Biochimica et Biophysica Acta, 1069 (1991) 123–126

ADONIS 000527369100371X

BBAMEM 70691

BBA Report

Nucleoside uptake by red blood cells from a primitive vertebrate, the pacific hagfish (*Eptatretus stouti*), is mediated by a nitrobenzylthioinosine-insensitive transport system

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(Received 23 July 1991)

Key words: Nucleoside transport; Nitrobenzylthioinosine; *p*-Chloromercuriphenylsulfonate; Dipyridamole; (Hagfish red blood cell)

Red blood cells from the Pacific hagfish (*Eptatretus stouti*) were found to possess a facilitated diffusion nucleoside transport system insensitive to inhibition by the nucleoside transport inhibitor nitrobenzylthioinosine (NBMPR). Uridine uptake by this route was saturable (apparent K_m 0.14 mM; V_{max} 2 mmol/l cells per h at 10°C), inhibited by inosine and adenosine, and blocked both by the vasodilator dipyridamole and by the thiol-reactive agent *p*-chloromercuriphenylsulphonate. The properties of this carrier resemble closely those of NBMPR-insensitive nucleoside transport systems in some mammalian neoplastic cell lines and in rat red cells. The presence of this type of carrier in a primitive vertebrate suggests that such transporters have a broad biological distribution and that they pre-date or arose at an early stage of vertebrate evolution.

To date, studies of the transmembrane permeation of nucleosides in vertebrate cells have been limited to cells of mammalian origin. A number of Na^+-dependent and Na^+-independent transport mechanisms for physiological and cytotoxic nucleosides have been described (for reviews, see Refs. 1–3). In human red blood cells, nucleoside transport is equilibrative and inhibited by nanomolar concentrations of nitrobenzylthioinosine (NBMPR) and related to S^6-derivatives of 6-thiopurine nucleosides such as nitrobenzylthioguanosine (NBTGR) [4–6]. Transport inhibition by NBMPR is associated with specific high-affinity binding of nucleoside analogue to the cell membrane [7,8]. Photoaffinity-labelling studies with [^{3}H]NBMPR have identified the human red cell transporter as a band 4.5 polypeptide (nomenclature of Steck [9]) (apparent M_r 45 000–66 000) [10,11] and the system has been purified to apparent homogeneity by a combination of ion-exchange and immunoaffinity chromatography [12]. Similar NBMPR-sensitive equilibrative nucleoside transporters are expressed in a wide range of other mammalian cell types and tissues [1–3]. In contrast, a number of mammalian cells possess Na^+-independent nucleoside transport mechanisms which have a low (micromolar) sensitivity to inhibition by NBMPR. These include some neoplastic cell lines [13–16] and mammalian CNS [17–19]. Unlike human red cells where nucleoside uptake is totally inhibited by NBMPR, rat red cells exhibit mixed NBMPR-sensitive and NBMPR-insensitive transport activity [20]. We report here that a similar NBMPR-insensitive nucleoside transport mechanism is present in red cells of the Pacific hagfish (*Epatatretus stouti*), generally regarded as amongst the most primitive of living vertebrates.

Hagfish were trapped at 160–200 m in Trevor Channel, Bamfield, B.C., Canada and maintained in running sea water until bled from the subcutaneous sinus into heparin. Red cells were prepared for transport experiments by washing three times with 20 vol. of an incubation medium containing 500 mM NaCl, 5 mM glucose and 15 mM Mops (morpholinepropanesulphonate; pH 7.5 at 10°C). The buffy coat was discarded. Uptake of [2-^{14}C]uridine (Amersham International, Amersham, Bucks., U.K.) at 10°C was measured by an oil-stop procedure as described previously [21]. In competition

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experiments, the inhibitory nucleoside and radioactive permeant were added simultaneously. Transport inhibitors obtained from Sigma Chemical, were preincubated with cells at 1°C for 30 min (*p*-chloromercuriphenylsulphonate (PCMBS)) or at 10°C for 1 h (NBMPR, NBTGR and dipyridamole) before assay of influx activity. [^{3}H]NBMPR (Moravek Biochemicals, Brea, CA, U.S.A.) binding to intact cells and isolated plasma membranes was determined by a centrifugation method [8]. Metabolism of [2-^{14}C]uridine by hagfish red cells was investigated in neutralised perchloric acid cell extracts [22] using a Beckman System Gold HPLC system in combination with a Beckman Model 171 Radiochemical Detector. Analysis was by reverse phase HPLC on a Beckman Ultraspere C_{18} 5 μm column (4.6 × 250 mm) by a multistep gradient program employing mobile phases containing methanol in solutions of potassium dihydrogen phosphate.

A representative time-course for [2-^{14}C]uridine (1 mM extracellular concentration) uptake by hagfish red cells is shown in Fig. 1. Influx was relatively slow, reaching a value of 0.47 mmol/l cells after 2 h incubation at 10°C. Since hagfish red cells contain 68% (v/v) water, this corresponds to an intracellular uridine concentration of 0.69 mM i.e. approximately two-thirds equilibration with intracellular water. At a lower extracellular uridine concentration of 0.1 mM, full equilibration with intracellular water occurred within 30 min incubation at 10°C (data not shown). In agreement with previous studies in mammalian red cells [23], no phosphorylation or phosphorolysis products of uridine were detected in medium or cells incubated with 1 mM nucleoside for 1 h at 10°C. Uptake of 0.1 mM uridine was unaffected by isosmotic replacement of Na^+ in the incubation medium with K^+ or by substituting NaCl with mannitol, establishing that nucleoside transport by hagfish red cells was not mediated by Na^+-dependent

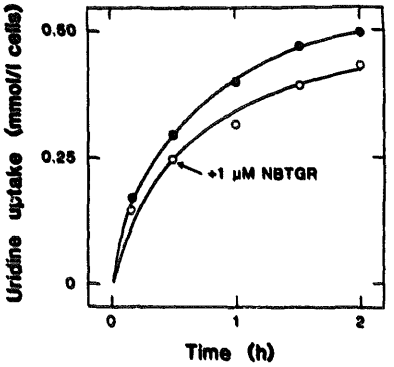

Fig. 1 Time course of uridine uptake by hagfish red blood cells. [2-^{14}C]Uridine uptake (1 mM extracellular concentration, 10°C) by NBTGR-treated (○, 1 μM final concentration) and control (●) red cells was determined as described in the text. Cells were pre-incubated with NBTGR for 1 h at 10°C before addition of radioactive permeant. Individual data points are means of duplicate determinations.

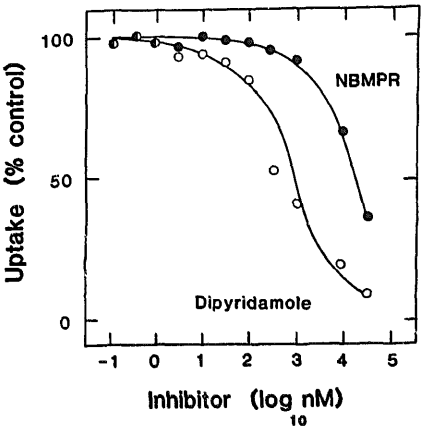

Fig. 2. Effects of NBMPR and dipyridamole on uridine uptake by hagfish red blood cells. Cells were pre-incubated with NBMPR (●) or dipyridamole (○) for 1 h at 10°C before assay of [^{14}C]uridine uptake (1 mM extracellular concentration, 10°C) using a 5 min incubation period. Results are given as a percentage of the control influx rate in the absence of inhibitor (1.64 mmol/l cells per h). Values are the means of duplicate estimates.

uptake mechanisms [24,25]. Although uptake of uridine by hagfish red cells was Na^+-independent and equilibrative, transport was only slightly inhibited in the presence of a high concentration of NBTGR (1 μM) (Fig. 1). Fig. 2 shows a full dose-response curve for inhibition of uridine uptake by NBMPR. In this and subsequent experiments, a 5 min incubation period was used to measure initial rates of transport. At the highest concentration of NBMPR tested (50 μM), there was a 62% reduction in 1 mM uridine uptake rate ($IC_{50} \approx 30$ μM). This contrasts markedly with the situation in human red cells for example, where NBMPR and NBTGR inhibit nucleoside transport with K_i values in the low nanomolar range (10^{-10}–10^{-9} M) [7,8]. The inhibition studies shown in Figs. 1 and 2 were complemented by direct [^{3}H]NBMPR binding experiments using both intact hagfish red cells and also isolated plasma membranes prepared by hypotonic lysis as described previously [26]. Protocols for these experiments involved equilibration of cells and membranes with graded concentrations of [^{3}H]NBMPR (0.1–20 nM, 1 h incubation at 20°C) in the absence and in the presence of excess nonradioactive NBTGR (10 μM). Bound and unbound radioligand were determined as described previously [8]. For intact cells, NBTGR caused a modest (13%) reduction in ligand binding at the highest [^{3}H]NBMPR concentration. However, as expected from the NBMPR inhibition results in Fig. 2, plots of bound vs. free radioligand were linear both in the absence and in the presence of NBTGR, indicating an absence of high-affinity [^{3}H]NBMPR binding sites. Similar negative results were obtained for isolated membranes.

To kinetically characterise nucleoside transport in hagfish red cells, initial rates of uridine influx were

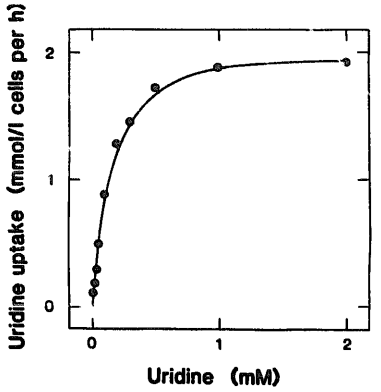

Fig. 3. Concentration-dependence of uridine uptake by hagfish red blood cells. [^{14}C]Uridine influx was measured at 10°C using a 5 min incubation period. Individual data points represent means of duplicate determinations.

measured as a function of extracellular uridine concentration in the range of 0.01–2.0 mM (Fig. 3). Uptake was saturable and conformed to simple Michaelis-Menten kinetics. Kinetic constants derived form a plot of s/v vs. s were; apparent K_m 0.14 mM, V_{max} 2 mmol/l cells per h. For comparison, the apparent K_m values for uridine uptake by the NBMPR-insensitive and NBMPR-sensitive nucleoside transporters of rat red cells are 0.16 and 0.05 mM, respectively (22°C) [20]. With respect to the substrate specificity of the hagfish nucleoside transport mechanism, we tested the effects of nonradioactive inosine and adenosine as inhibitors of 0.1 mM uridine uptake. The two nucleosides inhibited uridine uptake with equal efficacy, causing 80 and 98% inhibition at 0.1 and 1.0 mM extracellular nucleoside, respectively (control flux in the absence of competitor, 0.66 mmol/l cells per h). These results provide evidence that the hagfish red cell system accepts both purine and pyrimidine nucleosides, thereby exhibiting a broad substrate selectivity similar to equilibrative nucleoside transport systems found in mammalian cells [1–3].

NBMPR-sensitive and NBMPR-insensitive equilibrative nucleoside transporters from a wide range of mammalian cell types and species are susceptible to inhibition by the vasodilator dipyridamole [1–3]. As shown in Fig. 2, dipyridamole was a more effective inhibitor of uridine uptake by hagfish red cells than NBMPR, with an IC_{50} of ≈ 0.7 μM. This IC_{50} is higher than that found in most mammalian cell types, but is almost identical to that reported by us previously for dipyridamole inhibition of NBMPR-sensitive and NBMPR-insensitive uptake of uridine by rat red cells (IC_{50} 0.5 μM, 22°C) [20]. In a final experiment, we tested the effect of PCMBS on uridine transport by hagfish red cells. Cells were preincubated with extracellular PCMBS concentrations of 50 μM and 1 mM for 30 min at 1°C (10% haematocrit), then washed twice with ice-cold medium to remove unreacted orgamercurial. The initial rate of uridine influx (1 mM) under these conditions was inhibited 20 and 80%, respectively (control flux in cells pretreated with incubation medium only, 1.42 mmol/l cells per h, 10°C). These results contrast with 'classical' NBMPR-sensitive nucleoside transport in human, sheep and rat red cells [20,23,27] and in some mammalian cultured cell lines [14], which is unaffected by 1 mM PCMBS treatment at low temperature, conditions which minimise access of the organmercurial to the inner membrane surface. In contrast, the NBMPR-insensitive nucleoside transport mechanisms of rat red cells, Walker 256 rat carcinosarcoma and L1210 mouse leukaemia cells are characterised by the presence of an exofacial thiol group susceptible to PCMBS inhibition [13,14,20].

In conclusion, the present results suggest that hagfish red cells possess a single component of nucleoside transport which has the characteristics of NBMPR-insensitive equilibrative nucleoside transporters found in a number of mammalian cell types. The occurrence of such a transport mechanism in hagfish indicates that transporters of this type have a wide biological distribution and that they pre-date or arose at an early stage of vertebrate evolution. NBMPR-insensitive nucleoside transporters are not, however, ubiquitions in fish species. In parallel studies of teleosts, we have found that eel red cells (*Anguilla japonica*) possess a high density of conventional NBMPR-sensitive nucleoside transporters ($\approx 2 \cdot 10^5$ copies per cell, based upon [^{3}H]NBMPR-binding) (unpublished obervation). Photoaffinity-labelling experiments indicate that the eel transporter has an apparent molecular weight within the normal mammalian range of 45000–66000. The occurrence of nucleoside transporters in fish red cells may serve to make available plasma nucleosides for intracellular energy metabolism. In mammalian red cells, for example, there is both biochemical and genetic evidence that plasma adenosine is a physiological precursor of red cell ATP [28–30]. Also, inosine functions as a source of glycolytic intermediates in species which lack (pig) or have a low glucose transport activity (sheep) [29–31]. Glucose transport-deficiency has been reported in a number of teleost species (see, for example, Ref. 32), while hagfish red cells have a high capacity for hexose transport [33].

We thank the staff of the Bamfield Marine Station for providing laboratory and other facilities. This work was supported by the Natural Sciences and Engineering Research Council of Canada, the Alberta Heritage Foundation for Medical Research, the Royal Hong Kong Jockey Club and the Universities and Polytechnics Grants Committee, Hong Kong. In 1988, D.A.F. was supported as a Research Associate of the Western Canadian Universities Marine Biological Society. J.D.Y. is a Heritage Medical Scientist.

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