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Nucleoside transporter-mediated uptake and release of [^{3}H]L-adenosine in DDT$_1$ MF-2 smooth muscle cells

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Abstract

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

Keywords: Nitrobenzylthioinosine; Adenosine; Nucleoside transport; L-Adenosine

1. Introduction

At least seven nucleoside transporters have been identified to date, and characterized broadly as being either equilibrative or concentrative (see Geiger et al., 1996). These transporters function to regulate the influx of endogenous and exogenous nucleosides as well as nucleoside drugs across plasma membranes into cells (Cass, 1995). Additionally, bidirectional nucleoside transporters that are equilibrative and sensitive to inhibition by nitrobenzylthioinosine, so-called *es* transporters, appear to play an important role in the efflux of nucleoside drugs and intracellular nucleosides, the most important of which may be the physiologically active nucleoside adenosine. Thus, nucleoside transporters are important in regulating the extracellular levels and actions of adenosine; these actions are mediated, in the main, by cell surface adenosine receptors, and inhibitors of nucleoside transporters continue to be viewed as agents with therapeutic potential.

It is difficult to study the degree to which specific subtypes of nucleoside transporters mediate the efflux of endogenous D-adenosine formed intracellularly because relatively little is known about their types and functions, and because adenosine is so metabolically labile. To gain additional information about mechanisms of adenosine efflux, we showed that in heterogeneous rat brain preparations [^{3}H]L-adenosine, an enantiomer of the physiological D-adenosine, was (1) more metabolically stable than [^{3}H]D-adenosine, (2) a permeant of brain nucleoside transporters (Gu et al., 1991; Gu and Geiger, 1992), (3) released in a time- and temperature-dependent manner, and (4) 52% of the release was blocked by nitrobenzylthioinosine, thus implicating nucleoside transporters in the release of [^{3}H]L-adenosine (Gu et al., 1995).

Determining the type of transporter and the mechanism(s) by which [^{3}H]L-adenosine was being released was made difficult in brain by the heterogeneous nature of the tissue preparations, the presence of multiple subtypes of nucleoside transporters, including *es* transporters, and a large proportion of transporters described as being equilibrative and resistant (insensitive) to inhibition

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by nitrobenzylthioinosine, so-called *ei* transporters (Lee and Jarvis, 1988; Vijayalakshmi and Belt, 1988). Furthermore, it is unknown the degree to which our previous data was affected by the homogenized nature of the previously used rat brain preparations. To circumvent these potential problems, we used here a clonal cell line of DDT_1 MF-2 smooth muscle cells that appear to contain, almost exclusively, *es* transporters (Parkinson et al., 1996) in order to test the hypothesis that [^{3}H]L-adenosine is transported into and released from cells by *es* nucleoside transporters. In agreement with our previous work with rat brain preparations, we found that [^{3}H]D-adenosine and [^{3}H]L-adenosine were transported into and [^{3}H]L-adenosine was released from DDT_1 MF-2 cells, at least in part, by bidirectional *es* nucleoside transporters.

2. Materials and methods

2.1. Materials

[^{3}H]L-Adenosine (13 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA, USA), ^3H_2O (1.0 mCi/g) was from Amersham Canada (Oakville, Ontario, Canada), [^{14}C]- or [^{3}H]polyethylene glycol (10 and 2.0 mCi/g, respectively) and [^{3}H]D-adenosine (61.5 Ci/mmol) were from DuPont Canada (Mississuaga, Ontario, USA), and nitrobenzylthioinosine, dipyridamole and D-adenosine were from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were obtained from Gibco BRL. Generous gifts of L-adenosine and dilazep were supplied by Dr. K. Flora (Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute) and F. Hoffmann La Roche (Basel, Switzerland), respectively. Syrian hamster smooth muscle DDT_1 MF-2 cells were obtained from the American Type Culture Collection. Statistical analyses and non-linear regressions were conducted using Instat and GraphPad PRISM (Biosoft).

2.2. Cell culture

DDT_1 MF-2 smooth muscle cells, originally isolated from a steroid-induced leiomyosarcoma of Syrian hamster vas deferens, (Norris et al., 1974), were grown in suspension, at 37°C in 5% CO_2-95% humidified air, and maintained as exponentially proliferating cultures at cell densities of 3×10^4–3×10^5 cells/ml in DMEM supplemented with 5% fetal bovine serum, 4.5 g/l glucose and 2 mM L-glutamine (Gerwins et al., 1990). Cells were harvested by centrifugation ($120 \times g$ for 10 min), washed twice ($120 \times g$ for 5 min) and resuspended in Na^+ buffer (in mM: Tris, 20; K_2HPO_4, 3; NaCl, 136; $MgCl_2$, 0.5; $CaCl_2$, 0.9; glucose, 20; to pH 7.4 with HCl) to 1×10^6 cells/ml. Cell viability was determined by Trypan blue exclusion staining.

2.3. [^{3}H]Adenosine accumulation assays

[^{3}H]Adenosine accumulation was determined by an oil-stop centrifugation method (Parkinson et al., 1993). Briefly, a reaction mixture containing 100 μl of [^{3}H]D- or [^{3}H]L-adenosine (5 μCi/ml unless otherwise stated; 1 or 2 μM) in Na^+ buffer was layered over 100 μl oil (85 parts silicon oil: 15 parts paraffin oil) in a microcentrifuge tube. Uptake was initiated by the addition of 100 μl 1.0×10^6 cells/ml and terminated by addition of ice-cold dilazep (200 μM) and centrifugation at $13\,000 \times g$ for 30 s. Supernatants and oil were aspirated, tubes were washed once with 1 ml Na^+ buffer (4°C), pellets were digested in Triton X-100 (5%) overnight, and radioactivity was measured by scintillation spectroscopy. Assays were conducted at 25°C unless otherwise stated. To determine the degree to which adenosine accumulation was inhibited by nitrobenzylthioinosine, cells were pre-treated with nitrobenzylthioinosine, at concentrations ranging from 0.1 nM to 20 μM, or buffer for 15 min and incubated for 15 s with either 2 μM [^{3}H]D- or [^{3}H]L-adenosine. To determine enantiomeric inhibition of uptake, [^{3}H]D-adenosine (1 μM; 15 s) accumulation was measured in the absence or presence of L-adenosine (100 μM or 1 mM) and [^{3}H]L-adenosine (1 μM; 15 s) accumulation was measured in the absence or presence of D-adenosine (100 μM or 1 mM).

2.4. [^{3}H]L-Adenosine release assays

[^{3}H]L-Adenosine (10 μCi/ml; 10 μM) was incubated with cells (1×10^6 cells/ml) at 25°C for 1 h unless otherwise stated. Aliquots of 100 μl were added to microcentrifuge tubes, centrifuged at $13\,000 \times g$ for 20 s, supernatants were aspirated, and cell pellets were washed once with 1 ml Na^+ buffer (4°C) and kept on ice until taken for assay. [^{3}H]L-Adenosine release was initiated by resuspension of cell pellets in 500 μl Na^+ buffer (4° or 25°C) and terminated after 0–300 s by centrifugation at $13\,000 \times g$ for 20 s. Cell viability was ≥ 80%. To determine the degree to which nucleoside transport inhibitors blocked [^{3}H]L-adenosine release, cells pre-loaded with 10 μM [^{3}H]L-adenosine for 1 h were exposed for 15 min to dipyridamole or dilazep each at 30 μM, nitrobenzylthioinosine at concentrations ranging from 0.3 nM to 30 μM, or buffer prior to centrifugation and resuspension in release buffer alone or release buffer containing inhibitor.

3. Results

3.1. Accumulation

[^{3}H]D-Adenosine, and to a lesser degree [^{3}H]L-adenosine, was accumulated in a time- and temperature-dependent manner in DDT_1 MF-2 cells. Accumulations of [^{3}H]D-adenosine were significantly ($P < 0.01$) greater than

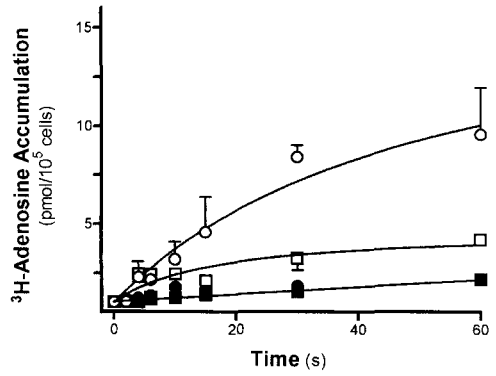

Fig. 1. Accumulations of [^{3}H]D-adenosine (circles) and [^{3}H]L-adenosine (squares) by DDT$_1$ MF-2 cells pre-incubated for 15 min in the absence (open symbols) or presence (solid symbols) of nitrobenzylthioinosine (10 μM). Incubations with 2 μM [^{3}H]D-adenosine or 2 μM [^{3}H]L-adenosine were for 0–60 s at 25°C. Symbols represent mean ± S.E.M. values from three experiments conducted in triplicate.

were those of [^{3}H]L-adenosine at incubations of 15 s or longer; [^{3}H]D-adenosine accumulations (pmol/10^5 cells) were, for example, 3.6 ± 1.8 at 15 s and 8.6 ± 2.4 at 60 s; [^{3}H]L-adenosine accumulations were, for example, 1.1 ± 0.2 at 15 s and 3.2 ± 0.2 at 60 s (Fig. 1). Following pre-incubation with 10 μM nitrobenzylthioinosine, 60 s accumulations were significantly ($P < 0.01$) inhibited by 86% for [^{3}H]D-adenosine and by 63% for [^{3}H]L-adenosine. Residual, nitrobenzylthioinosine-resistant, accumulations of 1.2 ± 0.2 pmol/10^5 cells were exactly the same for [^{3}H]D-adenosine and [^{3}H]L-adenosine (Fig. 1).

Significant ($P < 0.01$) dose-dependent inhibition of [^{3}H]L-adenosine accumulation was observed with nitrobenzylthioinosine (Fig. 2). The IC_{50} value for the nitrobenzylthioinosine-sensitive portion of [^{3}H]L-adenosine accumulation was 1.6 ± 0.1 nM (Fig. 2). In comparison, the IC_{50} value for the nitrobenzylthioinosine-sensitive portion

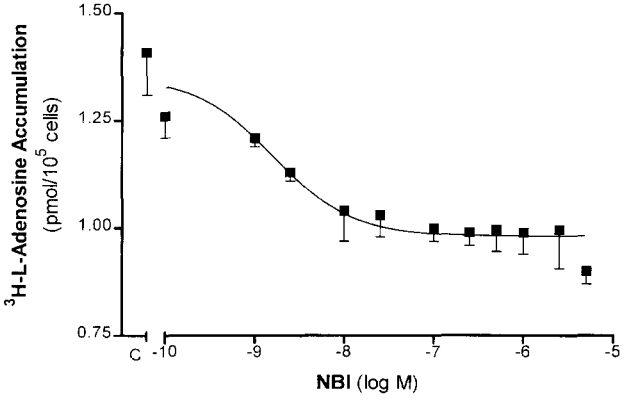

Fig. 2. Accumulation of [^{3}H]L-adenosine by DDT$_1$ MF-2 cells pre-incubated for 15 min with nitrobenzylthioinosine (NBI) at concentrations ranging from 0.1 nM to 20 μM. Incubations with 2 μM [^{3}H]L-adenosine were for 15 s at 25°C. Symbols represent mean ± S.E.M. values from three experiments conducted in triplicate.

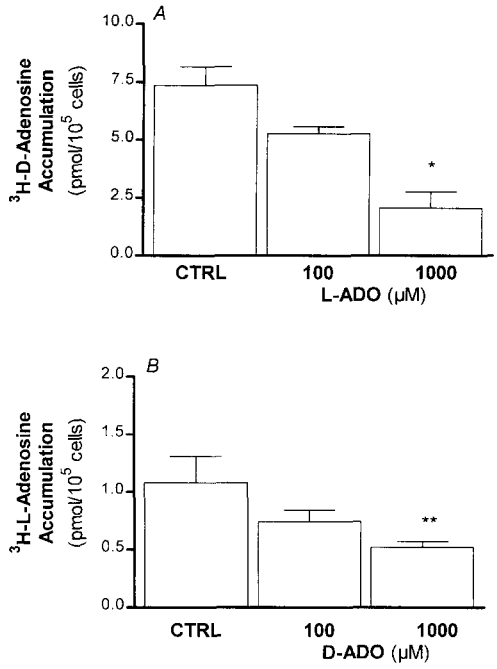

Fig. 3. Accumulation of A. [^{3}H]D-adenosine in the absence or presence of 100 μM or 1000 μM L-adenosine, and B. [^{3}H]L-adenosine in the absence or presence of 100 μM or 1000 μM D-adenosine. Incubations were with 1 μM [^{3}H]adenosine for 15 s at 25°C. Symbols represent mean ± S.E.M. values from three experiments conducted in triplicate. * $P < 0.05$ and ** $P < 0.01$ compared with control values.

of [^{3}H]D-adenosine accumulation was 2.0 ± 0.1 nM (data not shown). [^{3}H]D-Adenosine accumulation of 7.4 ± 0.8 pmol/10^5 cells was inhibited 29 ± 2% by 100 μM L-adenosine and was significantly ($P < 0.05$) inhibited 72 ± 5% by 1000 μM L-adenosine (Fig. 3A). [^{3}H]L-Adenosine accumulation of 1.2 ± 0.2 pmol/10^5 cells was inhibited 31 ± 1% by 100 μM D-adenosine and was significantly ($P < 0.01$) inhibited 52 ± 1% by 1000 μM D-adenosine (Fig. 3B).

3.2. [^{3}H]L-Adenosine release

To initiate release, pelleted cells were resuspended in buffer either at 25°C (Fig. 4A) or 4°C (Fig. 4B). [^{3}H]L-Adenosine in the supernatant increased and that remaining in pellets decreased with time at 25°C (Fig. 4). The release during the first 5 s at 4°C and at 25°C was rapid and amounted to 54–55% of the total amount of [^{3}H]L-adenosine loaded. During the 5 s–5 min time interval, release at 25°C was greater than at 4°C; of the total amount of [^{3}H]L-adenosine loaded, the amount recovered in the supernatants following 5 min was 88 and 58% at 25 and 4°C, respectively. [^{3}H]L-Adenosine release during 5 min was 43% greater at 25 than at 4°C. [^{3}H]L-Adenosine release during 5 min was significantly ($P < 0.05$) reduced by 45% with 30 μM nitrobenzylthioinosine and 47% with 30 μM

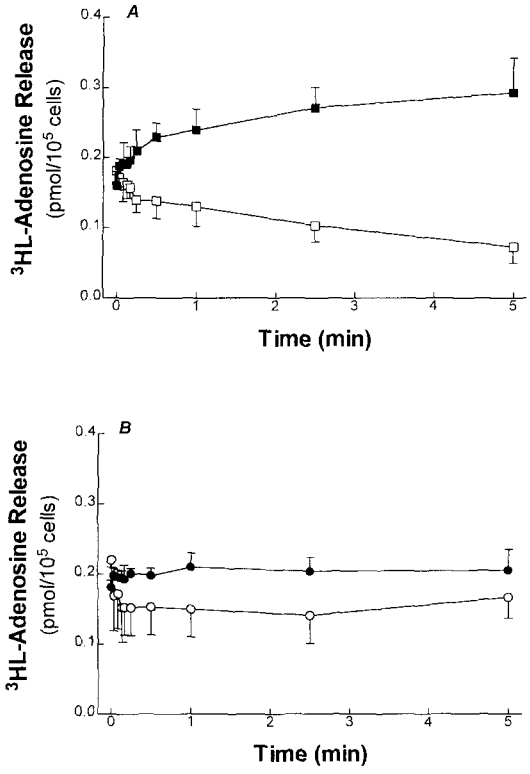

Fig. 4. Time- and temperature-dependent release of [^{3}H]L-adenosine. Cells were incubated with 10 μM [^{3}H]L-adenosine for 60 min and release was initiated following centrifugation and resuspension in buffer. [^{3}H]L-Adenosine was measured in cell pellets (open symbols) and supernatants (closed symbols) for incubations at 25°C (panel A) or 4°C (panel B). Symbols represent mean ± S.E.M. values from three experiments conducted in triplicate.

dipyridamole compared to DMSO vehicle, and 39% with dilazep compared to buffer vehicle (Fig. 5). In a separate series of experiments, nitrobenzylthioinosine inhibited 42%

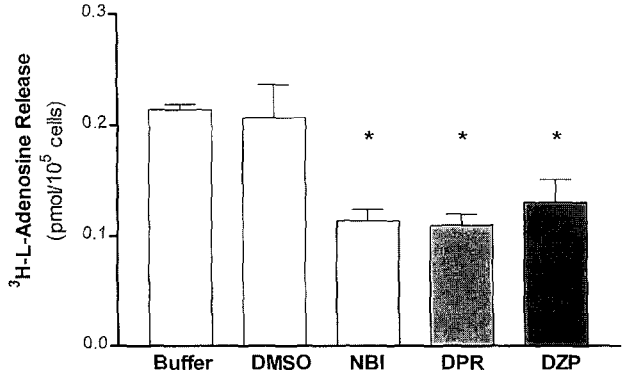

Fig. 5. Inhibition of [^{3}H]L-adenosine release by nucleoside transport inhibitors. Cells were incubated with 10 μM [^{3}H]L-adenosine for 60 min and release was measured over a 5-min period at 25°C in the presence of buffer (vehicle for dilazep), 0.3% DMSO (vehicle for nitrobenzylthioinosine and dipyridamole), or 30 μM nitrobenzylthioinosine, dipyridamole or dilazep. Bars represent mean ± S.E.M. values from three experiments conducted in triplicate. * $P < 0.05$ compared with respective vehicle controls. NBI, nitrobenzylthioinosine; DPR, dipyridamole; DZP, dilazep.

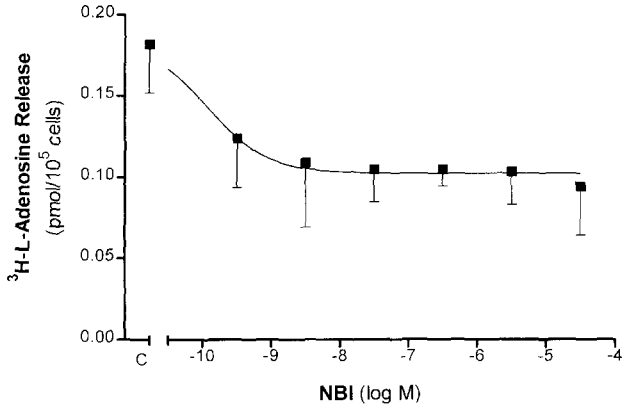

Fig. 6. Dose-dependent inhibition of [^{3}H]L-adenosine release by nitrobenzylthioinosine. Cells were incubated with 10 μM [^{3}H]L-adenosine for 60 min and release was measured over a 5-min period at 25°C in the absence or presence of nitrobenzylthioinosine (NBI) at concentrations ranging from 300 pM to 30 μM. Symbols represent mean ± S.E.M. values from three experiments conducted in triplicate.

of total [^{3}H]L-adenosine release with an apparent IC_{50} value of < 1 nM (Fig. 6).

4. Discussion

L-Adenosine, the stereo-enantiomer of the physiological D-adenosine, appears to be a useful probe for the study of nucleoside transport-mediated processes. In rat brain, it was more metabolically stable than D-adenosine, it did not inhibit adenosine kinase activity and only weakly inhibited adenosine deaminase activity, and it was not a substrate for either adenosine kinase or adenosine deaminase (Gu et al., 1991). Further, [^{3}H]L-adenosine was transported into rat brain synaptoneurosomes with rates (Gu et al., 1991) and K_T values (Gu and Geiger, 1992) that were similar to those observed for [^{3}H]D-adenosine. That [^{3}H]L-adenosine transport was mediated, at least in part, by nucleoside transporters was confirmed by showing that nitrobenzylthioinosine and unlabeled D-adenosine blocked uptake by approximately 50% (Gu et al., 1991; Gu and Geiger, 1992). More recently, we found that [^{3}H]L-adenosine was accumulated by and released from crude synaptosomal preparations of rat brain, and that the release of [^{3}H]L-adenosine was inhibited by three structurally dissimilar nucleoside transport inhibitors; inhibition was 40% by dipyridamole, 49% by dilazep, and 52% by nitrobenzylthioinosine (Gu et al., 1995). Thus, the release of [^{3}H]L-adenosine from rat brain preparations was mediated, at least in part, by what appear to be bidirectional equilibrative nucleoside transporters. However, the above studies were conducted with heterogeneous preparations of rat brain derived from homogenized tissue that contained multiple subtypes of nucleoside transporters. Therefore, DDT_1 MF-2 cells representing a homogeneous population of clonal cells that contain almost exclusively *es* type nucleo-

side transporters (Parkinson et al., 1996) were used to determine the degree to which [^{3}H]L-adenosine accumulation and release were mediated by specific bidirectional nucleoside transporters.

In rat brain preparations, we found that the rates of [^{3}H]D- and [^{3}H]L-adenosine accumulation were roughly equal (Gu et al., 1991). In contrast, accumulation of [^{3}H]D-adenosine was approximately 3 times higher than [^{3}H]L-adenosine in DDT_1 MF-2 cells, although at least part of this difference appeared due to metabolism of D-adenosine to impermeant nucleotides (Parkinson and Geiger, 1996). In cultured adrenal chromaffin cells L-adenosine was also found to be a permeant of nucleoside transporters (Casillas et al., 1993). However, in contrast, L-adenosine was a very poor permeant in mouse erythrocytes, L1210/AM cells, cultured armyworm ovary (Sf9) cells, human erythrocytes, Hela cells, Buffalo Green Monkey cells and parasitic protozoa (Dagnino et al., 1991; Hogue and Cass, 1994; Gati et al., 1989; Upston and Gero, 1995). Thus, in terms of stereoselectivity, adenosine transport in DDT_1 MF-2 was similar to findings in rat brain and adrenal chromaffin cells.

Stereoselectivity is also an important and emerging area for the pharmacology of nucleoside transport inhibitors. Pronounced stereoselectivity was observed between R 75231 and its stereo-enantiomers R 88016 and draflazine (Van Belle et al., 1993; Beukers et al., 1994). However, [^{3}H]D-adenosine uptake by DDT_1 MF-2 was equi-effectively inhibited by propentofylline, a racemic mixture of its hydroxy metabolite (±) A720287 and its stereoisomers (+) 833791 and (−) 844261 (Parkinson et al., 1996). Thus, it appears clear that stereoselectivity may be another property of nucleoside transporters that varies between species, tissues, cells and drugs (see Geiger and Fyda, 1991).

Following pre-incubations with 10 μM nitrobenzylthioinosine, accumulations of [^{3}H]D-adenosine and [^{3}H]L-adenosine were inhibited by 86 and 63%, respectively, to levels indistinguishable between [^{3}H]D- and [^{3}H]L-adenosine. This is similar to rat brain preparations, in which nitrobenzylthioinosine inhibited [^{3}H]L-adenosine accumulation by 52–65% (Gu et al., 1991). The IC_{50} value for the nitrobenzylthioinosine-sensitive portion of [^{3}H]L-adenosine accumulation in DDT_1 MF-2 cells was 1.6 nM and this value was similar to the IC_{50} value of 2.0 nM for the nitrobenzylthioinosine-sensitive portion of [^{3}H]D-adenosine accumulation found here and to values obtained for *es* transporters in a variety of tissues (Boumah et al., 1994; Jarvis and Young, 1986; Torres et al., 1990). That the accumulation of [^{3}H]L-adenosine was mediated by nucleoside transporters was supported further by findings that [^{3}H]D-adenosine accumulation was inhibited by up to 72% with L-adenosine and [^{3}H]L-adenosine accumulation was inhibited by up to 52% with D-adenosine. The nitrobenzylthioinosine-resistant accumulation of [^{3}H]L-adenosine may be mediated by passive diffusion or by nucleoside transporters that are resistant to inhibition by nitrobenzylthioinosine. However, our previous findings indicated that DDT_1 MF-2 express almost exclusively *es* nucleoside transporters (Parkinson et al., 1996).

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

In summary, nitrobenzylthioinosine inhibited a similar proportion of uptake and release of [^{3}H]L-adenosine, in DDT_1 MF-2 cells. Furthermore, nitrobenzylthioinosine had similar potencies for inhibiting [^{3}H]L-adenosine uptake and release. This indicates that nucleoside transport inhibitors may inhibit the receptor-mediated effects of adenosine produced intracellularly but potentiate the effects of adenosine produced extracellularly.

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