Uptake and Release of $[^3H]$Formycin B via Sodium-Dependent Nucleoside Transporters in Mouse Leukemic L1210/MA27.1 Cells

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Accepted for publication December 16, 1996

ABSTRACT

At least seven functionally distinct nucleoside transport processes exist; however, mouse leukemic L1210/MA27.1 cells possess only one subtype, a Na$^+$-dependent transporter termed N1/cif. The capacity of this transporter subtype to release nucleosides from L1210/MA27.1 cells was investigated with the poorly metabolized inosine analog $[^3H]$formycin B. Uptake of $[^3H]$formycin B into these cells was inhibited by replacement of Na$^+$ in the buffer with choline, or by blocking Na$^+$/K$^+$ ATPase with 2 mM ouabain, inhibiting glycolysis with 5 mM iodoacetic acid or inhibiting nucleoside transport with 1 mM phloridzin. Sodium stimulated uptake with an EC$_{50}$ value of 12 mM. To measure release of $[^3H]$formycin B, cells were loaded with $[^3H]$formycin B (10 $\mu$M) then washed and resuspended in buffer. Replacement of Na$^+$ in the buffer with choline enhanced $[^3H]$formycin B release by 20 to 47%, and significant stimulation of release was observed with Na$^+$ concentrations of 30 mM or less. Resuspending loaded cells into Na$^+$ buffer containing 2 mM ouabain or 10 $\mu$M monensin, a Na$^+$ ionophore, significantly enhanced $[^3H]$formycin B release during 20 min by 39% or 29%, respectively. Release of $[^3H]$formycin B into choline buffer was inhibited 26.5% by 10 mM phloridzin and 39.6% by 10 mM propentofylline, compounds known to inhibit various transporters including Na$^+$-dependent nucleoside transporters. Release was also inhibited significantly by 100 $\mu$M concentrations of dilazep, dipyridamole and nitrobenzylthioinosine, inhibitors with selectivity for Na$^+$-independent nucleoside transporters. In the absence of Na$^+$, the permeants adenosine and uridine enhanced $[^3H]$formycin B release by up to 40.5% and 21.4%, respectively. These data indicate that in the absence of an inwardly directed Na$^+$ gradient, Na$^+$-dependent nucleoside transporters can function in the release of nucleosides.

Nucleoside transport processes are membrane-bound carrier proteins that mediate the transfer of nucleosides across plasma membranes. Seven transporters have been characterized according to function (Cass, 1995) and are divided into two broad classes: Na$^+$-independent and Na$^+$-dependent processes. Na$^+$-independent transporters are facilitated diffusion processes that catalyze cellular influx or efflux of nucleosides with the direction of movement determined by the nucleoside concentration gradient. Two equilibrative transporters are distinguished by their sensitivity to the transport inhibitor NBMPR and are termed equilibrative sensitive (es) and equilibrative insensitive (ei), respectively (Vijayalakshmi and Belt, 1988). Na$^+$-dependent transporters couple the influx of Na$^+$ to the influx of nucleosides; thus, in the presence of a transmembrane Na$^+$-gradient nucleosides can be concentrated within cells to levels in excess of those in the extracellular environment. Five Na$^+$-dependent nucleoside transporters have been described and are termed N1 to N5. N1, also called cif, accepts purines and uridine as permeants, whereas N2, also called cit, and N4 are pyrimidine selective. N3 and N5, also called cib and cs, respectively, have broad permeant selectivity and accept both purines and pyrimidines. N5 (cs) is unique among the currently identified Na$^+$-dependent transporters for its sensitivity to inhibition by low nanomolar concentrations of NBMPR. Dipyridamole and dilazep inhibit both es and ei but are poor inhibitors of Na$^+$-dependent transporters (Cass, 1995).

Nucleoside transport processes are an important component of nucleoside salvage pathways and provide cells with nucleosides that are required for cellular metabolism. In addition, adenosine is an endogenous nucleoside that has autocrine and paracrine regulatory effects. In brain, adenosine is an inhibitory neuromodulator, and extracellular adenosine levels are regulated by nucleoside transport processes. Recent evidence indicates that glutamate transporters, which are dependent on Na$^+$ and normally function in cellular uptake, can mediate glutamate release after depolarization, ATP depletion or glycolytic inhibition (Madl and Burgess, 1993; Gemba et al., 1994). It has been proposed that

ABBREVIATIONS: NBMPR, nitrobenzylthioinosine or nitrobenzylmercaptopyrimidine riboside.
this is an important source of extracellular glutamate during conditions of abnormal metabolism, such as stroke (Sztawkowski and Attwell, 1994). Because adenosine levels also increase during stroke and cellular release of adenosine can be resistant to inhibitors of es and ei transporters (Geiger and Fyda, 1991), we investigated whether Na$^{+}$-dependent nucleoside transporters can mediate nucleoside release during conditions that perturb transmembrane Na$^{+}$ gradients.

MURINE Leukemia L1210 cells possess both Na$^{+}$-independent (es and ei) and Na$^{+}$-dependent (N1/cif) nucleoside transport activities (Crawford et al., 1990). Mutation strategies led to the isolation of L1210/MA27.1 cells which retain only an N1/cif nucleoside transporter (Crawford et al., 1990a); thus, these cells provide a model system to examine the function of Na$^{+}$-dependent nucleoside transporters. We investigated cellular release of $[^{3}$H]$\text{formycin B}$, a poorly metabolized inosine analog (Plagemann et al., 1990; Dagnino and Paterson, 1990; Wu et al., 1993) that is a substrate of N1/cif transporters present in L1210/MA27.1 cells (Crawford et al., 1990a), and found evidence for Na$^{+}$-dependent transporter-mediated release of $[^{3}$H]$\text{formycin B}$.

Materials and Methods

Materials. Mouse leukemic L1210/MA27.1 cells were provided by Dr. J.A. Belt. $[^{3}$H]$\text{formycin B}$ was purchased from Moravek Biochemicals (Brea, CA). $[^{3}$H]$\text{Adenosine}$, $[^{3}$H]$\text{H}_{2}\text{O}$ and $[^{3}$H]$\text{polyethylene glycol}$ were from DuPont NEN (Boston, MA). NBMPR was obtained from Research Biochemicals International (Natick, MA). RPMI 1640 and heat-inactivated horse serum were purchased from Gibco BRL (Burlington, Ontario). Dilazep was provided by F. Hoffmann-La Roche Ltd (Basel, Switzerland). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell culture. Mouse leukemic L1210/MA27.1 cells were maintained in logarithmic phase growth in RPMI 1640 culture medium with 10% heat-inactivated horse serum. Cells were harvested by centrifugation at 100 × g for 10 min, washed twice with Na$^{+}$ buffer (in mM: NaCl, 118; KCl, 4.9; MgCl$\text{2}$, 1.2; KH$_{2}$PO$_{4}$, 1.4; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 25; glucose, 11; CaCl$_{2}$, 1; pH 7.4, 300 ± 10 mOsm) then resuspended in Na$^{+}$ buffer to 10$^{6}$ cells/ml. For some experiments, cells were washed and resuspended in buffer in which NaCl was replaced with equimolar choline chloride (choline buffer). For experiments with ionic acid, glucose was omitted from the buffer. Osmolarity of buffers was adjusted, as necessary, to 300 ± 10 mOsm with NaCl or choline chloride.

Measurements of $[^{3}$H]$\text{formycin B}$ uptake. $[^{3}$H]$\text{formycin B}$ (10 μM; 6 μCi/μl) uptake into L1210/MA27.1 cells was measured by an oil-stop centrifugation method as described previously (Parkinson et al., 1993).

The effect of ouabain, an inhibitor of Na$^{+}/K^{+}$ ATPase, iodoacetic acid, an inhibitor of glycolysis, or phloridzin, an inhibitor of Na$^{+}$-dependent nucleoside transport (Lee et al., 1990), on $[^{3}$H]$\text{formycin B}$ uptake was assessed. Cells were preincubated with 2 mM ouabain for 40 min at 37°C (Dagnino et al., 1991), 5 mM iodoacetic acid for 20 min at 37°C (Plagemann and Aran, 1990) or 1 mM phloridzin for 15 min at 22°C (Huang et al., 1993) and $[^{3}$H]$\text{formycin B}$ uptake (22°C) was determined. The effect of nucleoside transport inhibitors on $[^{3}$H]$\text{formycin B}$ uptake was determined with cells preincubated for 15 min (22°C) with 100 μM concentrations of NBMPR, dilazep or dipyridamole.

The effect of graded Na$^{+}$ concentrations on $[^{3}$H]$\text{formycin B}$ uptake was determined by preparing and incubating (15 min, 22°C) cells in buffers containing 0, 6, 12, 30, 59 or 118 mM NaCl. Aliquots of cells were added to reaction mixtures containing $[^{3}$H]$\text{formycin B}$ in identical Na$^{+}$ concentrations. After uptake intervals of 180 sec, reactions were terminated and cell-associated radioactivity was determined.

Measurements of $[^{3}$H]$\text{formycin B}$ release. Cells were washed and resuspended at 5 × 10$^{6}$ cells/ml in Na$^{+}$ buffer and loaded with 10 μM (1 μCi/ml) $[^{3}$H]$\text{formycin B}$ for 30 or 70 min at 37°C. To determine total cellular loading of $[^{3}$H]$\text{formycin B}$, aliquots of cells (100 μl) were centrifuged (13,000 × g) through oil and associated radioactivity was determined. To assay cellular release of $[^{3}$H]$\text{formycin B}$, 100-μl aliquots of cells were transferred to 1.5-ml microcentrifuge tubes, centrifuged (13,000 × g) for 5 sec and loading buffer was aspirated. Cell pellets were cooled on ice and then resuspended in either Na$^{+}$ or choline buffer (22°C; 500 μl), and 400-μl aliquots were transferred to 1.5-ml microcentrifuge tubes containing 200 μl oil. After release intervals of 1 to 20 min, cells were centrifuged through oil and both supernatants (350 μl) and cell pellets were analyzed for radioactivity. Cells resuspended into buffer at 4°C were used to estimate release at 0 min. Cell viability after resuspension was determined by trypan blue exclusion assays and was routinely greater than 95%.

The effect of extracellular Na$^{+}$ concentrations on $[^{3}$H]$\text{formycin B}$ release was determined by resuspending $[^{3}$H]$\text{formycin B}$-loaded cells in 4°C or 37°C buffer containing 0, 30, 59 or 118 mM NaCl. Values of release at 0 min were subtracted from 10- and 20-min release values for each buffer.

To determine the effects of ouabain, iodoacetic acid or the Na$^{+}$-ionophore monensin on $[^{3}$H]$\text{formycin B}$ release, cells loaded for 30 min with $[^{3}$H]$\text{formycin B}$ were resuspended in Na$^{+}$ buffer (4°C or 37°C) alone or in Na$^{+}$ buffer containing 2 mM ouabain, 10 μM monensin or 5 mM iodoacetic acid. Release of $[^{3}$H]$\text{formycin B}$ during time intervals of 0, 10 or 20 min was measured as described above.

To test whether these treatments affected cell viability, trypan blue dye exclusion or intracellular water volume was measured. To determine intracellular volume, cells were incubated in Na$^{+}$ buffer for 30 min at 37°C, centrifuged and resuspended in buffer as described above. After 20 min at 37°C, $[^{3}$H]$\text{H}_{2}\text{O}$ (0.7 μCi/ml) or $[^{3}$H]$\text{polyethylene glycol}$ (0.7 μCi/ml) was added and cells were incubated for a further 3 min. Cells were then centrifuged through oil and cell pellets were assayed for tritium content.

The effects of inhibitors or permeants of nucleoside transport processes on release of $[^{3}$H]$\text{formycin B}$ were evaluated. Cells were loaded with $[^{3}$H]$\text{formycin B}$ in Na$^{+}$ buffer for 30 min at 37°C. Cell aliquots (100 μl) were centrifuged (13,000 × g) for 5 sec, supernatants were removed and pellets were resuspended in 500 μl choline buffer in the absence or presence of the nucleoside transport inhibitor phloridzin, dilazep, dipyridamole, NBMPR or propentofylline, or in the absence or presence of the N1/cif transporter permeant adenosine or uridine. Cells were incubated for 10 or 20 min at 37°C and then centrifuged through oil.

Measurements of $[^{3}$H]$\text{adenosine}$ release. The effect of iodoacetic acid on $[^{3}$H]$\text{adenosine}$ release was determined as described above, with cells loaded for 30 min (37°C) with $[^{3}$H]$\text{adenosine}$ (10 μM; 1 μCi/ml).

Results

Cellular accumulation of $[^{3}$H]$\text{formycin B}$ in L1210/MA27.1 cells. Cellular uptake of $[^{3}$H]$\text{formycin B}$ was greater with cells in Na$^{+}$ buffer than with cells in choline buffer; the rates of uptake were 7.6 ± 0.3 pmol/10$^{6}$ cells/min and 0.2 ± 0.4 pmol/10$^{6}$ cells/min, respectively. For cells in Na$^{+}$ buffer, uptake of $[^{3}$H]$\text{formycin B}$ was reduced by treatment of the cells with 2 mM ouabain, 5 mM iodoacetic acid or 1 mM phloridzin; the rates of uptake were 1.5 ± 0.2, 1.8 ± 0.4 and 0.6 ± 0.3 pmol/10$^{6}$ cells/min, respectively (fig. 1). Uptake of $[^{3}$H]$\text{formycin B}$ was inhibited 23.6% by 100 μM NBMPR, 59.2% by 100 μM dilazep and 56.6% by 100 μM dipyridamole (data not shown). Sensitivity of $[^{3}$H]$\text{formycin B}$ uptake to Na$^{+}$ was determined by measuring cellular accumulation in the presence of graded concentrations of NaCl. The EC$_{50}$
value obtained by nonlinear regression analysis was 12 mM Na$^+$ (fig. 2).

**Release of $[^{3}H]$formycin B from L1210/MA27.1 cells.**

Total $[^{3}H]$formycin B loaded in 70 min was 99,000 ± 12,000 dpm/10$^6$ cells (mean ± S.D.; n = 2). Release was stimulated by resuspending cells in Na$^+$ or choline buffer at 22°C. During 10-min intervals, the percent of total loaded $[^{3}H]$formycin B that was released into Na$^+$ or choline buffer was 31 ± 4% (mean ± S.D.) or 53 ± 7%, respectively (fig. 3). The rate of release of $[^{3}H]$formycin B at 22°C was 3.2 ± 0.3 pmol/5 × 10$^6$ cells/min in choline buffer and 1.1 ± 0.2 pmol/5 × 10$^6$ cells/min in Na$^+$ buffer (fig. 3).

Total $[^{3}H]$formycin B loaded in 30 min was 90,000 ± 3,000 dpm/10$^6$ cells (mean ± S.E.; n = 26). Release of $[^{3}H]$formycin B was examined at 37°C in the presence of several concentrations of Na$^+$. No effect of Na$^+$ concentration on release at 0 min was apparent (data not shown); however, release at 10 or 20 min in buffer containing 118 mM Na$^+$ was significantly (P < .05, ANOVA with Tukey’s HSD post tests) less than release in buffers containing 0 or 30 mM Na$^+$ (fig. 4). The percent of total loaded $[^{3}H]$formycin B that was released into Na$^+$ buffer (118 mM NaCl) during 0, 10 or 20 min was 16 ± 1%, 54 ± 1%, and 65 ± 1%, respectively. Release during 10 or 20 min (37°C) was 47% or 20% greater in buffer containing 118 mM choline chloride than in buffer containing 118 mM NaCl (fig. 4).

Release of $[^{3}H]$formycin B was enhanced by treatment of loaded cells with 2 mM ouabain or 10 μM monensin for 10 or 20 min (fig. 5). After 20 min treatment with ouabain or monensin, release of $[^{3}H]$formycin B was significantly (P < .05, paired t test) increased by 39% or 29%, respectively. In contrast, release was inhibited by treatment with 5 mM...
iodoacetic acid (fig. 5). Release was significantly (P < .05, paired t test) inhibited by 35% relative to control, after 20 min exposure to 5 mM iodoacetic acid (fig. 5). Because the glycolytic inhibitor iodoacetic acid may elevate endogenous adenosine levels, which could then competitively inhibit release of \([3H]\)formycin B, we tested the effect of iodoacetic acid treatment on tritium release after loading of cells with \([3H]\)adenosine (fig. 6). After 10- or 20-min treatments with iodoacetic acid, tritium release was significantly increased by 303% or 364%, respectively. Ouabain, monensin or iodoacetic acid treatment had no significant effect on intracellular volume or on cell viability (data not shown).

Inhibitors of nucleoside transport processes were examined for effects on \([3H]\)formycin B release from L1210/MA27.1 cells (table 1), and significant inhibition of \([3H]\)formycin B release was observed with each of the transport inhibitors used. Phloridzin, which inhibits nucleoside uptake by \(\mathrm{Na}^+\)-dependent but not by \(\mathrm{Na}^+\)-independent nucleoside transporters, produced significant inhibition of \([3H]\)formycin B release only at 10 mM, the highest concentration used. Propentofylline, which can inhibit adenosine uptake by both \(\mathrm{Na}^+\)-dependent and \(\mathrm{Na}^+\)-independent nucleoside transporters, significantly inhibited \([3H]\)formycin B release at both 1 and 10 mM. The classical inhibitors of \(\mathrm{Na}^+\)-independent nucleoside transport, dipyridamole, NBMPR and dilazep, also produced significant inhibition of \([3H]\)formycin B release; at 100 \(\mu\)M concentrations release was inhibited by approximately 10% with dilazep and 25% with NBMPR or dipyridamole. At concentrations of 10 \(\mu\)M, dipyridamole and NBMPR inhibited release by 0 to 10%. Of the inhibitors determined by resuspended cells into \(\mathrm{Na}^+\) buffer (4°C) in the absence or presence of ouabain, monensin or iodoacetic acid, was subtracted from 10- and 20-min release intervals. Bars represent S.E. for three separate experiments performed in quadruplicate (\(P < .05, \*P < .01\); paired t test comparing tritium release in the presence and absence of iodoacetic acid).
The main finding of this study was that release of \([\text{H}]\text{formycin B}\) from L1210/MA27.1 cells was \(\text{Na}^{+}\) dependent; removal of extracellular \(\text{Na}^{+}\) or disruption of transmembrane \(\text{Na}^{+}\)/\(\text{K}^{+}\) gradients enhanced \([\text{H}]\text{formycin B}\) release.

As shown previously (Parkinson et al., 1993; Crawford et al., 1990a), uptake of nucleosides by mouse leukemic L1210/MA27.1 cells, was inhibited by removal of extracellular \(\text{Na}^{+}\).

In the presence of physiological levels of \(\text{Na}^{+}\), the uptake of \([\text{H}]\text{formycin B}\) during a 5-min interval was 5-fold greater than in the absence of \(\text{Na}^{+}\). An \(EC_{50}\) value of 12 mM \(\text{Na}^{+}\) was obtained, which agrees with the value (13 mM) for nucleoside transporter-mediated uptake of 6-mercaptopurine in rat intestinal brush-border membrane vesicles (Iseki et al., 1996).

Phloridzin, an inhibitor of \(\text{Na}^{+}\)-dependent transporters for glucose as well as those for nucleosides (Lee et al., 1988, 1990), inhibited \([\text{H}]\text{formycin B}\) uptake by 73% over 5 min. Disruption of transmembrane \(\text{Na}^{+}\) gradients by blocking \(\text{Na}^{+}/\text{K}^{+}\) ATPase activity with ouabain or by depressing cellular ATP stores with the glycolytic inhibitor iodoacetic acid decreased \([\text{H}]\text{formycin B}\) uptake to 30 to 35% of control.

After loading of cells with \([\text{H}]\text{formycin B}\), release was enhanced by removal of extracellular \(\text{Na}^{+}\), or by treating cells with phloridzin, ouabain or monensin, which indicated that nucleoside release from these cells is stimulated by conditions that perturb transmembrane \(\text{Na}^{+}\) gradients.

In contrast to the stimulatory effects of ouabain, monensin and \(\text{Na}^{+}\) replacement, the glycolytic inhibitor iodoacetic acid decreased \([\text{H}]\text{formycin B}\) release. By depressing intracellular ATP levels, iodoacetic acid can depress \(\text{Na}^{+}/\text{K}^{+}\) ATPase activity and cause intracellular \(\text{Na}^{+}\) overload (Gembä et al., 1994); and it would thus be expected to have effects on nucleoside release similar to those of ouabain and monensin. We hypothesized that, by depressing ATP levels, iodoacetic acid elevated levels of intracellular adenosine which then competitively inhibited release of \([\text{H}]\text{formycin B}\). Consistent with this hypothesis, we found that iodoacetic acid stimulated tritium release in cells loaded with \([\text{H}]\text{adenosine}\).

The difference in release of these two compounds indicates that \([\text{H}]\text{adenosine}\) is the better permeant for outward transport. Previously, it had been shown that \(\text{Na}^{+}\)-dependent influx of 1 \(\mu\)M adenosine (190 pmol/10⁸ cells/sec) was approximately 8-fold faster than that of 1 \(\mu\)M formycin B (24 pmol/10⁸ cells/sec) in L1210 cells (Crawford et al., 1990b) and that adenosine has greater affinity than formycin B for \(\text{N}\text{1}/\text{cif}\) transporters (Vijayalakshmi and Belt 1988).

An interesting finding of these studies was that treatment of cells with phloridzin, ouabain or \(\text{Na}^{+}\)-replacement buffer was more effective in inhibiting \([\text{H}]\text{formycin B}\) uptake than in stimulating \([\text{H}]\text{formycin B}\) release. At least three factors may contribute to this difference. First, each of these treatments may elevate intracellular adenosine levels. In this case, total nucleoside release may be underestimated by measuring \([\text{H}]\text{formycin B}\) release, because simultaneous release of nonradioactive adenosine may competitively inhibit \([\text{H}]\text{formycin B}\) release. Second, uptake studies were performed with cells pretreated with the desired buffers and drugs; however, because pretreatment was not possible for release studies, release was measured from the beginning of exposure of cells to the various treatment conditions. Because the drugs were not at equilibration with their respec-

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**TABLE 1**

Effect of nucleoside transport inhibitors on release of \([\text{H}]\text{formycin B}\).

Cells were loaded with \([\text{H}]\text{formycin B}\), extracellular tritium was removed and cells were resuspended in choline buffer (37°C) in the absence or presence of test compounds. After 10- or 20-min release intervals, tiritium content of supernatants was expressed as a percent of control release, determined in the absence of added compounds. Experiments consisted of controls and two drug concentrations, and were performed in quadruplicate and repeated at least three times.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>10 min</th>
<th>20 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phloridzin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM</td>
<td>76.5 ± 1.5*</td>
<td>73.5 ± 3.0*</td>
</tr>
<tr>
<td>1 mM</td>
<td>93.6 ± 2.2</td>
<td>93.7 ± 4.8</td>
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<tr>
<td>Propentofylline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM</td>
<td>61.9 ± 2.5*</td>
<td>60.4 ± 1.9*</td>
</tr>
<tr>
<td>1 mM</td>
<td>81.5 ± 1.4*</td>
<td>78.8 ± 2.8*</td>
</tr>
<tr>
<td>Dilazep</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 (\mu)M</td>
<td>91.9 ± 3.2</td>
<td>88.6 ± 2.9*</td>
</tr>
<tr>
<td>Dipyridamole</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 (\mu)M</td>
<td>76.6 ± 4.6*</td>
<td>78.2 ± 2.8*</td>
</tr>
<tr>
<td>10 (\mu)M</td>
<td>91.6 ± 0.8</td>
<td>91.3 ± 5.1</td>
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<tr>
<td>NBMPR</td>
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<tr>
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<td>74.9 ± 3.6*</td>
<td>80.2 ± 1.8*</td>
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<tr>
<td>10 (\mu)M</td>
<td>89.3 ± 0.9*</td>
<td>103.1 ± 5.4</td>
</tr>
</tbody>
</table>

* \(P < .05\) ANOVA with Tukey’s HSD post test; relative to control values

**TABLE 2**

Effect of the nucleosides adenosine and uridine on release of \([\text{H}]\text{formycin B}\).

Cells were loaded with \([\text{H}]\text{formycin B}\), extracellular tritium was removed and cells were resuspended in choline buffer in the absence or presence of test compounds. After 10- or 20-min release intervals, tritium content of supernatants was determined. \([\text{H}]\text{formycin B}\) released in the presence of inhibitors is expressed as a percent of control release which was determined in the absence of nucleoside. Experiments, consisting of controls and two drug concentrations, were performed in quadruplicate and repeated at least three times.

<table>
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<th>Nucleoside</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Adenosine</td>
<td></td>
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</tr>
<tr>
<td>10 mM</td>
<td>140.5 ± 0.6*</td>
<td>135.9 ± 4.3*</td>
</tr>
<tr>
<td>1 mM</td>
<td>131.0 ± 4.7*</td>
<td>136.8 ± 5.1*</td>
</tr>
<tr>
<td>100 (\mu)M</td>
<td>140.9 ± 2.0*</td>
<td>128.9 ± 1.7*</td>
</tr>
<tr>
<td>10 (\mu)M</td>
<td>103.3 ± 2.06</td>
<td>110.4 ± 2.1*</td>
</tr>
<tr>
<td>Uridine</td>
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</tr>
<tr>
<td>10 mM</td>
<td>119.7 ± 2.1*</td>
<td>121.4 ± 1.7*</td>
</tr>
<tr>
<td>1 mM</td>
<td>110.8 ± 2.4*</td>
<td>111.9 ± 0.1*</td>
</tr>
<tr>
<td>100 (\mu)M</td>
<td>119.2 ± 2.1*</td>
<td>118.2 ± 0.7*</td>
</tr>
<tr>
<td>10 (\mu)M</td>
<td>102.5 ± 1.1</td>
<td>108.3 ± 1.7*</td>
</tr>
</tbody>
</table>

* \(P < .05\) ANOVA with Tukey’s HSD post test; relative to control values

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**Discussion**

tested and at the concentrations used, propentofylline produced the greatest inhibition of release (38%).

The effect of the nucleoside transporter permeants, adenosine and uridine, on release of \([\text{H}]\text{formycin B}\) was tested (table 2). In contrast to the inhibitory effects of nucleoside transport inhibitors, release of \([\text{H}]\text{formycin B}\) during 10 or 20 min exposure to adenosine or uridine at concentrations of 100 \(\mu\)M to 10 mM was significantly greater than release in choline buffer alone. At 10 \(\mu\)M, the lowest concentration tested, release was significantly greater than control after 20 min, but not 10 min, exposure to adenosine or uridine. At concentrations of 100 \(\mu\)M to 10 mM, adenosine produced greater elevation of \([\text{H}]\text{formycin B}\) release than did uridine.
tive target sites before initiation of release, this could lead to underestimation of the effects of the cell treatments on $[^3H]$formycin B release. Third, the finite intracellular volume of the cells meant that intracellular $[^3H]$formycin B concentrations were not constant for the duration of the release intervals. Each of these three factors would have the effect of lowering $[^3H]$formycin B release.

Differences were also observed in the Na$^+$ concentration dependence of $[^3H]$formycin B uptake and release; for example, uptake was unaffected but release was stimulated by reducing the buffer Na$^+$ concentration from physiological to 30 mM. This may indicate that intracellular levels of Na$^+$ are higher in cells used for release assays than in cells used for uptake assays. It is possible that intracellular Na$^+$ levels are elevated before initiation of release intervals, because cells are loaded with $[^3H]$formycin B in the presence of Na$^+$ buffer.

Release of $[^3H]$formycin B was depressed by millimolar concentrations of low-affinity inhibitors of Na$^+$-dependent nucleoside transporters, such as propentofylline (Parkinson et al., 1993) and phosphoribosylamine (Lee et al., 1988, 1990). Release was also decreased by 10 to 100 $\mu$M concentrations of NBMPR, diprydamole and dilazep, inhibitors that at nanomolar concentrations are selective for Na$^+$-independent nucleoside transporters (Cass, 1995). Several studies have measured adenosine release in the presence or absence of NBMPR or diprydamole at concentrations of 10 to 100 $\mu$M (Hoehn and White, 1990; Craig and White, 1993; Green, 1980; Cunha et al., 1996). Inhibition of release has been interpreted as evidence of release mediated by equilibrative transporters. However, the present study indicates that NBMPR, diprydamole and dilazep can inhibit nucleoside uptake and release mediated by Na$^+$-dependent transporters. Thus, high (>10 $\mu$M) concentrations of these compounds should be used with caution in investigations of cellular release mechanisms for nucleosides.

Stimulation of release by adenosine and uridine may indicate transacceleration in the absence of a Na$^+$ gradient. This phenomenon, commonly observed with Na$^+$-independent nucleoside transporters (Jarvis, 1986), can occur when transporter permeants are simultaneously present on both sides of the membrane. In the presence of a Na$^+$-gradient, Na$^+$-dependent transporters function as symporters and translocate nucleosides in an inward direction. As long as the Na$^+$ gradient is maintained, the intracellular accumulation of permeants does not appear to affect permeant uptake.

Our data suggest, however, that disruption of transmembrane Na$^+$ gradients may uncouple nucleoside transport from Na$^+$ translocation, and in this situation transport of nucleosides in one direction may accelerate the transfer in the opposite direction.

Carrier-mediated release of neurotransmitters, including glutamate, $\gamma$-aminobutyric acid and dopamine, has been demonstrated by elevating intracellular Na$^+$ levels, replacing extracellular Na$^+$, blocking Na$^+$/K$^+$ ATPase activity or inhibiting glycosylation (Gemb et al., 1994; Eshleman et al., 1994; Levi and Raiteri, 1993; Belhage et al., 1993). Furthermore, it has been suggested that carrier-mediated release of glutamate is a significant source of excitotoxic extracellular glutamate in cerebral ischemia (Sztakowski and Attwell, 1994). Adenosine released via reversal of Na$^+$-dependent nucleoside transporters may contribute to the micromolar levels of extracellular adenosine that arise during cerebral ischemia. Molecular evidence indicates that mRNA for N1/cif and N2/cit transporters is widely distributed in brain (Anderson et al., 1996). Other sources that may contribute to elevated extracellular adenosine levels include release via Na$^+$-independent transporters and release of ATP followed by enzymatic dephosphorylation to adenosine. In summary, we have demonstrated that by disrupting transmembrane Na$^+$ gradients, reversal of Na$^+$-dependent nucleoside transporters can mediate cellular release of nucleosides. The evidence that this release is transporter-mediated includes inhibition by transport inhibitors and stimulation by transporter permeants. Adenosine, a nucleoside with diverse receptor-mediated effects, may be released from cells by this process during conditions, such as ischemia, that depress cellular transmembrane Na$^+$ gradients by compromising intracellular ATP levels and/or Na$^+$/K$^+$ ATPase function.

Acknowledgments

We would like to thank Dr. J.D. Geiger, Dr. R. Bose, Ms. Wei Xiong, Mr. Kallof Mukherjee, Ms. Suzanne Delaney and Ms. Irene Foga for technical assistance.

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