Solubilization and reconstitution of a nucleoside-transport system from Ehrlich ascites-tumour cells

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Uptake of [³H]uridine by Ehrlich cells was mediated by both nitrobenzylthioinosine (NBMPR)-sensitive (75 %) and NBMPR-insensitive (25 %) mechanisms. Each cell contained approx. 26000 high-affinity ($K_D = 0.19$ nM) recognition sites for [³H]NBMPR, and binding was inhibited by dipyridamole and adenosine at concentrations similar to those required for inhibition of [³H]uridine uptake. Calculations show that each cell contains a total of about 35000 nucleoside transporters. Photoaffinity labelling of a partially purified preparation of plasma membranes with [³H]NBMPR resulted in a single broad ³H-labelled band on SDS/polyacrylamide gels, with an apparent molecular-mass peak of 42 kDa. This is in contrast with human erythrocyte membranes, where [³H]NBMPR photolabelled two broad bands with peaks at 55 and 80 kDa. Treatment of photoaffinity-labelled membranes with endoglycosidase F decreased the apparent molecular masses of both the Ehrlich-cell and erythrocyte [³H]NBMPR-labelled proteins to approx. 40 kDa. These results suggest that the human erythrocyte [³H]NBMPR-binding polypeptides are more extensively glycosylated than the corresponding Ehrlich-cell polypeptides. Octyl β -D-glucopyranoside [1.0 % (w/v) + asolectin] solubilized over 90 % of the [³H]NBMPR-binding sites, with near-complete retention of [³H]NBMPR-binding characteristics. The only major change was a 65-fold decrease in affinity for dipyridamole, which was partly reversed upon incorporation of the solubilized proteins into asolectin membranes. Proteoliposomes, prepared by using asolectin and the octyl glucoside-solubilized plasma membranes, were capable of accumulating [³H]uridine via a protein-dependent dipyridamole/nitrobenzylthioguanosine/dilazep-sensitive mechanism. We have thus demonstrated the efficient solubilization and functional reconstitution of a nucleoside-transport system from Ehrlich ascites-tumour cells.

INTRODUCTION

Nucleoside permeation of cell membranes occurs primarily by way of a non-concentrative facilitated-diffusion mechanism, which accepts as substrates a variety of purine and pyrimidine nucleosides [1,2]. This nucleoside-transport system (NTS), with subsequent intracellular metabolism, is the principal means by which the extracellular receptor-mediated actions of nucleosides, such as adenosine, are terminated [3,4]. Early studies with erythrocytes found that nucleoside transport could be inhibited specifically with nanomolar concentrations of compounds such as 6-[(4-nitrobenzyl)thio]-9- β -D-ribofuranosylpurine (nitrobenzylthioinosine, NBMPR) and dipyridamole [5–7]. Subsequent studies with [³H]NBMPR established it as both a reversible [8–14] and an irreversible [15–20] probe for membrane proteins associated with the NTS. In erythrocytes, the binding of [³H]NBMPR was correlated directly with the inhibition of nucleoside transport [8,21], and inhibition of [³H]NBMPR binding reflects the capacity of a compound to inhibit nucleoside transport [22]. More recent studies using a variety of cell types have, however, now established that multiple forms of nucleoside transporters exist in mammalian cell membranes, which differ in their sensitivities to inhibition by NBMPR [12,14,23–25] and

dipyridamole [11,13,18,19,26]. Recent work suggests that these transporters are products of different genes [27]; however, it is premature to conclude that differences in membrane organization and/or lipid environment may not affect the interaction of NBMPR with the transporter, which might appear as a genetic distinction between NBMPR-resistant and -sensitive transport [12,28]. The molecular nature of the mammalian NTS will not be fully appreciated until it proves possible to isolate the protein(s) responsible for nucleoside transport, and functionally reconstitute the putatively different systems in artificial lipid vesicles (liposomes).

Tse *et al.* [29] have solubilized, and re-incorporated in asolectin-vesicle membranes, the human erythrocyte nucleoside transporter. They showed that, upon removal of detergent, the binding of [³H]NBMPR and nucleoside transport were restored. However, the nucleoside transporter of human erythrocytes is uniquely homogeneous [8–10,21], and it may be necessary to study a more complex cell type to discern fully the heterogeneous molecular characteristics of the mammalian NTS.

The present study describes the characterization, solubilization and functional reconstitution of a nucleoside-transport mechanism from Ehrlich ascites-tumour cells which possess both NBMPR-sensitive and NBMPR-insensitive nucleoside transporters.

Abbreviations used: NTS, nucleoside-transport system; NBMPR, nitrobenzylthioinosine; NBTGR, nitrobenzylthioguanosine; OCTG, octyl β -D-glucopyranoside.

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EXPERIMENTAL

Materials

[¹⁴C]cholesteryl oleate (58 mCi/mmol) and [4(n)-³H]cytochalasin B (22 Ci/mmol) were purchased from Amersham Canada. [³H]uridine (40–45 Ci/mmol) and [³H]NBMPR (16 Ci/mmol) were purchased from ICN Canada and Moravsek Biochemicals Inc. (Brea, CA, U.S.A.) respectively, and ³H₂O (1 mCi/g), [¹⁴C]carboxymethyl-dextran (1 mCi/g) and Protosol were purchased from New England Nuclear. Endoglycosidase F, isolated from *Flavobacterium meningosepticum*, was purchased from Boehringer, Mannheim, West Germany. Asolectin (soybean phospholipids) was obtained from Associated Concentrates, Woodside, NY, U.S.A. and stored under N₂. Octyl β-D-glucopyranoside (OCTG) was purchased from Calbiochem, and CHAPS, digitonin, nitrobenzylthioguanosine (NBTGR), NBMPR and dipyrindamole were supplied by Sigma. Cholic acid, purchased from Eastman Kodak, was recrystallized three times from ethanol before use [30]. Dilazep was generously provided by Asta Werke, Germany. All other compounds were of reagent grade.

Isolation of Ehrlich-cell plasma membranes

Ehrlich ascites-tumour cells were grown as an intraperitoneal culture in mice (Swiss, male; approx. 30 g), and plasma membranes were prepared as described previously [31–33]. In brief, washed cells were swollen by suspension in 1 mM-ZnCl₂, fragmented by using a Polytron homogenizer PT-10-20, and resuspended in 9.25% (w/w) sucrose with removal of nuclei by centrifugation (three times) for 1 min at 900 g. The plasma membranes were then separated on a 30–60% sucrose gradient, and stored at –70 °C in 15% (v/v) dimethyl sulphoxide. In some cases, phenylmethanesulphonyl fluoride (final concn. 6 μM) and soybean trypsin inhibitor (final concn. 50 μg/ml) were included in the membrane isolation procedure. Sucrose solutions were in 0.5 mM-ZnCl₂/5 mM-K₂HPO₄, pH 7.5.

Solubilization of plasma membranes

Washed plasma membranes were incubated with detergent in either 50 mM-Tris/citrate or 100 mM-KCl/10 mM-Tris/HCl/0.1 mM-CaCl₂/0.1 mM-MgCl₂ (reconstitution buffer; pH 7.4) at 4 °C for 60 min. Comparable results were obtained with either buffer. After incubation, the mixtures were centrifuged at 120 000 g for 60 min and the supernatant was retained for immediate use for radioligand binding or reconstitution.

Reconstitution procedure

This was based on a method used for the functional reconstitution of the Ehrlich ascites-cell glucose and amino acid transport systems [34]. Asolectin was suspended in reconstitution buffer by vortex-mixing for 5 min, followed by sonication to clarity under N₂ at 4 °C (Branson Ultrasonic Cleaner, B-220). This suspension was then diluted with OCTG to give final concentrations of 0.8% OCTG and 62 mg of asolectin/ml. In experiments where vesicle recovery after gel filtration needed to be determined, a trace amount of [¹⁴C]cholesteryl oleate (~1000 d.p.m./μl) was included in the initial asolectin mixture. Preliminary experiments established that 26 ± 1 d.p.m. of [¹⁴C]cholesteryl oleate was incorporated per

nmol of lipid P in reconstituted vesicles (n = 12), determined by the method of Lowry & Tinsley [35]. The asolectin suspension (450 μl, containing ~28 mg of asolectin) was mixed with 3 ml of membranes solubilized with 1.0% OCTG/0.15% asolectin (~2 mg of protein/ml) and applied to a Sephadex G-50 column (1.5 cm × 28 cm) equilibrated with reconstitution buffer at 4 °C. Turbid fractions, eluted in the void volume, were frozen in ethanol/solid CO₂, thawed at 22 °C, then centrifuged at 40 000 g for 20 min and resuspended in 500 μl of reconstitution buffer. The suspended proteoliposomes were used without further treatment for radioligand-binding studies. To measure [³H]uridine uptake, the proteoliposomes were sonicated for 10 s at 4 °C before use. Brief sonication after the freeze/thaw cycle has been shown to enhance the activity of the reconstituted erythrocyte nucleoside transporter [29].

To obtain control (protein-free) liposomes, the reconstitution procedure was conducted exactly as described above but with a solution of 1.0% OCTG/0.15% asolectin rather than the solubilized membrane preparation.

[³H]uridine uptake

Ehrlich cells. To minimize intracellular metabolism of [³H]uridine, cells were depleted of ATP by sequential incubation with rotenone (20 ng/ml; 15 min at 37 °C) and 2-deoxyglucose (2 mM; 15 min at 37 °C), in Dulbecco's [36] phosphate-buffered saline. This decreased the ATP content of the cell suspensions by approx. 95%, as determined by a modification of the method of Stanley & Williams [37].

All assays were conducted at room temperature (~22 °C) in phosphate-buffered saline (pH 7.4). Where appropriate, cells were preincubated with transport inhibitors for 15 min before use for [³H]uridine uptake. Uptake was initiated by addition of cell suspension (~4 × 10⁷ cells/ml) to a solution of [³H]uridine (100 μM final concn.) layered over a 200 μl cushion of silicone oil/mineral oil (21:4, v/v) in 1.5 ml Eppendorf microcentrifuge tubes. Assays were terminated by centrifugation of the cells through the oil layer at 12 000 g for 5 s. The minimum reliable incubation time that could be achieved with this methodology was 3 s. The supernatants and oil were removed and the cell pellets digested with 1 M-NaOH for a minimum of 16 h at room temperature before determination of the ³H content of the NaOH digest after neutralization with HCl.

Parallel incubations were performed to determine the intracellular water volume of the cell pellet after centrifugation through oil. Cells were incubated with a combination of [¹⁴C]carboxymethyl-dextran and ³H₂O for 3 min at room temperature, and then separated from the extracellular medium by centrifugation through oil. Portions of the supernatants (100 μl) were counted in Aquasol, and cell pellets digested in NaOH were processed for counting as described above. The intracellular water space (V_i) of the cell pellet could then be derived as follows:

$$V_i = \frac{\text{total } ^3\text{H in pellet}}{^3\text{H}/\mu\text{l in supernatant}} - \frac{\text{total } ^{14}\text{C in pellet}}{^{14}\text{C}/\mu\text{l in supernatant}}$$

Proteoliposomes. Uptake of [³H]uridine by proteoliposomes and protein-free vesicles was assessed at 22 °C by using a centrifugal gel-filtration method [38]. Dis-

posable syringes (1 ml), fitted with polyethylene filter supports, were filled with Sephadex G-50 (fine grade) pre-equilibrated with 10 μ M-NBTGR/10 μ M-dilazep in reconstitution buffer at 4 °C. Excess fluid in these columns was removed, immediately before use, by centrifugation at 700 *g* for 45 s. Uptake was initiated by the addition of [³H]uridine (20 μ M final concn.) to a suspension of liposomes (containing [¹⁴C]cholesteryl oleate to calculate vesicle recovery). After a defined incubation time, a 100 μ l portion of this mixture was layered on a Sephadex column, and centrifuged for 45 s at 700 *g*, with the eluate collected directly into a preweighed glass tube. Samples were removed for analysis of protein [39] and ³H and ¹⁴C contents (dual-label counting), and the [³H]uridine content of each sample was normalized for intra-experimental variation in eluate volume and phospholipid content. Results are expressed as fmol of [³H]uridine accumulated per nmol of lipid P, or per mg of reconstituted membrane protein.

[³H]NBMPR binding

Equilibrium binding of [³H]NBMPR to intact Ehrlich cells (1×10^7 cells/ml) was determined at 22 °C in phosphate-buffered saline (pH 7.4) by using a 40 min incubation period. Cells were separated from the incubation medium by centrifugation (12000 *g* for 10 s), and the cell pellet was then washed and digested in 1 M-KOH, as described in previous studies involving human erythrocytes [22].

[³H]NBMPR binding to plasma membranes and liposomes was evaluated at 22 °C in either 50 mM-Tris/citrate or reconstitution buffer, by using a 30 min incubation period. Similar results were obtained with either buffer. The reaction was terminated by vacuum filtration through Whatman GF/B filters, followed by two washes each with 4 ml of buffer at 4 °C.

To measure [³H]NBMPR binding to solubilized membrane preparations, proteins were precipitated before filtration with a mixture of poly(ethylene glycol) (10 %, w/v) and γ -globulins (1.65 mg/ml) after exposure to [³H]NBMPR for 30 min at 22 °C. After filtration, the precipitates were rinsed twice with buffer containing 8 % poly(ethylene glycol), as described previously [40,41]. To compensate for the dilution and re-equilibration that occurred on addition of poly(ethylene glycol) (425 μ l) and γ -globulin (75 μ l) to the 1 ml incubation volume, [³H]NBMPR and inhibitors were present in the incubation media at 1.5 times the concentrations used for the membrane binding assays. Preliminary experiments established that the precipitation procedure did not affect [³H]NBMPR binding to intact membranes.

Non-specific binding of [³H]NBMPR was determined by using 10 μ M-NBTGR, 30 μ M-dipyridamole or 30 μ M-dilazep (15 min preincubation), depending on the specific assay (see the Results section).

Protein was measured by the method of Lowry *et al.* [42], except for reconstituted liposomes, where the method of Markwell *et al.* [39] was used. In all cases, bovine serum albumin was used as the standard, and appropriate controls were included for the effects of detergents and the presence of high concentrations of asolectin.

Preparation of human erythrocyte membranes

Human erythrocyte ghosts were prepared essentially as described by Dodge *et al.* [43], with the following

exceptions: (a) erythrocytes were washed initially with iso-osmotic saline, and (b) membranes were finally resuspended in 5 mM-sodium phosphate (pH 8.0 at 22 °C) and stored at -80 °C for a maximum of 4 weeks.

Photoaffinity labelling with [³H]NBMPR

Washed plasma membranes, from Ehrlich cells or erythrocyte ghosts (1–2 mg of protein/ml), were equilibrated for 90 min at 4 °C with [³H]NBMPR (25 nM) in either reconstitution buffer or 5 mM-sodium phosphate, respectively, in the absence or presence of dilazep (30 μ M). Membrane suspensions (600 μ l) in quartz cuvettes were then supplemented with 25 mM-dithiothreitol (final concn.), and exposed to u.v. light (5 cm from source) from a 100 W mercury arc lamp for 2.5 min (or as indicated in the Results section). For gel electrophoresis, photolabelled membrane suspensions were diluted with 15 vol. of reconstitution buffer, centrifuged at 20000 *g* for 20 min, resuspended in 30 ml of buffer at 4 °C, re-centrifuged as above, and finally resuspended in a minimum volume of buffer (~700 μ l). Samples were removed for protein and ³H analysis, and the membrane suspensions processed for gel electrophoresis as described by Laemmli [44]. For direct analysis of photoaffinity-labelling efficiency, samples were diluted 15-fold with buffer containing NBMPR (10 μ M) and adenosine (10 mM) at 22 °C. After a 30 min incubation at 22 °C, the preparation was centrifuged at 20000 *g* for 20 min and resuspended in 30 ml of buffer containing NBMPR and adenosine as indicated above. This wash procedure was repeated at least three times to remove all non-irreversibly bound [³H]NBMPR. The membranes were subsequently filtered and counted for radioactivity.

Treatment with endoglycosidase F

[³H]NBMPR-labelled membranes were treated with endoglycosidase F as described by Kwong *et al.* [45]. In summary, photolabelled membranes (0.25–0.5 mg of protein) were resuspended in 100 μ l of enzyme digestion buffer, containing 100 mM-sodium phosphate, pH 6.0, 75 mM- β -mercaptoethanol, 50 mM-EDTA, 0.5 % (w/v) Triton X-100, 0.05 % (w/v) SDS, and 1–2 units of endoglycosidase F. The mixture was incubated at 22 °C for 18 h with agitation, and terminated by addition of an equal volume of SDS/polyacrylamide-gel-electrophoresis sample buffer.

SDS/polyacrylamide-gel electrophoresis

Gradient polyacrylamide slab gels (5–15 %, v/v; 1.5 mm thick) were run as described by Laemmli [44]. Samples dissolved in SDS-electrophoresis buffer were heated at 50 °C for 3 min before application to the gel. Gel lanes were either cut into 2 mm slices and digested with Protosol for assay of ³H label, or stained with Coomassie Blue.

RESULTS

[³H]Uridine uptake by Ehrlich ascites-tumour cells

Uptake of [³H]uridine (100 μ M) by Ehrlich cells was rapid ($t_{1/2} \sim 1$ min) and non-concentrative (maximum intracellular concentration of 123 ± 4 pmol/ μ l; $n = 10$; Fig. 1), as expected for a facilitated transport system in an ATP-depleted system. NBMPR and dipyridamole, at

Fig. 1. Time course of $[^3\text{H}]$ uridine influx by Ehrlich ascites cells

Cells were incubated with $100\ \mu\text{M}$ - $[^3\text{H}]$ uridine, in the absence (total accumulation) or presence (non-mediated accumulation) of a combination of $10\ \mu\text{M}$ -NBMPR and $100\ \mu\text{M}$ -dipyridamole, at 22°C for the indicated times. Permeant influx was terminated by centrifugation of the cells through a layer of silicone oil/mineral oil as described in the text. Nucleoside-transporter-mediated influx ($\bullet$) was calculated as the total cellular accumulation of $[^3\text{H}]$ uridine minus the accumulation observed in the presence of NBMPR and dipyridamole ($\blacksquare$). Intracellular water volumes were calculated from $^3\text{H}_2\text{O}$ and $[^{14}\text{C}]$ carboxymethyldextran distributions as described in the text, and the intracellular concentrations of $[^3\text{H}]$ uridine are expressed as $\text{pmol}/\mu\text{l}$ of intracellular water. Each point represents the mean $\pm$ S.E.M. from 10 experiments.

concentrations greater than $10\ \mu\text{M}$, inhibited almost completely the cellular uptake of $[^3\text{H}]$ uridine. The uptake which remained was linear with incubation time, and probably represents passive diffusion and/or non-specific interactions of $[^3\text{H}]$ uridine with the cell membranes. The difference between $[^3\text{H}]$ uridine uptake in the absence and presence of $10\ \mu\text{M}$ -NBMPR or $100\ \mu\text{M}$ -dipyridamole is operationally defined as NTS-mediated uptake. At an external concentration of $100\ \mu\text{M}$ -uridine, the initial rate of uptake, deduced from the linear portion of the curve for mediated uptake (Fig. 1), was $1.38 \pm 0.09\ \text{pmol/s}$ per μl of intracellular water ($n = 10$).

Subsequent experiments revealed that dipyridamole inhibited the $[^3\text{H}]$ uridine uptake (20 s incubation) in a monophasic manner with an IC_{50} (concn. giving 50% inhibition) of $126\ \text{nM}$, whereas NBMPR showed a distinctly biphasic inhibition profile (Fig. 2). Cellular accumulation of $[^3\text{H}]$ uridine was inhibited by about 75% with $10\ \text{nM}$ -NBMPR, but concentrations of NBMPR in excess of $1\ \mu\text{M}$ were required to inhibit the residual transport activity. Adenosine also inhibited the influx of $[^3\text{H}]$ uridine ($\text{IC}_{50} = 53\ \mu\text{M}$), but behaved similarly to dipyridamole in that it did not discriminate between the NBMPR-sensitive and NBMPR-insensitive forms of the transporter (Fig. 2).

$[^3\text{H}]$ NBMPR binding to Ehrlich cells and plasma membranes

$[^3\text{H}]$ NBMPR bound with high affinity to a single class of specific sites in intact Ehrlich cells ($B_{\text{max.}} \approx 26000$ molecules/cell, $K_D = 0.84\ \text{nM}$) and partially purified plasma membranes ($B_{\text{max.}} \approx 13\ \text{pmol/mg}$ of protein, K_D

Fig. 2. Inhibition of $[^3\text{H}]$ uridine influx in Ehrlich ascites cells by NBMPR, dipyridamole and adenosine

Cells were preincubated with the indicated concentrations of inhibitor at 22°C for at least 15 min and then incubated with $100\ \mu\text{M}$ - $[^3\text{H}]$ uridine for 20 s. Cells were separated from the incubation medium by rapid centrifugation through an oil layer, and the ^3H content of the cell pellet was assessed by standard liquid-scintillation counting techniques. Influx in the presence of inhibitor is expressed as a percentage of the amount of $[^3\text{H}]$ uridine accumulated by the cells in the absence of drug (control uptake). Each point represents the mean $\pm$ S.E.M. from at least six independent experiments. IC_{50} values: adenosine, $53 \pm 6\ \mu\text{M}$; dipyridamole, $126 \pm 11\ \text{nM}$; NBMPR, $0.12 \pm 0.01\ \text{nM}$ and $1.6 \pm 0.2\ \mu\text{M}$ for the high- and low-affinity components respectively.

Table 1. Comparison of $[^3\text{H}]$ NBMPR-binding characteristics in intact Ehrlich cells, native plasma membranes, solubilized membranes and reconstituted proteoliposomes

Plasma-membrane proteins were solubilized with OCTG/asolectin and reconstituted in liposomal membranes as described in the text. Binding parameters (equilibrium dissociation constant, K_D ; maximum density of binding sites per mg of total protein, B_{max} ; Hill coefficient, h) were determined by mass-law analysis of data obtained from assays conducted at 22°C . Each value represents the mean $\pm$ S.E.M. from n experiments.

	n	K_D (nM)	$B_{\text{max.}}$ (pmol/mg of protein)	h
Cells	7	0.842 ± 0.098	0.51 ± 0.19	1.02 ± 0.06
Membranes	6	0.188 ± 0.043	12.71 ± 2.11	0.98 ± 0.07
Solubilized membranes	5	0.613 ± 0.122	13.35 ± 1.64	1.00 ± 0.01
Liposomes	6	0.188 ± 0.031	4.55 ± 1.19	0.91 ± 0.05

$= 0.19\ \text{nM}$) (Table 1, Fig. 3). Adenosine, dipyridamole and non-radiolabelled NBMPR inhibited the binding of $[^3\text{H}]$ NBMPR to Ehrlich-cell membranes in a monophasic manner, with K_i values of $87\ \mu\text{M}$ for adenosine, $0.18\ \mu\text{M}$ for dipyridamole and $0.15\ \text{nM}$ for NBMPR (Fig. 4). These values correspond well to the inhibitory potencies of these agents on $[^3\text{H}]$ uridine uptake by intact Ehrlich cells (see above).

Fig. 3. Binding of [3 H]NBMPR to plasma membranes prepared from Ehrlich ascites cells

Membranes were incubated with graded concentrations of [3 H]NBMPR at 22 °C in the absence (total) and presence (non-specific) of 10 μ M-NBMPR, 30 μ M-dipyridamole or 30 μ M-dilazep. Specific binding is defined as the total minus the non-specific binding. The [3 H]NBMPR concentrations represent the free concentrations present when equilibrium was attained (abscissa). This is a representative plot from the five experiments performed in duplicate. Inset: mass-law analysis (Scatchard plot) of the relationship between specific binding of [3 H]NBMPR and the equilibrium concentrations of free [3 H]NBMPR. Ordinate, ratio of bound to free ligand concentration (pmol bound/mg of protein per nM); abscissa, membrane-associated [3 H]NBMPR (pmol bound/mg of protein).

Table 2. Comparison of [3 H]NBMPR-binding characteristics of native Ehrlich-cell plasma membranes with those of a variety of detergent-solubilized membrane preparations

Membranes were solubilized with OCTG [1.0 %, w/v; with (+) or without (–) 0.15 % asolectin], cholate (2.5 %, w/v), CHAPS (0.5 %, w/v), or digitonin (0.1 %, w/v). Binding parameters (see Table 1 legend) were determined by mass-law analysis of data obtained from assays conducted at 22 °C as described in the text. All binding assays, including those using intact membranes, were terminated by the poly(ethylene glycol)-precipitation/filtration procedure (see the Experimental section). The relative solubilizing efficiency of each detergent is indicated by the percentage solubilization of [3 H]NBMPR-binding activity and protein from the plasma membranes. Each value represents the mean $\pm$ S.E.M. from n experiments.

	n	K_D (nM)	B_{max} (pmol/mg of protein)	h	Solubilization (%)	
					NBMPR	Protein
Membranes	12	0.20 ± 0.02	10.6 ± 0.6	0.94 ± 0.02		
OCTG (–)	5	0.46 ± 0.03	7.3 ± 1.2	1.00 ± 0.02	34 ± 4	49 ± 2
OCTG (+)	5	0.61 ± 0.12	13.4 ± 1.6	1.00 ± 0.01	90 ± 5	75 ± 7
Cholate	4	2.57 ± 0.51	3.6 ± 1.2	1.00 ± 0.01	38 ± 6	111 ± 6
CHAPS	4	0.35 ± 0.13	4.8 ± 0.6	1.03 ± 0.04	32 ± 8	66 ± 11
Digitonin	2	0.38	6.1	1.01	36	79

Photoaffinity labelling with [3 H]NBMPR

Ehrlich-cell plasma membranes. Although irreversible binding of [3 H]NBMPR increased with time of exposure to u.v. light, in both the presence and the absence of inhibitors, the percentage 'specific' photolabelling of proteins associated with the NTS, defined as the difference between [3 H]NBMPR binding in the absence and presence of 10 μ M-dilazep or NBMPR, decreased after exposure times in excess of 2.5 min. At 2.5 min exposure, [3 H]NBMPR bound irreversibly to approx. 10 % of the calculated NBMPR-sensitive transport sites, and 70 % of this binding was prevented by preincubation of the membranes with dilazep or NBMPR. Polyacrylamide-

gel electrophoresis showed a single major band of radio-labelled polypeptides with apparent molecular masses of 40–50 kDa, with a peak at 42 kDa (Fig. 5). This band of radiolabelling typically represented about 45 % of the total [3 H]NBMPR detected in the gel (excluding the radioactivity associated with the dye front), and was eliminated completely by incubating the membranes with dilazep before photolabelling. In some experiments, minor peaks of radiolabelling were also observed in the range of 100 kDa and 32 kDa (e.g. see Fig. 6), possibly representing aggregates and proteolytic products, respectively, of the major 42 kDa polypeptide. Similar results were obtained with plasma membranes prepared in the absence or presence of phenylmethanesulphonyl

Fig. 4. Inhibition of the site-specific binding of [^3H]NBMPR to intact plasma membranes, solubilized membranes and liposome-reconstituted membrane proteins by adenosine (a), dipyridamole (b) and non-radiolabelled NBMPR (c)

Ehrlich-cell plasma membranes (Memb, ▲) were solubilized with OCTG (1%)/asolectin (0.15%) (Sol, ●), and the solubilized material was then incorporated into asolectin vesicles (Lips, □) as described in the text. Each preparation was incubated with 0.5 nM [^3H]NBMPR for 40 min at 22 °C in the absence (control) or presence of inhibitor (abscissa). Site-specific binding in the presence of inhibitor (ordinate) is expressed as a percentage of control binding. The inhibition constants (K_i values, mean $\pm$ S.E.M.) for each compound are shown in the panels. K_i values were calculated from these data as: $\text{IC}_{50}/\{1 + ([L]/K_D)\}$, where $[L]$ and K_D represent the assay concentration and equilibrium dissociation constant of [^3H]NBMPR respectively; IC_{50} values were calculated from computer-generated best-fit curves to the data. Each point represents the mean $\pm$ S.E.M. from the number of experiments indicated in parentheses. Error bars are shown only when the S.E.M. exceeds the diameter of the graphical data point.

fluoride and soybean trypsin inhibitor. Interestingly, no significant incorporation of [^3H]NBMPR was apparent in the 50–65 kDa range, which is in contrast with previous studies on the photoaffinity labelling of [^3H]NBMPR-binding proteins in erythrocyte [16,20] (and see below), lung [18] and cardiac [46] membranes.

Human erythrocyte membranes. Erythrocyte membranes were photolabelled with [^3H]NBMPR and subjected to polyacrylamide-gel electrophoresis in parallel with the [^3H]NBMPR-photolabelled Ehrlich-cell membranes. Most of the [^3H]NBMPR-radiolabelled polypeptides in erythrocyte membranes (60% of the total [^3H]NBMPR detected on the gel) appeared as two broad bands with apparent molecular masses of 40–60 kDa (major) and 70–110 kDa (minor), with peaks at 55 and 80 kDa, respectively (Fig. 5). The minor high- M_r peak probably represents aggregates of the 40–60 kDa ^3H -labelled polypeptides. These results agree with previous studies on [^3H]NBMPR photolabelling of erythrocyte membranes [16,20,45].

Effect of membrane treatment with endoglycosidase F. The apparent molecular mass of the [^3H]NBMPR-photolabelled polypeptides in both Ehrlich-cell and human erythrocyte membranes was decreased to approx. 38–40 kDa after incubation with endoglycosidase F (Fig. 6). Furthermore, the low-molecular-mass band of radiolabelling observed for erythrocyte membranes was more discrete after incubation with endoglycosidase F (36–44 kDa), and was similar to the gel profile obtained for [^3H]NBMPR-labelled Ehrlich-cell membranes both before and after deglycosylation (Fig. 6). In erythrocyte membranes, the high-molecular-mass band of ^3H -labelled protein was also decreased after incubation with endoglycosidase F, to approx. 70 kDa.

Solubilization of [^3H]NBMPR-binding activity

Of a number of detergents examined, only membranes solubilized with Triton X-100 or Lubrol PX failed to show specific [^3H]NBMPR binding (Table 2). CHAPS and digitonin had the least effect on the binding affinity of [^3H]NBMPR for the solubilized membranes (compare data in Table 2). Although with cholate there was a 9-fold decrease in [^3H]NBMPR-binding affinity, the affinity was restored to 0.10 nM upon incorporation of the solubilized material into asolectin liposomes (results not shown). It is noteworthy that in the presence of asolectin (0.15%, w/v), OCTG solubilized 90% of the [^3H]NBMPR-binding sites and only 75% of the total membrane protein. Without asolectin relatively more protein than specific binding sites were solubilized. Asolectin did not influence the specific solubilization of [^3H]NBMPR-binding sites by any of the other detergents tested. Solubilization of [^3H]NBMPR-binding activity was optimal at 1.0% (w/v) OCTG (+ asolectin), but was dependent to some extent on the concentration of membrane protein used (Table 3). Subsequent experiments focused on the use of OCTG/asolectin for the solubilization and reconstitution of the NBMPR-sensitive NTS.

Compared with native membranes, solubilization decreased the affinity of adenosine and NBMPR for the [^3H]NBMPR-binding site by approx. 3-fold. For inhibition of [^3H]NBMPR binding by dipyridamole, however, the affinity fell 65-fold (Fig. 4). OCTG itself, when added to a [^3H]NBMPR-binding assay with plasma

Fig. 5. Photoincorporation of [3 H]NBMPR into Ehrlich-cell and human erythrocyte membranes

Ehrlich-cell plasma membranes (●) and erythrocyte membranes (○) were irradiated for 2.5 min with 25 nM-[3 H]NBMPR as described in the text. After extensive washing, the membrane samples were suspended in a 5% SDS/10% 2-mercaptoethanol sample buffer, heated for 3 min at 50°C, and applied to 5–15%-gradient polyacrylamide gels. Gels were sliced into 2 mm segments, and radioactivity was determined as described in the text. The numbers at the top of the Figure represent the location of protein standards in the gel and their respective molecular masses in kDa. The results shown are representative of those obtained from three similar experiments.

Fig. 6. Effect of endoglycosidase F on the electrophoretic mobility of the Ehrlich-cell (a) and human erythrocyte (b) [3 H]NBMPR-binding proteins

Membranes were radiolabelled with [3 H]NBMPR and then incubated for 18 h at 22 °C in either the presence (●) or the absence (□) of the enzyme (1 unit/250 μ g of protein). These preparations were then electrophoresed in parallel on the same slab gel (approx. 200 μ g of protein/lane), and the individual lanes were cut into 2 mm slices for 3 H analysis as described in the text. The numbers at the top of the Figure represent the location of protein standards in the gel and their respective molecular masses in kDa.

membranes, did not affect the ability of dipyridamole to inhibit [3 H]NBMPR binding to these membranes. The reason for this loss of dipyridamole-binding activity after solubilization is obscure, but may reflect the requirement of a lipid bilayer for high-affinity binding of dipyridamole.

Incorporation of OCTG-solubilized proteins into functional proteoliposomes

To assess the functional state of the NTS, the OCTG extract was reconstituted into proteoliposomes as described [34]. Proteoliposomes derived from the Sephadex

Table 3. Recoveries of membrane protein, [^{14}C]lipid and [^3H]NBMPR-binding activity upon solubilization and reconstitution of Ehrlich-cell plasma membranes

Plasma membranes were solubilized with OCTG (1%)/asolectin (0.15%) and incorporated into asolectin vesicles by removal of detergent via gel-exclusion chromatography in the presence of excess asolectin, followed by a freeze/thaw in ethanol/solid CO_2 . Protein and lipid concentrations were determined as described in the text. Specific [^3H]NBMPR (10 nM) binding activity (pmol bound/mg of protein) was assayed by the poly(ethylene glycol)-precipitation procedure at each stage of the preparation. Each value represents the mean $\pm$ S.E.M. from n experiments.

	n	Solubilization (%) [*]	Incorporation in liposomes (%) [†]	Percentage remaining in supernatant [‡]
Protein	10	63 $\pm$ 10	58 $\pm$ 6	28 $\pm$ 4
Lipid	10	(100) [§]	83 $\pm$ 3	9 $\pm$ 1
[^3H]NBMPR	6	74 $\pm$ 6	79 $\pm$ 7	19 $\pm$ 4

	Soluble	Proteoliposomes	Supernatant
[^3H]NBMPR binding (pmol/mg of protein)	9.6 $\pm$ 1.0	13.2 $\pm$ 1.6	7.2 $\pm$ 1.4

* Percentage solubilization of total membranous material with OCTG/asolectin.

† Percentage of the solubilized material added to the reconstitution procedure associated with the proteoliposomes after reconstitution.

‡ Percentage of the solubilized material added to the reconstitution procedure remaining in the soluble fraction (supernatant) after a 20 min/40000 g centrifugation.

§ [^{14}C]Cholesteryl-oleate was added to the preparation after membrane solubilization.

|| The specific [^3H]NBMPR (10 nM)-binding activity in intact Ehrlich-cell plasma membranes was 10.8 $\pm$ 1.3 pmol/mg of protein ($n = 6$).

G-50 column showed incorporation of approx. 58 % of the solubilized protein, 79 % of the [^3H]NBMPR-binding activity and 83 % of the lipid initially added to the reconstitution mixture. A slightly lower percentage of the added lipid was incorporated into liposomes formed in the absence of solubilized membrane proteins (63 $\pm$ 3 %; $n = 10$). Most of the unincorporated activity (protein, lipid or [^3H]NBMPR-binding activity) was recovered in the supernatant after separation of the proteoliposomes by centrifugation (Table 3).

[^3H]NBMPR binding. Proteoliposomes bound 4.5 pmol of [^3H]NBMPR/mg of protein (B_{max}) with a K_D of 0.19 nM. This K_D value is identical with that determined for [^3H]NBMPR binding to cell plasma membranes (Table 1). Adenosine and NBMPR inhibited the binding of [^3H]NBMPR, with K_i values similar to those reported for [^3H]NBMPR binding to cell plasma membranes (Fig. 4). The affinity of dipyridamole for the [^3H]NBMPR-binding site was higher in the proteoliposomes ($K_i = 0.8 \mu\text{M}$) than in the OCTG-solubilized preparations (12 μM), but still significantly lower than that seen in the intact

plasma membranes (0.18 μM). These results suggest that re-association of the [^3H]NBMPR-binding protein with a lipid (asolectin) bilayer partially restores the native binding properties of this membrane protein.

[^3H]Uridine uptake. The use of the centrifugal gel-filtration method to terminate uridine uptake by (proteo)-liposomes, as used in this study, resulted in the recovery from the columns of about 70 % of the added lipid and protein. In contrast, [^3H]uridine when added to columns in the absence of lipid vesicles was retained completely within the body of the column. Proteoliposomes accumulated significantly more [^3H]uridine (20 μM) than did protein-free liposomes at each incubation time tested (Fig. 7). Averages ($n = 5$) of 11.7 $\pm$ 0.2 and 7.1 $\pm$ 0.5 fmol of [^3H]uridine/nmol of lipid P were accumulated by proteoliposomes and protein-free liposomes respectively in 20 s. Preincubation of the proteoliposomes with NBTGR (100 nM)/dilazep (10 μM), adenosine (1 mM), or NBMPR (10 μM) decreased the uptake of [^3H]uridine by the vesicles to a value similar to that seen for protein-free liposomes (i.e. 7.8 $\pm$ 0.6 fmol of [^3H]uridine/20 s per nmol of lipid P). These inhibitors had no effect on the accumulation of [^3H]uridine by protein-free vesicles. It may be calculated that the rate of uptake of [^3H]uridine (20 μM) in the reconstituted system was 49 $\pm$ 8 ($n = 5$) pmol/20 s per mg of reconstituted protein.

Fig. 7. Time course of [^3H]uridine uptake by proteoliposomes and protein-free liposomes

Proteoliposomes, prepared as described in the text, were incubated either with 100 nM-NBTGR/10 μM -dilazep ($\square$) or dimethyl sulphoxide (solvent control, $\blacksquare$), and compared with protein-free liposomes ($\circ$) for their capacity to accumulate [^3H]uridine. Vesicles were incubated (22 $^{\circ}\text{C}$) with [^3H]uridine (20 μM) for the indicated times and then separated from the medium by centrifugal gel filtration. Nucleoside-transporter-mediated uptake of [^3H]uridine ($\blacklozenge$) was calculated as the difference in vesicular accumulation between the proteoliposomes preincubated with dimethyl sulphoxide and those preincubated with NBTGR/dilazep. Each point represents the mean $\pm$ S.E.M. from five experiments.

DISCUSSION

We have established that Ehrlich ascites-tumour cells possess both NBMPR-sensitive and NBMPR-insensitive nucleoside-transport mechanisms, as well as approx. 26000 [^3H]NBMPR-binding sites per cell. The ligand-binding characteristics of these sites are similar to those of the specific [^3H]NBMPR-binding sites in erythrocytes, where [^3H]NBMPR has been shown to bind specifically to functional nucleoside transporters [8,21]. Assuming that each NBMPR-sensitive transporter binds one molecule of [^3H]NBMPR, and that the NBMPR-insensitive transporters do not bind [^3H]NBMPR at concentrations less than 10 nM, it may be calculated that Ehrlich ascites cells contain approx. 35000 transporters/cell.

The apparent molecular mass (40–45 kDa) of the [^3H]NBMPR-binding protein in Ehrlich cells is significantly lower than that of the major [^3H]NBMPR-binding protein (50–60 kDa) in human erythrocyte membranes (the present study, and refs. [16,20]), lung [18] and cardiac [46] membranes, though it is similar to that observed in sheep reticulocytes [41]. Although it cannot be ruled out completely, it seems unlikely that the 42 kDa [^3H]NBMPR-binding peptide reported in the present study is a proteolytic fragment of a larger protein, as proposed for other systems studied [16,18,46], since the presence of both phenylmethanesulphonyl fluoride and soybean trypsin inhibitor during processing failed to alter the size of the final photolabelled peptide. It is possible that these molecular-mass differences reflect different extents of glycosylation of the [^3H]NBMPR-binding protein. Deglycosylation of the human erythrocyte nucleoside transporter with endoglycosidase F led to a 15–17 kDa decrease in its apparent molecular mass, which is in agreement with previous studies [45], whereas similar treatment decreased the apparent molecular mass of the Ehrlich-cell peptides by only 5 kDa.

OCTG, in combination with asolectin, affords a near-quantitative solubilization of [^3H]NBMPR-binding proteins from Ehrlich-cell plasma membranes, with the retention of many of the binding properties characteristic of the native membrane-bound protein, making it an ideal choice for further purification studies. In contrast, Triton X-100 extracts exhibited no [^3H]NBMPR-binding activity, and cholate extracts had a significantly lower affinity for [^3H]NBMPR. Although the binding affinities for NBMPR and adenosine in the OCTG-solubilized material were similar to those observed in the native membranes, the affinity of dipyrindamole for the solubilized [^3H]NBMPR-binding sites was markedly decreased. The binding affinity of dipyrindamole could be partly restored by re-incorporation of the solubilized material into lipid (asolectin) membranes. These results suggest that the binding of dipyrindamole, but not NBMPR or adenosine, is dependent on a lipid environment. Interestingly, there are wide species variations in the capacity of dipyrindamole to inhibit nucleoside transport and [^3H]NBMPR binding [13], and it is tempting to speculate that this variability may be due to differences in the membrane lipids and their interactions with the nucleoside-transport proteins.

Using a technique previously successful for reconstitution of the glucose and amino acid carriers [34], we have shown that proteoliposomes, prepared from OCTG-solubilized membrane proteins, were capable of specific mediated transport of [^3H]uridine, suppressed by known

inhibitors of the nucleoside-transport mechanism. It is unlikely that [^3H]uridine binding to the transport proteins is responsible for the observed accumulation by the proteoliposomes, since the difference in the amount of [^3H]uridine accumulated by the proteoliposomes and protein-free liposomes (~ 49 pmol/20 s per mg of protein) is 3-fold greater than the total number of nucleoside transporters in this preparation (17 pmol/mg of protein; calculated from the density of NBMPR-binding sites in the proteoliposomes with correction for the fact that 25% of transport activity in Ehrlich cells is NBMPR-insensitive, and hence these transporters are unlikely to possess high-affinity binding sites for NBMPR). Furthermore, based on the K_m (~ 250 μM ; J. R. Hammond, unpublished work) of uridine for this system, it would be expected that only a small proportion of these sites would be occupied at the concentration of [^3H]uridine (20 μM) used. The nucleoside-transport activity reported here is similar to that reported for a reconstituted preparation from Triton X-100-solubilized human erythrocyte membranes [29]. Given the fact that there is no accumulation against a gradient, and the small solute-accessible volume of these vesicles, the rapid equilibration observed is not surprising. This even pertains to liposomes which are carrier-free, since uridine has a relatively high lipid solubility.

In conclusion, we have described an efficient and effective solubilization procedure where nearly 100% of the [^3H]NBMPR-binding activity is recovered and the OCTG-solubilized proteins retain many of the ligand-binding characteristics of the native membrane. Reconstitution of the solubilized proteins into proteoliposomes with asolectin demonstrates recovery of a dilazep/NBTGR-sensitive transport system. Therefore, functional reconstitution of Ehrlich-cell nucleoside-transport activity has been achieved, and provides a basis for further purification to obtain a greater insight into the mechanism of action of this transporter at the molecular level.

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