

Research report

Use of a three-dimensional in vitro model of the rat blood–brain barrier to assay nucleoside efflux from brain

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Abstract

Extracellular adenosine is produced in brain during physiological and pathophysiological conditions. Once produced, this adenosine can undergo one or more of the following fates: it can interact with its receptors, it can be scavenged by astrocytes and/or neurons for ATP resynthesis, it can be transported across the blood–brain barrier and lost from the central nervous system, or it can be metabolized to inosine and hypoxanthine. The present study used a three-dimensional in vitro cell culture model of the rat blood–brain barrier, in which forebrain astrocytes and microvascular endothelial cells were cultured in cartridges containing multiple parallel polypropylene hollow fibers. Endothelial cells were cultured on the inner surfaces and astrocytes on the outer surfaces of these fibers. Growth medium was constantly perfused through the lumen of the fibers to mimic blood flow across endothelial cells in vivo. This co-culture system was used to examine the permeation of adenosine, and its metabolite inosine, from the astrocyte compartment to the endothelial cell compartment. Dipyridamole was added to the media perfusing the endothelial cell compartment to test whether it could decrease permeation of adenosine and inosine across the in vitro blood–brain barrier. Our results indicate that dipyridamole decreased permeation of total purines, especially inosine, across the barrier. Furthermore, permeation of fluorescein isothiocyanate-labeled albumin and radiolabeled sucrose, markers of the paracellular permeation pathway, were also decreased by dipyridamole. In conclusion, these data indicate that in addition to inhibiting nucleoside efflux across the barrier, dipyridamole can also improve blood–brain barrier function in this model.

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1. Introduction

Adenosine is an endogenous neuromodulator that activates a family of four G-protein coupled receptors, named A₁, A_{2A}, A_{2B} and A₃. 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 fluxes of adenosine across cell membranes [1]. Adenosine is produced at low levels during physiological conditions, but it increases during pathophysiological conditions such as hypoxia, ischemia and injury, due to

ATP dysregulation [2,3]. Under experimental cerebral ischemia in rats, adenosine levels in brain increase up to 100-fold but decrease to basal levels within 15 min of reperfusion [4,5].

There are two possible explanations for the rapid decrease in post-reperfusion adenosine levels. These are (1) salvage of adenosine, or its metabolites, for ATP resynthesis in brain; and (2) loss of adenosine, or its metabolites, from the brain following permeation of the blood–brain barrier. The present study investigates these two pathways through the use of a three-dimensional dynamic in vitro model of the blood–brain barrier (DIV-BBB; Fig. 1) [6,7]. This model consists of astrocytes and endothelial cells cultured on opposing surfaces of semi-permeable polypropylene capillaries. Multiple parallel capillaries are encased in a plastic cartridge and access

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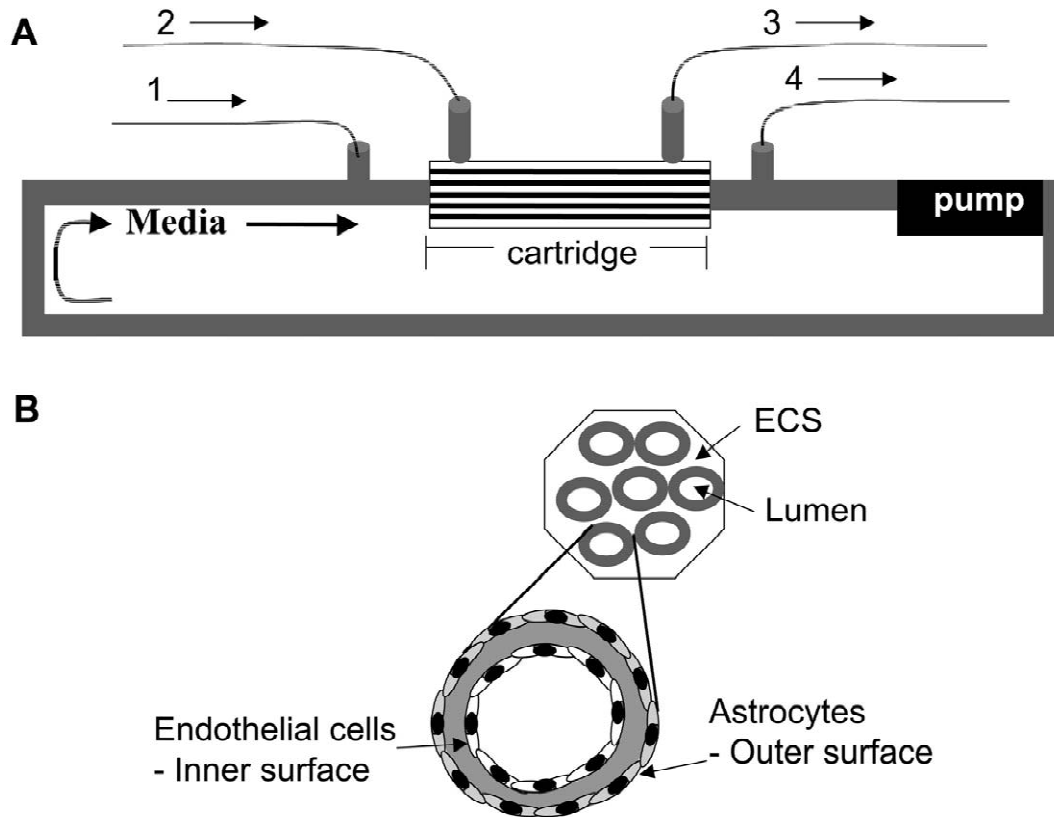


Fig. 1. Schematic diagrams of the culture system and permeability experiments. (A) Culture medium is contained in a closed flow path and is pumped through the lumen of parallel hollow fibers housed in a cell culture cartridge. (B) A cross-section illustrates that multiple parallel capillary fibers are contained within a cell culture cartridge. The extra-capillary space (ECS) is distinct from the luminal compartment. Using access ports 1 and 2, endothelial cells and astrocytes can be introduced into the luminal and the ECS compartments, respectively. Permeability experiments were performed by infusing FITC-albumin, [^{14}C]sucrose and [^3H]adenosine into the ECS via port 2 and measuring recovery of these substances in samples taken from the ECS and lumen via ports 3 and 4, respectively.

ports allow selective access to the luminal (endothelial) compartment or to the extra-capillary space (ECS, astrocyte compartment). Growth medium is constantly pumped across the surface of the endothelial cells. In the present experiments, adenosine was infused into the ECS compartment containing astrocytes, i.e., the 'brain' side of the DIV-BBB, and the subsequent appearance of adenosine and its metabolites was measured in the luminal compartment containing endothelial cells, i.e., the 'blood' side of the DIV-BBB. Dipyridamole, a nucleoside transport inhibitor, was perfused through the 'blood' to evaluate the contribution of these transporters to the changes in nucleoside levels on the two sides of the DIV-BBB.

Dipyridamole does not appear to cross the blood–brain barrier (BBB) [8–10] and peripheral administration of dipyridamole slows the decrease in brain adenosine levels during post-ischemic reperfusion [11]. Dipyridamole, used prophylactically for stroke prevention, has been proposed to produce neuroprotection by elevation of brain adenosine levels via effects at the BBB [12]. In the present study, we have used the DIV-BBB model to test the hypothesis that peripheral administration of dipyridamole can inhibit adenosine movement from the brain into the blood.

2. Materials and methods

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.

2.1. Rat brain microvascular endothelial cell isolation and culture

The methods used for isolation and culture of rat brain microvascular endothelial cells (RBMECs) have been described [7,13]. Briefly, forebrains from Sprague–Dawley rats (50 g) were homogenized using a Dounce homogenizer then treated with protease (50 $\mu\text{g}/\text{ml}$) and DNase (7 U/ml) for 1 h at 37 $^{\circ}\text{C}$. After centrifugation (200 g, 7 min), pellets were resuspended in 15% dextran to remove lipids. Collagenase treatment (1 mg/ml; 2 h at 37 $^{\circ}\text{C}$) followed by Percoll gradient centrifugation was performed to isolate and purify endothelial cells. Cells were cultured in endothelial cell media in T-175 tissue culture flasks pre-coated with bovine fibronectin (3 $\mu\text{g}/\text{cm}^2$). Endothelial cells

achieved 70–80% confluence by days 5–7. Cells were trypsinized, resuspended into a volume of 3 ml culture medium (5×10^6 cells) and inoculated into the luminal compartment of the DIV-BBB.

2.2. Endothelial cell culture media

Rat primary endothelial cells were cultured in media (medium 1) containing Dulbecco's modified Eagle medium (DMEM) with 1 mg/ml glucose, 20% plasma derived equine serum, 75 $\mu\text{g/ml}$ endothelial cell growth supplement, 16 $\mu\text{g/ml}$ heparin, 50 $\mu\text{g/ml}$ bovine insulin, 50 $\mu\text{g/ml}$ human transferrin, 50 ng/ml sodium selenite, 325 $\mu\text{g/ml}$ glutathione, 5 $\mu\text{g/ml}$ ascorbic acid and $1 \times$ penicillin–streptomycin–amphotericin B. An alternate medium formulation (medium 2) was used in some experiments and consisted of DMEM containing 1 mg/ml glucose and enriched with: 4% fetal bovine serum (FBS), 15% plasma-derived equine serum, $1 \times$ penicillin–streptomycin–amphotericin B, 2 mM L-glutamine, 1% non-essential amino acids, $1 \times$ MEM vitamins, 1 mM sodium pyruvate, 100 $\mu\text{g/ml}$ endothelial cell growth supplement and 50 $\mu\text{g/ml}$ heparin.

2.3. Rat forebrain astrocyte cell culture

Rat primary forebrain astrocytes were cultured from E19 Sprague–Dawley rat fetuses as previously described [14]. Cortical tissue was dissected free of meninges, hippocampus and basal structures, then cut up, triturated numerous times and centrifuged at 50 g for 10 min. The supernatant was aspirated and the pellet was resuspended in DMEM-F12 with 10% FBS and $1 \times$ penicillin–streptomycin–amphotericin B. Cells were plated in 10 ml of media in T-175 flasks pre-coated with polylysine (100 μg). After 5–7 days in culture, the flasks were shaken vigorously at 250 rpm for ~16 h to remove neurons, type II astrocytes, microglia and oligodendrocytes. The medium was aspirated and the cells were trypsinized (3 ml; 25 $\mu\text{g/ml}$ trypsin) for 10 min at 37 °C. Fresh medium was added and cells were split 1:1 into fresh T-175 flasks. After reaching confluence, the cells were trypsinized and loaded into cartridges as described below.

2.4. [^3H]Adenosine uptake

RBMECs and rat astrocytes (RAs) were cultured in 12-well plates until confluent. Cells were washed with buffer [in mM: NaCl, 118; 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (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] then preincubated with dipyrindamole (0–30 μM ; 0.5 ml/well), a nucleoside transport inhibitor, for 15 min at room temperature. Cells were then incubated with solutions containing the same dipyrindamole concentrations supplemented with 1 μM [^3H]adenosine (1 $\mu\text{Ci}/$

ml). After 3 min, solutions were aspirated and the cells were rapidly washed twice with ice-cold buffer. Cellular protein was dissolved by incubating cells overnight with NaOH (1 M; 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.

2.5. Immunohistochemistry

Aliquots of the endothelial cells and astrocytes were cultured on chamber slides. To assess uptake of low-density lipoprotein, endothelial cells were incubated with acetylated low density lipoprotein labeled with 10 $\mu\text{g/ml}$ 1,1'-dioctadecyl-3,3,3',3'-tetramethyl indo-carbocyanine perchlorate (Dil Ac-LDL) for 4 h at 37 °C. Cells were then washed with buffer, fixed with buffered formaldehyde, rinsed with water and mounted with glycerol.

Endothelial cells and astrocytes were fixed with 3% phosphate buffered formaldehyde for 20 min, washed, then incubated in blocking buffer [3% normal goat serum, 0.1% Triton X-100 and 1% bovine serum albumin (BSA), pH 7.4] for 1 h at room temperature. Cells were then incubated overnight at 4 °C with antibodies to zona occludins 1 (ZO-1; 0.5 $\mu\text{g/ml}$), glial acidic fibrillary protein (GFAP; 1:1000 dilution) or α -actin (1:1000 dilution). Reactivity to ZO-1 or GFAP was detected with fluorescein isothiocyanate (FITC) conjugated to donkey anti-rabbit antibody at a dilution of 1:200. Reactivity to α -actin was detected with a 1:100 dilution of Texas Red conjugated sheep anti-mouse antibody.

2.6. Metabolism of [^3H]adenosine by media components

[^3H]Adenosine, 10 μM , 0.5 $\mu\text{Ci/ml}$, was incubated with complete medium, buffer or individual medium components for 30 min at 22 °C. Samples were analyzed for [^3H]adenine nucleotides, [^3H]inosine, [^3H]hypoxanthine and [^3H]adenosine content by thin-layer chromatography (TLC). The method of Schrader and Gerlach was used to identify the [^3H]purines [15]. Briefly, *n*-butanol–ethyl acetate–methanol–ammonium hydroxide (7:4:3:4) were mixed, placed in a TLC tank and allowed to equilibrate for 90 min. Samples (20 μl) were spotted onto Silica Gel GF plates with 5 μl of cold carrier. Cold carrier consisted of 15 mM adenosine, 15 mM inosine, 15 mM hypoxanthine, 15 mM adenine, 15 mM AMP, 7.5 mM uric acid and 6.5 mM xanthine. Plates were run for 3 h, and standards 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 h, scintillation fluid (5 ml) was added and radioactivity was determined using scintillation spectrometry. The levels of tritiated uric acid, xanthine and adenine were at or below our detection limit (0.25 pmol)

in all experiments and are therefore not reported. ATP, ADP and AMP are not resolved with this system; thus, tritium-containing compounds co-migrating with the AMP standards are identified as adenine nucleotides.

2.7. Dynamic *in vitro* model of the rat blood–brain barrier

Rat brain microvascular endothelial cells and astrocytes were cultured in a three dimensional model of the blood–brain barrier as previously described [6,7,16]. This model consists of a cartridge, containing 50 hollow Pronectin F-treated polypropylene capillaries, connected by gas permeable tubing to a flow path for culture media and a pulsatile pump (Fig. 1). Four sampling ports on the cartridge allow upstream and downstream (relative to the direction of media flow) access to the lumen (inner surface of the capillaries) or ablumen (ECS), respectively. The capillaries within the cartridge contain 0.5 μm pores that allow gas and nutrient exchange between the lumen and ECS but do not permit cells to cross. Lumen and ECS surfaces were coated with 3 $\mu\text{g}/\text{cm}^2$ fibronectin and ECS surfaces were additionally coated with polylysine (3 $\mu\text{g}/\text{cm}^2$).

Endothelial cells were first inoculated into the lumen and allowed to adhere under static conditions over a 3-h period. Flow was then initiated through the lumen, allowing endothelial cells to be under low shear stress of 2 dyne/cm^2 for 24 h and then exposed to shear stress of 4 dyne/cm^2 for the duration of the experiment. Astrocytes were added to the ECS compartment 7–10 days after endothelial cells. Medium was replaced three times per week and permeability experiments were performed after 7 days of co-culture.

2.8. Permeability experiments

Medium 1 was used for all permeability experiments. After 1 week of co-culture, cartridges were attached to an SP260P dual injection–withdrawal syringe pump with 10 ml syringes and tubing (1 mm I.D.). The tubing was attached to the downstream and upstream ECS sampling ports and to the downstream lumen sampling port via blunted 18 g needles and luer lock adapters (Fig. 1). The cartridges remained in the cell culture incubator and the tubing was passed through the incubator door to the syringe pump located adjacent to the incubator. 2-Amino-1,5-dihydro-7-(3-pyridinylmethyl)-4H-pyrrolo[3,2-*d*]pyridin-4-one (BCX-34; 10 μM), a purine nucleoside phosphatase inhibitor, alone or together with dipyridamole (10 μM) was added to the media and perfused through the lumen. After 30 min, the syringe pump was used to infuse (0.1 ml/min) a mixture containing 0.5 mg/ml FITC-conjugated bovine albumin (FITC-albumin), [^{14}C]sucrose (50 nCi/ml), 10 μM [^3H]adenosine (0.5 $\mu\text{Ci}/\text{ml}$) and 10 μM BCX-34 in media into the upstream port of the ECS.

Down-stream samples were collected from the ECS and lumen at 10 min intervals and were analyzed for FITC, [^{14}C] and [^3H] content. Tritium samples were further analyzed by TLC for [^3H]adenine nucleotides, [^3H]inosine, [^3H]hypoxanthine and [^3H]adenosine. At the end of each experiment, RBMECs and RAs were trypsinized to remove them from the cartridges and viability was assessed by trypan blue exclusion.

2.9. Materials

Protease and collagenase were purchased from Roche Diagnostic (Laval, Canada). DNase 1, dextran, bovine fibronectin, heparin, insulin–transferrin–sodium selenite media supplement, glutathione, polylysine, trypan blue, FITC-albumin, mouse monoclonal anti- α -actin antibody and trypsin-EDTA were purchased from Sigma–Aldrich (Oakville, Canada). DMEM, DMEM-F12, FBS, L-glutamine, non-essential amino acids, MEM vitamins, penicillin-streptomycin-amphotericin B, sodium pyruvate and ascorbic acid were purchased from Invitrogen (Burlington, Canada). Plasma derived equine serum was purchased from Atlantic Biologicals (Norcross, GA, USA). Endothelial cell growth supplement and Dil Ac-LDL were purchased from Biomedical Technologies (Stoughton, MA, USA). Rabbit anti-ZO-1 antibody was purchased from Zymed Laboratories (San Francisco, CA, USA), rabbit anti-cow GFAP antibody was purchased from Dako Diagnostics Canada (Mississauga, Canada), Texas Red conjugated sheep anti-mouse antibody and FITC-conjugated donkey anti-rabbit antibody were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). [^3H]Adenosine and [^{14}C]sucrose were purchased from Perkin-Elmer Life Sciences Canada (Woodbridge, Canada). BCX-34 was a generous gift from Dr. Philip Morris of Biocryst Pharmaceuticals (Birmingham, AB, USA). Dipyridamole was purchased from Research Biochemicals International (Natlick, MA, USA). Silica Gel GF TLC plates were purchased from Fisher Scientific (Whitby, Canada). Pronectin F-treated cell culture cartridges and peristaltic pump were purchased from Spectrum Laboratories (Rancho Dominguez, CA, USA). SP260P syringe pump was purchased from World Precision Instruments (Sarasota, FL, USA).

3. Results

3.1. Characterization of cell cultures

Up to 14 days in culture, approximately 90% of cells in RBMEC cultures were positive for LDL uptake and ZO-1 immunoreactivity, characteristic of endothelial cells. GFAP immunoreactivity, a feature of astrocytes, was routinely $\leq 1\%$. Immunoreactivity for α -actin, a characteristic of smooth muscle cells and pericytes, was $\leq 2.5\%$ after 3

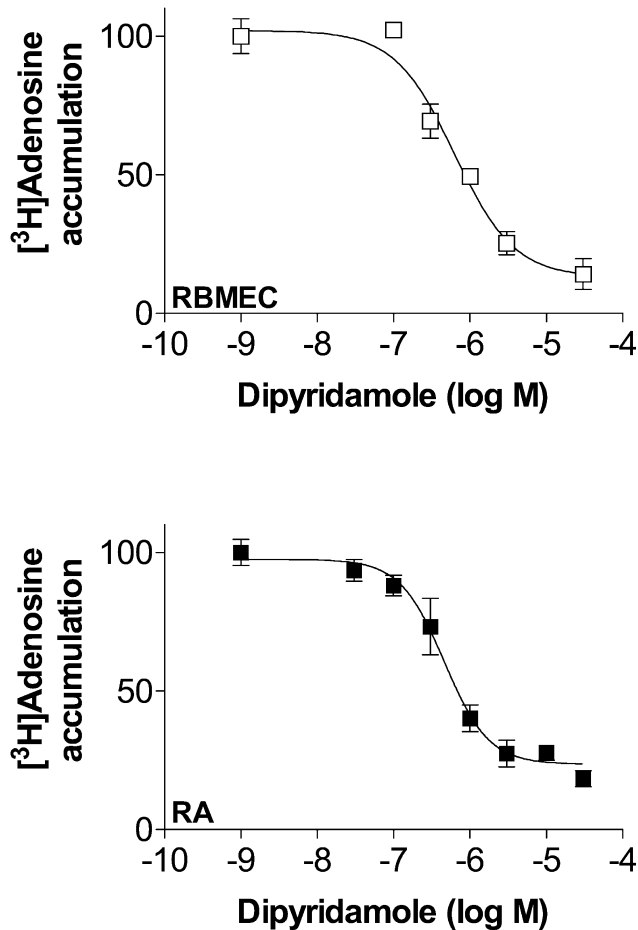


Fig. 2. Inhibition of [^3H]adenosine accumulation by brain microvascular endothelial cells (RBMECs, upper panel) or rat astrocytes (RAs, lower panel) by dipyrindamole. Cells were pretreated with dipyrindamole or buffer for 15 min then incubated with 1 μM [^3H]adenosine \pm dipyrindamole for 1 min. Data are expressed as % control and are means \pm S.E.M. for 6–8 determinations.

days in culture and increased up to 10% by 14 days in culture.

3.2. [^3H]Adenosine uptake

To verify that equilibrative nucleoside transporters are the predominant mechanism for nucleoside accumulation by RBMECs and RAs, the concentration-dependent effects of dipyrindamole on [^3H]adenosine uptake were assessed. [^3H]Adenosine uptake into RBMEC and RA cultures was inhibited by dipyrindamole with IC_{50} values of 640 ± 80 and 540 ± 130 nM, respectively (Fig. 2). At 30 μM , dipyrindamole inhibited 91 and 81% of [^3H]adenosine uptake into RBMECs and RAs, respectively.

3.3. Metabolism of [^3H]adenosine by media components

Preliminary experiments indicated very low recovery of [^3H]adenosine from DIV-BBB cultures. Therefore, metabolism of [^3H]adenosine by two different formulations of endothelial cell culture media was assayed (Table 1). After 30 min at 22 $^{\circ}\text{C}$, medium 1 reduced a 10 μM concentration of [^3H]adenosine by 69% and medium 2 caused an 84% reduction in [^3H]adenosine. The decrease in [^3H]adenosine was recovered as [^3H]inosine and [^3H]hypoxanthine with only small amounts of [^3H]adenine nucleotides detected. BCX-34 decreased [^3H]hypoxanthine and increased [^3H]inosine production; the addition of dipyrindamole did not alter this recovery profile. Each of the media components was tested individually to identify the source of [^3H]adenosine metabolism. Endothelial cell growth supplement and FBS had greater effects on [^3H]adenosine metabolism than equine serum. Endothelial cell growth supplement potentially contains adenosine deaminase,

Table 1
Metabolism of [^3H]adenosine by individual components and complete RBMEC growth media

	Adenine nucleotides	Inosine	Hypoxanthine	Adenosine
Buffer	0.7 \pm 0.1	1.3 \pm 0.4	4.5 \pm 1.7	91.9 \pm 3.1
Endothelial cell growth supplement	25.7 \pm 13.0	7.6 \pm 0.9	59.1 \pm 6.2	2.1 \pm 0.6
Equine serum	1.6 \pm 1.4	9.1 \pm 1.3	16.6 \pm 15.2	70.4 \pm 18.4
FBS	7.4 \pm 6.2	76.6 \pm 9.1	14.6 \pm 4.2	1.8 \pm 0.7
Medium 1 ^a	1.7 \pm 1.4	32.1 \pm 0.3	27.9 \pm 11.0	22.8 \pm 14.1
Medium 1+10 μM BCX-34	0.9 \pm 0.1	66.1 \pm 13.7	5.4 \pm 2.8	25.4 \pm 11.6
Medium 1+10 μM BCX-34+10 μM DPR	0.9 \pm 0.1	69.2 \pm 12.4	3.7 \pm 0.1	26.3 \pm 12.4
Medium 2 ^b	1.1 \pm 0.4	73.9 \pm 0.9	9.5 \pm 2.2	8.2 \pm 5.0
Medium 2+10 μM BCX-34	0.8 \pm 0.1	82.4 \pm 7.5	3.4 \pm 0.7	11.2 \pm 4.7
Medium 2+10 μM BCX-34+10 μM DPR	0.8 \pm 0.0	84.2 \pm 9.6	2.7 \pm 0.3	12.3 \pm 9.9

Data are means \pm S.D. ($n=2-4$) expressed as a percentage of initial [^3H]adenosine added to the incubation mixtures.

^a Medium 1 contained 75 $\mu\text{g}/\text{ml}$ endothelial cell growth supplement, 16 $\mu\text{g}/\text{ml}$ heparin, 5 $\mu\text{g}/\text{ml}$ vitamin C, 325 $\mu\text{g}/\text{ml}$ glutathione, 50 $\mu\text{g}/\text{ml}$ insulin, 50 $\mu\text{g}/\text{ml}$ transferrin, 50 ng/ml selenium, 1 mg/ml glucose, 20% plasma derived equine serum and 1 \times penicillin–streptomycin–amphotericin B in DMEM.

^b Medium 2 contained 1 \times non-essential amino acids, 1 \times MEM vitamins, 1 \times penicillin–streptomycin–amphotericin B, 1 mg/ml glucose, 2 mM glutamine, 1 mM pyruvate, 50 $\mu\text{g}/\text{ml}$ heparin, 4% FBS, 15% plasma derived equine serum and 100 $\mu\text{g}/\text{ml}$ endothelial cell growth supplement in DMEM.

purine nucleoside phosphorylase and adenosine kinase as [^3H]inosine, [^3H]hypoxanthine and [^3H]adenine nucleotides were all elevated. FBS appeared to have substantial adenosine deaminase activity as [^3H]inosine was the predominant metabolite detected. Heparin, vitamin C, insulin, transferrin and selenite had no effect on [^3H]adenosine metabolism (data not shown).

3.4. Permeability experiments

DIV-BBB cultures were assessed for permeability to FITC-albumin and [^{14}C]sucrose by infusing these substances at a rate of 0.1 ml/min into the ‘upstream’ sampling port for the ECS. Downstream samples were obtained from the ECS and lumen and analyzed for the presence of FITC and [^{14}C]. The time when FITC or [^{14}C] began to increase in ECS samples was approximately 50 min and was dictated by the combined volumes of (a) the tubing between the infusion syringe pump and the up-

stream ECS port, (b) the ECS, and (c) the tubing between the downstream ECS port and the sample withdrawal pump.

Each experiment consisted of a pair of DIV-BBB cultures. For 30 min prior to the initiation of infusions into the ECS and throughout the duration of the experiment the cartridges were perfused through the lumen with media containing 10 μM BCX-34. One cartridge of each pair was perfused with media containing both 10 μM BCX-34 and 10 μM dipyridamole. Dipyridamole significantly ($P \leq 0.05$) decreased the permeability of DIV-BBB cultures to FITC-albumin, [^{14}C]sucrose, total [^3H]purines and [^3H]inosine (Fig. 3A–D). Dipyridamole significantly inhibited the permeation of FITC-albumin at 90 and 100 min after initiation of the ECS infusion by 52 and 61%, respectively. The permeation of [^{14}C]sucrose, measured at 100, 110 and 120 min, was significantly inhibited by 70, 74 and 72%, respectively. The permeation of total [^3H]purines, measured at 110 and 120 min, was reduced by 67 and 64%,

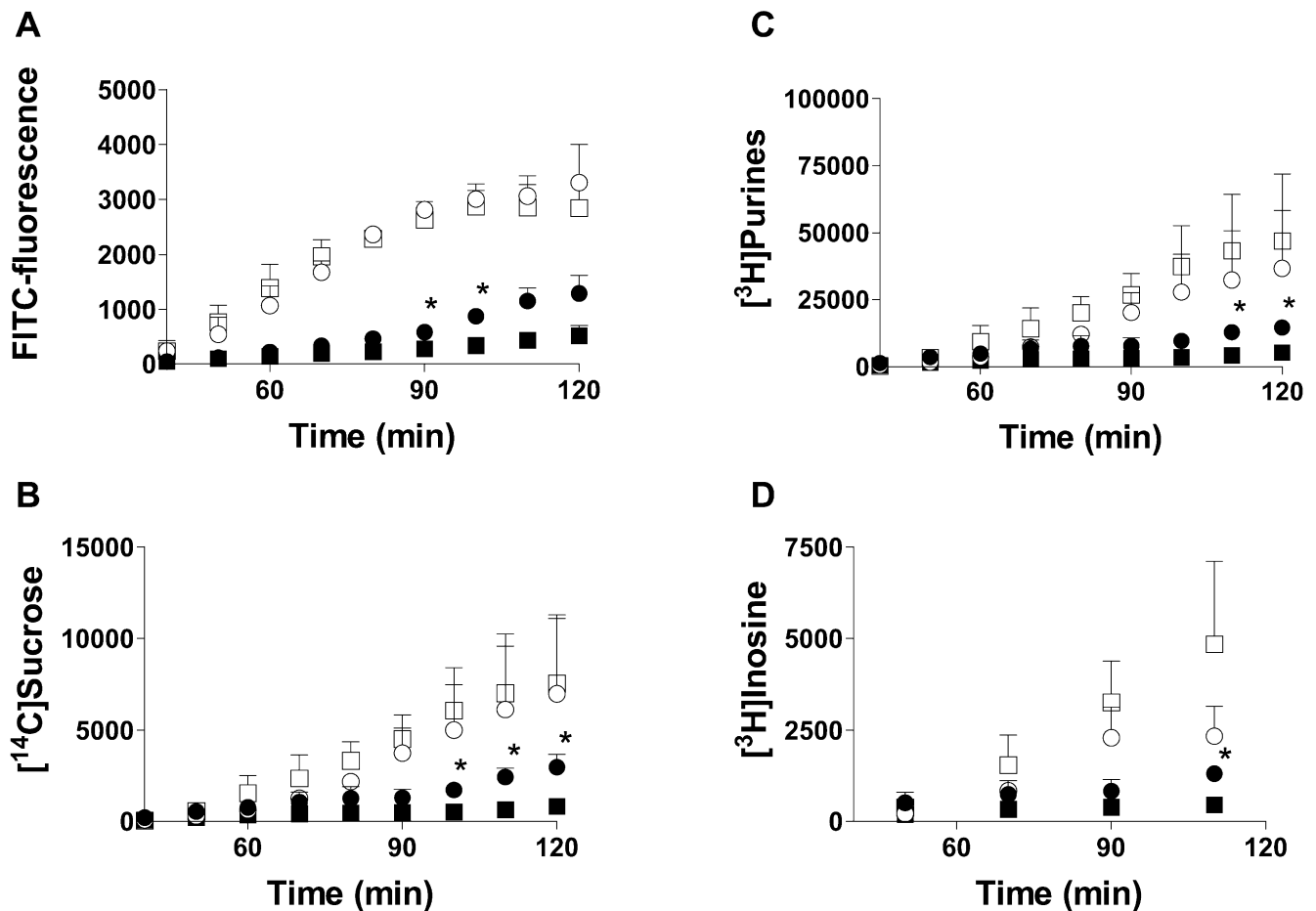


Fig. 3. DIV-BBB cultures were established with RBMEC and RA cultures as described in the text. FITC-albumin, [^{14}C]sucrose, [^3H]adenosine and BCX-34 were infused at a rate of 0.1 ml/min into the upstream ECS port of cartridges perfused through the lumen with medium 1 containing BCX-34 (circles) or medium 1 containing both BCX-34 and dipyridamole (squares). Samples were obtained from the downstream ECS (open symbols) and lumen (closed symbols) ports and analyzed for FITC-albumin (A), [^{14}C]sucrose (B), total [^3H]purines (C) or [^3H]inosine (D) content. Data are expressed as fluorescence units (A) or d.p.m. (B–D) and are means from three independent experiments. S.E.M. bars are shown only in one direction for clarity. * $P < 0.05$, t -test comparing lumen \pm dipyridamole.

respectively, in the presence of dipyridamole. The appearance of inosine in the lumen at 110 min was significantly reduced, by 65%, in the presence of dipyridamole. Due to metabolism by media during the course of the experiments, [^3H]adenosine levels were low, 5–10% of [^3H]inosine levels, and were not significantly affected by dipyridamole (data not shown).

4. Discussion

The main findings of this study were that (1) dipyridamole inhibits [^3H]adenosine uptake into RAs and RBMECs with IC_{50} values of 540–640 nM, (2) the RBMEC growth media components FBS, equine serum and endothelial cell growth supplement contain enzymes capable of metabolizing adenosine, and (3) perfusing the endothelial cell compartment of a three dimensional cultured rat blood–brain barrier with dipyridamole-supplemented culture media decreased permeation across the barrier, from the ECS to the lumen, of the nucleoside transporter permeant inosine as well as the paracellular diffusion markers sucrose and albumin.

Primary cultures of RBMECs were positive for LDL-uptake and ZO-1 immunostaining. GFAP staining was consistently low (<1%), indicating that few astrocytes contaminated our RBMEC cultures. α -Actin positive cells were initially at low abundance but increased with time in culture, indicating the presence of pericytes in our RBMEC cultures. Many researchers use clonal cell lines instead of primary cultures. This strategy ensures that contaminating cell types do not affect the integrity of the barrier; however, clonal cell lines may lack the ability to form a barrier with all the characteristics of the in vivo structure. The small proportion of contaminating cell types may have contributed to the variability among cartridge co-cultures.

Several components of RBMEC culture media, including endothelial cell growth supplement, FBS and plasma derived equine serum, contained enzymes capable of metabolizing [^3H]adenosine. BCX-34, a purine nucleoside phosphatase inhibitor, reduced or prevented hypoxanthine formation and was, thus, used to prevent metabolism of [^3H]inosine to hypoxanthine. In the presence of BCX-34, the predominant tritium-labeled compound was [^3H]inosine. Erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA; 1 μM), an inhibitor of adenosine deaminase, was used in preliminary experiments. However, as EHNA produced only partial inhibition of the decrease in [^3H]adenosine and the increase in [^3H]inosine, EHNA was not used routinely.

Our observation that cell culture media can metabolize adenosine is an important note of caution to other researchers. Replacing culture media with buffer or serum-free and endothelial cell growth supplement-free medium is a simple solution for many experiments. In these

experiments, however, media replacement posed the risk of a deterioration of blood–brain barrier phenotype and was not attempted.

Dipyridamole is an inhibitor of the two subtypes of equilibrative (sodium-independent) nucleoside transporters termed ENT1 and ENT2. It inhibited [^3H]adenosine accumulation by RAs and RBMECs by 81–91%, indicating that these transporters are the primary route of [^3H]adenosine influx in these cells. The remaining 10–20% of [^3H]adenosine accumulation may be due to a small component of non-specific association of [^3H]adenosine with the cells and/or uptake by concentrative nucleoside transporters. In fact, RBMECs have been shown to express CNT2 [17], a purine selective concentrative nucleoside transporter. The potency of dipyridamole to inhibit cellular accumulation of [^3H]adenosine was greater than was previously observed with recombinant rat ENT1 and ENT2 expressed in *Xenopus* oocytes [18] but was similar to that reported for rat synaptosomes [19] and rat C6 glioma cells [20]. These studies indicate that dipyridamole is an effective inhibitor of ENTs but estimates of its potency vary among cell types and cell preparations.

Albumin and sucrose are normally excluded from the brain as they exhibit low lipophilicity and are not substrates for receptor-mediated endocytosis or transport processes. Albumin and sucrose are markers of paracellular diffusion, which is very low in the blood–brain barrier due to the presence of tight junctions. Dipyridamole added to culture media flowing through the lumen decreased the permeation of FITC-albumin, [^{14}C]sucrose and [^3H]purines, including the nucleoside transporter permeant [^3H]inosine. The decrease in [^3H]inosine permeation was predicted because dipyridamole is an inhibitor of ENT1 and ENT2. Both of these transporters accept inosine as a permeant. Therefore, the effect of dipyridamole to increase [^3H]inosine in the ECS and to decrease [^3H]inosine in the lumen, relative to control cartridges, is consistent with dipyridamole-mediated inhibition of inosine transport down its concentration gradient. The effects of dipyridamole on FITC-albumin and [^{14}C]sucrose permeation were not expected as neither of these compounds are nucleoside transporter permeants.

Many of the pharmacological effects of dipyridamole are due to inhibition of the cellular uptake of adenosine and the consequent increase in adenosine receptor activation. We have provided data demonstrating that adenosine is largely metabolized by media during the course of our experiments. Thus, it is unlikely that dipyridamole is enhancing the receptor-mediated effects of exogenous adenosine to produce the observed effects on FITC-albumin and [^{14}C]sucrose permeation. Although it is possible that the RAs and RBMECs can produce sufficient localized quantities of endogenous adenosine to activate adenosine receptors, a recent report indicates that abluminal adenosine does not affect paracellular BBB permeability [21]. Alternatively, the effects of dipyridamole on FITC-

albumin and [^{14}C]sucrose permeability may be due to the enhanced inosine levels evoked by dipyridamole. Inosine, while not as active a signaling molecule as adenosine, nevertheless has biological functions in addition to its role as an intermediate in cellular metabolism [22]. However, to our knowledge no effects of inosine on the blood–brain barrier have been reported. In addition to inhibiting nucleoside transporters, dipyridamole has also been reported to have antioxidant properties [23] and to inhibit phosphodiesterase activity [24]. As the present studies were performed under normoxic conditions, an antioxidant effect is unlikely to explain our findings. Inhibition of phosphodiesterase activity and elevation of cAMP levels could explain our findings as cAMP analogs and drugs that increase cAMP can decrease paracellular diffusion through endothelial cell monolayers [25–27]. Thus, dipyridamole may decrease paracellular diffusion of FITC-albumin and [^{14}C]sucrose across the DIV-BBB subsequent to increasing cAMP production in RBMECs.

In summary, the main finding of this study was that dipyridamole decreased permeation of FITC-albumin and [^{14}C]sucrose across an in vitro model of the rat blood–brain barrier. Whether this effect of dipyridamole contributes to its clinical efficacy in secondary stroke prevention remains to be determined.

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