BIOCHEMICAL LETTERS

Conflicting evidence regarding the transport of α -glutamyl-dipeptides by human erythrocytes

We write concerning recent ^1H spin-echo n.m.r. studies published in this journal (King & Kuchel, 1984, 1985) and elsewhere (King *et al.*, 1983; Vandenberg *et al.*, 1985) reporting the presence of high-capacity transport mechanisms for di- and tri-peptides in human erythrocytes. It has been proposed that these systems function to scavenge small peptides from plasma, allowing erythrocyte peptidases to contribute to whole body peptide metabolism and perhaps also the catabolism of peptide hormones and/or their degradation products (Vandenberg *et al.*, 1985). It has also been suggested that plasma α -glutamyl-peptides may be the physiological source of glutamate for erythrocyte glutathione (GSH) biosynthesis (King & Kuchel, 1985). Glutamate for this purpose is required at a relatively modest rate of 12 $\mu\text{mol/h}$ per litre of cells. However, in contrast with cysteine and glycine, the two other constituent amino acids of GSH, human erythrocytes exhibit a low permeability towards free glutamate (Young *et al.*, 1980).

King & Kuchel (1985) investigated α -glutamyl-dipeptide transport across the human erythrocyte membrane by an indirect method employing n.m.r. to monitor hydrolysis of peptides added to cell suspensions at high haematocrit. Kinetic constants for steady-state trans-membrane peptide fluxes were computed from progress curves of peptide hydrolysis by using the integrated Michaelis-Menten equation, data analysis relying on n.m.r. evidence that hydrolysis occurred intracellularly and that peptide transport was the rate-limiting step of the process. Reported apparent K_m and V_{max} values for glutamylalanine transport at 37 °C were 2.4 mM and 3.6 mmol/h per litre of cells, respectively, compared with 11.2 mM and 19.7 mmol/h per litre of cells, respectively, for alanylglutamate transport.

Our long-standing interest in erythrocyte GSH metabolism and amino acid transport prompted us to attempt to study glutamyl-peptide transport by human erythrocytes directly by using conventional transport methodology (Young & Ellory, 1982). In the experiment shown in Fig. 1, fresh human erythrocytes collected into heparin were washed free of plasma and incubated at 37 °C (10% haematocrit) in substrate-free buffered iso-osmotic NaCl medium [150 mM-NaCl, 5 mM-glucose and 15 mM-Mops (pH 7.5 at 37 °C)] or in medium containing 10 mM of either α -L-glutamyl-L-alanine, L-alanyl- α -L-glutamate, L-glutamate or L-alanine. At predetermined time intervals (30 min–3 h), aliquots (1.0 ml) were removed and immediately centrifuged at 12000 g for 15 s in an Eppendorf 5414 microcentrifuge. Supernatants were retained, and the cells rapidly washed four times with ice-cold medium to remove extracellular

Fig. 1. Intracellular alanine and glutamate concentrations in human erythrocytes incubated in substrate-free medium (▲) or in the presence of 10 mM amino acid (●), glutamylalanine (○) or alanylglutamate (△)

Values are means of duplicate determinations. See the text for experimental details.

peptide and/or amino acid. Samples were then deproteinized with 5% (w/v) sulphosalicylic acid and the extracts analysed on an LKB 4400 amino acid analyser using the manufacturer's lithium buffer system for physiological fluids. Glutamylalanine was eluted from the analyser column between citrulline and α -aminobutyrate, whereas alanylglutamate was eluted between α -aminobutyrate and valine.

Under these experimental conditions, there was no evidence of large-scale glutamylalanine or alanylglutamate uptake during the 3 h incubation period. Thus, intracellular alanine and glutamate concentrations at the start of the incubation were 0.26 and 0.40 mmol/litre of cells, respectively, compared with 0.27 and 0.73 mmol/litre of cells after 3 h in the presence of 10 mM extracellular glutamylalanine, 0.43 and 0.60 mmol/litre of cells in the presence of alanylglutamate and 0.14 and 0.41 mmol/litre of cells after 3 h in the absence of peptide. None of the cell samples contained detectable intracellular levels of dipeptide (< 0.01 mmol/litre of

cells). In marked contrast, we were able to demonstrate the expected large increase in intracellular alanine in erythrocytes incubated in the presence of 10 mM extracellular free amino acid (6.6 mmol/litre of cells after 3 h). The observed time-course of L-alanine uptake in this experiment is in good agreement with previously published rates of L-alanine transport in human erythrocytes determined either by tracer (Young *et al.*, 1980, 1983) or spin-echo n.m.r. (Brindle *et al.*, 1979) methods. This uptake is mediated by the amino acid transport systems ASC and L.

The poor ability of cells to transport free glutamate (Fig. 1) is also in agreement with previous studies (Young *et al.*, 1980) and eliminates the possibility of substantial peptide-derived glutamate efflux from the cells either during the 37 °C incubation or during the subsequent ice-cold washing steps. Intracellular glycine remained unchanged at 0.31 mmol/litre of cells during incubation of erythrocytes with glutamate and the two dipeptides. Thus, there was no significant metabolism of intracellular glutamate to GSH. Analysis of the extracellular media revealed that there was < 5% hydrolysis of alanylglutamate or glutamylalanine during the course of the 3 h incubation. Repeat experiments with cells from additional unrelated donors provided essentially identical results. In a control experiment, we incubated glutamylalanine (10 mM) for 1 h at 37 °C in the presence of a 10% erythrocyte lysate. There was 27% peptide hydrolysis under these conditions, confirming that haemolysates are capable of peptide breakdown.

The presence of peptide transporters in erythrocytes is an attractive concept, not least because erythrocytes would provide a convenient model system for detailed kinetic and molecular studies of peptide transporter function. As reviewed by Matthews & Payne (1980), the occurrence of physiologically important peptide transport systems in tissues such as the small intestine is well established. Unlike King & Kuchel (1985), our results suggest that human erythrocytes have a low capacity for α -glutamyl-peptide transport. Based on the intracellular glutamate levels in Fig. 1, we estimate maximum rates of glutamylalanine and alanylglutamate transport of 0.08 and 0.06 mmol/h per litre of cells respectively (10 mM extracellular peptide), 35-fold and 155-fold less than predicted rates calculated from the n.m.r.-derived kinetic constants.

With regard to GSH biosynthesis, peptide-derived glutamate uptake was significantly less than glutamate uptake from 10 mM free amino acid which occurred at 0.11 mmol/h per litre of cells. Thus, alanylglutamate and glutamylalanine were less efficient than glutamate as a source of intracellular amino acid. The concentrations of α -glutamyl-peptides in human plasma are not known, but are likely to be in the low micromolar range. Even for free glutamate, the observed rate of transport corresponds to only 1 μ mol/h per litre of cells at its physiological concentration of 60 μ M (Fasman, 1976), i.e. < 10% of the erythrocyte's glutamate requirement. It is therefore highly doubtful whether plasma glutamyl-peptides can contribute significantly towards the provision of glutamate for erythrocyte GSH biosynthesis. The source of glutamate for this purpose remains to be resolved.

We have also carried out similar transport experiments with glycylglycine, one of the other peptides studied by Kuchel and co-workers (King *et al.*, 1983). Again, we

found no evidence of large-scale peptide or peptide-derived amino acid uptake by the cells (0.07 mmol/h per litre of cells at 10 mM extracellular peptide, compared with the published rate of 7.2 mmol/h per litre of cells), raising general doubts about the presence of dipeptide transport systems in human erythrocytes. The low permeability of human erythrocytes to glycylglycine was confirmed in a parallel series of experiments in which cells were incubated with 10 mM peptide for up to 3 h as described above, washed free of extracellular peptide with ice-cold medium and samples of the washed packed erythrocytes (0.5 ml) analysed by ¹H-n.m.r. (Rabenstein *et al.*, 1985). In those experiments we were unable to detect either an increase in cellular glycine concentration or the presence of glycylglycine in erythrocytes. The reasons for the discrepancies between the present results and those of King *et al.* (1983) and King & Kuchel (1985) are unclear, but may be related to the methodologies used in their n.m.r. experiments which permit the calculation of steady-state parameters for peptide transport without direct demonstration of peptide translocation across the cell membrane. Finally, we note that the incubation medium used by King *et al.* (1983) contained amphotericin B. This channel-forming antibiotic is known to alter dramatically the permeability characteristics of erythrocyte membranes [see for example Kim & McManus (1971)]. It is not clear whether amphotericin B was also present in the glutamyl-peptide transport experiments.

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No evidence of high capacity α -glutamyl-dipeptide transport into human erythrocytes

In a recent letter Young *et al.* (1987) reported results which led them to contradict our claim (King & Kuchel, 1985) that human erythrocytes are highly permeable to α -L-glutamyl-L-alanine and L-alanyl- α -L-glutamate. They deduced, therefore, that these peptides cannot serve as a physiological source of glutamate for glutathione

synthesis in human red cells. We agree with the new conclusions and confirm that the interpretation of our earlier experimental results was incorrect. The method we used to infer peptide transport was based on measuring the appearance of signals from free amino acids in the ^1H spin-echo n.m.r. spectra of red cells treated with peptide; the high concentration of intracellular peptidases catalyse the hydrolysis of the peptides, and the rate of formation of free amino acids was interpreted as being due to peptide transport across the cell membrane into the cytoplasm.

It is now realized that, especially in high-haematocrit suspensions (even in non-spinning n.m.r. tubes), invariably approx. 1% of the cells haemolyse over a 2 h period. This releases peptidases to the extracellular medium where they catalyse peptide hydrolysis. The phenomenon is illustrated in Fig. 1. Fresh, washed red cells (haematocrit 32%; this is less than half that which was used in our previous n.m.r. experiments) were incubated with peptide at 37 °C. N.m.r. spectra were recorded periodically at a probe temperature of 37 °C. Spectrum A was obtained 7 min after addition of L-alanyl- α -L-glutamate; the spectral features are similar to those reported previously (King & Kuchel, 1985). The small inverted peak a indicates the presence of free alanine, whilst the large (truncated) resonance, adjacent to it at higher frequency, is from the corresponding methyl group in the peptide. Spectrum B, which was obtained after 291 min of incubation, contains two obviously new features; the alanine resonance has increased in intensity as has that denoted g, namely, the glutamate- H^γ . Spectrum C is of red cells (haematocrit 80%) obtained after washing the sample immediately after the acquisition of spectrum B. The small glucose peaks (the main complex centred at 3.4 p.p.m.) and lactate (methyl resonance at 1 p.p.m.) in spectrum C, relative to the

Fig. 1. ^1H spin-echo n.m.r. spectra of human erythrocytes

Sample preparation: venous blood was withdrawn (from B.E.C.) and immediately washed by two cycles of centrifugation ($\sim 3000\text{ g}$ for 5 min, 4 °C) in 0.154 M-NaCl/5 mM-glucose and three cycles ($\sim 2000\text{ g}$ for 5 min, 4 °C) in the same medium constituted with $^2\text{H}_2\text{O}$. The buffy coat was aspirated along with the supernatant at each washing step. The final haematocrit was adjusted to 32% and 2.5 ml of the suspension was added to a 5 mm (o.d.) glass n.m.r. tube. At the start of the reaction time-course 70 μl of 500 mM-L-alanyl- α -L-glutamate was added to the cell suspension and immediately mixed by five times inverting and re-inverting the tube with movement of the trapped air bubble along the full extent of the tube.

N.m.r. conditions: we used a Varian XL-400 spectrometer operating in the pulse Fourier transform mode; the probe temperature was 37 °C; the residual $^1\text{HO}_2\text{H}$ resonance in the spectra was suppressed by selective irradiation prior to the spin-echo pulse sequence (e.g., King & Kuchel, 1985). Data was stored in 8192 memory locations. Each spectrum was derived from 128 free-induction decays and a line broadening factor of 1 Hz was used. The three spectra above were plotted so that the intensity of the glutathione-glutamyl- H^γ resonances (denoted by GSH) were of equal amplitude. A, Spectrum of cell suspension 7 min after addition of the peptide; B, the same sample after 291 min of incubation; C, cells from the sample in B obtained by washing four times in the $^2\text{H}_2\text{O}$ medium using the method of Young *et al.* (1987). Spectral assignments are not given in detail but 'a' denotes the free alanine- H^β , 'g' denotes free glutamate- H^γ . The chemical-shift scale was assigned with the ergothionine $-\text{N}-(\text{CH}_3)_3$ resonance set to 3.100 p.p.m.