Enantiomeric selectivity of adenosine transport systems in mouse erythrocytes and L1210 cells

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In mediating the entry of adenosine into mouse erythrocytes and mouse leukaemia L1210 cells, nucleoside transport systems were stereoselective, showing a marked preference for the D-enantiomer of adenosine (D-Ado). Inward zero-*trans* fluxes of the mirror-image isomer, L-adenosine (L-Ado), in those cells were slow relative to those of D-Ado. Contributing to L-Ado fluxes in both cell types were (i) a transporter-mediated process of high nitrobenzylthioinosine-sensitivity and (ii) simple diffusion.

INTRODUCTION

In animal cells, several distinct processes contribute to plasma-membrane fluxes of nucleosides [1,2]. Erythrocytes from several species express equilibrative nucleoside transport (NT) systems of high sensitivity to nitrobenzylthioinosine (NBMPR) [3,4], whereas in some cultured cells nucleoside fluxes are mediated by multiple systems of high or low NBMPR-sensitivity, including Na⁺-linked concentrative NT systems [5,6]. In mouse leukaemia L1210/AM cells, three entry processes contribute to fluxes of adenosine [6]: an Na⁺-linked concentrative NT system that is insensitive to 10 μ M-NBMPR and accounts for about 20% of D-adenosine (D-Ado) fluxes (at 22 °C and extracellular concentration of 10 μ M), and two non-concentrative NT systems of (i) high NBMPR-sensitivity (IC₅₀ about 1 nM), and (ii) low NBMPR-sensitivity (IC₅₀ about 1 μ M) that are not Na⁺-linked. Non-concentrative fluxes of adenosine in L1210/AM cells are completely inhibited by dipyrindamole at concentrations greater than 3 μ M [6].

Erythrocytic NT systems accept as substrates nucleosides that differ widely in nucleobase structure, although certain modifications in the ribofuranosyl moiety are not tolerated [7,8]. We show here that the enantiomeric configuration of the ribofuranosyl moiety of adenosine is a determinant of the interaction of that nucleoside with NT systems of mouse erythrocytes (mRBCs) and mouse leukaemia L1210/AM cells; the natural isomer, D-Ado was preferred over the L-enantiomer, L-adenosine (L-Ado).

EXPERIMENTAL

Cells

Erythrocytes were obtained from female Ha/ICR mice (Health Sciences Animal Services, University of Alberta) by cardiac puncture after CO₂ asphyxiation. The mRBCs were washed three times in Tris-buffered saline (TBS) and resuspended in that medium at 2 × 10⁸ cells/ml.

L1210/AM cells, a line deficient in deoxycytidine kinase, 2'-deoxyadenosine kinase and adenosine kinase activities [9], were cultured in Fischer's medium with 10% horse serum at cell densities less than 5 × 10⁵ cells/ml to ensure exponential growth. For transport studies, cells were resuspended in Dulbecco's phosphate-buffered saline [10] with 5 mM-glucose (DPBS).

Measurement of nucleoside fluxes

Inward zero-*trans* fluxes were measured at 22 °C by a time-course method employing replicate assay mixtures. Microcentrifuge tubes containing 188 μ l portions of ³H-labelled permeants (D-Ado or L-Ado) in TBS, or cell suspensions (188 μ l or 200 μ l) in TBS or DPBS, layered over 100 μ l of silicone oil, were placed in the rotor of an Eppendorf Model 5412 microcentrifuge. To begin influx intervals, 188 μ l portions of mRBC suspension (or 188 μ l or 100 μ l portions of medium with ³H-labelled permeant) were rapidly added to the tubes at 1.5 or 1.2 s intervals by means of an automatic sample dispenser (Digiflex-CX Automatic Pipette model 33010; Micromedex Systems, Horsham, PA, U.S.A.). Cells were pelleted under the oil layer by centrifugation (16000 g, 30 s) as soon as possible after addition of the last component of the assay mixture and the ³H content of cell pellets was determined as described previously [11]. Zero-time values for the cell content of ³H-labelled permeants were estimated by extrapolation of time courses for cellular uptake of D- and L-Ado to points of intersection; this procedure allowed estimation of the shortest interval of cell exposure to permeant. That interval included permeant uptake during (i) sample delivery with the automatic dispenser, (ii) the interval between the end of sample delivery and centrifuge switch-on, and (iii) cell-pelleting time (equivalent to about 2 s of incubation under these conditions [12]).

Cell water spaces

Intracellular water volumes were determined by subtraction of the extracellular pellet water volume (de-

Abbreviations used: NT, nucleoside transport; NBMPR, nitrobenzylthioinosine {6-[(4-nitrobenzyl)thio]-9- β -D-ribofuranosylpurine}; mRBCs, mouse erythrocytes; D-Ado, D-adenosine ('physiological adenosine', 9- β -D-ribofuranosyladenine); L-Ado, L-adenosine (9- β -L-ribofuranosyladenine); TBS, Tris-buffered saline (166 mM-NaCl/10 mM-glucose/10 mM-Tris/HCl, pH 7.4 at 22 °C); DPBS, Dulbecco's phosphate-buffered saline [10] with 5 mM-glucose, IC₅₀, concentration causing half-maximal inhibition.

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terminated with [^{14}C]sucrose) from the total pellet water volume (determined with $^3\text{H}_2\text{O}$).

Materials

L-Ado [13] was generously provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD, U.S.A. This material was eluted as a single peak that co-chromatographed with D-Ado on a Whatman Magnum Partisil 10 ODS-3 h.p.l.c. column with a methanol/water gradient. In addition, the identity and purity of the L-Ado preparation were confirmed by determining the melting point (observed: 234–236 °C; literature values: 232–235 °C [13] and 230–231 °C [14]) and the ^1H n.m.r. spectrum, the latter with a Bruker AM-400 spectrometer. The enantiomeric identity of L-Ado was confirmed by measurement (Perkin–Elmer model 241 polarimeter) of the optical rotation, $[\alpha]_{\text{D}}^{20} + 59.2^\circ$ (c 0.05 in water) [literature values: $[\alpha]_{\text{D}}^{25} + 61.4$ (c 0.5 in water) [13] and $[\alpha]_{\text{D}}^{27} + 59.4$ (c 1.2 in water) [14]]. The enantiomeric purity of the L-Ado preparation was demonstrated by enzymic deamination of a mixture of 10 μCi of L- ^3H Ado (33 Ci/mmol; prepared from the above L-Ado sample by Moravsek Biochemicals, Brea, CA, U.S.A.) and non-radioactive D-Ado (600 nmol), using 0.1 unit of adenosine deaminase Type II from calf intestinal mucosa (Sigma). Adenosine deaminase is known to deaminate L-Ado very slowly [14]. Inosine isolated from the deaminase reaction mixture by h.p.l.c. was virtually free of ^3H radioactivity, showing that the L-Ado preparation was enantiomerically pure.

^3H Nucleosides were purified by h.p.l.c. before use. $[2, 8\text{-}^3\text{H}]\text{D-Ado}$ (33 Ci/mmol), $^3\text{H}_2\text{O}$ (100 mCi/ml) and $[\text{U-}^{14}\text{C}]\text{sucrose}$ (499–671 mCi/mmol) were from Moravsek Biochemicals, ICN Radiochemicals, Irvine, CA, U.S.A., and DU PONT Canada (NEN Products), Lachine, Que., Canada, respectively, and NBMPR was prepared in this laboratory [15].

RESULTS AND DISCUSSION

Time courses for inward zero-*trans* fluxes of 5 μM -D- and L-Ado in mRBCs are shown in Fig. 1. The shortest interval of cell exposure to permeant, determined as described in the Experimental section, was about 4 s in the experiment shown. Initial rates of permeant uptake, which measure unidirectional inward fluxes of D- or L-Ado, were estimated from the time courses of Fig. 1. Fluxes of 5 μM -D-Ado (1.3 ± 0.1 pmol/s per μl of cell water; mean $\pm$ S.E.M.) were about 20-fold greater than those of L-Ado (0.078 ± 0.021 pmol/s per μl of cell water), showing that the mRBC NT system was highly stereoselective.

The possibility that trace amounts of D-Ado in the L-Ado preparation might be responsible for the observed L-Ado fluxes was excluded through analysis of the L-Ado preparation (see under 'Materials' in the Experimental section). It appeared, therefore, that the slow, inward fluxes of L-Ado were real, and, to explore the process(es) involved, fluxes were measured at high L-Ado concentrations. Fig. 2 shows time courses for inward fluxes of 100 μM - and 200 μM -L-Ado in mRBCs and the effects of NBMPR and D-Ado. In the presence of 200 μM -D-Ado [K_m for inward flux in mRBCs about 8 μM (W. P. Gati & A. R. P. Paterson, unpublished work)], the inward flux of 100 μM -L- ^3H Ado was decreased by about 60 %, from 1.2 ± 0.1 to 0.44 ± 0.11 pmol/s per μl of cell

Fig. 1. Enantiomeric selectivity of NT in mRBCs

Inward zero-*trans* fluxes of 5 μM -D- and -L-Ado were initiated by the addition, at 1.5 s intervals, of 188 μl portions of cell suspension (3.8×10^7 cells) to equal volumes of ^3H -labelled permeant solution by means of an automatic pipetting device. Permeant uptake intervals were ended by pelleting the cells under an oil layer as described in the text. Incubation mixtures containing D-Ado or L-Ado were completed in an alternating sequence, so that points on the two progress curves were related on the time scale as shown. Results are means from duplicate assays.

water; further, the presence of 5 μM -NBMPR (sufficient to block entry of D-Ado) decreased the inward flux of 200 μM -L-Ado by about 85 %, from 1.4 ± 0.2 to 0.17 ± 0.09 pmol/s per μl of cell water. These inhibitory effects indicated that entry of the L-enantiomer into mRBCs was partly carrier-mediated. Incomplete inhibition of the slow L-Ado fluxes by these agents suggested that a component of L-Ado permeation was attributable to diffusion. After prolonged (2 h) incubation of mRBCs with 100 μM -L-Ado, a substantial component of NBMPR-insensitive entry of L-Ado was evident (results not shown). Thus high concentrations of the L-enantiomer of adenosine apparently entered mRBCs slowly by (i) an NT-mediated process and (ii) simple diffusion.

Fig. 3(a) shows time courses for the entry of 5 μM -D- and -L-Ado in L1210/AM cells in Na^+ -containing buffer (DPBS), a condition in which the Na^+ -linked adenosine entry process and two non-concentrative entry processes are expressed in these cells [6]. D-Ado fluxes proceeded at a substantial rate under these conditions (2.9 ± 0.1 pmol/s per μl of cell water), whereas L-Ado fluxes (0.13 ± 0.01 pmol/s per μl of cell water) were barely measurable, indicating that all three nucleoside entry processes were highly stereoselective. Measurement of D- and L-Ado fluxes (extracellular concn. 10 μM) in L1210/AM cells under conditions that excluded adenosine entry through the non-concentrative systems (Na^+ -containing buffer in the presence of 20 μM -dipyridamole) yielded a similar result, indicating that the

Fig. 2. Partial inhibition of inward L-Ado fluxes in mRBCs by D-Ado and NBMPR

Time courses for inward zero-*trans* fluxes of L-Ado were determined as in Fig. 1. Cells were exposed simultaneously to permeant and inhibitor. Results are means from triplicate assays. (a) Inhibition of 100 μM-L-Ado fluxes by 200 μM-D-Ado (4.9×10^7 cells/assay). (b) Inhibition of 200 μM-L-Ado fluxes by 5 μM-NBMPR (3.9×10^7 cells/assay).

Fig. 3. Enantiomeric selectivity of NT systems in L1210/AM cells

Replicate incubation mixtures were completed by using an automatic sample dispenser to add 188 μl portions (a) or 100 μl portions (b) of ^3H -labelled permeants in Na^+ -containing buffer (DPBS) to cell suspensions in DPBS. (a) shows time courses for the zero-*trans* influx of 5 μM-D- and L-Ado in cells (6.8×10^8) in a final volume of 376 μl. Results are means for duplicate or triplicate assay mixtures. In the experiment of (b) 100 μM-L-Ado fluxes were measured in Na^+ -containing buffer (final volume 300 μl) in either the absence or presence of 5 μM-NBMPR. Cells (2.5×10^8) were exposed to the inhibitor for 2–3 min before addition of permeant. Results are means from quadruplicate assays.

Na^+ -linked NT system was stereoselective (results not shown). In the Na^+ -containing medium, fluxes of 100 μM-L-Ado (1.3 ± 0.1 pmol/s per μl of cell water) were reduced to 0.45 ± 0.16 pmol/s per μl of cell water by 5 μM-NBMPR (Fig. 3b), although entry of the L-enantiomer was not abolished under these conditions. Residual entry of L-Ado in the presence of NBMPR could be attributed to (i) diffusion or (ii) mediated entry through the Na^+ -linked NT system. The latter possibility

is unlikely, since the presence of 400 μM-L-Ado did not affect influx of 10 μM-D- ^3H Ado in these cells in Na^+ medium containing buffer in the presence of 20 μM-dipyridamole (results not shown); that result indicates that interaction of high concentrations of L-Ado with the Na^+ -linked NT system was negligible. On the other hand, 500 μM-L-Ado decreased the influx of 5 μM-D-Ado by about 60% in Na^+ -containing buffer in the absence of transport inhibitors [from 3.4 ± 0.1 to 1.5 ± 0.1 pmol/s

Fig. 4. Partial inhibition of inward D-Ado fluxes in L1210/AM cells by L-Ado

Time courses for inward fluxes of D-Ado were determined as in the experiment of Fig. 3(a). L1210/AM cells (6.4×10^6 /assay) were suspended in Na^+ -containing buffer. Results are means from triplicate assays.

per μ l of cell water (Fig. 4)], suggesting that, at such high concentrations of L-Ado, inhibitor-sensitive NT processes appear to contribute to L-Ado fluxes.

The present study indicates that the enantiomeric configuration is a structural determinant of nucleoside interaction with NT systems. Substrate enantiomeric selectivity has been demonstrated in various systems, including glucose transporters of rat adipose cells, for which L-glucose is a poor substrate [16], and P_1 -purinoceptors of smooth muscle, which are stereospecific for D-Ado [17].

Despite the present demonstration of selectivity in NT systems for the D-enantiomer of adenosine, we suggest caution in the use of L-Ado to measure diffusional fluxes of adenosine, because the low inward fluxes of L-Ado in mRBCs and leukaemia L1210 cells shown in the present study appear to be partially mediated by nucleoside transporters, judging by the inhibitory effects of NBMPR and D-Ado (Figs. 2 and 3). Consistent with this con-

clusion is the inhibition of D-Ado fluxes in L1210 cells by 500 μ M-L-Ado (Fig. 4).

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