhibitors. 1. Synthesis, enzyme inhibition, and antiseizure activity of 5-iodotubercidin analogues. J Med Chem **43** (15): 2883-93, 2000b.

Ujfalusi, A., Cseppento, A., Nagy, E., Szabo, J. Z., Kovacs, P., and Szentmiklosi, A. J.: Sensitization by chronic diazepam treatment of A2A adenosine receptor-mediated relaxation in rat pulmonary artery. Life Sci **64** (2): L19-25, 1999.

Valerio, D., Duyvesteyn, M. G., Dekker, B. M., Weeda, G., Berkvens, T. M., van der Voorn, L., van Ormondt, H., and van der Eb, A. J.: Adenosine deaminase: characterization and expression of a gene with a remarkable promoter. Embo J **4** (2): 437-43, 1985.

van Calker, D., Muller, M., and Hamprecht, B.: Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. J Neurochem **33** (5): 999-1005., 1979.

Van den Berghe, G., Bontemps, F., Vincent, M. F., and Van den Bergh, F.: The purine nucleotide cycle and its molecular defects. Prog Neurobiol **39** (5): 547-61, 1992.

Van der Weyden, M. B., and Kelley, W. N.: Human adenosine deaminase. Distribution and properties. J Biol Chem **251** (18): 5448-56, 1976.

Van Reempts, J., Van Deuren, B., Haseldonckx, M., Van de Ven, M., Thone, F., and Borgers, M.: Purine nucleoside phosphorylase: a histochemical marker for glial cells. Brain Res **462** (1): 142-7, 1988.

Van Wylen, D. G., Park, T. S., Rubio, R., and Berne, R. M.: Increases in cerebral interstitial fluid adenosine concentration during hypoxia, local potassium infusion, and ischemia. J Cereb Blood Flow Metab **6** (5): 522-8., 1986.

Vijayalakshmi, D., and Belt, J. A.: Sodium-dependent nucleoside transport in mouse intestinal epithelial cells. Two transport systems with differing substrate specificities. J Biol Chem **263** (36): 19419-23, 1988.

von Lubitz, D. K.: Adenosine A3 receptor and brain. A culprit, a hero, or merely yet another receptor? Ann N Y Acad Sci **825:** 49-67, 1997.

Von Lubitz, D. K.: Adenosine and cerebral ischemia: therapeutic future or death of a brave concept? Eur J Pharmacol **365** (1): 9-25, 1999.

Von Lubitz, D. K., Beenhakker, M., Lin, R. C., Carter, M. F., Paul, I. A., Bischofberger, N., and Jacobson, K. A.: Reduction of postischemic brain damage and memory deficits following treatment with the selective adenosine A1 receptor agonist. Eur J Pharmacol **302** (1-3): 43-8, 1996.

Wallman-Johansson, A., and Fredholm, B. B.: Release of adenosine and other purines from hippocampal slices stimulated electrically or by hypoxia/hypoglycemia. Effect of chlormethiazole. Life Sci **55** (9): 721-8, 1994.

Wang, T. F., Rosenberg, P. A., and Guidotti, G.: Characterization of brain ecto-apyrase: evidence for only one ecto-apyrase (CD39) gene. Brain Res Mol Brain Res **47** (1-2): 295-302., 1997.

Wang, Y., Roman, R., Lidofsky, S. D., and Fitz, J. G.: Autocrine signaling through ATP release represents a novel mechanism for cell volume regulation. Proc Natl Acad Sci U S A **93** (21): 12020-5, 1996.

Wang, Y., and White, T. D.: Effect of protein kinase C activation on N-methyl-D-aspartate-evoked release of adenosine and [3H]Norepinephrine from rat cortical slices. J Pharmacol Exp Ther **285** (1): 105-9, 1998.

Ward, J. L., Sherali, A., Mo, Z. P., and Tse, C. M.: Kinetic and Pharmacological Properties of Cloned Human Equilibrative Nucleoside Transporters, ENT1 and ENT2, Stably Expressed in Nucleoside Transporter-deficient PK15 Cells. Ent2 exhibits a low

affinity for guanosine and cytidine but a high affinity for inosine. J Biol Chem **275** (12): 8375-8381, 2000.

Wauquier, A., Van Belle, H., Van den Broeck, W. A., and Janssen, P. A.: Sleep improvement in dogs after oral administration of mioflazine, a nucleoside transport inhibitor. Psychopharmacology **91** (4): 434-9, 1987.

White, T. D.: Potentiation of excitatory amino acid-evoked adenosine release from rat cortex by inhibitors of adenosine kinase and adenosine deaminase and by acadesine. Eur J Pharmacol **303** (1-2): 27-38, 1996.

Whittingham, T. S.: Aspects of brain energy metabolism and cerebral metabolism. *In* Cerebral Ischemia and Resuscitation, ed. by A. Schurr and B. M. Rigor, pp. 101-121, CRC Press, Boca Raton, Fl, 1990.

Wiesner, J. B., Ugarkar, B. G., Castellino, A. J., Barankiewicz, J., Dumas, D. P., Gruber, H. E., Foster, A. C., and Erion, M. D.: Adenosine kinase inhibitors as a novel approach to anticonvulsant therapy. J Pharmacol Exp Ther **289** (3): 1669-77, 1999.

Wiginton, D. A., Adrian, G. S., Friedman, R. L., Suttle, D. P., and Hutton, J. J.: Cloning of cDNA sequences of human adenosine deaminase. Proc Natl Acad Sci U S A **80** (24): 7481-5, 1983.

Wilson, D. K., and Quiocho, F. A.: A pre-transition-state mimic of an enzyme: X-ray structure of adenosine deaminase with bound 1-deazaadenosine and zinc-activated water. Biochemistry **32** (7): 1689-94., 1993.

Wilson, D. K., Rudolph, F. B., and Quiocho, F. A.: Atomic structure of adenosine deaminase complexed with a transition-state analog: understanding catalysis and immunodeficiency mutations. Science **252** (5010): 1278-84., 1991.

Worku, Y., and Newby, A. C.: Nucleoside exchange catalysed by the cytoplasmic 5'-nucleotidase. Biochem J **205** (3): 503-10, 1982.

Wu, P. H., Barraco, R. A., and Phillis, J. W.: Further studies on the inhibition of adenosine uptake into rat brain synaptosomes by adenosine derivatives and methylxanthines. Gen Pharmacol **15** (3): 251-4, 1984.

Yamazaki, Y., Truong, V. L., and Lowenstein, J. M.: 5'-Nucleotidase I from rabbit heart. Biochemistry **30** (6): 1503-9, 1991.

Yao, S. Y., Ng, A. M., Muzyka, W. R., Griffiths, M., Cass, C. E., Baldwin, S. A., and Young, J. D.: Molecular cloning and functional characterization of nitrobenzylthioinosine (NBMPR)-sensitive (es) and NBMPR-insensitive (ei) equilibrative nucleoside transporter proteins (rENT1 and rENT2) from rat tissues. J Biol Chem **272** (45): 28423-30, 1997.

Yao, S. Y., Ng, A. M., Ritzel, M. W., Gati, W. P., Cass, C. E., and Young, J. D.: Transport of adenosine by recombinant purine- and pyrimidine-selective sodium/nucleoside cotransporters from rat jejunum expressed in Xenopus laevis oocytes. Mol Pharmacol **50** (6): 1529-35, 1996.

Yao, S. Y., Sundaram, M., Chomey, E. G., Cass, C. E., Baldwin, S. A., and Young, J. D.: Identification of Cys140 in helix 4 as an exofacial cysteine residue within the substrate-translocation channel of rat equilibrative nitrobenzylthioinosine (NBMPR)-insensitive nucleoside transporter rENT2. Biochem J **353** (Pt 2): 387-393., 2001.

Zhang, G., Franklin, P. H., and Murray, T. F.: Anticonvulsant effect of N-ethylcarboxamidoadenosine against kainic acid-induced behavioral seizures in the rat prepiriform cortex. Neurosci Lett **114** (3): 345-50., 1990.

Zhang, G., Franklin, P. H., and Murray, T. F.: Manipulation of endogenous adenosine in the rat prepiriform cortex modulates seizure susceptibility. J Pharmacol Exp Ther **264** (3): 1415-24, 1993.

Zhou, Q. Y., Li, C., Olah, M. E., Johnson, R. A., Stiles, G. L., and Civelli, O.: Molecular cloning and characterization of an adenosine receptor: the A3 adenosine receptor. Proc Natl Acad Sci U S A **89** (16): 7432-6, 1992.

Zimmermann, H.: 5'-Nucleotidase: molecular structure and functional aspects. Biochem J **285** (Pt 2): 345-65, 1992.

Zimmermann, H.: Biochemistry, localization and functional roles of ecto-nucleotidases in the nervous system. Prog Neurobiol **49** (6): 589-618, 1996.

Zimmermann, H.: Are mechanisms of exocytosis neurotransmitter specific? Neurochem Int **31** (6): 759-61., 1997.

Zimmermann, H.: Extracellular metabolism of ATP and other nucleotides. Naunyn Schmiedebergs Arch Pharmacol **362** (4-5): 299-309., 2000.

Zoref-Shani, E., Bromberg, Y., Lilling, G., Gozes, I., Brosh, S., Sidi, Y., and Sperling, O.: Developmental changes in purine nucleotide metabolism in cultured rat astroglia. Int J Dev Neurosci **13** (8): 887-96, 1995.

Zoref-Shani, E., Kessler-Icekson, G., and Sperling, O.: Pathways of adenine nucleotide catabolism in primary rat cardiomyocyte cultures. J Mol Cell Cardiol **20** (1): 23-33, 1988.