Photoaffinity labelling of a nitrobenzylthioinosine-binding polypeptide from cultured Novikoff hepatoma cells

Wendy P. GATI,* Judith A. BELT,† Ewa S. JAKOBS,* James D. YOUNG,§ Simon M. JARVIS† and Alan R. P. PATERSON*

* Cancer Research Group (McEachern Laboratory) and † Department of Physiology, University of Alberta, Edmonton, Alberta, Canada T6G 2H7; ‡ Division of Biochemical and Clinical Pharmacology, St. Jude Children’s Research Hospital, Memphis, TN 38101, U.S.A.; and § Department of Biochemistry, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong

Site-specific binding of nitrobenzylthioinosine (NBMPR) to plasma membranes of some animal cells results in the inhibition of the facilitated diffusion of nucleosides. The present study showed that nucleoside transport in Novikoff UA rat hepatoma cells is insensitive to site-saturating concentrations of NBMPR. Equilibrium binding experiments demonstrated the presence of high-affinity sites for NBMPR in a membrane-enriched fraction from these cells. In the presence of uridine or dipyrimidamole, specific binding of NBMPR at these sites was inhibited. When Novikoff UA membranes were covalently labelled with [3H]NBMPR by using photoaffinity techniques, specifically bound radioactivity was incorporated exclusively into a polypeptide(s) with an apparent Mr of 72000–80000, determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Covalent labelling of this polypeptide was abolished in the presence of excess nitrobenzylthioguanosine (NBTGR) and reduced in the presence of adenosine, uridine, or dipyrimidamole. The apparent Mr of the NBMPR-binding polypeptide in Novikoff UA cells is significantly higher than that reported for corresponding polypeptides in other cell types (Mr, 45000–66000). When membrane-enriched preparations from S49 mouse lymphoma cells were photo labelled and mixed with labelled Novikoff UA membrane-enriched preparations, gel electrophoresis resolved the NBMPR-binding polypeptides from the two preparations.

INTRODUCTION

Photoaffinity labelling techniques, which have been useful in molecular studies of receptor binding site polypeptides, have been applied recently to the study of nucleoside transporter polypeptides. In animal cells, this transport mechanism mediates the passage of a diverse group of nucleosides across the plasma membrane. NBMPR, a potent inhibitor of nucleoside transport in some cells, is bound tightly at specific membrane sites which are on, or associated with, transporter polypeptides. Upon exposure to u.v. light, site-bound NBMPR becomes covalently bound to a transporter polypeptide, allowing identification (on electrophoretograms) in human erythrocyte membranes (Young et al., 1983), in a plasma membrane-enriched fraction from S49 mouse lymphoma cells (Young et al., 1984), in membrane-containing preparations from rat and guinea pig lung (Shi et al., 1984) and liver (Wu & Young, 1984), and in crude membrane preparations from guinea pig heart (Kwan & Jarvis, 1984) and brain (Jarvis & Ng, 1985).

This report describes the electrophoretic identification of an NBMPR-binding polypeptide in membranes from a line of cultured Novikoff rat hepatoma cells. The nucleoside transport mechanism in Novikoff cells is of low sensitivity to NBMPR, requiring micromolar concentrations of the inhibitor to effect substantial inhibition of nucleoside transport (Wohlhueter et al., 1978; Plagemann & Wohlhueter, 1984), whereas nanomolar concentrations of the inhibitor block nucleoside transport in erythrocytes and cultured S49 mouse lymphoma cells (Cass et al., 1974; Plagemann & Wohlhueter, 1984) and saturate the high affinity transporter sites at which the inhibitor is bound on the latter cells (Cass et al., 1974, 1981). Unlike some Novikoff cell lines (e.g. the N151-67 wild type; Plagemann & Wohlhueter, 1980), the Novikoff UA line used in the present study has binding sites for NBMPR. The nucleoside transporter of these cells differs from that of Walker 256 rat carcinomas or cells in that the latter cells not only have a nucleoside transport system with low sensitivity to NBMPR (Paterson et al., 1983a; Belt & Noel, 1985), but also lack NBMPR binding sites (Paterson et al., 1983a). The identification of high affinity NBMPR binding sites in Novikoff UA cells (in which the inhibitor affects transport only at very high concentrations) suggests that the interactions of NBMPR and of permeants with the nucleoside transporter occur in these cells at separate sites which might be on the same molecular components of the transporter, or possibly on separate components.

An NBMPR-binding polypeptide(s) from a membrane-enriched fraction of Novikoff UA cells has been covalently labelled by u.v. photoactivation of site-bound [3H]NBMPR and identified on polyacrylamide gels. The apparent Mr of this polypeptide, 72000–80000, is different from that of nucleoside transporter polypeptides identified in several other cell types, which have an apparent Mr of 45000–66000.

Abbreviations used: NBMPR (nitrobenzylthioinosine), 6-[[4-nitrobenzyl]thioj]-9-β-D-ribofuranosylpurine; NBTGR (nitrobenzylthioguanosine), 2-amino-6-[[4-nitrobenzyl]thioj]-9-β-D-ribofuranosylpurine.
MATERIALS AND METHODS

Cell culture

Wild-type Novikoff rat hepatoma cells (NIS1-67), provided by P. G. W. Plagemann and R. M. Wohlueter, were adapted to growth in Eagle's minimal essential medium supplemented with 5% horse serum, 5% calf serum, non-essential amino acids (0.1 mM), glutamine (4 mM), and antibiotics. The adapted line referred to as Novikoff UA. Cultures for preparation of a membrane containing fraction were expanded to 800 ml in large roller bottles rotated at 1.5 rev./min. Cell concentrations were kept below 4 x 10^5 cells/ml. Other cell types (S49 mouse lymphoma, L1210 mouse leukaemia, and Walker 256 rat carcinosarcoma) were maintained in Fischer's medium supplemented with 10% heat-inactivated horse serum. Sources of cell lines have been acknowledged (Cass & Au-Yeung, 1976; Paterson et al., 1983a).

Nucleoside transport measurements

To determine the NBMPR sensitivity of cellular nucleoside transport systems, cells (4-8 x 10^6) were incubated for 10 min at 22 °C in transport medium (serum-free growth medium without bicarbonate, supplemented with 20 mM-Hepes) containing the indicated concentrations of the nucleoside transport inhibitor. A portion of each of these cell suspensions was then centrifuged (16,000 g, 30 s) to obtain a cell-free medium fraction to which [3H]adenosine was added to achieve a final concentration of 20 μM. Portions (100 μl) of the remaining cell suspension were layered over 100 μl of an oil mixture (Harley et al., 1982) in 1.5 ml microcentrifuge tubes, and intervals of [3H]adenosine uptake were initiated by the rapid addition of 100 μl of the [3H]adenosine solutions in medium. Uptake intervals (4 s) were determined by pelleting the cells (16,000 g, 30 s) under the oil layers. After the completion of all assays, supernatants were aspirated and tubes washed above the oil layer with water. The wash water was aspirated together with most of the oil, and cell pellets were dissolved in 0.5 ml of 1% Triton X-100 and assayed for 3H content in a liquid-scintillation system. Assays were conducted in triplicate. In each experiment, the extracellular space in cell pellets was determined by substituting [14C]sucrose (5-10 μCi/ml) for [3H]adenosine in some assays. The measured 3H content of each cell pellet was corrected for the presence of [3H]adenosine in the extracellular space of the pellet. The rates of adenosine uptake determined from 4 s intervals are not presented as rigorously determined initial rates of permeant uptake that by definition are transport rates, but as reasonable approximations of the latter.

Preparation of membrane-enriched fraction

The method of Ross et al. (1977) described for S49 mouse lymphoma cells was used. The applicability of this procedure to Novikoff UA cells was confirmed by examination of Wright stained cytospin preparations of cell homogenates. All steps were performed at 4-8 °C. Washed cells were disrupted by N2 cavitation and the homogenate was centrifuged at 900 g for 5 min. The supernatant was then centrifuged at 41,000 g for 20 min to pellet a membrane-enriched fraction. This fraction was gently resuspended in 50 mM-Tris/HCl (pH 7.4 at 22 °C) at a protein concentration of 14-16 mg/ml by using a Dounce homogenizer, and stored in a liquid-N2 refrigerator. The membrane fraction from 10^6 cells contained about 25 mg of protein, or about 12% of total cell protein. Protein was determined by a modification (Peterson, 1977) of the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Equilibrium binding experiments

Portions of the membrane-containing preparation (20 μl, 300 μg of protein) were added to 1.0 ml of 50 mM-Tris/HCl (pH 7.4 at 22 °C) containing graded concentrations of [3H]NBMPR (0.04-4 nM) in the presence or absence of NBMPR (0.1 μM), the former condition providing determinations of non-specific binding. NBMPR is a very potent inhibitor of nucleoside transport that is tightly bound and competes with NBMPR at the high-affinity, transporter-associated binding sites (Paterson et al., 1983b). In some experiments, other substances were included as inhibitors of NBMPR binding. After incubation of the suspensions at 22 °C for 30 min, the crude membranes were collected onto Whatman GF/C glass fibre filters (prewashed with 5 ml of ice-cold Tris buffer). The filters were washed twice with 5 ml portions of ice-cold buffer and incubated in a Triton-based liquid-scintillation cocktail (Pande, 1976) for at least 2 h before assay of radioactivity. Equilibrium concentrations of free [3H]NBMPR were determined by subtraction of bound radioactivity from the total determined for each incubation suspension. Specific binding was determined as the difference between binding in the absence (total binding) and in the presence (non-specific binding) of 10 μM-NBMPR.

Photoaffinity labelling

Portions of the membrane-containing preparation (about 1.5 mg of protein) were incubated in 50 mM-Tris/HCl (pH 7.4 at 22 °C) containing 50 nM-[3H]-NBMPR and 50 mM-dithiothreitol in a total volume of 200 μl. In some experiments, nucleoside permeants or other transport inhibitors were included to determine their effects on NBMPR binding. The suspensions were incubated for 15 min at 22 °C, then chilled to 4 °C and transferred to 1 mm light path quartz cuvettes. The cuvettes were kept chilled during a 1 min irradiation with u.v. light at a distance of 4 cm from the quartz cooling sleeve of a 450 W mercury-arc lamp (Canrad-Hanovia Inc., Newark, NJ, U.S.A.). The suspension was then diluted 75-fold in Tris buffer containing 10 μM-NBMPR and allowed to stand at room temperature for 20 min. The membrane fraction was recovered by centrifugation at 41,000 g for 25 min and dissolved at room temperature in gel sample buffer [5% (w/v) SDS, 5 mM-Na2 EDTA, 10% (w/v) glycerol, 0.01% Bromphenol Blue, 2 mM-phenylmethanesulphonyl fluoride, 10 mM-dithiothreitol, and 20 mM-Tris/HCl (pH 8.0 at 22 °C)].

Gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed in 1.5 mm thick acrylamide slab gels in the discontinuous buffer system of Laemmli (1970) according to the method of Thompson & Maddy (1982). The acrylamide concentration in the resolving gel was 12%. Migration distances of standard proteins or membrane proteins were determined after staining with Coomassie Blue. Radioactivity was measured in a liquid-scintillation system after extraction of 2 mm slices of the gel lanes into
Photoaffinity labelling of a nucleoside transport polypeptide

a solution of 3% Protosol in Econofluor. Recovery of radioactivity from the gels was about 80%.

Chemicals

[3H]NBMPR (16 Ci/mmol) and [2,8-3H]adenosine (33 Ci/mmol) were purchased from Moravek Biochemicals, Brea, CA, U.S.A. [U-14C]Sucrose (584 Ci/mol) was from Amersham, Oakville, Ont., Canada. Dipyridamole was a gift from Boehringer Ingelheim (Canada), Burlington, Ont., Canada. Molecular mass standards for gel electrophoresis were purchased from Bio-Rad Laboratories (Canada), Mississauga, Ont., Canada. Materials for cell culture were purchased from Gibco (Canada), Burlington, Ont., Canada. Protosol and Econofluor were purchased from NEN (Canada), Lachine, Que., Canada.

RESULTS

Inhibition of adenosine transport by NBMPR

The effect of prior equilibration with NBMPR on adenosine transport in Novikoff UA cells is shown in Fig. 1. Results of identical experiments with S49, L1210, and Walker 256 cells were included for comparison. In S49 cells, the transport of adenosine was sensitive to NBMPR with an IC50 value (that concentration of NBMPR capable of reducing the rate of adenosine transport to 50% of the control value) of 2 nM, and inhibition of transport was virtually complete when cells were exposed to 100 nM-NBMPR. In contrast, both Walker 256 and Novikoff UA cells were relatively insensitive to NBMPR with 50% inhibition of transport resulting from exposure to NBMPR concentrations greater than 10 µM. These high IC50 values are in agreement with previous reports (Wohlhueter et al., 1979; Paterson et al., 1983a; Belt, 1983) that both Novikoff and Walker cells have nucleoside transport mechanisms of low sensitivity to

Fig. 1. NBMPR inhibition of adenosine transport

Cells were incubated with the indicated concentration of NBMPR for 10 min and assayed for adenosine transport activity as described in the Materials and methods section. S49 cells (○), L1210 cells (●), Walker 256 cells (■), and Novikoff UA cells (□).

NBMPR. The concentration-effect curve for NBMPR inhibition of adenosine transport in L1210 cells was biphasic with a broad plateau spanning the range from 10 to 1000 nM-NBMPR, indicating that these cells have two types of transport activity that differ in sensitivity to NBMPR (Belt, 1983). The IC50 value for NBMPR inhibition of the sensitive component of transport (5 nM) was similar to that seen in S49 cells.

Equilibrium binding experiments

Saturable, high-affinity binding of [3H]NBMPR to the membrane-enriched fraction from Novikoff UA cells was demonstrated. A component of binding that did not saturate and was evident in the presence of 10 µM-NBTGR represented non-specific binding at the level of about 2% of the total binding when the free ligand concentration equaled its KD. Fig. 2 shows the results of a typical equilibrium binding experiment and a Scatchard analysis of the data. The average values for the binding constants (+ S.D.) determined in five experiments (representing four membrane preparations) were: KD, 0.60 ± 0.40 nM; Bmax, 1.0 ± 0.6 pmol/mg of protein. The membrane preparations were enriched about 4-fold over intact Novikoff UA cells in terms of the molar content of site-bound NBMPR per mg of protein.

The experiment of Fig. 3 demonstrates inhibition of NBMPR binding to Novikoff UA membranes by dipyridamole, a potent inhibitor of nucleoside transport structurally dissimilar to NBMPR, and by uridine.

Fig. 2. Equilibrium binding of [3H]NBMPR to the membrane-enriched fraction from Novikoff UA cells

Membranes were incubated with graded concentrations of [3H]NBMPR in the absence (○) or presence (□) of 10 µM-NBTGR for 30 min at 22°C. Radioactivity bound to the membranes collected on glass fibre filters was determined, and the equilibrium free [3H]NBMPR concentration was determined as the difference between total and bound radioactivity. Specifically bound [3H]NBMPR was determined as the difference between bound ligand at equilibrium in the absence and in the presence of 10 µM-NBTGR. Values are the means of duplicate estimates. The inset shows a Scatchard analysis of the relationship between specifically bound [3H]NBMPR (B) and equilibrium concentration of free [3H]NBMPR (F). This analysis yielded these binding constants: KD, 0.48 nM; Bmax, 0.55 pmol/mg of protein.
Equilibrium binding of [H]NBMPR to a membrane-containing preparation was assayed as described in the text, with uridine (1 mM) or dipyridamole (3 µM) included in the [H]NBMPR solution to which the membrane preparation was added. B refers to specifically bound [H]NBMPR and F to the equilibrium concentration of free [H]NBMPR. Values are the means of duplicate estimates. The convergence of the plotted data at the ordinate shows that the site-specific binding of NBMPR is inhibited by both uridine and dipyridamole in an apparently competitive manner.

Interaction of these compounds with the nucleoside transport mechanism of Novikoff cells has been described (Plagemann & Wohlhueter, 1980). The double reciprocal plot in Fig. 3 shows that both dipyridamole and uridine inhibited binding of NBMPR to the membrane preparation from Novikoff UA cells in an apparently competitive manner.

**Photoaffinity labelling**

Covalent labelling of Novikoff UA membrane polypeptides with [H]NBMPR was demonstrated by SDS/polyacrylamide-gel electrophoresis. In Fig. 4, the gel radioactivity profiles of membrane preparation polypeptides photolabelled in the absence and presence of NBTGR indicate total and non-specific covalent labelling, respectively. Site-specific NBMPR labelling was restricted to a single, sharp, symmetrical peak of radioactivity, which migrated with polypeptides of apparent Mr 72000–80000. A second peak of radioactivity migrated just behind the tracking dye and apparently represented non-specific labelling and/or unbound ligand because this peak was not reduced in the presence of NBTGR. Approx. 40% of the available NBMPR-binding sites were covalently labelled under the experimental conditions described.

When the ligand binding and photoactivation procedures were performed in the presence of 5 mM adenosine or 50 µM dipyridamole, specific covalent labelling of the Mr 72000–80000 polypeptide(s) was reduced to 25% and 35% of the control value, respectively. The presence of 10 mM uridine resulted in a small (90% of control) but reproducible reduction in labelling of the polypeptide. No decrease in specific labelling of the NBMPR-binding polypeptide was observed when adenosine or uridine solutions at the above concentrations were present in a separate cuvette between the u.v. light source and the sample cuvette during photoactivation, indicating that absorption ('shielding') of u.v. light by adenosine or uridine in the photoactivation mixture did not account for the adenosine and uridine effects on NBMPR labelling.

The apparent Mr of the [H]NBMPR-photolabelled polypeptide from the membrane preparation from Novikoff UA cells differs from the values (45000–66000) reported for the labelled binding site polypeptides from other cell types. It is unlikely that the higher Mr of the Novikoff UA polypeptide reported here is an artifact resulting from aggregation of proteins in the presence of u.v. light, since Coomassie Blue staining of membrane preparations from Novikoff UA cells before and after u.v. irradiation showed no apparent differences in protein band patterns in the SDS-gels.

A membrane-enriched fraction from S49 cells was photolabelled under the same conditions in which Novikoff UA membrane preparations were labelled, with the result that site-specific radioactivity was restricted to a polypeptide with an apparent Mr of about
DISCUSSION

An NBMPR-binding polypeptide from a membrane-enriched fraction of Novikoff UA cells has been identified by covalent labelling with $[^3H]$NBMPR. Although these cells have binding sites for NBMPR, nucleoside transport remains unaffected in the presence of site-saturating concentrations of the inhibitor. The apparent $M_r$ of this polypeptide determined by SDS/polyacrylamide-gel electrophoresis was 72000–80000, a value that differs from those of transport polypeptides in several other cell types (45000–66000), determined by the same photoaffinity labelling procedures. Identification of the NBMPR-photolabelled polypeptides as nucleoside transporter components has been based mainly on the concept that the high affinity membrane sites at which NBMPR is bound are part of the nucleoside transport mechanism. Evidence for this concept includes (a) the observed proportionality between binding site occupancy by NBMPR and fractional inhibition of uridine transport by NBMPR in human erythrocytes (Cass et al., 1974), (b) instances of the joint presence of functional nucleoside transporters and of high affinity NBMPR binding sites in some cell types, or the joint absence of these properties in other cell types (Cass et al., 1981; Young & Jarvis, 1983), (c) the proportionality between the nucleoside transport capacity ($V_{\text{max}}$) and the abundance of the high affinity binding sites in erythrocytes from various species (Jarvis et al., 1982; Jarvis & Young, 1982), and (d) the joint reconstitution of uridine transport activity by the incorporation into phospholipid vesicles of partially purified NBMPR-binding protein from human erythrocytes (Tse et al., 1985).

While some types of cells (notably erythrocytes and S49 cells) possess NBMPR-sensitive nucleoside transporters with sites that bind NBMPR with high affinity (as noted above), the existence of nucleoside transport mechanisms of low sensitivity to NBMPR has been recognized in other cell types (for example, Novikoff UA and Walker 256 cells) in which substantial inhibition of transport occurs only in the presence of micromolar concentrations of NBMPR. Thus, while nucleoside permeation is mediated in cultured cells of two lines, the Walker 256 carcinosarcoma (Belt & Noel, 1985) and the Novikoff hepatoma N1S1-67 wild type (Plagemann & Wohlhueter, 1980), cells of both lines lack sites that bind NBMPR with high affinity (Paterson et al., 1983a; Plagemann & Wohlhueter, 1984). Other cell types (for example, L1210, HeLa and CHO cells) appear to possess nucleoside transporters of both low and high NBMPR sensitivity. The latter are apparent in the biphasic responses (inhibition) that are seen when cells are exposed to graded concentrations (from micromolar to nanomolar) of NBMPR (Belt, 1983; Dahlg-Harley et al., 1981; Plagemann & Wohlhueter, 1984). Sites on these cells that bind NBMPR with high affinity are likely associated with nucleoside transporters of high NBMPR sensitivity. The Novikoff UA cells used in this study present a further complication in that, while the nucleoside transport mechanism in these cells is of low NBMPR sensitivity, sites that bind NBMPR with high affinity are present.

The uncloned cell stock (Novikoff N1S1-67 wild type; Plagemann & Wohlhueter, 1984), from which our Novikoff UA line was derived, evidently contains variants which synthesize polypeptides that bind NBMPR with high affinity (as in S49 cells and

---

50000–54000. Fig. 5 compares gel radioactivity profiles obtained with membrane preparations from S49 cells and Novikoff UA cells. These preparations were photolabelled separately and then subjected to electrophoretic analysis, each in separate lanes (Fig. 5a) in the same gel slab, or together in a third lane (Fig. 5b). These Figures show that, in the mixed sample, two specifically labelled polypeptide bands were evident, and that the $M_r$ values of these corresponded to those in the preparations from each cell line.

Vol. 236
erythrocytes). The presence of binding site variants in the uncloned Novikoff UA cell line has been shown (J. A. Belt, unpublished work) by the isolation of clones that (a) lack NBMPR binding sites, or (b) possess such sites at several different levels of cellular abundance. Covalent NBMPR labelling (W. P. Gati & A. R. P. Paterson, unpublished work) of a membrane-enriched preparation from one of the latter clones (in which cells had nine times as many binding sites as cells of the parental Novikoff UA line) with [3H]NBMPR resulted in identification of a polypeptide with an Mr similar to that found in membranes from Novikoff UA cells in this study (Figs. 4 and 5).

Our identification of the NBMPR-binding polypeptide in Novikoff UA cells as a transporter polypeptide is supported by the observed blockade of NBMPR photolabelling in the presence of adenosine, uridine, and dipyridamole, substances that interact with the nucleoside transport mechanism in Novikoff cells (Plagemann & Wohlhueter, 1980). The apparently competitive inhibition by uridine and dipyridamole of NBMPR binding to Novikoff UA membrane sites does not necessarily mean that these substances interact at the nucleoside permeation site, as we have argued elsewhere (Koren et al., 1983; Jarvis et al., 1983).

Although high affinity binding of NBMPR to the Novikoff UA polypeptide and inhibition by nucleoside permeants of this binding support the idea that this polypeptide is part of the transport mechanism, its role in nucleoside permeation remains unclear. The high Mr of the Novikoff UA NBMPR-binding polypeptide may be related to the low NBMPR sensitivity of nucleoside transport in these cells. Additional studies are required to determine whether this apparently distinctive molecular property restricts the transport inhibitory effects of bound NBMPR.

This work was supported by the National Cancer Institute of Canada (grant to A. R. P. P.), the Alberta Heritage Savings Trust Fund: Applied Cancer Research Program (Alberta Cancer Board) (grant to A. R. P. P., S. M. J. and J. D. Y.), and the National Cancer Institute (U.S.A.) (grant CA33362 to J. A. B.). J. D. Y. thanks the Cancer Research Campaign, U.K., for a project grant. S. M. J. is a Scholar of the Alberta Heritage Foundation for Medical Research. A. R. P. is a Research Associate of the National Cancer Institute of Canada. We thank Dr. R. M. Wohlhueter for karyotypic confirmation of the identity of our line of Novikoff cells. The skilled technical assistance of Michael Prendergast is acknowledged.

REFERENCES

Received 21 October 1985/6 January 1986; accepted 7 February 1986