GLIA 52:25–35 (2005)

Nucleoside Transporter Expression and Function in Cultured Mouse Astrocytes

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KEY WORDS
adenosine; CNT2; ENT1; ENT2; guanosine; thymidine

ABSTRACT
Uptake of purine and pyrimidine nucleosides in astrocytes is important for several reasons: (1) uptake of nucleosides contributes to nucleic acid synthesis; (2) astrocytes synthesize AMP, ADP, and ATP from adenosine and GTP from guanosine; and (3) adenosine and guanosine function as neuromodulators, whose effects are partly terminated by cellular uptake. It has previously been shown that adenosine is rapidly accumulated by active uptake in astrocytes (Hertz and Matz, Neurochem Res 14:755–760, 1989), but the ratio between active uptake and metabolism-driven uptake of adenosine is unknown, as are uptake characteristics for guanosine. The present study therefore aims at providing detailed information of nucleoside transport and transporters in primary cultures of mouse astrocytes. Reverse transcription-polymerase chain reaction identified the two equilibrative nucleoside transporters, ENT1 and ENT2, together with the concentrative nucleoside transporter CNT2, whereas CNT3 was absent, and CNT1 expression could not be investigated. Uptake studies of tritiated thymidine, formycin B, guanosine, and adenosine (3-s uptakes at 1–4°C to study diffusional uptake and 1–60-min uptakes at 37°C to study concentrative uptake) demonstrated a fast diffusional uptake of all four nucleosides, a small, Na^+-independent and probably metabolism-driven uptake of thymidine (consistent with DNA synthesis), larger metabolism-driven uptakes of guanosine (consistent with synthesis of DNA, RNA, and GTP) and especially of adenosine (consistent with rapid nucleotide synthesis), and Na^+-dependent uptakes of adenosine (consistent with its concentrative uptake) and guanosine, rendering neuromodulator uptake independent of nucleoside metabolism. Astrocytes are accordingly well suited for both intense nucleoside metabolism and metabolism-independent uptake to terminate neuromodulator effects of adenosine and guanosine.

INTRODUCTION

Uptake of purine and pyrimidine nucleosides in astrocytes is important for several reasons: (1) even in the adult brain astrocytes are capable of cell division and may partly utilize cellular uptake to acquire nucleosides for synthesis of not only RNA but also DNA (Fontana et al., 1980; Huff et al., 1990; Korr et al., 1994; Kornack and Rakic, 2001); (2) AMP, ADP, and ATP are avidly synthesized from adenosine in astrocytes (Matz and Hertz, 1989) and play important roles in energy homeostasis and signaling; (3) astrocytes synthesize guanine nucleotides (Pelled et al., 1999); (4) ATP is released from astrocytes during the propagation of $Ca2^+$ waves (Guthrie et al., 1999) and degraded extracellularly; and (5) adenosine and guanosine function as neuromodulators, acting on P1 purinergic receptors of different subtypes in neurons and/or astrocytes (van Calker and Hamprecht, 1979; Kim et al., 1991; Rathbone et al., 1999; Ciccarelli et al., 2001; Chen et al., 2001; Di Iorio et al., 2004; Wittendorp et al., 2004), and cellular uptake contributes to the termination of their neuromodulator effects. However, except for adenosine (Hertz, 1978; Thampy and Barnes, 1983; Bender and Hertz, 1986; Hösli and Hösli, 1988; Matz and Hertz, 1990; Bender et al., 1994; Othman et al., 2002), only little information is available about the mechanisms by which purine and pyrimidine nucleosides are accumulated into astrocytes, and even in the case of adenosine the relative importance of different uptake mechanisms is unknown.

Three major Na^+-dependent nucleoside transport systems are expressed in nontransformed cells: *cif* (N1) accepts purine nucleosides, including the inosine derivative formycin B, and uridine as permeants; *cit* (N2 and N4) is pyrimidine selective, but it also transports adenosine and, in the case of N4, guanosine; and *cib* (N3) accepts both purine and pyrimidine nucleosides (Cass et al., 1999; Ritzel et al., 2001a). Additional transporters have been described in transformed cells, including a guanosine-specific Na^+-dependent transporter, *csg* (Flanagan and Mecklin-Gill, 1997). The Na^+ gradient provides the driving force for the uptake, and *cif* and *cit* transport the nucleoside and Na^+ in a 1:1 ratio, whereas *cib* requires uptake of 2 Na^+ for each molecule nucleoside accumulated. Both *cif* and *cit* are widespread in brain, whereas *cib* is expressed to a minor extent in brain and only in selected regions (Ritzel et al., 1998, 2001b). In addition, one or both of the two equilibrative, non-Na^+-dependent nucleoside transporters *es* and *ei* are expressed rather ubiquitously and are also present

Grant sponsor: Canadian Spinal Research Organization.

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Received 8 December 2004; Accepted 22 February 2005

DOI 10.1002/glia.20216

Published online 12 May 2005 in Wiley InterScience (www.interscience.wiley.com).

in brain (Jennings et al., 2001) and in C-6 cells, an astrocytic model system (Sinclair et al., 2000), although little *es* is found in the latter (Sinclair et al., 2001). The difference between *es* and *ei* is that *es*, but not *ei*, is inhibited by low nanomolar concentrations of nitrobenzyl-thio-inosine (NBTI). With the exception of *cgs*, none of the Na^+-dependent transporters is NBTI-sensitive.

The facilitated diffusion mediated by the equilibrative transporters *es* and *ei* is only capable of bringing the intracellular nucleoside concentration up to the same level as the extracellular concentration (or vice versa during nucleoside efflux). Nevertheless, they can facilitate maintained uptake, if the accumulated nucleoside is metabolized, thereby reducing the concentration of free nucleoside and allowing continuous facilitated diffusion along the resulting concentration gradient (e.g., Hertz, 1991a,b). Such a "metabolism-driven" uptake is capable of supplying nucleosides for synthesis of RNA, DNA, and adenosine nucleotides at the rates at which these are formed, but it cannot be used for efficient uptake of neuromodulator adenosine or guanosine independently of subsequent intracellular metabolism. It may therefore be consistent with the neuromodulator activity of adenosine that accumulation of extracellular adenosine into adjacent astrocytes and neurons (Hertz, 1978; Hösli and Hösli, 1988; Bender et al., 1994; Othman et al., 2002) can occur as a metabolism-independent, concentrative uptake (Hertz and Matz, 1989; Matz and Hertz, 1990). However, it has not been established whether a similar uptake mechanism exists for guanosine.

The existence of different Na^+-dependent nucleoside transport systems is reflected by the demonstration of three concentrative nucleoside transporters (CNTs) in nontransformed cells: CNT1, which mediates uptake by system *cit* and N2 (but not N4); CNT2, which is the transporter responsible for the *cif* system, and CNT3, corresponding to *cib*. The two equilibrative, nonconcentrative adenosine transporters ENT1 and ENT2 mediate the uptake by facilitated diffusion (systems *es* and *ei*), with ENT1 sensitive, and ENT2 resistant, to low nanomolar concentrations of NBTI. The complete nucleotide base sequence has been determined for mouse ENT1 and ENT2 as well as for mouse CNT2 and CNT3 (Choi et al., 2000; Kiss et al., 2000; Patel et al., 2000; Handa et al., 2001; Ritzel et al., 2001b; Strausberg et al., 2002), whereas the complete sequence for CNT1 has not been determined in the mouse, and the transporter responsible for N4 is unknown. The available information allows demonstration of expression in any mouse tissue of nucleoside transporters known to be present in nontransformed cells with the exception of CNT1 by reverse transcription-polymerase chain reaction (RT-PCR) and primers of a composition selectively recognizing subtype-specific sequences of the transporter genes in the mouse.

In the present study, we have investigated the expression of CNT and ENT genes in primary cultures of mouse astrocytes by RT-PCR. To establish that the expressed genes are also functionally important in astrocytes, we have combined the gene expression study with a study of Na^+-dependence (determining uptake in the presence and absence of Na^+) and a kinetic analysis of the nucleoside transport systems operating in these cultures. Kinetics and Na^+ dependence of the uptakes were determined for the purine nucleosides guanosine and adenosine, the poorly metabolizable inosine analogue formycin B, and the pyrimidine nucleoside thymidine, which has no known neuromodulator action and is a precursor of DNA but not of RNA. Uptakes were determined as total accumulation of radioactivity in the presence of tritiated adenosine, guanosine, formycin B, or thymidine during either a 3-s incubation period in ice-cold medium or long-term (1–60 min) incubation at 37°C. The former approach provides information about diffusional uptake (simple [passive] or facilitated diffusion, distinguished in the present study by saturability or delay at high concentrations and in the literature by sensitivity to specific inhibitors) in the more or less complete absence of metabolism of the accumulated nucleoside (on account of the short uptake period and the low temperature), the second about total uptake (diffusional uptake plus Na^+-independent metabolism-driven uptake or Na^+-dependent, concentrative uptake).

Primer sequences:

A: ENT1- Forward:5'-AGC CTG TGC AGT TGT CAT TTT-3'
Reverse: 5'-TCT TCC TTT TGG CTC CTC TCC-3'

B: ENT2- Forward:5'- CGA GTC GGT GCG GAT TCT G-3'
Reverse: 5'- GAC AGC TTC TCG GTC AGG TAG-3'

C: CNT2-Forward:5'-AGG CCT GGA ACT CAT GGA AGG-3'
Reverse:5'-GAG TCC TAT AAA CAC CCG CTT TGC-3'

D: CNT3-Forward:5'-GGT TCC CTA GGA ATC GTG AT-3'
Reverse:5'-CCA ATT GCA GTT CAG CGT TGG T-3'

E: β-Actin- Forward: 5'-ACA GCT GAG AGG GAA ATC GTG CG-3'
Reverse: 5'-ACT TGC GCT CAG GAG GAG CAA TG-3'

Fig. 1. Primer sequences used for RT-PCR of mRNA for ENT1 (**A**), ENT2 (**B**), CNT2 (**C**), CNT3 (**D**) and β-actin (**E**). The primers were constructed based on the published nucleobase sequences for ENT1, ENT2, CNT2 and CNT3 and the publications by Leung et al. (2001) and Ritzel et al. (2001b), and the primer sequence for β-actin was from Hayashi et al. (1999).

MATERIALS AND METHODS

Materials

All chemicals for preparation of tissue culture medium, most other nonradiolabeled chemicals, and radiolabeled [8-[^{3}H]adenosine (6.9 Ci/mmol) were purchased from Sigma Chemical Company (St. Louis, MO); [8-[^{3}H]guanosine (15 Ci/mmol) and [^{3}H(G)]formycin B (14 Ci/mmol) were obtained from Moravek Biochemical (Brea, CA), and [methyl-[^{3}H]thymidine (200 Ci/mmol) was purchased from Du Pont (Boston, MA). Reagents for RT-PCR were from the sources detailed below. The sequence of the nucleoside transporter primers and the β-actin primer, used for quantitation of β-actin as a housekeeping gene to ascertain that approximately equal amounts of PCR product had been applied in different gels, are shown in Figure 1.

Cell Cultures

Cultures of astrocytes were prepared in Falcon Primaria culture dishes as described by Hertz et al. (1982, 1998) and Juurlink and Hertz (1992): the parts of the cerebral hemispheres above the lateral ventricles were dissected out of the brains from newborn Swiss mice and mechanically dissociated. They were grown for 2 weeks under a CO_2/air (5%/95%) atmosphere in a tissue culture medium (Hertz et al., 1982; Juurlink and Hertz, 1992) modified from Eagle's MEM (Eagle, 1959) by doubling the concentrations of amino acids, except glutamine (2 mM) and quadrupling the concentrations of vitamins. The medium contains 7.5 mM glucose, no pyruvate, glutamate, nucleoside, or antibiotics, and it is almost identical to Dulbecco's low-glucose medium. The culturing medium was fortified with horse serum (initially 20% and later declining to 10%). After 2 weeks, the culturing was continued for another 2–3 weeks in a similar medium which in addition contained 0.25 mM dibutyryl cyclic AMP, a procedure known to evoke a pronounced morphological and functional differentiation of the cells (Meier et al., 1991; Schubert et al., 2000) without leading to the formation of reactive astrocytes (Wandosell et al., 1993). Such cultures constitute an excellent model for many functional characteristics of astrocytes in situ (Juurlink and Hertz, 1992; Hertz et al., 1998).

RT-PCR

For determination of mRNA expression of the four nucleoside transporters by RT-PCR, a cell suspension was prepared by discarding the culturing medium, adding Trizol (Gibco-BRL, Life Technologies, NY) to cultures on ice (1-ml/100-mm culture dish), and scraping the cells off the culture dish. The resulting cell suspension was incubated at room temperature for 5 min (to complete cell lysis); 200 μl of chloroform was added, and the mixture was vigorously vortexed and centrifuged at 12,000*g* for 15 min at 4°C. The upper, aqueous layer was removed, 500 μl isopropanol was added, and the solution was incubated at room temperature for 10 min, and then at −20°C for 1 h and centrifuged at 12,000*g* for 10 min at 4°C. The supernatant was washed once with 75% ethanol, and centrifuged at 7,500*g* for 5 min at 4°C. The pellet containing the cellular RNA was air dried and dissolved in 10 ml sterile, distilled water, and an aliquot was used for spectrophotometric determination of the amount of RNA.

RT was initiated by a 5-min incubation at 65°C of 5 μl of RNA extract diluted with sterile, distilled water to a total volume of 13 μl and fortified with Random Hexamer (Gibco-BRL) at a final concentration of 12.5 ng/μl and dNTPs (a mixture of dATP, dGTP, dCTP, and dTTP [from Pharmacia Biotech, Uppsala, Sweden] at a final concentration of 0.5 mM. The mixture was rapidly chilled on ice (for ≥2 min) and briefly spun, and 4 μl 5× First-Strand Buffer, 2 μl 0.1 M dithiothreitol [DTT] and 1 μl RNaseOUT Recombinant RNase Inhibitor (40 U/μl) were added, giving a final volume of 20 μl. After the mixture had been incubated at 42°C for 2 min, 1 μl (200 U) of Superscript II (Gibco) was added, and the incubation at 42°C continued for another 50 min. Subsequently the reaction was inactivated by heating to 70°C for 15 min, and the mixture was chilled on ice for ≥2 min and briefly centrifuged. Finally, RNA complementary to the cDNA formed during the reaction was removed by incubation with 1 μl of *Escherichia coli* RNaseH (2 U) at 37°C for 20 min.

PCR was performed in a thermocycler (from Robocycler, Stratagene, Amsterdam, The Netherlands); 2 μl 10× PCR Buffer, pH 8.4 (Gibco), 12.5 μl 25 mM $MgCl_2$ (Pharmacia Biotech, Sweden), 0.4 μl 10 mM dNTPs, 2 μl 10 μM of one of the nucleoside transporter primers or of the β-actin primer, 0.1 μl *Taq* polymerase (Gibco), and 1.0 μl cDNA template were mixed with sterile distilled water to a final volume of 20 μl. Initially the template was denatured by heating to 94°C for 2 min, followed by 30 2-min amplification cycles, each consisting of three 40-s periods, the first at 94°C, the second at 57°C, and the third at 72°C. The final step was extension at 72°C for 10 min. Finally the PCR products were separated by 1% agarose gel electrophoresis. Positive controls for mRNA of all the transporters were brain tissue (Ritzel et al., 1998, 2001a; Anderson et al., 1999; Choi et al., 2000; Kiss et al., 2000) and intestine (Ritzel et al., 2001a; Lu et al., 2004) from adult mice. Negative controls were prepared from the cultures by omission of the reverse transcriptase from the RT mixture. A DNA ladder was also included. The brightest band in its middle represents 500 bp, the other bands 400, 300, 250, 200, 150, 100, and 50 bp. PCR products of ENT1, ENT2, and CNT2 from astrocytes and of CNT3 from cortex were confirmed by sequencing.

Uptake Rates

To determine diffusional uptake of tritiated adenosine, guanosine, formycin B and thymidine (simple diffusion or facilitated diffusion mediated by ENT1 or ENT2, albeit at reduced intensity at the lowered temperature [Okhubo et al., 1991]), individual cultures, containing cells corresponding to ~0.15 mg protein (in a 35-mm culture dish), were washed three times with ice-cold phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 7.3 mM Na_2HPO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 7.5 mM glucose; pH 7.3) and subsequently exposed for 3 s to 1, 10, or 100 μM of a labeled nucleoside in 1 ml of ice-cold serum-free tissue culture medium, which had been aerated with 5% CO_2 in air. The 3-s period was the time from which the culture was exposed to the labeled medium until the medium had been removed, and the culture was washed for the first time (out of a total of five) with a similar PBS as before the exposure to the radioisotope. Very small standard errors of the uptakes (see Results) show the reproducibility of this procedure.

For determination of time courses of total uptakes at 37°C, i.e., rates of accumulation of radioactivity, regard-

less of uptake mechanism (with concentrative uptake being either metabolism-driven or Na^+-dependent active uptake), each culture was washed 3 times with PBS at 37°C and incubated under a CO_2/air (5%/95%) atmosphere at 37°C (in a CO_2 incubator) for 1, 2, 5, 10, 15, 20, 30, or 60 min in 1 ml of aerated, preheated, serum-free tissue culture medium, which contained the radiolabeled nucleoside at a concentration of either 1 μM or 250 μM (the minimum and maximum concentrations used in the subsequent more detailed investigation of concentration dependence). After the incubation, the cultures were washed 5 times with ice-cold PBS to remove adherent radioactive medium. For the determination of concentration dependence of the uptake, each culture was washed as described above and subsequently incubated at 37°C under a CO_2/air (5%/95%) atmosphere in 1 ml aerated, preheated tissue culture medium with radiolabeled adenosine, guanosine, formycin B, or thymidine at concentrations of 1, 5, 10, 25, 50, 100, and 250 μM. The length of the incubation period was 15 min for adenosine; 30 min for guanosine; and 2 min for formycin B and thymidine, time periods during which the uptakes had been found to occur rectilinearly as a function of time. After the incubation the cultures were washed as described above. For determination of Na^+ dependence, the cultures were washed 3 times at 37°C with nonradioactive tissue culture medium of a similar composition as the medium to be used during the incubation. Subsequently, each culture was incubated at 37°C for 2 min for formycin B and thymidine or 15 min for adenosine and guanosine in either normal tissue culture medium with 116 mM NaCl or tissue culture medium in which NaCl had been replaced by 116 mM choline chloride. Both media contained radiolabeled adenosine, guanosine, formycin B or thymidine at a concentration of 25 μM. After the incubation the cultures were washed 5 times with ice-cold medium of similar composition as before the incubation.

In all studies, the specific radioactivity of the medium as well as accumulated radioactivity and protein content of the cultures were determined after the cells in the incubated and washed cultures had been dissolved by overnight incubation at room temperature in 1 ml 1 M NaOH solution. Part of the NaOH digest and medium samples were separately introduced in a Rack Beta liquid scintillation counter (LKB-Wallac 1217-001) for determination of radioactivities; another part of the NaOH solution was used to determine protein by the aid of the conventional Lowry technique (Lowry et al., 1951), using bovine serum albumin (BSA) as the standard. Uptakes were expressed as nmol/mg protein, calculated by dividing the measured radioactivity per mg protein (DPM/mg) with the specific activity of the incubation medium (DPM/nmol).

Statistical Analysis

Statistical analysis was performed by the aid of the computer software package StatView SE+Graphics™, version 1.03. One-factor analysis of variance (ANOVA) was used followed by Fisher's PLSD (protected least significant difference) test. Differences were considered significant at $P < 0.05$.

RESULTS

Gene Expression

Figure 2 shows agarose gels displaying astrocytic cDNAs, obtained by RT-PCR using the primers shown in Figure 1 for mouse ENT1 (Fig. 2A), ENT2 (Fig. 2B), CNT2 (Fig. 2C), and CNT3 (Fig. 2D), respectively. The first lane, to the far left (M), represents a DNA ladder, as described under Materials and Methods; the next lane (1) the PCR product in cultured astrocytes, with intense bands for ENT1 (A) and ENT2 (B) at ~150 and 400 base pairs (bp), respectively; less intense staining for CNT2 (C) at ~400 bp; and no staining for CNT3 (D). The following lane (2) is a PCR-negative control of astrocytes in the absence of reverse transcriptase. The two next lanes show the PCR product of cerebral cortex as a positive control for all four transporters in the presence of the reverse transcriptase (3) and as a negative control in its absence (4). The two last lanes lane represent a more intense staining of CNT2 and CNT 3 in the intestine in the presence of the reverse transcriptase (5), and not in its absence (6). To ensure that comparable amounts of mRNA were investigated for astrocytes, cerebral cortex, and intestine the expression of β-actin, used as a housekeeping gene in the same samples was also determined (Fig. 3). The staining for astrocytes (1), cerebral cortex (2), and intestine (3) at ~400 bp were similar.

Nucleoside Uptakes

For all four nucleotides diffusional uptakes, measured in ice-cold medium during a 3-s period as described in Materials and Methods, increased with the concentration of the nucleoside (Fig. 4). The increase followed a rectilinear course as a function of the concentration over the entire range in the case of formycin B and thymidine, which were accumulated at identical rates (0.19 nmol/mg protein at 100 μM). This finding does not differentiate between simple diffusion and facilitated diffusion with low affinity. Diffusional uptake rates of guanosine were slightly larger and those of adenosine much larger (1.58 nmol/mg protein at 100 μM). For both of purine nucleosides, there was a tendency toward saturation of the uptakes with increasing concentrations, suggesting that at least parts of the uptakes occurred by facilitated rather than simple diffusion. This is consistent with the hydrophilicity of nucleosides and their resulting poor transport through cell membranes by simple diffusion.

All remaining uptake measurements were carried out at 37°C and continued for ≥1 min. Figure 5 shows the uptake of 1 μM adenosine and guanosine (upper panel)

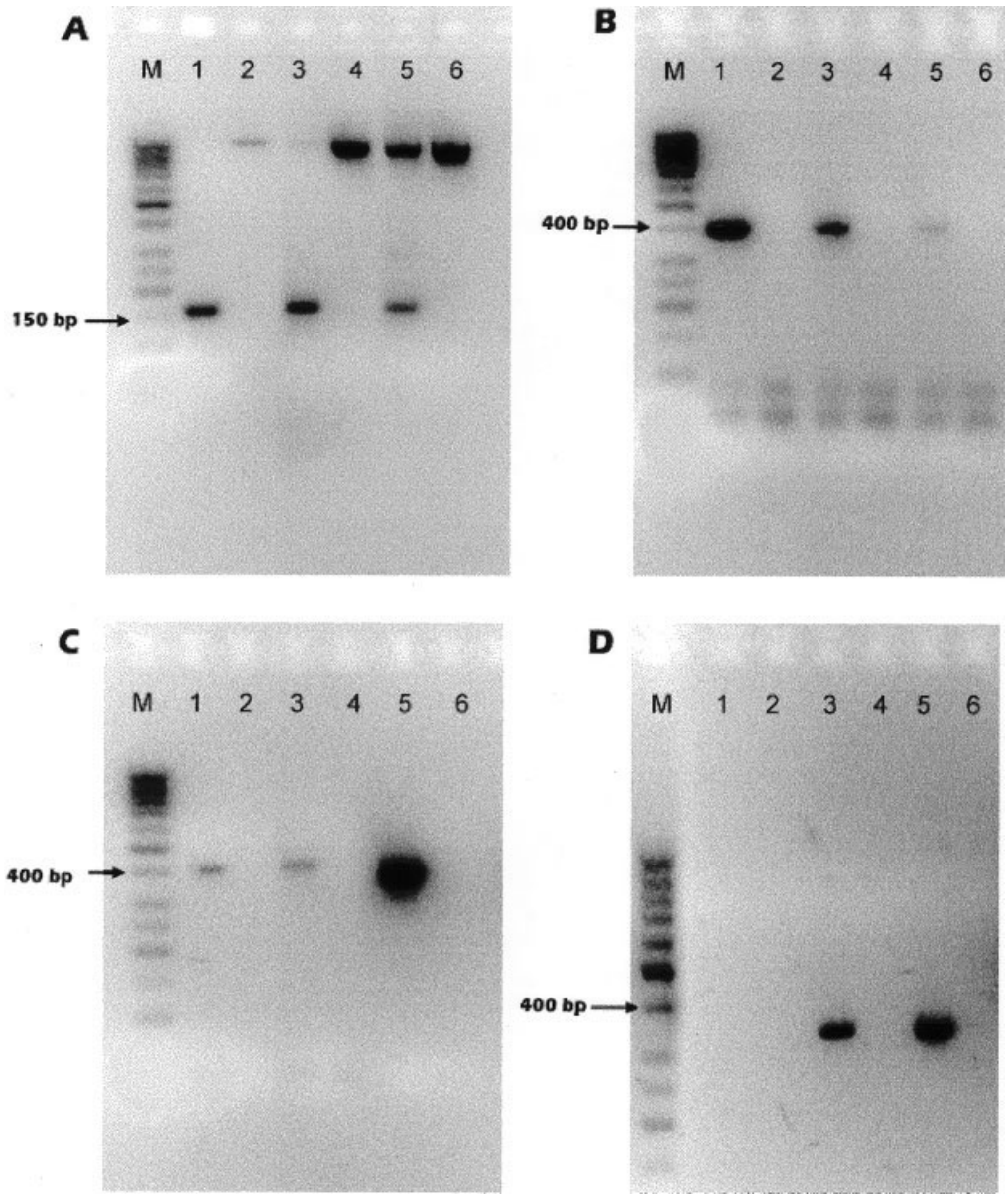

Fig. 2. Presence of nucleoside transporter family RNA in primary cultures of mouse astrocytes. **A:** Gel of the RT-PCR product generated using the ENT1 primer shown. **B:** Corresponding gel of the RT-PCR product generated using the ENT2 primer shown. **C:** Corresponding gel of the RT-PCR product generated using the CNT2 primer shown. **D:** Corresponding gel of the RT-PCR product generated using the CNT3 primer shown. In all panels, the first lane (M), to the far left, represents a DNA ladder, in which the brightest band in the middle represents 500 bp, and the other bands 400, 300, 250, 200, 150, 100, and 50 bp, **lanes 2–3** show the PCR product from astrocyte cultures in the presence (**lane 1**) or absence (**lane 2**) of the reverse transcriptase (RT); **lanes 3–6**, controls for each transporter (**lanes 3 and 4**, mouse brain; **lanes 5 and 6**, mouse intestine), likewise in the presence (**lanes 3 and 5**) and absence (**lanes 4 and 6**) of RT.

as well as formycin B and thymidine (lower panel) during incubation periods of up to 60 min. The uptake of adenosine was rectilinear for 20 min, indicating the initial uptake rate, and it subsequently declined slightly, probably mainly reflecting gradually developing labeling of intracellular adenosine resulting in concomitant efflux of label (Hertz, 1991a,b). Guanosine uptake rate was slightly slower, but after 15–30 min, there was a slight increase in uptake rate, possibly due to trans-accelerated self-exchange, so that the uptake velocities of adenosine and guanosine at 1 μM became virtually identical during the second 30-min period. In contrast, the uptake of formycin B was completed after only 2 min, when the total accumulation of accumulated radioactivity reached a plateau, indicating that unidirectional influx and efflux of label were equal. Thymidine resembled formycin B in showing a very low uptake rate. However, in contrast to formycin B, the uptake continued at a reduced rate between 2 and 30 min, after which time it reached a plateau, suggesting equilibration of a pool of very limited size. Accordingly the total accumulation of radioactivity was about twice that of formycin B, but still much less than those of adenosine and guanosine.

Similar experiments were also carried out at nucleoside concentrations of 250 μM (Fig. 6). Accumulation of radioactivity after 60 min was much higher for all nucleosides except guanosine, the accumulation of which only increased by a factor of ~3 (indicating a low K_m value for the uptake). Both adenosine and guanosine showed initial uptake rates for 15–30 min, but the uptake rate of adenosine was approximately 10 times higher than that of guanosine. Again, accumulation of formycin B was rapidly completed, but it took slightly longer to reach a plateau than at 1 μM formycin B (con-

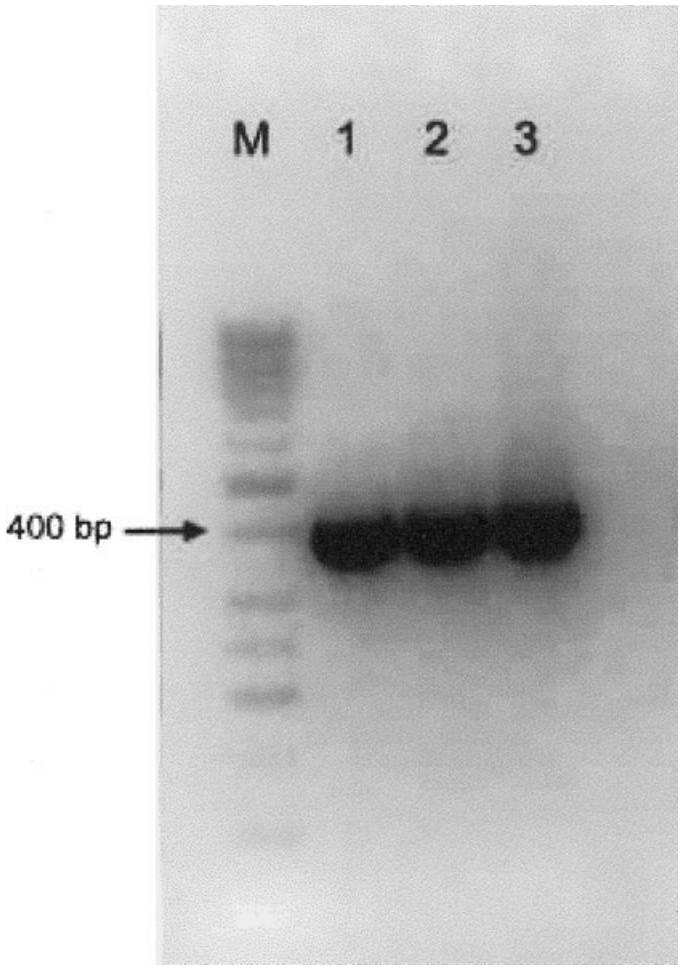

Fig. 3. RT-PCR analysis of expression of RNA for β-actin at ~450 bp (lane M) in the samples of mouse astrocytes, mouse cerebral cortex and mouse intestine used in Fig. 2. RNA from astrocytes (**lane 1**), cortex (**lane 2**), and intestine (**lane 3**). The RT-PCR products were generated using the β-actin primer shown in Fig. 1E.

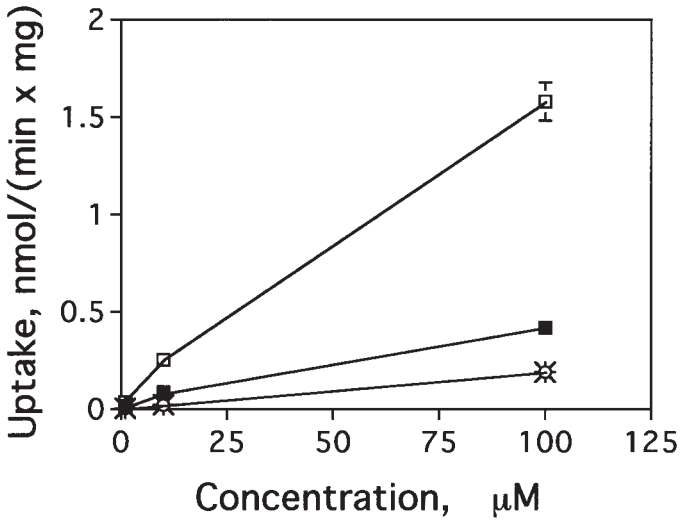

Fig. 4. Uptake of [^{3}H]adenosine (open squares), [^{3}H]guanosine (filled squares), [^{3}H]formycin B (open circles), or [^{3}H]thymidine (stars) as a function of their extracellular concentration into cultured mouse astrocytes during a 3-s incubation period in ice-cold tissue culture medium. Results are averages of six individual experiments, using cultures from two different batches, with SEM. indicated by vertical bars if they extend beyond the symbols.

sistent with facilitated rather than simple diffusion). Most thymidine accumulation had also occurred after 5 min. Although the subsequent increase in isotope content was ~10 times larger than at 1 μM thymidine (indicating an increase in the slowly filling pool), it constituted a much smaller fraction of total uptake, which was increased more than 100 times, reflecting a much larger diffusional uptake.

During incubation in Na^+-free media the uptakes of adenosine and guanosine, measured during a 15 min period at concentrations of 25 μM, were reduced (indicating a sodium-dependent part of the uptake), whereas Na^+ deprivation had no significant effect on the uptake of either formycin B or thymidine (Fig. 7). The omission of Na^+ lowered guanosine uptake from 0.96 nmol/min per mg protein to 0.50 nmol/min per mg protein, i.e., by ~ 50%, but the uptake of adenosine was only reduced by ~25%. However, due to the higher uptake rate for adenosine than for guanosine at the concentration of 25 μM, the Na^+-dependent adenosine uptake (~2.5 nmol/min per mg protein) was larger than the Na^+-dependent uptake of guanosine (0.46 nmol/min per mg protein), although it was smaller than the Na^+-independent adenosine uptake (~7.0 nmol/min per mg protein).

The concentration dependence of the uptake of the two nucleosides showing a Na^+-dependent uptake, i.e., adenosine and guanosine during time intervals when the uptakes occurred at their initial rates (adenosine: 15 min; guanosine: 30 min) is shown in Figure 8. Both nucleosides showed saturable uptakes combined with a second uptake that either was nonsaturable or had a low affinity. The slope of the nonsaturable uptake was considerably steeper for adenosine than for guanosine. The parts of the curves showing the nonsaturable uptakes between 100 and 250 μM in Figure 8 were extrapolated to zero concentration (not shown in the Figure) and the extrapolated values at 1, 5, 10, 25, and 50 μM subtracted from the actually measured uptakes at each of these concentrations in order to deduct the nonsaturable uptakes at each concentration and thus estimate the rates of the saturable uptakes. The reciprocal values of the saturable uptakes were plotted against the reciprocal values of the concentrations in a conventional Lineweaver–Burk plot (Fig. 9) to obtain V_{max} and K_m values. The validity of this procedure is indicated by the rectilinear courses of the plotted values. The reciprocal values of their intercepts with the y-axis and the extension of the x-axis indicate, respectively, the V_{max} and the numerical value of the K_m. Both K_m and V_{max} values were several times larger for adenosine (K_m ~12 μM; V_{max} ~1 nmol/min per mg protein) than for guanosine (K_m ~2 μM; V_{max} ~ 0.05 nmol/min per mg protein). However, due to the higher affinity of the uptake of guanosine, the uptake rates of adenosine and guanosine approached each other at submicromolar nucleoside concentrations, e.g., 0.2 μM (Table 1). They were also con-

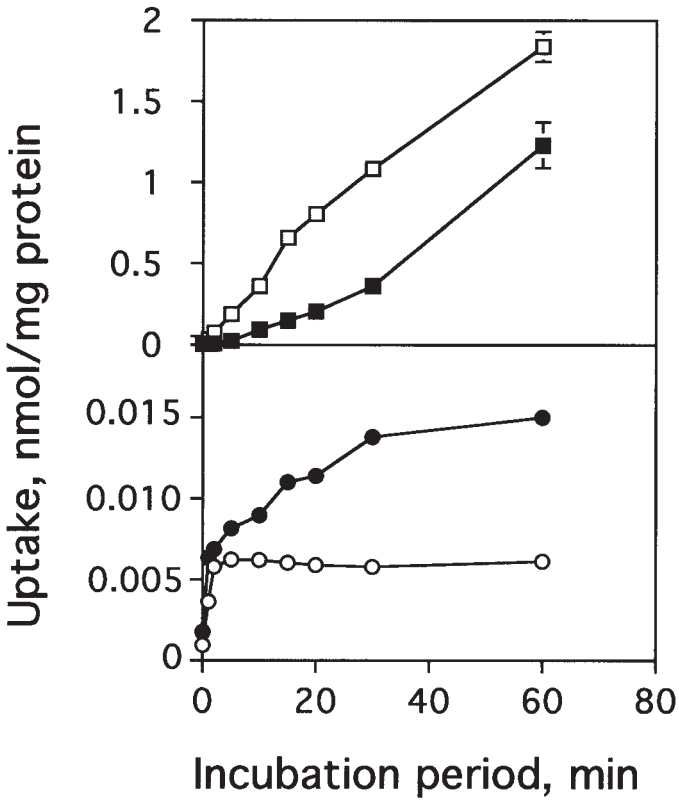

Fig. 5. Time course for uptake of [^{3}H]adenosine (open squares, upper) and [^{3}H]guanosine (filled squares, upper), as well as [^{3}H]formycin B (open circles, lower) and [^{3}H]thymidine (filled circles, lower) into cultured mouse astrocytes during incubation periods of 1–60 min in tissue culture medium at 37°C. The initial concentration of the nucleoside in the incubation medium was 1.0 μM. Results are averages of six individual experiments, using cultures from two different batches, with SEM indicated by vertical bars if they extend beyond the symbols.

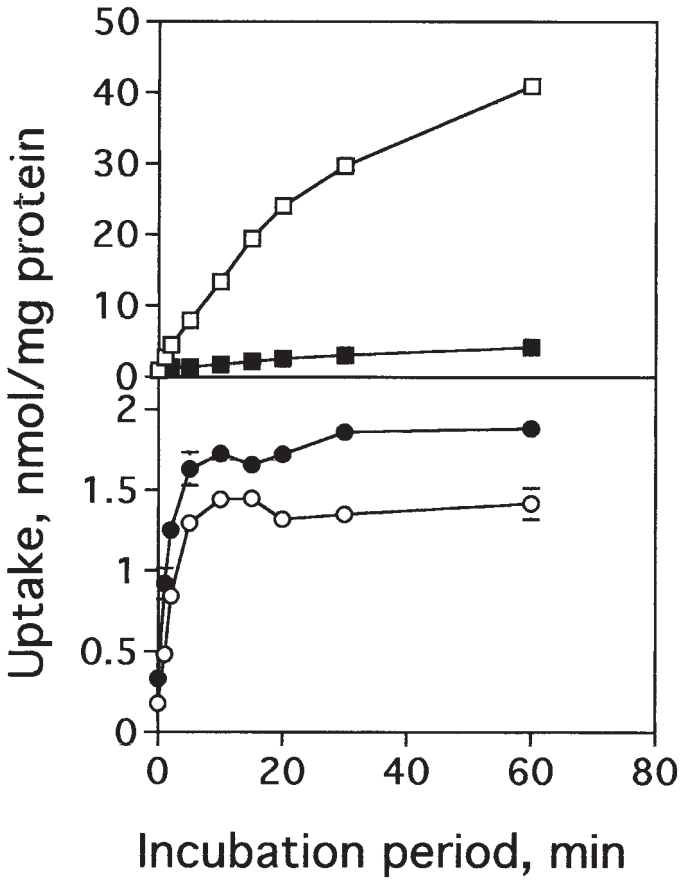

Fig. 6. Time course for uptake of [^{3}H]adenosine (open squares, upper) and [^{3}H]guanosine (filled squares, upper), as well as [^{3}H]formycin B (open circles, lower) and [^{3}H]thymidine (filled circles, lower) into cultured mouse astrocytes during incubation periods of 1 to 60 min in tissue culture medium at 37°C. The initial concentration of the nucleoside or formycin B in the incubation medium was 250 μM. Results are averages of six individual experiments, using cultures from two different batches, with SEM indicated by vertical bars if they extend beyond the symbols.

siderably higher than the uptake rates by facilitated diffusion, which can be calculated for thymidine and formycin B. Thus, at physiologically occurring concentrations of adenosine and guanosine (Dobolyi et al., 1998a,b), both nucleosides are efficiently accumulated into astrocytes.

DISCUSSION

Transporter Expression and Na^+ Dependence of Uptake

Since the expression of β-actin was similar in the different gels, the intensity of the different bands may be used as a direct estimate of transporter mRNA expression, with the caveat that the efficiency of different primers may not be the same. The presence of mRNA for each of the two ENTs is marked, consistent with the presence of diffusional uptakes for all four nucleosides, with the tendency toward a saturation of the uptake at high concentrations observed for the 3-s uptakes of adenosine and guanosine at low temperature, with the somewhat slower uptake of formycin B and of the initial phase of the thymidine uptake at 250 than at 1 μM and with the observation by Gu et al. (1996) that part of the adenosine uptake in cultured human astrocytes is potently inhibited by NBTI. However, the lack of any tendency for the 3-s uptake of formycin B at low temperature to approach a plateau within the range of 1–100 μM may suggest a high K_m value, which is consistent with published data for formycin B by the ENT1 and especially the ENT2 transporter (Burke et al., 1998). The presence of both ENTs is also consistent with previous uptake studies in human astrocytes by Gu et al. (1996), but the distinct expression of mRNA for both ENT2 (*ei*) and ENT1 (*es*) is in contrast to C-6 glioma cells, where ENT2 accounts for >95% of the total equilibrative nucleoside transporter expression (Sinclair et al., 2001).

Expression of CNT2 but not of CNT3 is consistent with Na^+-dependent uptakes of adenosine and guanosine, but not of thymidine. It is accordingly not necessary to assume the presence of a specific guanosine transporter, which presently only has been established in transformed cells (Flanagan and Mecklin-Gill, 1997). Moreover, this transporter is sensitive to NBTI, which

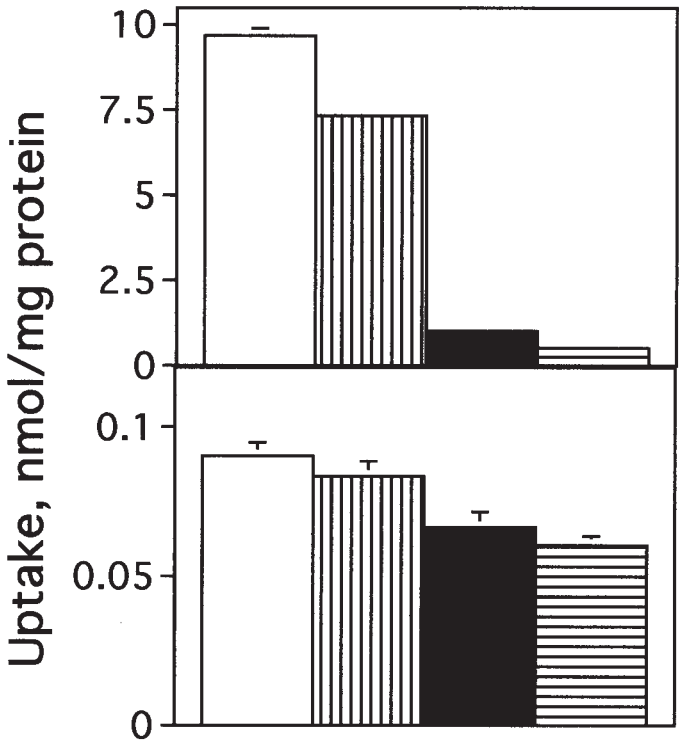

Fig. 7. Upper: Sodium dependence of [^{3}H]adenosine and [^{3}H]guanosine uptakes. The cultures were incubated for a 15-min period in tissue culture medium containing 25 μM of the nucleoside and either a normal NaCl concentration (116 mM), indicated by an open column for adenosine and a filled column for guanosine, or corresponding medium in which 116 mM choline chloride had been substituted for NaCl, indicated by a vertically striped column for adenosine and a horizontally striped column for guanosine. Results are averages of six individual experiments, using cultures from two different batches, with SEM indicated by vertical bars, if extending beyond the columns. The differences between uptakes in Na^+-containing and Na^+-depleted media were statistically significant for both adenosine and guanosine. Lower panel: Sodium dependence of [^{3}H]formycin and [^{3}H]thymidine uptakes. The cultures were incubated for a 2-min period in tissue culture medium containing 25 μM of formycin B or thymidine and either a normal NaCl concentration (116 mM), indicated by an open column for formycin B and a filled column for thymidine, or corresponding medium in which 116 mM choline chloride had been substituted for NaCl, indicated by a vertically striped column for formycin B and a horizontally striped column for thymidine. Results are averages of six individual experiments, using cultures from two different batches, with SEM indicated by vertical bars, if extending visibly beyond the columns. There was no statistically significant difference between uptakes in Na^+-containing and Na^+-depleted media.

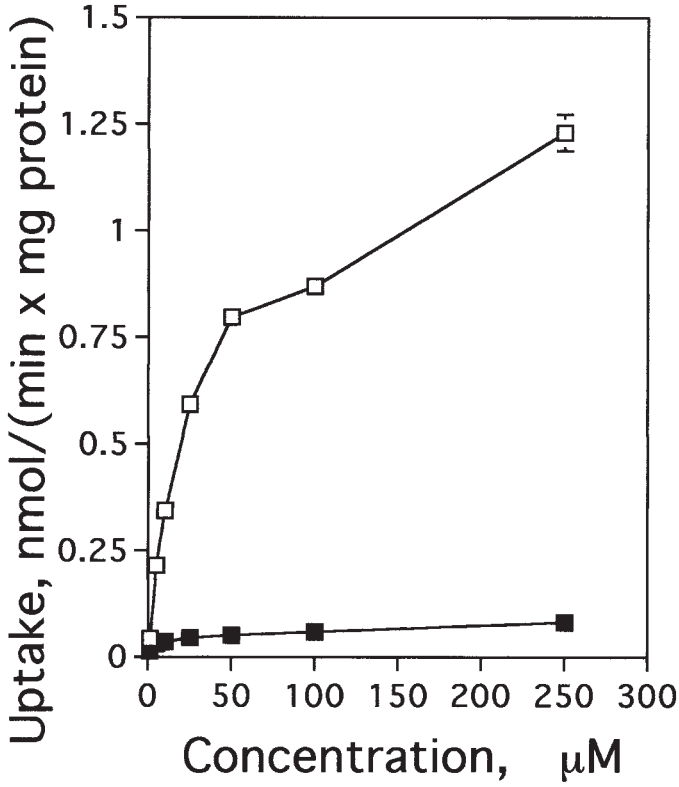

Fig. 8. Uptake of [^{3}H]adenosine (open squares) and [^{3}H]guanosine (filled squares) into cultured mouse astrocytes as a function of their extracellular concentration during an incubation period during which the uptakes occurred at their initial velocity, i.e., following a rectilinear course as a function of time (adenosine: 15 min, guanosine: 30 min) in tissue culture medium at 37°C. Results are averages of six individual experiments, using cultures from two different batches, and with SEM indicated by vertical bars, if they extend beyond the symbols.

does not abolish the increase in extracellular adenosine exerted by administration of guanosine (Di Iorio et al., 2002). Quantitatively all three transporters are well expressed, compared with whole brain. The expression of CNT1 could not be directly examined but the absence of a Na^+-dependent thymidine uptake justifies the conclusion that this transporter is absent. The Na^+-dependence of adenosine uptake is in agreement with a previous observation by Bender et al. (1994). A Na-dependent uptake of adenosine is also consistent with our previous unequivocal demonstration of a concentrative uptake of adenosine in astrocytes, indicated by an increase in the intracellular concentration of labeled, nonmetabolized adenosine many times above the extracellular adenosine concentration, when adenosine deaminase activity was inhibited (Matz and Hertz, 1990). However, the presence of a Na^+-dependent component is in contrast to the absence of concentrative adenosine transporters in C-6 glioma cells (Sinclair et al., 2000) but, from other cell types, it is known that transformed cells may lose concentrative nucleoside transporter expression expressed by their nontransformed counterparts (Dragan et al., 2000).

There was also a Na^+-dependent uptake of guanosine, which may be consistent with the recently established role of its neuromodulator function (Chen et al., 2001; Traversa et al., 2002). Uptake of formycin B, a poorly metabolizable structural inosine analogue (Plagemann and Woffendin, 1989), is also mediated by CNT2 in many cell types (Plagemann et al., 1990; Borgland and Parkinson, 1997), but in the present cells there was no evidence that formycin B is accumulated by any other mechanism than diffusion.

Nondiffusional Na^+-Independent Uptake

In spite of the lack of Na^+-dependent uptake of thymidine there was after the first 2 min a further, continuous uptake during the next 30 min, which was most conspic-

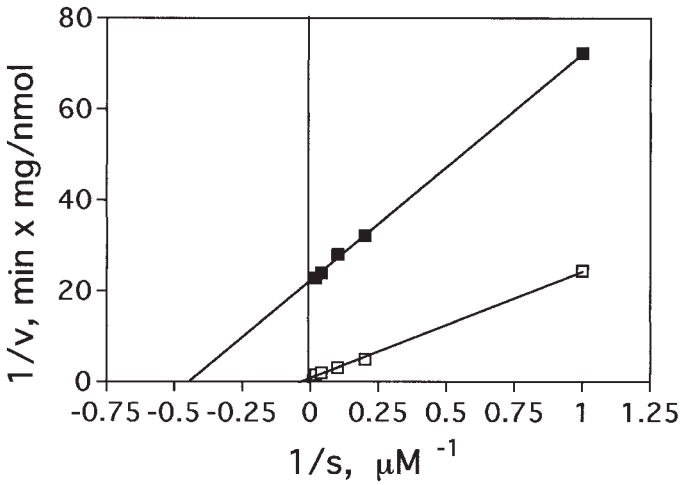

Fig. 9. Lineweaver–Burk plot showing kinetics for the saturable uptakes of adenosine (open squares) and guanosine (closed squares). For construction of the plot, the slopes of the nonsaturable uptakes of 100–250 μM in Fig. 8 were extrapolated to zero concentration and the extrapolated values at 1, 5, 10, 25, and 50 μM were subtracted from the actually measured uptakes at each of these concentrations, in order to deduct the nonsaturable uptakes at each concentration and thus determine the rates of saturable uptake. The Lineweaver–Burk plot shows the reciprocal values of the saturable uptakes of adenosine (open squares) and guanosine (filled squares) plotted against the reciprocal values of the concentrations. Each of these sets of values followed a straight line (regression coefficients for adenosine 0.999 and for guanosine 0.998), and the reciprocal values of their intercepts with the y-axis and the extension of the x-axis indicate, respectively, the V_{max} and the numerical value of the K_m, which are summarized in Table 1.

uous during the uptake of 1 μM thymidine, but was ~10 times larger, when its concentration was increased 250 times. These characteristics of the uptake suggest a saturable metabolism-driven component of the uptake, which was very slow compared to the saturable uptakes of guanosine and especially adenosine. Since thymidine is a precursor for DNA, but not for RNA this finding is in agreement with a small precursor pool for DNA synthesis and a very low rate of cell proliferation in these, confluent, cultures.

Metabolism-driven uptakes also of adenosine and guanosine were indicated by the finding that their saturable, concentrative uptakes exceeded their Na^+-dependent uptakes. The Na^+-independent uptake of guanosine after 15 min of incubation with 25 μM guanosine (0.50 nmol/mg protein) is larger than the amount of guanosine accumulation to be expected after equilibration between extracellular guanosine and guanosine in an intracellular water phase amounting to 3.9 μl/mg protein (Chen et al., 1992). This suggests a metabolism-driven component of the uptake, which is of approximately similar quantitative importance as the Na^+-dependent concentrative uptake for total uptake of guanosine. The metabolism-driven uptake may reflect RNA synthesis, which is likely to be more intense than DNA synthesis. Moreover, guanosine deaminase is present in astrocytes (Zoref-Shani et al., 1995), and phosphorylation to GTP occurs (Pelled et al., 1999).

The uptake of adenosine resembled that of guanosine by consisting of a saturable and a nonsaturable component with approximately similar kinetics as have generally been observed previously (Hertz, 1978; Thampy and Barnes, 1983; Bender and Hertz, 1986). However, the uptake is ≥10 times more intense than the adenosine uptake into primary cultures of rat astrocytes reported by Othman et al. (2002). Both the saturable and the nonsaturable uptake rates were considerably higher than in the case of guanosine. Based on the water space of 3.9 μl/mg protein, the 3-s uptake of 1.58 nmol/mg protein at 100 μM is 4 times larger than should be expected from diffusion alone (simple and/or facilitated), and it is likely to include a component which is either active or metabolism-driven. Similarly, the apparently nonsaturable part of the uptake, which was subtracted from the total uptake to calculate the saturable uptake, is larger than can be explained by diffusion alone. The saturable, concentrative uptake was also much larger than Na^+-dependent uptake, indicating a large Na^+-independent component with roughly similar affinity. This entire component can be explained as metabolism-driven, since it has previously been demonstrated that ATP synthesis from accumulated adenosine in astrocytes at an extracellular adenosine concentration of 10 μM proceeds at a rate of ≥0.2 nmol/min per mg protein (Matz and Hertz, 1989), i.e., almost similar to the rate of the Na^+-independent part of the saturable uptake, which can be calculated to occur at 10 μM adenosine (7 nmol/mg protein during a 15 min period at 25 μM corresponds to 0.2–0.3 nmol/min per mg protein at 10 μM adenosine). Maintenance of such a high rate of metabolism-driven adenosine uptake requires a rate of facilitated diffusion, which is at least equally high. An uptake rate of 0.4–0.5 nmol/min per mg protein during a 3-s period corresponds to an uptake of almost 10 nmol/min per mg protein, which is more than high enough to keep pace with metabolic conversion of adenosine.

TABLE 1. K_m, V_{max} and uptake rates at 0.2 μM ($V_{0.2}$) for adenosine, guanosine, formycin B and thymidine into mouse astrocytes in primary cultures

Nucleoside	K_m μM	V_{max} nmol/(min × mg)*	$V_{0.2}$ nmol/(min × mg)*
Adenosine	12.0	0.89	0.02
Guanosine	2.3	0.048	0.005
Formycin B			0.0004
Thymidine			0.0004

K_m and V_{max} values for adenosine and guanosine were determined from the Lineweaver-Burk plot shown in Fig. 6, and uptake rates at 0.2 μM ($V_{0.2}$) calculated from these kinetic constants. Uptake rate at 0.2 μM ($V_{0.2}$) for formycin B and thymidine were obtained from the non-saturable uptakes shown in Fig. 4.
*nmol/min per mg protein.

Uptake Kinetics

The saturable uptake of both guanosine and adenosine adhered to simple Michaelis–Menten kinetics suggesting one component only. This is possible if the Na^+-dependent active uptake and the Na^+-independent metabolism-driven uptake have approximately similar kinetics. The affinity of the guanosine uptake was high, possibly reflecting the guanosine concentrations normally occur-

ring in brain extracellular fluid. The concentration dependence of a guanosine effect on free cytosolic Ca^{2+} ($[Ca^{2+}]_i$) concentration in astrocytes (half-maximum effect at 1 μM guanosine, peak response at 10 μM) together with the establishment that the resting extracellular guanosine concentration in the rat brain of ~0.2 μM is doubled or tripled after K^+-induced depolarization (Dobolyi et al., 1998a,b) suggest that the guanosine receptor reacts to physiologically occurring fluctuations in extracellular guanosine concentration. The high affinity of the uptake, securing high uptake rates at the low micromolar and submicromolar level, is in agreement with these observations. The affinity is more than one order of magnitude higher than that of the specific guanosine transporter found in transformed cells (Flanagan and Mecklin-Gill, 1997).

The K_m value for the saturable adenosine uptake was considerably higher than that for guanosine uptake. This difference does not reflect a higher resting extracellular concentration of adenosine than of guanosine, but rather that ATP is an important neuromodulator released from astrocytes during stimulation (Guthrie et al., 1999) and energy failure and hydrolyzed by ectonucleotidases (Parkinson and Xiong, 2004), so that extracellular adenosine may reach high levels (Rudolphi et al., 1992; Pedata et al., 2001).

CONCLUSIONS

The present results have shown the expression of both ENT1 and ENT 2 (*es* and *ei*) together with CNT2, and thus of *cif*, in astrocytes. However, CNT3 was not expressed and the lack of Na^+-dependent thymidine uptake suggests the absence of not only *cit*, but also *cib*, and thus of CNT1. The uptake system accumulated all four nucleosides studied by facilitated diffusion and both adenosine and guanosine by metabolism-driven uptake and by concentrative, Na^+-dependent uptake, which was larger for adenosine than for guanosine. This system is far more complex than that expressed in C-6 cells and it makes astrocytes well suited for both intense nucleoside metabolism and metabolism-independent uptake to terminate neuromodulator effects of adenosine and guanosine.

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