Uptake and metabolism of dipeptides by human red blood cells

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A function of the abundant cytoplasmic peptidases in red blood cells could be hydrolysis of oligopeptides circulating in plasma. To investigate whether human red blood cells actively transport dipeptides for this purpose, these cells were incubated with ^{14}C -labelled glycylproline, glycylsarcosine, glycylalanine, glycine, proline and alanine. There was uptake of each dipeptide, as indicated by their recovery as dipeptides in the cell cytoplasm. However, after a brief time (1–2 min) uptake of dipeptides abruptly ceased, while that of amino acids continued. As a result, after 30 min red blood cell uptake of amino acids was 5–13-fold greater than that of any dipeptide. Investigation of intracellular contents after 1 min of incubation revealed different metabolism for different dipeptides. The composition of intracellular radioactivity was 19–71 % as intact dipeptides, 0–20 % as free amino acids and 8–77 % as neither dipeptides nor constituent amino acids. Investigation of the mechanism of dipeptide uptake by red blood cells showed: (1) a lack of hydrolysis by the plasma membrane, (2) no non-specific binding to the plasma membrane, and (3) a lack of saturation over a wide range of concentrations (0.05–50 mM). The data suggest that the mechanism of uptake of trace amounts of dipeptides by human red blood cells is either by simple diffusion or by a carrier system which has a very weak affinity for dipeptides. Upon entry, depending on the molecular structure, dipeptides are either hydrolysed or transformed into new compounds. The red blood cell uptake, however, does not appear to play any appreciable role in clearance of dipeptides from the plasma in the human.

INTRODUCTION

Oligopeptides may enter the bloodstream as products of protein digestion, hormones, enkephalins, glutathione and others. The cytoplasm of red blood cells contains considerable hydrolase activity against oligopeptides [1,2], the function of which remains uncertain. One possibility could be that it is involved in the hydrolysis of oligopeptides circulating in plasma. The above possibility requires entry of oligopeptides into red blood cells. Indeed, the results of recent studies have suggested the presence of transport systems for dipeptides and tripeptides in human red blood cells [3–6]. The method used to infer peptide transport was based on measuring the appearance of signals from free amino acids in the proton n.m.r. spectrum of red blood cells incubated with dipeptides and tripeptides [3–6]. However, a number of methodological problems make uncertain the conclusion that oligopeptides are taken up into red blood cells [7]; for example, the incubation period was maintained for 3 h, and during such a prolonged incubation period there was some haemolysis of red blood cells, resulting in the release of intracellular peptidases into the incubation medium [8]. Therefore free amino acid formation from peptides could have occurred outside rather than inside the red blood cells.

The purpose of the present experiments was to investigate the entry of dipeptides into human red blood cells (1) by a technique which directly measures transport, and (2) in the absence of extracellular hydrolysis. The technique involved the incubation of red blood cells with ^{14}C -labelled dipeptides and measurement of intracellular radioactivity as dipeptide. The problem of extracellular hydrolysis was eliminated by maintaining a short incubation period (e.g. 1 min) and by using dipeptides which are resistant to membrane hydrolysis. Glycylproline and glycylsarcosine have been previously used for the assay of peptide transport activity in tissues [9–11].

MATERIALS AND METHODS

Uptake studies

Blood for all experiments was obtained by venous puncture from healthy volunteers. Red cells were washed at least three times with 5 vol. of buffer containing 140 mM-NaCl, 5 mM-KCl, 2 mM-MgCl₂ and 15 mM-Tris, pH 7.5, by centrifugation for 10 min at 500 g. Supernatant and buffy coat were removed by aspiration. The final suspension was diluted with buffer to a haematocrit of approx. 50 %.

The method used to study dipeptide uptake by red blood cells was similar to that previously used for amino acid uptake, with some modifications [12]. Briefly, equal volumes of prewarmed red blood cell suspension and a buffer containing a dipeptide or amino acid were mixed. The buffer was the same as described above, except for appropriate decreases in NaCl concentration to maintain iso-osmolarity with each concentration of dipeptide or amino acid. Incubations were performed at 37 °C in a shaking water bath. At various time intervals, 300 μl of incubation mixture was removed into a 1.8 ml centrifuge tube containing 1 ml of ice-cold buffer and centrifuged for 10 s at 12800 g in an Eppendorf microcentrifuge. The supernatant was removed by aspiration and the cells were resuspended in 1 ml of buffer containing 2 % sucrose, and recentrifuged for 10 s at 12800 g. Again the supernatant was removed; part of it was kept for sucrose determination and the remainder was diluted with 10 ml of Aquasol-2 (New England Nuclear, Boston, MA, U.S.A.) and counted for radioactivity in a Packard liquid scintillation counter. The tubes with the pellets were weighed and the cells were then extracted by addition of 1 ml of 5 % trichloroacetic acid. The precipitates were centrifuged for 1 min at 12800 g and the supernatant was removed. Part of it was again kept for sucrose determination and the rest was counted for radioactivity. The tubes with the pellets were dried overnight at 100 °C and

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then weighed. Blanks were produced by adding ice-cold red cell suspension and peptide or amino acid solution to the centrifuge tube and immediately adding buffer.

Intracellular volume

Intracellular volume was determined as previously described [13]. Briefly, total water content per g of cells was determined from the difference between wet and dry weights. The portion of trapped extracellular water was determined by sucrose measurement and the intracellular water per g of cells was calculated as the difference between total and extracellular water.

Non-specific binding

Two methods were used to assess non-specific binding. First, the red blood cells after incubation with dipeptides were lysed with 1 ml of 5% trichloroacetic acid, water or 0.5% Triton X-100. The samples were centrifuged and the precipitate was solubilized by addition of Soluene-350 (Packard Instruments, Downers Grove, IL, U.S.A.) and counted for radioactivity content. Second, after incubation of red blood cells with dipeptides (0.05–0.5 mM) as described above, either the usual buffer or one containing 10 mM dipeptide was used to terminate the uptake. The remainder of the procedure was as described above.

Intracellular content

For the analysis of intracellular content after incubation with each [^{14}C]dipeptide or [^{14}C]amino acid, 6% sulphosalicylic acid was added to the red blood cells. The procedures for incubation of red blood cells and preparation of supernatant were the same as described above for uptake studies. The radioactivity content of the supernatant was fractionated by means of a cation-exchange chromatography technique utilizing an amino acid analyser (Varian, Walnut Creek, CA, U.S.A.). Fractions were collected from the amino acid analyser at 4 min intervals, diluted with 10 ml of Aquasol-2 and counted for radioactivity content. Identification of the radioactivity was made by comparison with the elution times of known standards.

Calculations and statistics

In order to calculate the first-order rate constant (K_d) as well as the K_m and $V_{\max}$, the initial rates of uptake (v) were fitted to the following equation:

$$v = (V_{\max} \cdot s) / (K_m \cdot s) + K_d \cdot s$$

where s is concentration in mM. Derivative-free non-linear regression analysis was performed using the AR segment of the BMDP Statistical Software [14]. Data are given as means $\pm$ S.E.M. Differences between rates of uptake were evaluated by the non-paired t test for two groups or by analysis of variance [14] followed by the Student–Newman–Keuls test [15] in the case of three or more groups.

Materials

Amino acids and peptides were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). L-[U- ^{14}C]Alanine, L-[U- ^{14}C]proline, [U- ^{14}C]glycine, glycyl-L-[U- ^{14}C]proline, and glycyl-L-[U- ^{14}C]alanine were purchased from New England Nuclear (Boston, MA, U.S.A.). [U- ^{14}C]Glycylsarcosine was a gift from Dr. Frederick Leibach of the Medical College of Georgia, Augusta, GA, U.S.A.

RESULTS

Uptake versus time

Initially, assuming that accumulation of radioactivity within the red blood cells represents uptake, we investigated the time

Fig. 1. Time course of peptide and amino acid uptake by human red cells over 2 min (a) and over 30 min (b)

The initial concentration of all substrates in the incubation medium was 0.5 mM. The intracellular concentration was calculated from radioactivity on the assumption that the intracellular radioactivity was present as dipeptide or amino acid respectively. The standard error at each point was approx. 5% of the mean value. ●, Ala; ■, Gly; ▲, Pro; ○, Gly-Ala; □, Gly-Pro; △, Gly-Sar.

course of uptake of glycylalanine, glycine and alanine. The concentration of each substrate in the medium was 0.5 mM. There was uptake of each substrate, which was linear over at least 60 s (Fig. 1a). After this time interval, the uptake of the dipeptide slowed dramatically. In contrast, the uptake of each amino acid was linear for at least 5 min and continued for the entire duration of incubation, which was 30 min (Fig. 1b). Even during the first 60 s the uptake of glycylalanine was less than that of either alanine or glycine. This pattern persisted as the incubation period was prolonged; for example, at 30 min the uptakes of alanine and glycine were 9- and 5-fold greater respectively than that of glycylalanine.

To determine whether this phenomenon also applies to non-hydrolysable dipeptides, we investigated the red cell uptake of glycylproline and glycylsarcosine (Fig. 1). Essentially the pattern of red cell uptake of these peptides was similar to that of glycylalanine, except that the uptake of glycylproline was linear for only 30 s. In contrast with glycylproline, the uptake of proline continued for the duration of the incubation period and was linear during the initial 10 min (Fig. 1b). At 30 min the uptake of proline was 13-fold greater than that of glycylproline (Fig. 1b).

Mechanism of uptake

To determine whether there is non-specific binding, two sets of experiments were performed. In one experiment, after incubation

Table 1. First-order rate constants for non-saturable uptake

Except for glycylproline, measurements were made after 60 s of incubation of human red blood cells with a range of concentrations of substrates. The incubation period was 20 s for glycylproline. The kinetic constants were calculated by derivative-free non-linear regression using a BMDP program. Data are presented as means $\pm$ S.E.M.

Substrate	<i>n</i>	Rate constant (min ⁻¹)
Glycylsarcosine	6	0.049 $\pm$ 0.002
Glycylproline	6	0.009 $\pm$ 0.002
Glycylalanine	6	0.007 $\pm$ 0.002
Glycine	3	0.010 $\pm$ 0.001
Proline	6	0.029 $\pm$ 0.009
Alanine	7	0.012 $\pm$ 0.002

of red cells with dipeptides and subsequent lysis by water, trichloroacetic acid or Triton X-100, only background levels of radioactivity were associated with the particulate fraction. Since the possibility remained that lysis by these agents might also have released peptides bound to the cell membrane, an additional study was performed. Addition of excess peptide to the wash buffer was ineffective in displacing radioactivity; rates of uptake were equal whether or not the buffer contained a 20–200 molar excess of peptide. Therefore the results of these studies do not provide any evidence for non-specific binding of dipeptides to human red blood cells.

To investigate whether there is mediation of a carrier system in the uptake of dipeptides, we determined the rates of red cell uptake of glycylproline, glycylsarcosine, glycylalanine, glycine, proline and alanine over a range of concentrations (0.05–50 mM; Table 1). The incubation period was 60 s for all amino acids and

dipeptides, except glycylproline, for which it was 20 s. With all three peptides and with proline, uptake was linear over the entire range of concentrations. In contrast, glycine and alanine showed saturable as well as non-saturable components of uptake.

Kinetic constants

The first-order rate constants for the non-saturable uptake of dipeptides and amino acids are given in Table 1. Among the dipeptides the order of the first-order rate constants was: glycylsarcosine > glycylproline = glycylalanine. Among the amino acids the value was greatest for proline, and not significantly different for the other two amino acids.

The kinetic parameters of the saturable components of alanine and glycine uptake were also calculated. The K_m values of glycine and alanine uptake were 0.19 ± 0.013 and 0.26 ± 0.08 mM respectively, and the V_{max} values of glycine and alanine uptake were 3.77 ± 1.24 and 5.16 ± 1.23 mmol $\cdot$ min⁻¹ $\cdot$ l⁻¹ respectively. Both K_m and V_{max} values were significantly ($P < 0.01$) greater for alanine than for glycine. Furthermore, the kinetic constants for saturable as well as non-saturable uptake of glycine and alanine are in excellent agreement with previously published results [12,16,17].

Intracellular metabolism

To investigate intracellular metabolism of dipeptides, we analysed the intracellular contents of the red blood cells after 1 min of incubation with [¹⁴C]glycylsarcosine, glycyl[¹⁴C]proline and glycyl[¹⁴C]alanine. Fractionation of the intracellular content after incubation with [¹⁴C]glycylsarcosine revealed the presence of two peaks of radioactivity, which accounted for the total radioactivity content of the red cells (Fig. 2). One peak was identified as glycylsarcosine, but the other peak indicated formation of a new compound from glycylsarcosine. There was no formation of free [¹⁴C]glycine. The amount of radioactivity was much greater in the unknown peak than in the glycylsarcosine

Fig. 2. Composition of radioactivity in red blood cell intracellular contents after 1 min of incubation

Sulphosalicylic acid extracts were prepared as described in the Materials and methods section. Aliquots containing approx. 2500 d.p.m. were applied to the ion-exchange column of the amino acid analyser and fractions were collected every 4 min for 1 h. For the purpose of comparison, the elution positions of the amino acids and peptides studied are indicated by arrows. In each case, the material referred to as 'unknown' is shown to elute within the first three fractions, i.e. within 12 min. Data are presented as means of between three and six experiments.

peak ($77 \pm 4\%$ versus $19 \pm 5\%$). Fractionation of the intracellular content of red blood cells after incubation with glycyl[^{14}C]proline showed three peaks of radioactivity (Fig. 2): one peak corresponded to proline, one to glycylproline and one indicated formation of a new compound from glycylproline. As with glycylsarcosine, the amount of radioactivity was much greater in the unknown than in the dipeptide peak (69 ± 2 versus $26 \pm 1\%$). There was only a trace amount of radioactivity in the free proline peak ($3 \pm 1\%$). Fractionation of the intracellular content after incubation of red cells with glycyl[^{14}C]alanine also revealed three peaks of radioactivity (Fig. 2), one corresponding to [^{14}C]alanine, one to glycylalanine and the other indicating formation of a new compound from glycylalanine. The metabolism of glycylalanine differed from that of the other two dipeptides, in that the amount of radioactivity in the unknown peak was much smaller than that in the dipeptide peak ($8 \pm 0.3\%$ versus $71 \pm 1\%$). Furthermore, a substantial amount of radioactivity appeared in the free amino acid peak ($20 \pm 1\%$).

For comparison we performed similar studies with [^{14}C]glycine, [^{14}C]alanine and [^{14}C]proline (Fig. 2). Fractionation of the red blood cell content after 1 min of incubation with [^{14}C]proline showed only one peak, which corresponded to free proline. Analysis of intracellular content of red blood cells after 1 min of incubation with [^{14}C]alanine revealed two peaks; one peak, a large fraction ($84 \pm 5\%$), corresponded to alanine and the other peak, which had a small amount of radioactivity ($16 \pm 5\%$), indicated formation of a new compound from alanine. Intracellular metabolism of glycine differed from that of the other two amino acids. Fractionation of intracellular content after 1 min of incubation with [^{14}C]glycine revealed two peaks, but the largest amount of activity ($56 \pm 2\%$) was in the peak indicating formation of a new compound from glycine. The remainder of radioactivity, in the second peak ($44 \pm 2\%$), corresponded to free glycine.

Extracellular metabolism

The accumulation of radioactivity in the red blood cells could represent uptake of unhydrolysed dipeptides or the uptake of free amino acids resulting from dipeptide hydrolysis in the incubation medium, or both. To investigate whether there was hydrolysis of dipeptides outside the cell, we analysed the incubation fluid for the presence of free [^{14}C]glycine, [^{14}C]alanine or [^{14}C]proline after 1 and 5 min of incubation of red blood cells with labelled glycylsarcosine, glycylalanine and glycylproline respectively. The results showed no free amino acids in the incubation medium. This indicates that the accumulation of radioactivity in the red cells as shown above was entirely the result of entry of unhydrolysed dipeptides into the cells.

To determine whether there was release of products of intracellular metabolism (Fig. 2) we also analysed the above incubation fluids for the unidentified ^{14}C -labelled metabolites. None was detected after 1 or 5 min of incubation of red blood cells with any dipeptide.

DISCUSSION

The present experiment provides evidence for the uptake of glycyl dipeptides by human red blood cells. The evidence includes the finding of unhydrolysed dipeptides in the cell cytoplasm. The results also show a large difference between rates of uptake of dipeptides and of amino acids. After a brief exposure (1 min), dipeptide uptake abruptly ceased, whereas that of amino acids continued. As a result red blood cells accumulated substantial amounts of amino acids, but only trace amounts of dipeptides.

One explanation for this phenomenon could be that dipeptides are non-specifically bound to the plasma membrane, which

rapidly becomes saturated. This possibility, however, was not supported by the results of studies usually performed to investigate membrane binding. We found neither recovery of dipeptides in the membrane fraction of red blood cells nor saturation in uptake in response to large increases in dipeptide concentration in the incubation, as would have been expected with non-specific binding. In addition, non-specific binding to the plasma membrane would not account for hydrolysis of glycylalanine and glycylproline by the cytoplasm of human red blood cells (Fig. 2). The fact that the cytoplasm was the site of hydrolysis for these dipeptides was indicated by lack of hydrolase activity in either the incubation medium or the plasma membrane. Another explanation could be that dipeptide influx was coupled with an efflux process which allowed very little intracellular accumulation. The investigation of the composition of the incubation fluid, however, did not reveal any evidence to support this possibility.

Investigation of the mechanism of uptake indicated that the entry was either by simple diffusion or by a carrier system which has a very weak affinity for dipeptides, since over a wide range of concentrations (0.05–50 mM) there was no evidence of saturation in the uptake of any dipeptide. In contrast, red blood cell uptake of glycine and alanine showed a saturable component of entry with increasing concentration, their K_m values being 0.19 and 0.26 mM respectively. An unexpected finding was lack of saturation in the red cell uptake of proline. However, at all concentrations, uptake of proline was significantly greater than uptake of either glycine or alanine. Indeed, the non-saturable kinetic constant was over 2-fold greater for proline than for either alanine or glycine (Table 1).

Our investigation of the fate of dipeptides after entry into red blood cells showed, as expected, a greater hydrolysis of glycylalanine than of either glycylproline or glycylsarcosine. The unexpected finding was the formation of new compounds which were neither dipeptides nor their constituent amino acids. The formation was most pronounced with glycylsarcosine and least pronounced with glycylalanine (Fig. 2). The formation of new compounds was not unique to dipeptides, but also occurred with amino acids. Among the three amino acids tested, the formation was considerable with glycine, but little or none was observed with alanine and proline (Fig. 2). Since erythrocytes are rich in glutathione, the intracellular metabolism may suggest formation of γ -glutamyl compounds. However, the relative amounts of new compounds formed from these substrates do not reflect the specificity of γ -glutamyl transpeptidase [18], and, in addition, red cells are believed to be devoid of γ -glutamyl transpeptidase activity [19]. An implication of the above findings is that measurement of the concentration of a dipeptide or its amino acid residues or both is not adequate to determine dipeptide uptake by red cells, since a substantial fraction of the dipeptide might be present in another form. Indeed, this may have accounted for the failure of a previous study [7] to find dipeptides within human red blood cells.

Finally, it is pertinent to consider the physiological importance of red blood cells in the metabolism of dipeptides in man. If the phenomenon of rapid cessation of uptake, as we observed *in vitro* (Fig. 1), also occurs *in vivo*, there would be very little opportunity for entry of dipeptides across the red cell plasma membrane. Absence of an active transport system should not necessarily compromise the capacity of a tissue for dipeptide clearance if the plasma membrane is substantially active in peptide hydrolysis, as is the situation in the liver [20,21]. However, human red blood cells appear to have very little capacity for dipeptide hydrolysis by the plasma membrane. This was suggested by the failure to find any free [^{14}C]alanine in the medium after incubation of red blood cells with glycyl[^{14}C]alanine. Therefore it appears that red

blood cells do not play an appreciable role in the clearance of dipeptides from the plasma in man.

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