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NUCLEOSIDE TRANSPORT. PHOTOAFFINITY LABELLING OF HIGH-AFFINITY NITROBENZYLTHIOINOSINE BINDING SITES IN RAT AND GUINEA PIG LUNG

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SUMMARY: Binding of the potent nucleoside transport inhibitor [^{3}H]nitrobenzylthioinosine to rat and guinea pig lung membranes was investigated. Reversible high-affinity binding was found in both species (apparent $K_D \sim 0.3$nM). Binding was inhibited by nitrobenzylthioguanosine, adenosine and uridine. Dipyridamole was also an effective inhibitor of [^{3}H]nitrobenzylthioinosine binding to guinea pig membranes. In contrast, rat membranes were relatively insensitive to dipyridamole. Exposure of site-bound [^{3}H]nitrobenzylthioinosine to high intensity U.V. light resulted in the photoaffinity labelling of lung proteins with apparent molecular weights similar to that of the human erythrocyte nucleoside transporter (45,000-65,000).

Kinetic studies of nucleoside transport in a variety of cell types have established that physiological and cytotoxic nucleoside molecules cross the plasma membrane of animal cells mainly by a nucleoside-specific facilitated-diffusion process which is independent of subsequent intracellular nucleoside metabolism (1-3). Transport by this route is selectively inhibited by nanomolar concentrations of the 6-substituted thiopurine ribonucleoside NBMPR[1]. In erythrocytes, NBMPR inhibition of nucleoside transport is competitive with respect to substrate concentration and is associated with high-affinity binding of inhibitor to the cell membrane (apparent K_D 0.1-1 nM). A variety of experimental approaches have established that this binding represents a specific interaction with functional nucleoside transporters (3). Binding of NBMPR to the carrier is reversible, but exposure of human erythrocyte 'ghosts' to high intensity U.V. light in the presence of [^{3}H]NBMPR results in selective covalent labelling of the transporter, a band 4.5 polypeptide with an apparent molecular weight on SDS-polyacrylamide gels of 45,000-65,000 (4-6).

[1]NBMPR, nitrobenzylthioinosine

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The objective of the present study was to extend these photolysis experiments to another tissue as a first step towards a molecular comparison of nucleoside transporters in different cell types. The lung was chosen for these experiments because of its high density of high-affinity NBMPR binding sites (7) and because of this tissue's physiological role as a major site for the removal of circulating adenosine, a potent endogenous vasodilator (8, 9). Our results suggest that the nucleoside transporters from rat and guinea pig lung have similar apparent molecular weights to that of the human erythrocyte carrier.

MATERIALS AND METHODS

Materials. [G-^{3}H]NBMPR (specific radioactivity 17 Ci/mmol and >98% radiochemically pure) was purchased from Moravek Biochemicals, Brea, CA, U.S.A. NBTGR[2] was a generous gift from Professor A.R.P. Paterson, University of Alberta Cancer Research Group.

Tissue and Membrane Preparation. Male Sprague-Dawley rats (400-450 g) and Dunkin-Hartley guinea pigs (700-800 g) were anaesthetised with ether and the lungs perfused in situ with heparinized saline to remove trapped erythrocytes. Lung tissue was then removed from the animals, washed twice in ice-cold saline and homogenised in 25 vol (w/v) of ice-cold 50 mM Tris-HCl (pH 7.4 at 22°C) using a Brinkmann Polytron PT-10 (setting 5, 6 s). Samples were centrifuged at 45,000 g for 10 min, and the pellets washed 3 times in 25 vol of Tris-HCl before resuspension in the same volume of buffer. This crude total membrane preparation was used without further purification. Human erythrocyte 'ghosts' were prepared from fresh heparinized blood as described previously (10). Protein was determined by the method of Lowry et al., (11).

High-Affinity NBMPR Binding. Rat and guinea pig lung membranes in 50 mM Tris-HCl (0.05-0.08 mg protein) were incubated with graded concentrations of [^{3}H]NBMPR (final equilibrium concentrations 0.05-10 nM) in a total volume of 1 ml for 30 min at 22°C in the presence or absence of 20 μM NBTGR as competing nonradioactive ligand. Incubations were terminated by filtration on glass fibre filters (Whatman GF/B, which were washed with ice-cold buffer before sample filtration) under suction. The filters were washed four times with 3 ml aliquots of ice-cold buffer, the entire procedure being completed within 15 s. The filters were dried and added to 10 ml of scintillation fluid. Vials were shaken at room temperature overnight before counting. [^{3}H]NBMPR binding to membranes was expressed per mg protein. Nonspecific binding was defined as binding in the presence of 20 μM NBTGR (corrected for absorption of radioactive ligand by the filters). Specific binding was calculated as the difference in binding in the presence and absence of NBTGR. Equilibrium concentrations of unbound [^{3}H]NBMPR were calculated from the differences between the starting [^{3}H]NBMPR concentrations and the amounts of ligand bound to membranes and filters.

Photoaffinity Labelling. Rat and guinea pig lung membranes (final protein concentration 0.8 mg/ml) and human erythrocyte 'ghosts' (final protein concentration 2 mg/ml) were equilibrated at room temperature for 30 min with

[2]NBTGR, nitrobenzylthioguanosine

a saturating concentration of [^{3}H]NBMPR (75 nM for erythrocyte 'ghosts' and 25 nM for lung membranes) in the presence and in the absence of 20 μM NBTGR. Samples were then cooled to 4°C and supplemented with 50 mM dithiothreitol, added as a free-radical scavenger. Photolysis was carried out in conventional 3 ml silica spectrophotometer cuvettes (10 mm light path) with continous stirring at 4°C using a 450-watt mercury arc lamp (Conrad-Hanovia Inc., Newark, New Jersey, U.S.A.). U.V. exposure was for 45 s at a distance of 6.5 cm from the lamp's silica cooling sleeve. Samples were then diluted 10-fold with buffer containing 20 μM NBTGR and allowed to stand at room temperature for 10 min before recovery of the membrane fraction by centrifugation. The membranes were washed twice more in NBTGR-containing buffer and then extracted at room temperature with gel electrophoresis buffer. Insoluble material was removed by centrifugation (15,000 *g*, 5 min). SDS-polyacrylamide gel electrophoresis was carried out in 1 mm-thick slab gels by the method of Thompson and Maddy (12) using the Laemmli buffer system (13). Samples dissolved in gel sample buffer were not heated before application to the gel. The [^{3}H] content of 2 mm-gel slices was measured by liquid scintillation counting as described previously (6). The recovery of applied radioactivity was typically 65-75%.

RESULTS AND DISCUSSION

Fig. 1 shows the concentration-dependence of [^{3}H]NBMPR binding to rat and guinea pig lung membranes, demonstrating the presence of both high-affinity saturable (NBTGR-sensitive) and nonsaturable (NBTGR-insensitive) binding components in the two species. Specific binding saturated at about 2.5 nM [^{3}H]NBMPR. Scatchard analyses of the data indicated a single population of binding sites with a similar dissociation constant (K_D) in the two species but a different number of binding sites (B_{max}). Mean (± SEM) apparent K_D and B_{max}

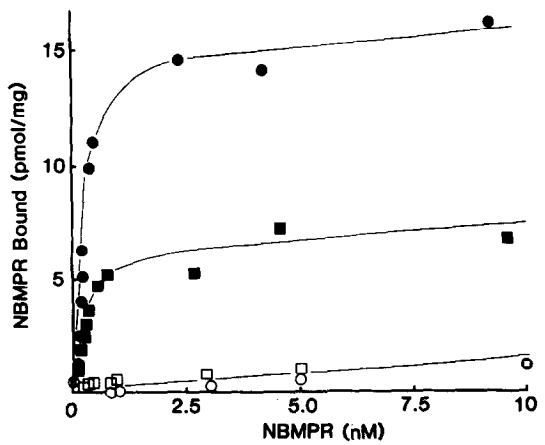

Figure 1. Concentration dependence of nitrobenzylthioinosine binding to rat and guinea pig lung membranes. [^{3}H]NBMPR binding to rat (■,□) and guinea pig (●,○) lung membranes was measured in the absence (■,●) and in the presence (□,○) of 20 μM NBTGR as competing ligand. Values are means of duplicate estimates.

values from a number of experiments were 0.22 ± 0.08 nM and 8.1 ± 1.1 pmol/mg protein for rat (n = 3), respectively, and 0.38 ± 0.12 nM and 19.3 ± 5.2 pmol/mg protein for guinea pig (n = 4), respectively. Both species exhibited the same amount of nonspecific (NBTGR-insensitive) [^{3}H]NBMPR binding (1.5 pmol/mg protein at 10 nM NBMPR). NBTGR-displacement experiments confirmed that high-affinity NBMPR binding to lung membranes was reversible (data not shown).

As expected from previous studies with the erythrocyte nucleoside transporter, high-affinity NBMPR binding activity in lung membranes was inhibited by mM concentrations of the physiological nucleosides, adenosine and uridine (Fig. 2). Adenosine was the more effective inhibitor, correlating with the known relative affinities of these two nucleosides for transport in a variety of cell types, adenosine having the lower apparent K_m (3). The properties of NBMPR binding to rat and guinea pig lung membranes were investigated further by studying the effects of dipyridamole on high-affinity binding activity in the two species. Dipyridamole is a potent structurally unrelated inhibitor of nucleoside transport and NBMPR binding. High-affinity

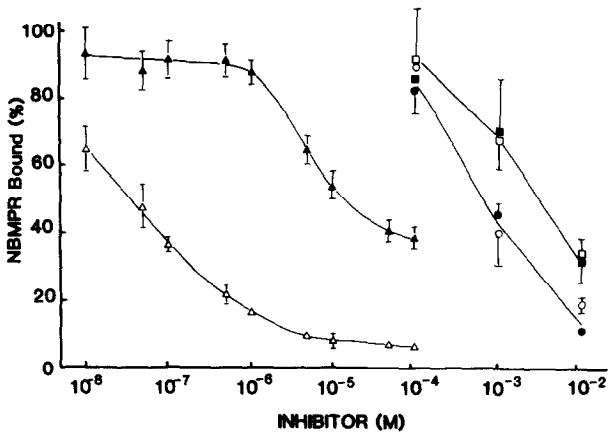

Figure 2. Dipyridamole, adenosine and uridine inhibition of nitrobenzylthioinsoine binding to rat and guinea pig lung membranes. The effects of varying concentrations of dipyridamole (△,▲), adenosine (○,●) and uridine (□,■) on equilibrium NBTGR-sensitive [^{3}H]NBMPR binding (NBMPR concentration 0.5 nM) to rat (closed symbols) and guinea pig (open symbols) lung membrane preparations were determined as described in the text. Data are expressed as percentages of control values and are means (± SEM) of triplicate estimates.

NBMPR binding activity in guinea pig lung was essentially abolished in the presence of 1 μM dipyridamole (Fig. 2). In marked contrast, this concentration of dipyridamole had no detectable effect on NMBPR binding to rat lung membranes. Higher concentrations of dipyridamole caused only partial inhibition of binding activity. IC_{50} values in the two preparations differed by more than two orders of magnitude. It has previously been shown that adenosine uptake by rat lung is approximately 100 times less sensitive to dipyridamole than in guinea pig lung (14). Lung NBMPR binding sites in the two species therefore have the properties expected of nucleoside transport proteins in this tissue. Pharmacokinetic experiments in mice have shown that accumulation of the cytotoxic nucleoside tubercidin in lung tissue is inhibited by preadmininstration of NBMPR-phosphate, a prodrug form of NBMPR (15).

As shown in Figs. 3 and 4, [^{3}H]NBMPR photoaffinity labelling experiments under equilibrium binding conditions resulted in substantial radiolabelling of both rat and guinea pig lung membrane protein. Photolysis was carried out in the presence of dithiothreitol to minimise the possibility of nonspecific labelling (4-6). For both species, the radiolabelled protein migrated as single symmetrical peaks on SDS-polyacrylamide gels with apparent molecular weights in the same range as the human erythrocyte nucleoside transporter (45,000-65,000). The rat lung radioactive peak was broader than that in the guinea pig and had a significantly higher apparent molecular weight. Covalent incorporation of [^{3}H]NBMPR into these proteins was abolished when photolysis was carried out in the presence of NBTGR. The minor high and low molecular peaks observed with the erythrocyte membranes correspond to aggregates of the transporter and low molecular weight degradation products, respectively (5, unpublished results). All preparations exhibited nonspecific labelling in the lipid region of the gel. Approximately 10% of the available high-affinity NBMPR binding sites in the lung membrane preparations were photoaffinity labelled under our experimental conditions.

In conclusion, the present results suggest that nucleoside transport in rat and guinea pig lung is mediated by polypeptides with broadly similar

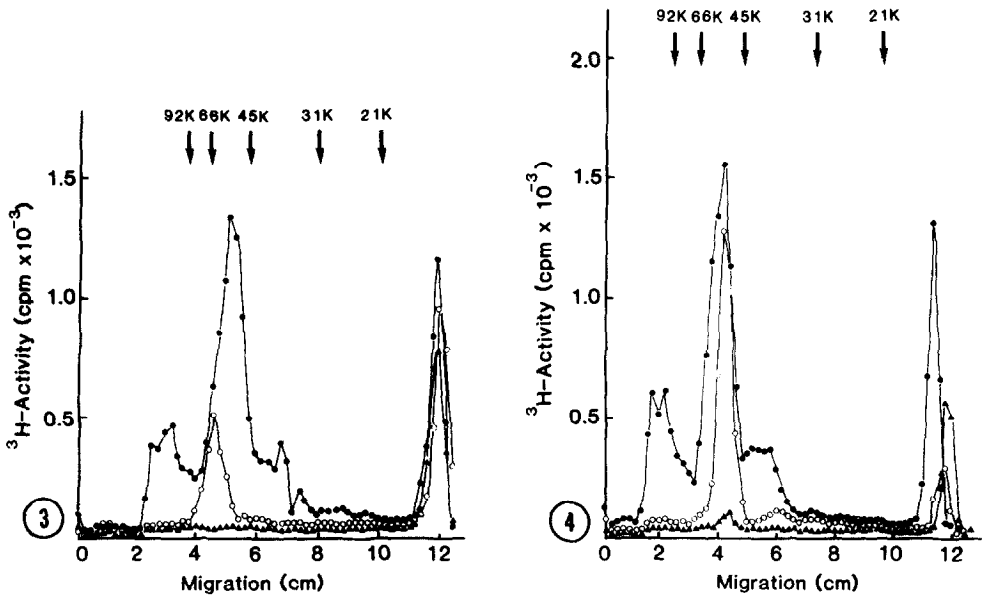

Figure 3. Photoaffinity labelling of rat lung membranes and human erythrocyte 'ghosts' with [^{3}H]nitrobenzylthioinosine under equilibrium binding conditions. Membranes were equilibrated with saturating concentrations of [^{3}H]NBMPR in the presence and in the absence of 20 μM NBTGR and exposed to U.V. light for 45 s. Unreacted [^{3}H]NBMPR was removed by washing. Samples were subjected to SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. [^{3}H]Profiles and positions of molecular weight standards are from the same slab gel. Membranes from rat lung (○) and human erythrocytes (●). Photolysis of rat lung membranes in the presence of NBTGR (▲). Data for human erythrocyte membranes in the presence of NBTGR coplotted with the rat lung NBTGR counts (not shown).

Figure 4. Photoaffinity labelling of guinea pig lung membranes and human erythrocyte 'ghosts' with [^{3}H]nitrobenzylthioinosine under equilibrium binding conditions. Photolysis and SDS-polyacrylamide gel electrophoresis of guinea pig lung membranes and human erythrocyte 'ghosts' in the presence of [^{3}H]nitrobenzylthioinosine were carried under identical conditions to those described in the legend to Figure 3. Guinea pig lung membranes in the absence (○) and in the presence of NBTGR (▲). Human erythrocyte membranes in the absence of NBTGR (●).

molecular weights to that responsible for nucleoside permeation in the erythrocyte. Our [^{3}H]NBMPR photolysis experiments achieved a high degree of radiolabelling specificity, even with crude lung membrane preparations. We anticipate that photoaffinity labelling experiments with this reagent will

find widespread application in molecular studies of nucleoside transport in this and other systems.

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