Expression of high levels of nitrobenzylthioinosine-sensitive nucleoside transport in cultured human choriocarcinoma (BeWo) cells

Christine E. BOUMAH, Douglas L. HOGUE and Carol E. CASS*

Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

We have examined binding of [³H]nitrobenzylthioinosine (NBMPR) and influx of [³H]thymidine in adherent cultures of human choriocarcinoma (BeWo) cells and, for comparison, cervical-carcinoma (HeLa) cells. Specific association of NBMPR with BeWo cells at 22 °C required 1.5 h to reach an equilibrium between free and bound ligand, whereas association with HeLa cells required 20–30 min. Scatchard analysis of NBMPR binding to low-density cultures of BeWo cells revealed a total of 27×10^6 sites per cell, consisting of two distinct populations that differed in their affinities for NBMPR. One population bound NBMPR with 'high' affinity ($B_{\max,1}$ 15.0 pmol/10⁶ cells; K_{d1} 0.6 nM) and the other, larger, population bound NBMPR with 'low' affinity ($B_{\max,2}$ 29.0 pmol/10⁶ cells; K_{d2} 14.5 nM). By contrast, HeLa cells possessed only 4.1×10^5 sites per cell, and these sites all bound NBMPR with the same affinity ($B_{\max}$ 0.7 pmol/10⁶ cells; K_d 0.5 nM). Interaction of NBMPR with both populations of sites in BeWo cells could be blocked by nitrobenzylthioguanosine (NBTGR), dilazep or dipyrindamole. Concentration–effect relationships for dilazep inhibition of binding of 1 nM- and 25 nM-NBMPR to BeWo cells were monophasic, with virtually complete inhibition achieved at 0.1 μ M and 1 μ M respectively. Plasma-membrane preparations from BeWo cells also had high numbers of NBMPR-binding sites, and u.v. irradiation of site-bound [³H]NBMPR in such preparations labelled polypeptides that migrated in electrophoretograms as a broad band with a peak M_r of 60000. The concentration–effect relationship for NBMPR inhibition of thymidine transport by BeWo cells was biphasic, with an IC_{50} for inhibition of the 'NBMPR-sensitive' component of 1.6 nM and a substantial (15–20 %) component of flux that was not inhibited by 10 μ M-NBMPR and was thus 'NBMPR-insensitive'. $V_{\max}$ values for thymidine transport by BeWo cells were 20–30-fold larger than the corresponding values for transport by HeLa cells. Elimination of the Na⁺ gradient had no effect on initial rates of thymidine fluxes measured in either the presence or the absence of NBMPR. Our results demonstrate that BeWo cells have an unusually large capacity for NBMPR-sensitive nucleoside transport, apparently resulting from high levels of expression of 'erythrocyte-like' transport elements, identified by their high-affinity interaction with NBMPR. The relationship of the low-affinity binding sites to NBMPR-sensitive transporter elements is uncertain.

INTRODUCTION

The entry of nucleosides into mammalian cells is a complex process that involves transport systems of several types (for reviews, see [1–4]). There are two equilibrative and at least two concentrative nucleoside transport (NT) systems. The equilibrative sensitive (*es*) system (nomenclature of Vijayalakshmi & Belt [5]) is inhibited by nanomolar concentrations of the nucleoside analogue nitrobenzylthioinosine (NBMPR), and the equilibrative insensitive (*ei*) system is unaffected by NBMPR concentrations below 1 μ M. The concentrative Na⁺-dependent NT systems, first identified in kidney and intestinal tissues [6,7] and more recently in blood cells and leukaemic cell lines [8–10], are insensitive to NBMPR and differ in their substrate specificities [5,11].

The best-characterized NT systems are the *es* transporters of erythrocytes. In human erythrocytes, NBMPR binds with high affinity (K_d 1 nM) to a single population of transport-inhibitory sites (1.1×10^4 per cell) on the surface of the plasma membrane [12,13]. Exposure of site-bound [³H]NBMPR to high-intensity u.v. light results in covalent labelling of NBMPR-binding glycoproteins, which migrate on SDS/polyacrylamide gels in the 'band 4.5' region (nomenclature of Steck [14]) with an apparent M_r of 55000 [15]. The abundance of NBMPR-binding sites varies

greatly in erythrocytes from one species to another [16], with $B_{\max}$ values ranging from 2×10^4 sites per erythrocyte in rabbits to just 27 binding sites per erythrocyte in guinea pigs. Among a group of mammalian erythrocytes that exhibit only *es* NT activity, uridine influx rates are proportional to cellular abundance of NBMPR-binding sites, and the ratios of $V_{\max}$ to $B_{\max}$ values are constant [16]. NBMPR-binding proteins purified from human erythrocytes confer both uridine transport and NBMPR-binding activities when reconstituted in unilamellar-phospholipid vesicles [17], indicating that the NBMPR-binding protein is the *es* transporter.

High-affinity NBMPR-binding sites (usually about 10^5 sites per cell) have also been detected on membranes of many cultured cell lines, and it has been assumed that these sites represent functional *es* transporters, similar to those of erythrocytes. Although there are exceptions [18], most cell lines exhibit concentration–effect relationships for inhibition of NT activity by NBMPR that are biphasic, indicating the presence of NT processes that are insensitive to NBMPR. The co-existence of *es* NT activity with *ei* and/or other NBMPR-insensitive NT activities has made it difficult to establish the extent to which the *es* transporters of such cell lines resemble transporters of erythrocytes.

The human choriocarcinoma (BeWo) cell line, derived from

Abbreviations used: NBMPR (nitrobenzylthioinosine), 6-[(4-nitrobenzyl)thio]-9-(β -D-ribofuranosyl)purine; NT, nucleoside transport; *es*, equilibrative NBMPR-sensitive; *ei*, equilibrative NBMPR-insensitive; NBTGR (nitrobenzylthioguanosine), 2-amino-6-[(4-nitrobenzyl)thio]-9-(β -D-ribofuranosyl)purine.

* To whom reprint requests should be addressed.

placental trophoblasts [19], has been studied mainly as a model for differentiation of syncytiotrophoblasts from cytotrophoblasts. Proliferating BeWo cultures are predominantly (96–99%) small mononuclear cells, morphologically and functionally similar to cytotrophoblasts *in utero*. Exposure of BeWo cultures to methotrexate inhibits proliferation and induces differentiation into large multinucleated syncytiotrophoblastic cells [20], which express high levels of choriogonadotropin, lactogen and other placental hormones [21]. BeWo cells have also been used as a model system to study transferrin-mediated uptake and release of iron from maternal to fetal blood [22].

Preliminary experiments in our laboratory [23] suggested that the NBMPR-binding activities of BeWo cells are considerably higher than those of other cultured cell types. This work was undertaken first to quantify the capacity of BeWo cells to bind NBMPR and then to establish if the large numbers of NBMPR-binding sites are associated with elevated 'NBMPR-sensitive' NT activity, as would be expected for an erythrocyte-like *es* transporter. Binding of [^3H]NBMPR was examined under pseudo-first-order conditions to adherent cultures and membrane preparations of BeWo cells and, for comparison, cells of another human line (HeLa cervical carcinoma) previously shown to exhibit NBMPR-sensitive NT activity [24]. NBMPR-binding-site densities were quantified in both cell types, and, since BeWo cells exhibited two populations of binding sites, the effects of several NT inhibitors and permeants on NBMPR binding to BeWo cells were assessed in an attempt to determine if the two populations were related with respect to their interaction with other ligands. BeWo membranes were photolabelled with [^3H]NBMPR and analysed electrophoretically to determine if there were also two populations of NBMPR-binding polypeptides. Finally, the kinetics of thymidine influx in BeWo and HeLa cells were compared, by using concentration–effect relationships for NBMPR inhibition to estimate the proportion of transport that occurred via the *es* NT system. We report here results that demonstrate (i) high NBMPR-binding capacity and NBMPR-sensitive thymidine transport activity in BeWo cells, relative to HeLa cells, and (ii) the first demonstration of multiple NBMPR-binding sites in cells with NBMPR-sensitive NT activity.

MATERIALS AND METHODS

Cell culture

The origin and characteristics of human choriocarcinoma (BeWo) cells have been described [19,25]. HeLa S3 cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Cultures were initiated from frozen stocks that were free of *Mycoplasma*, as determined with the *Mycoplasma* detection kit (Gen-Probe Inc., San Diego, CA, U.S.A.), and were routinely re-started from frozen stocks after 30–36 subculture generations.

Stock cultures, maintained in plastic T-25 or T-75 flasks, were grown in antibiotic-free growth media, which consisted of RPMI 1640 supplemented with 5% fetal-bovine serum plus 5% Nuserum type IV for BeWo cells or 10% calf serum for HeLa cells. BeWo and HeLa cells are adherent and were removed from culture flasks by dissociation with 0.05% trypsin/0.02% EDTA (20 min, 37 °C). For experiments, BeWo and HeLa cells were harvested from actively proliferating cultures and were plated in 60 mm $\times$ 15 mm tissue-culture dishes at densities of 0.5×10^5 – 2×10^5 and 5×10^5 cells per dish respectively. The cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air and used 3–4 days later. Cell numbers were determined with an electronic particle counter (model Zf; Coulter Electronics,

Hialeah, FL, U.S.A.) after dissociation with trypsin/EDTA and resuspension in 0.15 M-NaCl solution.

Preparation of membranes

Fractions enriched in plasma membranes were prepared from trypsin-treated cultures of BeWo and HeLa cells by differential centrifugation [26]. Briefly, cells were disrupted by sonication in the presence of phenylmethanesulphonyl fluoride, the lysates were fractionated in a 20% Percoll gradient and the resulting preparations (enriched in plasma membranes) were washed twice (40000 g, 15 min, 4 °C) in 50 mM-Tris/HCl (pH 7.4) to remove Percoll. Protein content was measured by the Lowry protein assay [27].

NBMPR binding by intact cells

Binding of NBMPR was determined at 22 °C by using replicate adherent cultures (3 per condition) that were grown in 60 mm culture dishes as described above. Cultures were cooled to room temperature and rinsed twice with fresh 'cell-binding medium', which consisted of RPMI 1640 (without bicarbonate) supplemented with 9.6 mM-NaCl, 5 mM-glucose and 20 mM-Hepes (pH 7.4). [^3H]NBMPR was dissolved in cell-binding medium and used at concentrations between 1 and 25 nM. Binding assays were initiated by addition of [^3H]NBMPR (2 ml/dish or, in some experiments with BeWo cells, 10 ml/dish) in the absence or, to determine non-specific binding, in the presence of an excess (10 μM) of either non-radioactive NBMPR or the competing ligand nitrobenzylthioguanosine (NBTGR). Cultures were incubated at room temperature for intervals that ranged from 10 min to 4 h, and the binding assays were ended by removing the medium by aspiration. The cells were drained, air-dried and solubilized by treatment with 0.5 M-KOH (1 ml/dish) for 1 h at 37 °C or, where indicated, rinsed once with 2 ml of phosphate-buffered saline, air-dried and solubilized. Phosphate-buffered saline consisted of 137 mM-NaCl, 5.36 mM-KCl, 1.1 mM- KH_2PO_4 and 1.1 mM- Na_2HPO_4 (pH 7.2). Cell-associated radioactivity was determined by liquid-scintillation counting of 200 μl samples mixed with 8 ml of xylene/Triton X-100 scintillant [28]. Free NBMPR was determined from the radioactivity present in the media at the end of the binding assays. Protein content was determined by using the Bio-Rad microassay on 10- μl samples of KOH-solubilized cells that had been neutralized with 0.5 vol. of 1.0 M-HCl.

The effects of NT permeants and inhibitors on binding of NBMPR to BeWo cells under conditions of complete site occupancy were determined as follows. Cultures were incubated with 25 nM-[^3H]NBMPR (10 ml/dish) in the presence of the competing ligand at room temperature for 2 h. In the determination of concentration–effect relationships for inhibition of NBMPR binding of dilazep, cultures were incubated with either 1 or 25 nM-[^3H]NBMPR alone or in the presence of graded concentrations of dilazep. Non-specific binding was determined in replicate cultures that were incubated in the presence of 10 μM non-radioactive NBMPR. The radioactive content of cultures was analysed as described above.

Specifically bound NBMPR was determined by subtracting the amounts of radioactivity associated with cells in the presence of excess NBMPR or NBTGR (non-specific binding) from the amounts of radioactivity in their absence (total binding). NBMPR-binding parameters were obtained graphically from Scatchard plots of bound/free versus bound NBMPR [29]. Linear plots were analysed by computer 'best fit', and K_d and B_{max} values were obtained from the slopes and abscissa intercepts respectively. Non-linear plots were resolved graphically by the method of Rosenthal [30].

NBMPR binding by membranes

Binding of NBMPR by plasma-membrane-enriched fractions was determined at 22 °C by using membranes prepared as described above. Membrane samples (10 µg of protein, 3 samples per condition) were suspended in 'membrane-binding medium' (50 mM-Tris/HCl, pH 7.4, 20 mM-dithiothreitol) in the absence or, to determine non-specific binding, the presence of 10 µM-NBTGR. Binding assays were initiated by addition of 50 µl of [³H]NBMPR (final concns. 50 and 200 nM) and ended after incubation for 5 or 30 min by centrifugation (40000 g, 15 min, 4 °C). Membrane pellets were solubilized by treatment with 1 ml of 5 % Triton X-100 for 30 min at room temperature, and membrane-bound radioactivity was determined by liquid-scintillation counting tubes in 10 ml of xylene scintillant.

NBMPR-binding polypeptides in plasma-membrane fractions of BeWo cells were identified by electrophoretic analysis of proteins after site-specific photolabelling with [³H]NBMPR by a procedure described in detail elsewhere [26].

Thymidine transport

Transport was measured at 22 °C by determination of initial rates of uptake of [*methyl*-³H]thymidine by replicate adherent cultures (3 per condition). 'Transport medium' consisted of 20 mM-Tris/HCl (pH 7.4), 3 mM-K₂HPO₄, 1 mM-MgCl₂·6H₂O, 1.8 mM-CaCl₂, 5 mM-glucose and 130 mM-NaCl, and, in experiments that assessed the Na⁺-dependence of thymidine transport, NaCl was replaced with 140 mM-choline chloride. Each culture was processed individually, and metronome timing was used for uptake intervals of ≥ 3 s. The growth medium was removed by aspiration just before the assay, which was initiated by rapid addition of transport medium that contained various concentrations of [³H]thymidine (10–400 µM, 2 µCi/ml). Transport assays were stopped by aspiration of the medium, followed immediately by immersion of the culture dish in a large volume of ice-cold phosphate-buffered saline that contained 100 µM-dilazep; this procedure provided virtually instantaneous quenching of uptake reactions. The dishes were drained, and the cells were dissolved in 1.25 ml of 0.5 M-KOH at 37 °C for 1 h and assayed for radioactivity by liquid-scintillation counting of 0.2–1 ml samples that were mixed with 8 ml of xylene/Triton X-100 scintillant. The amount of [³H]thymidine associated with cells at zero time was determined by incubating cells for 10 min at 4 °C with transport medium that contained 100 µM-dilazep and, immediately thereafter, for ≤ 3 s at 4 °C with transport medium that contained the appropriate concentration of [³H]thymidine.

The effects of NBMPR on thymidine transport were determined as follows. Cultures (3 per condition) were cooled to room temperature, rinsed twice with cell-binding medium and incubated for 2 h in binding medium alone or binding medium that contained non-radioactive NBMPR at final concentrations between 0.1 nM and 20 µM. Transport of 100 µM-[³H]thymidine was measured as described above, either in the absence or in the presence of NBMPR at the same concentrations as in the binding step. For determination of cell-associated thymidine at zero time, cultures were cooled to 4 °C after the NBMPR-binding step and incubated for 10 min at 4 °C in transport medium that contained 100 µM-dilazep and the appropriate concentration of NBMPR.

Initial rates of uptake and kinetic constants were determined by direct computer fit of linear or non-linear (second-order polynomial regression) rate equations. The initial rates were obtained as the coefficients of the first-order term, which expresses the slope of the tangent at *t* = 0.

Materials

[G-³H]NBMPR (23 Ci/mmol, 1 Ci = 37 GBq) and [*methyl*-

³H]thymidine (8 Ci/mmol) were obtained from Moravsek Biochemicals (Brea, CA, U.S.A) and were repurified by h.p.l.c. using a reverse-phase Partisil 10/25 ODS-3 column (Whatman, Clifton, NJ, U.S.A.) and methanol/water gradients. NBMPR and NBTGR were prepared in the laboratory of Dr. A. R. P. Paterson (Edmonton, Alberta, Canada) from 6-thioinosine and 6-thioguanosine as described previously [31] or were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dilazep dihydrochloride was generously given by F. Hoffman-La Roche and Co. (Basel, Switzerland). Cell-culture reagents were obtained from GIBCO (Burlington, Ont., Canada). Other reagents were purchased from standard commercial sources.

RESULTS

Time courses of NBMPR binding of adherent cultures

In the binding of NBMPR to cultured cells at 22 °C, an equilibrium between cell-associated and free NBMPR is usually achieved within 30 min of incubation [24,32]. In this work,

Fig. 1. Time course of binding of NBMPR by BeWo and HeLa cells

Cultures of BeWo cells (2.3×10^5 cells/dish; 0.52 mg of protein/dish) and HeLa cells (4.5×10^6 cells/dish; 0.56 mg of protein/dish) were incubated at 22 °C for the time intervals indicated in binding media (10 ml for BeWo and 2 ml for HeLa) that contained 20 nM-[³H]NBMPR alone (□) or with 10 µM-NBTGR (○). Each point represents the mean ($\pm$ S.D.) of three measurements of cell-associated radioactivity. Specific binding (●) was calculated by subtracting the amount of cell-associated radioactivity obtained in the presence of NBTGR from the amount obtained in the absence of NBTGR. The medium content of NBMPR (determined at the end of binding assays) was decreased by $\leq 1\%$ for both BeWo and HeLa cells.

Fig. 2. Scatchard analysis of NBMPR binding of BeWo and HeLa cells

Cultures of BeWo cells (1.7×10^5 cells/dish; 0.47 mg of protein/dish) and HeLa cells (5.1×10^6 cells/dish; 0.62 mg of protein/dish) were incubated at 22 °C for 120 min in transport media that contained graded concentrations of [^{3} H]NBMPR (12 concentrations between 1 and 25 nM) alone or in the presence of 10 μ M-NBTGR. Cultures were processed as described in the Materials and methods section, except that, after binding intervals were terminated, the cultures were analysed for their radioactive content without washing. Assays were performed in triplicate. Specifically bound NBMPR is the difference between radioactivity in the absence of 10 μ M-NBTGR (total binding) and in the presence of NBTGR (non-specific binding). Free NBMPR was determined from the radioactivity present in the medium at equilibrium.

preliminary experiments (results not shown) with trypsin-treated cells from cultures of BeWo cells suggested that binding of NBMPR (i) required long time periods to reach equilibrium, and (ii) was unusually high. The experiments of Fig. 1 compared time courses of binding of 20 nM-[^{3} H]NBMPR to adherent cultures of BeWo cells and HeLa cells under pseudo-first-order conditions that were achieved by incubating cells in volumes large enough to maintain the concentration of NBMPR nearly constant during binding reactions. NBMPR binding by BeWo cells occurred slowly, and after 30 min only 50% (approx. 5 pmol/ 10^6 cells) of maximum binding was achieved; thereafter, nearly 60 min was required for occupancy of the remaining binding sites. For HeLa cells, binding of NBMPR proceeded rapidly, and > 95% of binding had occurred within 20–30 min. Qualitatively similar time courses were obtained for both cell lines with 1 nM- and 40 nM-[^{3} H]NBMPR (results not shown).

In the experiments of Fig. 1, specific binding of NBMPR was determined by also measuring binding in the presence of a high concentration of the competing ligand NBTGR. At any time after incubation with a given concentration of NBMPR, there was significantly more NBMPR specifically bound by BeWo cells than by HeLa cells. At equilibrium after exposure to 20 nM-[^{3} H]NBMPR, the amounts of NBMPR specifically bound to BeWo and HeLa cells were 10 and 0.063 pmol/ 10^6 cells respectively. BeWo cells are larger than HeLa cells [33], and, when equilibrium binding at 20 nM-NBMPR was expressed in terms of cellular protein content, there was a 10-fold difference in site-specific binding between BeWo and HeLa cells (4.8 and 0.5 pmol/mg of protein respectively). The amounts of [^{3} H]NBMPR bound non-specifically to BeWo cells were also much higher than the amounts bound to HeLa cells.

Determination of NBMPR-binding sites

The results of the preceding experiments suggested that BeWo cells possess an unusually large number of NBMPR-binding sites. In the experiments of Fig. 2, analyses of site-specific binding of [^{3} H]NBMPR were conducted for both BeWo and HeLa cells under the conditions (120 min incubation at 22 °C) that were required to achieve equilibrium in BeWo cells. Although a linear

Scatchard plot was obtained for HeLa cells, a non-linear plot was obtained for BeWo cells, suggesting the existence of more than one type of NBMPR-binding site. Graphic resolution of the non-linear plot by the method of Rosenthal [30] yielded two straight lines, from which were derived estimates of the binding parameters for two distinct populations of sites. The smaller population bound NBMPR with greater affinity (K_{d1} 0.6 nM; $B_{max,1}$ 15.0 pmol/ 10^6 cells) than the larger population (K_{d2} 14.5 nM; $B_{max,2}$ 29.0 pmol/ 10^6 cells).

In the experiments of Fig. 2, the total number of NBMPR-binding sites in BeWo cells was 27.5×10^6 sites per cell. However, different values for the total number of NBMPR-binding sites were obtained in other equilibrium-binding experiments, which also yielded non-linear Scatchard plots. These differences in abundance of NBMPR-binding sites were shown to be related to the number of BeWo cells present in culture dishes. In Table 1 are summarized results of experiments in which Scatchard analyses were conducted with BeWo cultures established at different culture densities. For both the 'high-affinity' and 'low-affinity' sites, there was an inverse relationship between the culture density and the number of NBMPR-binding sites, when expressed as the number of sites per cell. Burres & Cass [25,33] have previously shown that BeWo cells are smaller in more crowded cultures, and, when the data were expressed as the number of sites per mg of protein, the density-dependent differences disappeared, with the total number ranging from 8.0×10^{12} to 9.6×10^{12} per mg of protein.

The K_d values shown in the experiments of Table 1 differed, depending on the procedure used to process cultures for analysis of radioactivity, and the greatest difference was seen in the values obtained for the low-affinity sites. When the cultures were washed with phosphate-buffered saline, K_{d2} values were 2.7–2.9 nM, whereas when the washing step was omitted, K_{d2} values were 14.5–16.6 nM. These differences probably resulted from a loss of site-bound [^{3} H]NBMPR during the washing step. The more accurate estimates of K_d and B_{max} values were those obtained with the protocol in which cells were analysed for radioactive content without disturbing the equilibrium by washing, as in the experiments of Fig. 2.

Table 1. NBMPR binding by BeWo cultures of different densities

BeWo cultures were established at different initial densities (0.5×10^5 , 1.0×10^5 and 2.0×10^5 cells/dish) and were assayed for NBMPR binding on day 3 as outlined in Fig. 2. Culture densities at the time of assay are given for each set of data, and the units for $B_{\max}$ and K_d values are respectively pmol/ 10^6 cells and nM.

Culture density (cells/dish) ($\times 10^{-5}$)	Binding parameters				Total NBMPR-binding sites	
	$B_{\max.1}$	(K_{d1})	$B_{\max.2}$	(K_{d1})	per cell ($\times 10^{-6}$)	per mg ($\times 10^{-12}$)
1.7*	15.0	(0.6)	29.0	(14.5)	27.5	9.6
2.4	8.0	(1.1)	20.0	(16.6)	16.8	8.1
2.1†	8.0	(0.1)	22.6	(2.7)	18.4	8.6
4.4†	8.7	(0.4)	11.7	(2.9)	12.2	8.9
9.6†	4.5	(0.4)	7.1	(2.7)	7.0	8.2

* Data from Fig. 2.

† Cultures were washed once with phosphate-buffered saline before analysis of cell-associated [^{3} H]NBMPR.

Table 2. Effect of nucleoside transporter substrates and inhibitors on specific binding of NBMPR by BeWo and HeLa cells

Cultures of BeWo cells (2.2×10^5 cells/dish) and HeLa cells (4.2×10^6 cells/dish) were incubated at 22 °C for 2 h in binding media (10 ml for BeWo cells; 2 ml for HeLa cells) that contained 25 nM-[^{3} H]NBMPR alone or in the presence of the competing ligand at the concentrations indicated. Non-specific binding was determined in the presence of non-radioactive NBMPR as described in the Materials and methods section. Results for binding in the presence of competing ligands are expressed as percentages of specific binding (6.9 and 0.05 pmol/ 10^6 cells for BeWo and HeLa cells respectively) in the absence of competing ligands (control). Results are shown from two experiments; values represent averages from three determinations.

Competing ligand (concn.)	NBMPR binding (% of control value)	
	HeLa	BeWo
Adenosine (10 mM)	71*	58, 57
Thymidine (10 mM)	74, 45	76, 57
Uridine (10 mM)	75*	80, 77
Dilazep (10 μ M)	8, 22	6, 0
Dipyridamole (10 μ M)	26, 19	26, 16
NBTGR (10 μ M)	0, 0	0, 0

* Value for a single experiment.

Analysis of site-specific binding of [^{3} H]NBMPR to HeLa cells (Fig. 2) yielded linear Scatchard plots, indicating a homogeneous population of binding sites ($B_{\max}$ 0.7 pmol/ 10^6 cells). HeLa cells possessed a total of 4.1×10^5 NBMPR-binding sites per cell, a number that is similar to that obtained in previous studies in which HeLa cells were grown in suspension culture [24]. The K_d value (0.5 nM) was in the same range as the value (K_d 0.6 nM) obtained for the high-affinity population of NBMPR-binding sites of BeWo cells.

Inhibition of NBMPR binding

Studies with human erythrocytes and HeLa cells have shown that various nucleoside permeants and transport inhibitors compete with NBMPR for binding to *es* transporter elements, apparently by interaction with the same, or overlapping, sites [24,34,35]. The experiments of Table 2 examined the effects of several NT permeants and inhibitors on specific binding of

Fig. 3. Concentration-effect relationship for dilazep inhibition of binding of low and high concentrations of NBMPR by BeWo cells

Cultures of BeWo cells (4.2×10^5 cells/dish) were incubated at 22 °C for 120 min in binding media (10 ml) that contained low (1 nM; $\square$) or high (25 nM; $\triangle$) concentrations of [^{3} H]NBMPR and dilazep at the concentrations indicated. Non-specific binding was determined in the presence of non-radioactive 10 μ M-NBMPR, and specific binding was calculated as described in the Materials and methods section. Results are expressed as percentages of specific binding (8.1 pmol/ 10^6 cells) obtained in the absence of dilazep (control).

[^{3} H]NBMPR under conditions that saturated both populations of BeWo binding sites. Since the low-affinity sites were 2-fold more abundant than the high-affinity sites, decreases of > 40–50 % in specific binding could only occur if the test ligand blocked binding to both populations. The NT inhibitors (NBTGR, dilazep, dipyridamole) decreased NBMPR binding by 70–100 %, suggesting interaction of these agents with the low-affinity as well as the high-affinity sites. The NT permeants, although present in reaction mixtures at very high concentrations, decreased NBMPR binding only partly and it was not possible to draw conclusions about their interaction with the low-affinity sites. In control experiments that were conducted with HeLa cells, the pattern of inhibition by NT inhibitors and permeants was similar to that reported in previous studies [24,34,35].

The experiments of Fig. 3 examined the concentration-effect relationships for inhibition by dilazep of specific binding of NBMPR to the high- and low-affinity sites of BeWo cells. Cultures were incubated with [^{3} H]NBMPR at either (i) a sub-saturating concentration (1 nM) to assess dilazep inhibition of binding to the high-affinity sites, or (ii) a saturating concentration (25 nM) to assess dilazep inhibition of binding to both populations of sites. Both inhibition curves were monophasic, with complete inhibition of high-affinity binding and total binding at 0.1 and 1.0 μ M respectively. Binding at the lower concentration of NBMPR was more sensitive (roughly 10-fold) to inhibition by dilazep than was binding at the higher concentration.

NBMPR binding by plasma membranes

The experiments of Table 3 compared specific binding of NBMPR under pseudo-first-order conditions by plasma-membrane-enriched fractions from BeWo and HeLa cells. The results demonstrated that the differences in NBMPR binding exhibited by the two cell lines were due, in large part, to differences in the binding capacity of plasma membranes. After a 30 min incubation with 50 nM-[^{3} H]NBMPR, the amount of NBMPR specifically associated with membranes from BeWo cells was 5-fold greater than that associated with membranes from HeLa cells. When the concentration of [^{3} H]NBMPR was raised from 50 to 200 nM, there was a 3-fold increase in site-

Table 3. NBMPR binding by plasma membranes of BeWo and HeLa cells

Membranes were isolated and enriched for plasma membranes, and site-specific binding of [^3H]NBMPR was measured at 22 °C, under the conditions listed below, as described in the Materials and methods section. Results presented are of triplicate determinations from a single experiment, which was repeated with qualitatively similar results three times. Values (mean $\pm$ S.D.) represent the difference between the amount of NBMPR bound in the absence of 10 μM -NBTGR (total binding) and the amount bound in the presence of NBTGR (non-specific binding).

Binding conditions	Specifically bound NBMPR (pmol/mg of protein)	
	HeLa	BeWo
50 nM-NBMPR, 30 min	1.9 $\pm$ 0.3	8.8 $\pm$ 1.1
200 nM-NBMPR, 5 min	1.1 $\pm$ 0.3	6.7 $\pm$ 0.4
200 nM-NBMPR, 30 min*	1.8 $\pm$ 0.5	26.0 $\pm$ 1.3

* The concentration of free NBMPR in reaction mixtures at 30 min was 199 nM (HeLa) and 195 nM (BeWo).

Fig. 4. Identification of NBMPR-binding polypeptides in plasma-membrane-enriched fractions from BeWo cells

Plasma-membrane fractions were prepared from cells (10^8 trypsin-treated cells) and then photolabelled with 100 nM-[^3H]NBMPR under equilibrium binding conditions in the absence ($\circ$) or presence (Δ) of 10 μM -NBTGR. Irradiated membranes (250 μg) were solubilized and subjected to electrophoresis in 12% polyacrylamide gels containing 5% SDS. After staining, the gel was cut in 2 mm slices, and their radioactivity was determined by liquid-scintillation counting. The positions of proteins of known M_r are indicated: SG, stacking gel; TD, tracking dye.

specific binding by BeWo membranes, whereas binding by HeLa membranes was unchanged. Under the latter conditions, which appeared to be saturating for the membrane preparations from both cell types, there was a 13-fold difference between BeWo and HeLa cells in specific binding of NBMPR. In addition, the time required to reach equilibrium was evidently greater for BeWo cells than for HeLa cells, since an increase in specific binding was seen in BeWo cells between 5 and 30 min.

Photolabelling experiments with [^3H]NBMPR have indicated that protein(s) that bind NBMPR are present on plasma membranes of many cell types [36]. Fig. 4 shows the electrophoretic profile of proteins after photolabelling of plasma membranes from BeWo cells with [^3H]NBMPR. Labelled polypeptides

Fig. 5. Time course of uptake of [methyl- ^3H]thymidine by BeWo and HeLa cells

Cultures of BeWo cells (1.9×10^5 cells/dish; 0.52 mg of protein/dish) and HeLa cells (4.6×10^6 cells/dish; 0.55 mg of protein/dish) were used to assay uptake at 22 °C of ($\square$) 20 μM -, ($\circ$) 100 μM - and ($\bullet$) 200 μM - thymidine as described in the Materials and methods section. Each point represents the mean ($\pm$ S.D.) of three determinations.

migrated as a single broad band, with a peak apparent M_r of 60000. The photolabelling was specific, since the labelled polypeptides disappeared almost completely when photolabelling was performed in the presence of an excess of NBTGR, a structurally related NT inhibitor, and there was no obvious indication of two separate populations of NBMPR-binding polypeptides. Thus the NBMPR-binding proteins of BeWo cells were similar to those of human erythrocytes and several other cell types [15,36–38] in that they migrated in the band 4.5 region of electrophoretograms with M_r between 45000 and 66000.

Transport of thymidine

To determine if the high NBMPR-binding capacity of BeWo cells was accompanied by high NT activity, thymidine transport rates were determined in both cell types grown at population densities that produced cultures with equivalent amounts of protein. Fig. 5 shows time courses of cellular uptake of 20 μM , 100 μM and 200 μM -[^3H]thymidine, which yielded respectively total transport rates of 27, 123 and 162 pmol/s per 10^6 cells for BeWo cells and 1.9, 4.9 and 6.1 pmol/s per 10^6 cells for HeLa cells.

That the transport activity of BeWo cells was greater than that

Fig. 6. Kinetics of total thymidine transport in BeWo and HeLa cells

Initial rates of transport were determined as described in the Materials and methods section from time courses (3–12 s) of uptake of graded concentrations of [^3H]thymidine by BeWo cells (1.8×10^5 cells/dish) and HeLa cells (5.3×10^6 cells/dish). Reciprocals ($1/v$ versus $1/s$) of transport rates and thymidine concentrations are plotted in the insets.

Table 4. Kinetic parameters of total thymidine transport in BeWo and HeLa cells

Presented are results of a series of experiments in which thymidine transport kinetics were determined as described in Fig. 6. Thymidine transport rates were determined from time courses from 0 to 20 s in Expts. 1 and 6 and from 0 to 10 s in Expts. 2, 3, 4, 5 and 7.

	Culture density (cells/dish) $\times 10^{-5}$	K_m (μM)	$V_{\max}$ (pmol/s per 10^6 cells)
BeWo cells			
Low density:			
Expt. 1	1.9	125	215
Expt. 2	2.4	95	221
Expt. 3	2.2	95	185
Expt. 4*	1.8	107	196
Mean $\pm$ s.d.		105 ± 14	204 ± 16
High density:			
Expt. 5	9.3	100	27
HeLa cells			
Expt. 6	46	125	8.2
Expt. 7*	53	97	9.0
Mean $\pm$ s.d.		108 ± 16	8.6 ± 0.5

*Data from Fig. 6.

Fig. 7. Concentration-effect relationship for NBMPR inhibition of thymidine transport in BeWo cells

Cultures of BeWo cells (2.4×10^5 cells/dish) were incubated for 120 min in transport media that contained NBMPR at the concentrations indicated, after which uptake of $100 \mu\text{M}$ -[^3H]thymidine was determined in the presence of the same NBMPR concentrations. Transport rates were calculated from time courses (3–12 s) and are presented as percentages of the value ($128.1 \text{ pmol/s per } 10^6$ cells) obtained in the absence of NBMPR (control).

respectively. Table 4 summarizes the results from a series of such kinetic studies which, to control for differences in size between the two cell types, were performed with BeWo and HeLa cells at population densities corresponding to 0.4–0.5 mg of protein per dish. The K_m and $V_{\max}$ values varied slightly in different experiments, but the conclusion remained unchanged; although both cell types transported thymidine with similar K_m values, the maximal rates of transport were 20–25 times greater in BeWo cells than in HeLa cells. In addition, as was observed for NBMPR-binding activity in BeWo cells, transport activity decreased dramatically in crowded cultures, with $V_{\max}$ values of 204 and 27 pmol/s per 10^6 cells for cells grown at low and high population densities respectively.

NBMPR-sensitive transport of thymidine in BeWo cells

Although BeWo cells possessed a large number of NBMPR-binding sites and transported thymidine at rapid rates, the extent to which thymidine permeation occurs by an NBMPR-sensitive process remained to be established. In the experiment of Fig. 7, thymidine transport rates were determined in low-density cultures of BeWo cells that had been incubated with graded concentrations of NBMPR for a period of time sufficient to achieve an equilibrium with the NBMPR-binding sites. A large component of transport activity was highly sensitive to NBMPR, with maximum inhibition at 10 nM. Approx. 85% (about 110 pmol/s per 10^6) of the thymidine entering BeWo cells was transported by an NBMPR-sensitive route(s), and the concentration of NBMPR that decreased the sensitive component by 50% (IC_{50} value) was 1.6 nM. The remaining 15% (about 20 pmol/s per 10^6 cells) represented thymidine permeation by an NBMPR-insensitive route(s).

Table 5 lists published values for numbers of NBMPR-binding sites and $V_{\max}$ of NBMPR-sensitive thymidine transport for S49 and CHO cells, together with the values for BeWo and HeLa cells derived in this study. BeWo cells not only possessed a much larger number of NBMPR-binding sites than did the other cell types, but the $V_{\max}$ value for NBMPR-sensitive thymidine transport was substantially greater than in the other cell types. In

of HeLa cells was confirmed by analysis of the concentration-dependence of total thymidine transport rates. In the experiments shown in Fig. 6, the thymidine transport process had $V_{\max}$ values for BeWo and HeLa cells of 196 and 9 pmol/s per 10^6 cells

Table 5. NBMPR-binding sites and $V_{\max}$ values of 'NBMPR-sensitive' thymidine transport in different cell lines

Numbers of NBMPR-binding sites per cell for BeWo and HeLa cells were obtained from Fig. 2, and $V_{\max}$ values for 'NBMPR-sensitive' thymidine transport were derived from data in Table 4, assuming that the 'NBMPR-sensitive component' of transport was 55% for HeLa cells and 85% for BeWo cells (the present work). The $V_{\max}$ values are expressed in terms of cell water, by using intracellular water volumes determined previously [33] for BeWo and HeLa cells grown under conditions similar to those used in this study.

Cell type	No. of NBMPR-binding sites per cell	$V_{\max}$ of 'NBMPR-sensitive' thymidine transport (pmol/s per μ l of cell water)
BeWo*	27.5×10^6	91.3
HeLa	4.5×10^6	3.0
S49†	6.6×10^4	5.1
CHO‡	6.0×10^4	3.5

* Values for NBMPR-binding sites represents both populations.
† Values from [39].
‡ Values from [40,41].

computing the $V_{\max}$ values in Table 5, the proportion of total transport represented by the NBMPR-sensitive component was determined from concentration-effect relationships for NBMPR inhibition of NT activity in each of the cell lines.

Since total thymidine transport rates were much decreased in crowded cultures (see Table 4), a kinetic study was undertaken with BeWo cultures of high population density (8×10^5 – 10^6 cells per dish) to assess further the effects of culture conditions on transport activity. Transport rates were determined for different thymidine concentrations in the absence and presence of $10 \mu\text{M}$ -NBMPR, and the kinetic analysis was performed on NBMPR-sensitive transport rates calculated from the difference between total transport and transport in the presence of NBMPR (results not shown). The K_m values (about $100 \mu\text{M}$) were the same for low- and high-density cultures, whereas the $V_{\max}$ values differed considerably (22 and $173 \text{ pmol/s per } 10^6$ cells respectively), although the proportion of transport that was sensitive to NBMPR was unchanged (82 and 85% respectively).

Absence of Na^+ -dependent transport of thymidine in BeWo cells

Cellular uptake of thymidine by passive diffusion is generally a slow process [42] and seemed unlikely to be responsible for the large component of NBMPR-insensitive uptake seen in BeWo cells in the experiment of Fig. 7. Also, the NBMPR-insensitive component was blocked by the cold stopping solution that contained dilazep (results not shown). Mediated influx of thymidine that occurred in the presence of NBMPR could be due to transport via (i) equilibrative NBMPR-insensitive processes, (ii) Na^+ -dependent processes, or (iii) a combination of both. The experiments of Fig. 8 examined the effects of replacing Na^+ with choline on the initial rates of uptake of $5 \mu\text{M}$ -thymidine by BeWo cells in the absence and presence of NBMPR, and on uptake during 30 min incubations (see inset), since low levels of Na^+ -dependent NT processes are sometimes revealed during long-term uptake assays in the presence of inhibitors of the *es* transporter [43]. Na^+ replacement had no effect on thymidine transport rates in either the absence or presence of NBMPR and, since Na^+ replacement also had no effect on long-term uptake of thymidine in NBMPR-treated cells, the insensitive component did not appear to be Na^+ -dependent. Na^+ replacement did result in a decrease in long-term uptake of thymidine in the absence of NBMPR, possibly from indirect effects on thymidine metabolism.

Fig. 8. Uptake of thymidine by BeWo cells in the absence of Na^+

Cells were incubated for 120 min in Na^{2+} -containing ($\circ$, $\bullet$) or choline-containing ($\triangle$, $\blacktriangle$) transport media, with ($\circ$, $\triangle$) or without ($\bullet$, $\blacktriangle$) $10 \mu\text{M}$ -NBMPR. Uptake of $5 \mu\text{M}$ - ^3H thymidine (in the appropriate transport medium) was then determined as described in the Materials and methods section. Each point represents the mean ($\pm$ S.D.) of three measurements, and the results obtained at intervals of 5–30 min are shown in the inset. Initial rates in the absence of NBMPR were $1.51 \text{ pmol/s per } 10^6$ cells in both Na^+ - and choline-containing media, and in the presence of NBMPR were $0.20 \text{ pmol/s per } 10^6$ cells in either Na^+ - or choline-containing media.

DISCUSSION

NBMPR is a tight-binding and specific inhibitor of equilibrative transport of nucleosides in mammalian cells that has been used as a probe to identify transporter proteins in the membranes of human erythrocytes (for reviews, see [2,36]). Among erythrocytes from different species, a higher number of NBMPR-binding sites is associated with a proportionally higher uridine transport activity, and the ratio of values of $V_{\max}$ (uridine *zero-trans* influx) to $B_{\max}$ (NBMPR-binding sites) remains constant [16]. NBMPR-binding proteins have been shown in reconstitution studies to mediate *es* NT activity for human erythrocytes [17,44], cultured leukaemia (CEM) cells [45] and Ehrlich ascites-tumour cells [46]. For other cell types, the inhibition by NBMPR of equilibrative NT processes, combined with the demonstration of NBMPR-binding polypeptides in plasma membranes by site-specific photolabelling, has provided strong evidence for the idea that NBMPR-binding proteins mediate *es* NT activity. In such studies, it has usually been assumed that quantification of the total number of NBMPR-binding sites provides an indication of the abundance of *es* transporter proteins. Exceptions have been observed, as in the recent report of differences in NT activity, without differences in NBMPR-binding activity, between normal and transformed fibroblasts [47].

In the present work, we report results that demonstrate high NBMPR-binding capacity in BeWo cells, relative to HeLa cells.

The time courses of association of NBMPR with intact BeWo cells differed from those of HeLa cells when determined under pseudo-first-order conditions. Specific binding of NBMPR occurred slowly and was not complete in BeWo cells until after 1.5–2 h of incubation at 22 °C, whereas binding of NBMPR to HeLa cells occurred much more rapidly. The difference in rates of NBMPR binding between BeWo cells and HeLa cells cannot be simply explained by differences in NBMPR uptake (and/or intracellular metabolism), since it was also seen with plasma-membrane preparations; maximum binding to HeLa membranes was achieved within 5–10 min, whereas at least 30 min was necessary to reach maximum binding of NBMPR to BeWo membranes. For HeLa cells, the rapid occupancy of the NBMPR-binding sites suggested a superficial location, at or near the cell surface, as has been shown for erythrocytes [48]. Although the basis of the slow occupancy of NBMPR-binding sites of BeWo cells is uncertain, it seems reasonable to speculate that a proportion of the sites may be relatively inaccessible to NBMPR.

Scatchard analyses suggested heterogeneity of the NBMPR-binding sites in BeWo cells. The non-linear plots were consistent with the existence of at least two distinct populations of high-affinity NBMPR-binding sites, although they could also arise from negative co-operativity between binding sites, such that binding to some sites results in decreased binding to other sites. One population of sites bound NBMPR with an affinity similar to that of the binding sites of HeLa cells, whereas the other population bound NBMPR with somewhat lower affinity. Since the second low-affinity population of NBMPR-binding sites has not been described previously, its characteristics were assessed by analysing the ability of other ligands to block binding of NBMPR to BeWo cells. In a previous study with HeLa cells [24], binding of NBMPR was almost completely inhibited by low concentrations of dipyradamole (K_i 30 nM) and partially inhibited by uridine (K_i 1.7 mM). In the present study, we compared the effects of relatively high concentrations of several NT inhibitors and permeants on NBMPR binding to BeWo and HeLa cells and found no obvious differences between the two cell types. We also determined the concentration–effect relationships for dilazep inhibition of binding of NBMPR to BeWo cells at (i) a sub-saturating concentration (high-affinity sites partially occupied), and (ii) a saturating concentration (high- and low-affinity sites completely occupied). The dose–response curves were monophasic, with a difference of about 10-fold in sensitivity to inhibition by dilazep. These results indicated that the low-affinity sites interact with some of the same ligands as the high-affinity sites.

The existence of two populations of NBMPR-binding sites in BeWo cells could have been due to the presence of two different populations of cells in the cultures used for binding assays. The BeWo cultures used in this study contained predominantly small, undifferentiated, cytotrophoblast-like cells, and the numbers of larger syncytiotrophoblasts arising spontaneously in such cultures varied between 1 and 4%, a number too low to account for the additional population of NBMPR-binding sites. Furthermore, an analysis of NBMPR binding to cells of a genetically homogenous subclone of the BeWo line isolated during the course of this study also yielded non-linear Scatchard plots (results not shown) that could be resolved into two distinct components. In recent studies of NBMPR binding by freshly isolated human placenta, a single population of high-affinity binding sites was seen in syncytiotrophoblasts [49,50]. We have not yet examined the NBMPR-binding characteristics of BeWo cells grown under conditions that result in conversion of cytotrophoblast-like cells into syncytiotrophoblast-like cells.

After [^{3} H]NBMPR photolabelling and SDS/PAGE of plasma membranes from BeWo cells, a single broad band of ^{3} H-labelled

polypeptides was detected. These polypeptides migrated in electrophoretograms in a similar fashion to the *es* transporter proteins of human erythrocytes [51], suggesting similarities between the BeWo and erythrocyte proteins. If the binding-site heterogeneity of BeWo cells was due to interaction of NBMPR with different proteins, their differences could not be resolved electrophoretically. It is also possible that NBMPR bound to a protein species that was not photolabelled.

The present results do not establish which population of sites, or to what extent each population of sites, participates in the NBMPR-dependent transport of thymidine by BeWo cells. The NBMPR-inhibition studies demonstrated that 80–85% of thymidine transport in BeWo cells occurred via NBMPR-sensitive process(es), and the remainder apparently via Na^+ -independent process(es). To determine the significance of the high number of NBMPR-binding sites in BeWo cells, we compared total and NBMPR-sensitive thymidine transport activity in BeWo and HeLa cells. Although the two cell types exhibited similar K_m values (approx. 100 μM) for the NBMPR-sensitive process, the V_{max} value for BeWo cells was much greater than the V_{max} value for HeLa cells. The concentration–effect relationship for inhibition of the NBMPR-sensitive component of thymidine transport in BeWo cells exhibited a low IC_{50} value (1.6 nM), suggesting that the transport inhibition was due to interaction with the high-affinity sites. The functional significance of the low-affinity sites is as yet uncertain.

The NBMPR-insensitive component of thymidine transport in BeWo cells was also large, relative to that of other cell types. Since thymidine transport rates were not dependent on a Na^+ gradient, it seems likely that the NBMPR-insensitive component of transport represents *ei*-mediated process(es), similar to those identified in other cell types [5]. Experiments with a non-metabolized permeant such as formycin B are needed to demonstrate rigorously the presence of *ei* activity.

In summary, we have demonstrated that BeWo cells possessed an elevated number of NBMPR-binding sites and exhibited high thymidine transport activity, as compared with HeLa cells (this study) and other cultured cell lines [39–41]. There appeared to be at least two populations of NBMPR-binding sites in cultured BeWo cells, one with high affinity and the other with low affinity for NBMPR. Since NBMPR interaction with both populations was blocked by other NT inhibitors, including two that are structurally unrelated to nucleosides, it seems likely that the low-affinity sites are also associated with nucleoside-transporter elements. The IC_{50} value for NBMPR inhibition of thymidine influx in BeWo cells was closer to the K_d value of the high-affinity sites, suggesting that the low-affinity sites may not represent functional transport elements. The identity of the low-affinity sites was not evident from site-specific photolabelling of plasma membranes, which identified a broadly migrating population of NBMPR-binding polypeptides with electrophoretic mobilities similar to that of the *es* transporter of human erythrocytes. Further characterization of the low-affinity NBMPR-binding sites is required.

The preliminary studies that led to this work were conducted by K. Hodgson and D. Petaya. We acknowledge the excellent technical assistance of Mrs. S. Rasmussen. This work was supported by the National Cancer Institute of Canada and the Medical Research Council of Canada. Salary support for C.E.B. (Postdoctoral Fellow), D. L. H. (Studentship) and C.E.C. (Senior Research Scientist) was from the National Cancer Institute of Canada.

REFERENCES

1. Paterson, A. R. P. & Cass, C. E. (1986) in *Membrane Transport of Antineoplastic Agents* (Goldman, I. D., ed.), pp. 309–329, Pergamon Press, Oxford.

2. Jarvis, S. M. (1988) in *Adenosine Receptors* (Cooper, D. M. F. & Londos, C., eds.), pp. 113–123, Alan R. Liss, New York
3. Plagemann, P. G. W., Wohlhueter, R. M. & Woffendin, C. (1988) *Biochim. Biophys. Acta* **947**, 405–443
4. Gati, W. P. & Paterson, A. R. P. (1989) in *Red Blood Cell Membranes: Structure, Function, Clinical Implications* (Agre, P. & Parker, J. C., eds.), pp. 635–661, Marcel Dekker, New York
5. Vijayalakshmi, A. & Belt, J. A. (1988) *J. Biol. Chem.* **263**, 19419–19423
6. Le Hir, M. & Dubach, U. C. (1984) *Pflugers Arch.* **401**, 58–63
7. Schwenk, M., Hegazy, E. & Lopez del Pino, V. (1984) *Biochim. Biophys. Acta* **805**, 370–374
8. Darnowski, J. W., Holridge, C. & Handschumacher, R. E. (1987) *Cancer Res.* **47**, 2614–2619
9. Dagnino, L., Bennett, L. L., Jr. & Paterson, A. R. P. (1991) *J. Biol. Chem.* **266**, 6308–6311
10. Lee, C. W., Sokoloski, J. A., Sartorelli, A. C. & Handschumacher, R. E. (1991) *Biochem. J.* **274**, 85–90
11. Williams, T. C. & Jarvis, S. M. (1991) *Biochem. J.* **274**, 27–33
12. Cass, C. E., Gaudette, L. A. & Paterson, A. R. P. (1974) *Biochim. Biophys. Acta* **345**, 1–10
13. Jarvis, S. M. & Young, J. D. (1980) *Biochem. J.* **190**, 377–383
14. Steck, T. L. (1974) *J. Cell Biol.* **62**, 1–19
15. Young, J. D., Jarvis, S. M., Robins, M. J. & Paterson, A. R. P. (1983) *J. Biol. Chem.* **258**, 2202–2208
16. Jarvis, S. M., Hammond, J. R., Paterson, A. R. P. & Clanachan, A. S. (1982) *Biochem. J.* **208**, 83–88
17. Kwong, F. Y. P., Davies, A., Tse, C. M., Young, J. D., Henderson, P. J. F. & Baldwin, S. A. (1988) *Biochem. J.* **255**, 243–249
18. Young, J. D., Jarvis, S. M., Belt, J. A., Gati, W. P. & Paterson, A. R. P. (1984) *J. Biol. Chem.* **259**, 8363–8365
19. Pattilo, R. A. & Gey, G. O. (1968) *Cancer Res.* **28**, 1231–1236
20. Burres, N. S. & Cass, C. E. (1987) *Cancer Res.* **47**, 5059–5064
21. Friedman, S. J. & Skehan, P. (1979) *Cancer Res.* **39**, 1960–1967
22. van der Ende, A., du Maine, A., Simmons, C. F., Schwartz, A. L. & Strous, G. J. (1987) *J. Biol. Chem.* **262**, 8910–8916
23. Cass, C., Boumah, C. & Hogue, D. (1991) *Int. J. Purine Pyrimidine Res.* **2**, 39
24. Dahlig-Harley, E., Eilam, Y., Paterson, A. R. P. & Cass, C. E. (1981) *Biochem. J.* **200**, 295–305
25. Burres, N. S. & Cass, C. E. (1986) *J. Cell. Physiol.* **128**, 375–382
26. Hogue, D. L., Hodgson, K. C. & Cass, C. E. (1989) *Biochem. Cell Biol.* **68**, 199–209
27. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
28. Pande, S. V. (1976) *Anal. Biochem.* **74**, 25–34
29. Scatchard, G. (1949) *Ann. N. Y. Acad. Sci.* **51**, 660–672
30. Rosenthal, H. E. (1967) *Anal. Biochem.* **20**, 525–532
31. Paul, B., Chen, M. F. & Paterson, A. R. P. (1975) *J. Med. Chem.* **18**, 968–973
32. Paterson, A. R. P., Yang, S., Lau, E. Y. & Cass, C. E. (1979) *Mol. Pharmacol.* **16**, 900–908
33. Burres, N. S. & Cass, C. E. (1989) *In Vitro Cell. Dev. Biol.* **25**, 419–423
34. Jarvis, S. M. (1986) *Mol. Pharmacol.* **30**, 659–665
35. Jarvis, S. M. & Ng, A. S. (1985) *J. Neurochem.* **44**, 183–188
36. Jarvis, S. M. & Young, J. D. (1987) *Pharmacol. Ther.* **32**, 339–359
37. Kwong, F. Y. P., Baldwin, S. A., Scudder, P. R., Jarvis, S. M., Choy, Y. M. & Young, J. D. (1986) *Biochem. J.* **240**, 349–356
38. Almeida, A. F., Jarvis, S. M., Young, J. D. & Paterson, A. R. P. (1984) *FEBS Lett.* **176**, 444–448
39. Cass, C. E., Kolassa, N., Uehara, Y., Dahlig-Harley, E., Harley, E. R. & Paterson, A. R. P. (1981) *Biochim. Biophys. Acta* **649**, 769–777
40. Wohlhueter, R. M., Marz, R. & Plagemann, P. G. W. (1978) *J. Membr. Biol.* **42**, 247–264
41. Wohlhueter, R. M., Marz, R. & Plagemann, P. G. W. (1979) *Biochim. Biophys. Acta* **553**, 261–268
42. Zimmerman, T. P., Mahony, W. B. & Prus, K. L. (1987) *J. Biol. Chem.* **262**, 5748–5754
43. Dagnino, L. & Paterson, A. R. P. (1990) *Cancer Res.* **50**, 6549–6553
44. Tse, C.-M., Belt, J. A., Jarvis, S. M., Paterson, A. R. P., Wu, J.-S. & Young, J. D. (1985) *J. Biol. Chem.* **260**, 3506–3511
45. Crawford, C. R., Ng, C. Y. C., Ullman, B. & Belt, J. A. (1990) *Biochim. Biophys. Acta* **1024**, 289–297
46. Hammond, J. R. & Johnstone, R. M. (1989) *Biochem. J.* **262**, 109–118
47. Meckling-Gill, K. A. & Cass, C. E. (1992) *Biochem. J.* **282**, 147–154
48. Agbanyo, F. R., Cass, C. E. & Paterson, A. R. P. (1988) *Mol. Pharmacol.* **33**, 332–337
49. Yudilevich, D. L. & Barros, L. F. (1990) *Biochem. Soc. Trans.* **18**, 1136–1140
50. Barros, L. F., Bustamante, J. C., Yudilevich, D. L. & Jarvis, S. M. (1991) *J. Membr. Biol.* **119**, 151–161
51. Wu, J.-S. R., Kwong, F. Y. P., Jarvis, S. M. & Young, J. D. (1983) *J. Biol. Chem.* **258**, 13745–13751