Biochimica et Biophysica Acta, 597 (1980) 183—188

BBA Report

BBA 71443

IS INOSINE THE PHYSIOLOGICAL ENERGY SOURCE OF PIG ERYTHROCYTES?

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(Received August 13th, 1979)

Key words: Adenosine; Inosine; Adenosine deaminase; Nucleoside transport; ATP; (Pig erythrocyte)

Summary

Pig erythrocytes are unable to metabolize glucose and their physiological energy source is unknown. These cells have a high-capacity nucleoside transport system with similar properties to that responsible for nucleoside transport in other species. Nucleoside transport is sufficiently rapid to allow the possibility that inosine and/or adenosine may represent major energy substrates for pig erythrocytes in vivo. Normal and adenosine deaminase-deficient pig erythrocytes have similar ATP levels, suggesting that adenosine is not important in this respect. However, it was calculated that an extracellular inosine concentration of only 40 nM could support the cells' entire energy requirement, a value 40-fold lower than plasma levels of this nucleoside.

Erythrocytes from the pig, unlike other mammalian red cells, are unable to metabolize glucose [1—4]. As a consequence, glucose cannot support important physiological processes such as ATP regeneration [5], active cation transport [6—9] or methaemoglobin reduction [10,11]. Treatment of pig cells with amphotericin B, an antibiotic which considerably increases membrane permeability, allows glucose conversion to lactate at a rate comparable to that found with untreated human erythrocytes, demonstrating that the inability of normal pig cells to utilize glucose is not due to a lack of glycolytic enzymes or co-factors, but that it results from an inability of the cell mem-

brane to transport glucose [5,12]. In contrast, erythrocytes from newborn pigs possess a functional glucose transport system [13,14] and are consequently highly permeable to the hexose. These glucose-permeable foetal cells are able to use glucose as an energy substrate [15], but are replaced by glucose-impermeable adult erythrocytes within a month after birth. Despite considerable investigation over the last 50 years, the energy source of adult pig erythrocytes is unknown. In the present communication, we reassess some existing evidence and present additional new data to suggest that the nucleoside inosine may fulfill this role.

There is already existing evidence to suggest that a whole variety of compounds including inosine, adenosine, ribose, deoxyribose, glyceraldehyde and dihydroxyacetone can maintain ATP levels in adult pig erythrocytes in vitro [4,5,7,11,12,16]. It is also true, however, that these compounds have been used in concentrations far higher than those normally found to exist in pig plasma [4,5,12] and that pig plasma itself is unable to sustain ATP levels in adult pig erythrocytes [5,12]. It follows from this that a critical balance must exist between supply and utilization of energy substrate and that the erythrocyte must be able to take up substrate from low concentrations in circulating blood. Such a mechanism has already been demonstrated for nucleoside-permeable sheep erythrocytes which maintain ATP levels 40% higher than do similar cells having a genetically-controlled nucleoside transport deletion [17]. It was decided, therefore, to look for the presence of such a nucleoside transport system in adult pig erythrocytes with the aim of relating its properties to the concentration of nucleosides found in circulating plasma.

Experiments performed at 37°C established that nucleoside transport in adult pig erythrocytes was extremely rapid. Subsequent kinetic experiments were therefore performed at the lower temperature of 25°C. Fig. 1 shows the concentration dependence of inosine uptake at this temperature. Transport was saturable with apparent K_m and V values of 0.18 mM and 57.7 mmol/l cells per h, respectively. Uridine and adenosine transport also conformed to Michaelis-Menten kinetics (apparent K_m 0.25 and 0.08 mM, respectively: V 50.5 and 25.2 mmol/l cells per h, respectively). Inosine was an effective inhibitor of uridine uptake with an apparent K_i of 0.14 mM (Fig. 2). Lineweaver-Burk analysis of the data established that inhibition was competitive. Adenosine also competitively inhibited uridine influx (apparent K_i 0.048 mM). Temperature-dependence studies for adenosine, inosine and uridine gave a mean Q_{10} value for V of 4.1 with an apparent activation energy of 24 kcal/mol. In contrast to V, the apparent K_m for inosine influx was relatively insensitive to temperature (0.11 and 0.12 mM at 25 and 15°C, respectively).

Various vasodilator drugs and S-substituted 6-thiopurine ribonucleosides are potent nucleoside transport inhibitors in a variety of cell types [17—22]. Fig. 3 shows that nitrobenzylthioinosine and dipyridamole (2,6-bis-(diethanolamino)-4,8-dipiperidinopyrimido(5,4-*d*)pyrimidine) are good inhibitors of uridine uptake by pig erythrocytes. The concentrations required to induce 50% inhibition of 1 mM nucleoside uptake were 0.07 and 0.5 μM for nitrobenzylthioinosine and dipyridamole, respectively (final haematocrit 8%). Binding studies at 25°C with ^{3}H-labelled nitrobenzylthioinosine (Movarek

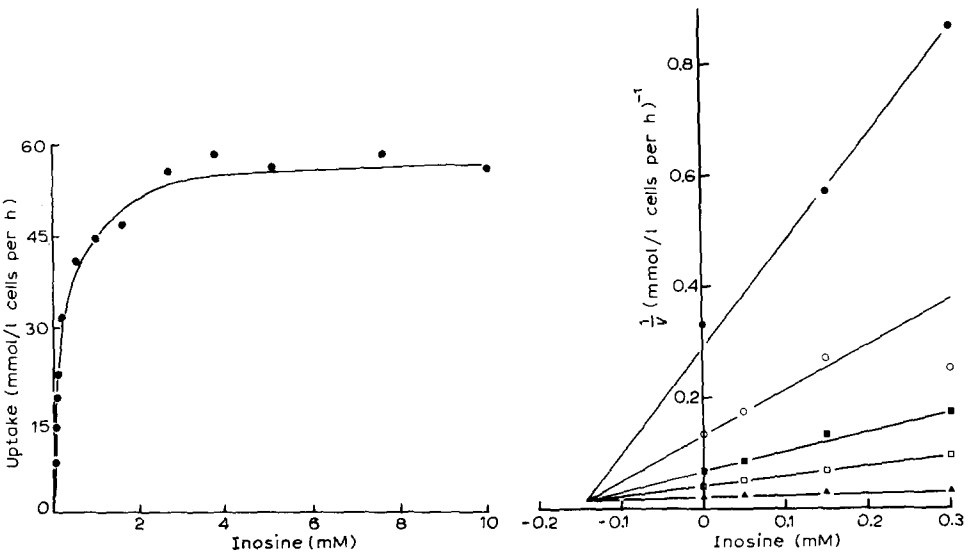

Fig. 1. Concentration dependence of inosine uptake by adult pig erythrocytes. Freshly-drawn pig erythrocytes were washed three times with 20 vols. of a medium containing 140 mM NaCl, 5 mM KCl, 20 mM sodium phosphate (pH 7.2 at 25°C), 2 mM $MgCl_2$ and 0.1 mM EDTA (disodium salt). The buffy coat was discarded. Nucleoside uptake at 25°C was measured by mixing 0.2 ml pre-warmed washed erythrocytes (haematocrit approx. 20%) with 0.2 ml pre-warmed medium containing 0.054—20 mM inosine (including [U-^{14}C]inosine (0.5 Ci/mol), Radiochemical Centre, Amersham). Incubations were stopped after 3 s by transferring 0.2 ml of the cell suspension to 0.8 ml ice-cold stopping solution (ice-cold medium containing 10 μM nitrobenzylthioinosine, a generous gift from Professor A.R.P. Paterson, University of Alberta Cancer Research Unit, Edmonton, Canada) layered on top of 0.5 ml ice-cold *n*-dibutyl phthalate in an Eppendorf microcentrifuge tube (vol. 1.5 ml). The tubes were immediately centrifuged (15 000 × *g*, 15 s) using an Eppendorf 3200 microcentrifuge. The upper layer of cell-free aqueous medium and the majority of *n*-dibutyl phthalate were removed by suction, leaving the cell pellet at the bottom of the tube. After carefully wiping the inside of the centrifuge tube with absorbent tissue, the cells were processed for scintillation counting as previously described [17]. Blank values were obtained using erythrocyte samples which had been mixed with radioactive inosine at 1°C. Transport rates were calculated after subtraction of these blanks (usually less than 10% of the transport rate). Uptake for all inosine concentrations was linear for at least 5 s. The data were analyzed as a two-parameter hyperbola by the method of Bliss and James [37] to give apparent K_m and V values of 0.18 ± 0.02 mM and 57.7 ± 1.1 mmol/l cells per h, respectively. These constants were used to draw the fitted curve.

Fig. 2. Effect of inosine on uridine uptake by pig erythrocytes. The reciprocals ($1/v$) of uridine uptake rates (means of duplicate estimates) at 1.0 (▲), 0.2 (□), 0.1 (■), 0.05 (○) and 0.025 (●) mM are plotted against the respective concentrations of inhibitor (inosine). Apparent K_i 0.14 mM. See legend to Fig. 1 for other experimental details.

Biochemicals, CA) gave a high-affinity binding component of $(850 \pm 70) \cdot 10^{-23}$ mol/cell (mean ± S.E. [5]) (apparent K_D 1.3 nM). Similar results were obtained at 37°C.

A number of studies have postulated that nucleosides cross cell membranes by group-translocation mechanisms [23—26]. One investigation has suggested that adenosine deaminase is involved in adenosine uptake by human erythrocytes [27]. The present findings are not consistent with these hypotheses. The apparent K_i values for inosine and adenosine inhibition of uridine uptake are sufficiently close to the apparent K_m values for their own uptake to suggest that all three nucleosides are transported by the same system. We further tested the involvement of adenosine deaminase in nucleoside transport by investigating pig erythrocytes with an inherited deficiency of this enzyme [28, 29]. These cells have normal kinetic constants for nucleoside influx

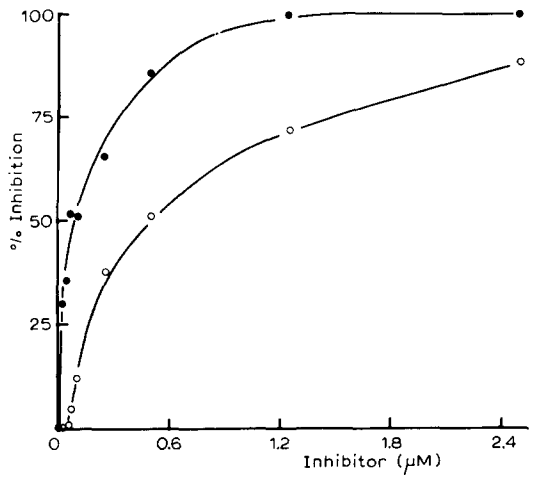

Fig. 3. Effect of nitrobenzylthioinosine and dipyridamole on uridine uptake by normal pig erythrocytes. Uridine (1 mM) and inhibitor were added to cell suspensions at the same time. ●—●, Nitrobenzylthioinosine; ○—○, dipyridamole. Values are means of duplicate estimates. Other experimental details are given in the legend to Fig. 1.

despite a 180-fold reduction in enzyme activity (apparent K_m for adenosine, inosine and uridine 0.091, 0.16 and 0.35 mM, respectively: V 27.3, 56.8 and 64.1 mmol/l cells per h, respectively). Adenosine and inosine competitively inhibited uridine uptake with apparent K_i values of 0.063 and 0.14 mM, respectively, and the apparent activation energy for adenosine influx was 22.4 kcal/mol. Nucleoside uptake by adenosine-deaminase deficient cells was readily inhibited by nitrobenzylthioinosine and dipyridamole and binding experiments with ^{3}H-labelled nitrobenzylthioinosine gave a high affinity binding component of $(840 \pm 70) \cdot 10^{-23}$ mol/cell (mean ± S.E. [5]) (apparent K_D 1.4 nM). It is therefore unlikely that group-translocation mechanisms are responsible for nucleoside transport by pig erythrocytes. Evidence supporting group-translocation mechanisms in other cell types has been critically reviewed and rejected by Plagemann and co-workers [30].

These results establish that adult pig erythrocytes possess a high-capacity medium-affinity transport system for nucleoside uptake which does not discriminate between purine and pyrimidine nucleosides. The properties of this system are similar to those of nucleoside transport in human and nucleoside-permeable sheep erythrocytes, although sheep cells have a considerably lower V [17,22,31—33]. We have previously shown that the saturable high-affinity binding of nitrobenzylthioinosine to intact erythrocytes or isolated cell membranes represents specific interaction of inhibitor with functional nucleoside transport sites [33]. Assuming that each high-affinity binding site represents a single nucleoside transport system, the binding data indicate that each pig erythrocyte has approx. 5000 nucleoside transport sites per cell, giving estimated turnover numbers for inosine and adenosine influx of 137 and 60 molecules/s per site, respectively, at 25°C or approx. 560 and 250 molecules/s per site, respectively, at 37°C. Adenosine and inosine fluxes in the pig are therefore sufficiently rapid to allow the possibility of extremely low plasma concentrations providing sufficient glycolytic intermediates to maintain the required level of ATP synthesis.

The possible role of adenosine in this process can be checked using adenosine deaminase-deficient pig erythrocytes. These cells were found to have normal ATP levels (2.23 ± 0.16 and 2.14 ± 0.18 mmol/l cells for normal and adenosine deaminase-deficient cells, respectively (mean ± S.E. [8]), estimated by an NADH-enzyme coupled assay (Boehringer Test Combination Diagnostics)). This strongly suggests that adenosine deaminase is not directly involved in ATP metabolism, a conclusion supported by Perrett and Dean [34] who found that adenosine kinase, rather than adenosine deaminase, is the preferred route for adenosine metabolism in human erythrocytes at physiological (μM) substrate concentrations.

Assuming, therefore, that inosine is the relevant nucleoside, knowledge of the kinetic constants of transport allows the influx rate at any external nucleoside concentration to be estimated and vice versa. If pig erythrocytes in vivo utilize ATP at a similar rate to that found in vitro (0.125 mmol/l cells per h [5]), the maximum nucleoside requirement is 47 μmol/l cells per h (2.67 ATPs per molecule, assuming no recycling). Using estimated apparent K_m and V values for inosine influx at 37°C of 0.17 mM and 235 mmol/l cells per h, respectively, it can be calculated from the Michaelis-Menten equation that a steady-state plasma inosine concentration of 40 nM will supply the cells' entire energy requirements, provided that transport is rate-limiting, an assumption we have verified by net uptake studies. We therefore measured plasma and erythrocyte inosine concentrations in blood samples (ear vein) from unanaesthetized animals taking precautions to avoid inosine uptake and metabolism during sample preparation. Blood was collected into syringes containing dipyridamole (final concentration 25 μM), immediately chilled and centrifuged. Plasma and erythrocytes were deproteinated with ice-cold trichloroacetic acid within 5 min of collection. Analysis by high-performance liquid chromatography [35] gave a plasma inosine concentration of 1.67 ± 0.23 μM (mean ± S.E. [4]), lower than a previous literature value of 11 μM obtained under experimental surgical conditions [36], but still more than 40-fold greater than the required steady-state concentration. The erythrocyte inosine concentration (corrected for trapped plasma using [^{3}H]inulin as space marker) was 0.054 ± 0.027 μmol/l cell water giving an [erythrocyte]:[plasma] ratio of 0.032, additional direct evidence that erythrocyte inosine metabolism is limited by membrane transport.

The amount of inosine in circulating blood and the ability of adult pig erythrocytes to remove and metabolize this substrate are both consistent with the notion that inosine acts normally as a major energy source for maintaining ATP levels in pig erythrocytes in vivo.

S.M.J. is in receipt of an M.R.C. postgraduate studentship.

Note added in proof: (Received February 12th, 1980). An independent study by Watts et al. (Life Sci. 25, 1577—1582), published after the present manuscript was submitted, provides additional evidence that inosine is a physiological energy source of pig erythrocytes. This inosine is possibly derived from the liver (Kim et al. (1980) Biochim. Biophys. Acta (in the press), cited by Watts et al.). Pig erythrocytes may therefore experience local high concentrations of plasma inosine even greater than 2 μM.

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