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UPTAKE OF PEPTIDES CONTAINING Tyr-Pro BY HUMAN AND MOUSE ERYTHROCYTES

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Abstract—Red blood cells (RBCs) harvested from mice were used to investigate the possible existence of an uptake system for peptides in these cells. The radioactively iodinated tetrapeptide Tyr-MIF-1 (Tyr-Pro-Leu-Gly-amide) was incubated with RBCs for varying lengths of time with or without inhibitors. The RBCs showed saturable uptake that could be inhibited by Tyr-Pro containing peptides. Uptake was also found in human RBCs, but was more robust in the mouse. Uptake by mouse RBCs was temperature dependent and magnesium sensitive but did not require sodium, potassium, or glucose. With the exception of some enkephalin- and dynorphin-related peptides that partially inhibited uptake, most substances tested were without effect. The results of HPLC showed internalization of the N-Tyr-Pro containing peptides, with accumulation of degradation products over time. The degradation products, however, did not inhibit transport, suggesting that peptides were transported intact into the RBCs with degradation occurring after internalization. This suggestion was strengthened by the finding that only the cytosol of the RBC, not its membranes, rapidly degraded Tyr-MIF-1 to free iodine and iodotyrosine. Nevertheless, the cytosol contained a large amount of immunoreactive material that eluted at the position of intact Tyr-MIF-1 on HPLC. These findings show that RBCs can take up, store, and degrade Tyr-Pro containing peptides.

Binding and transport of peptides by CNS tissue is receiving increasing attention. Inaccessibility of CNS tissue has led to the search for more readily available sources of material. Circulating red blood cells (RBCs†) may constitute such a source. RBCs have been used in the search for alterations in pathological conditions. For example, there have been investigations of the uptake of choline by RBCs in Down's syndrome [1], Friedrich's ataxia [2], Alzheimer's disease [2–5], and Huntington's chorea [2], the binding to RBCs of insulin in obesity, diabetes mellitus, and pregnancy [6, 7], the binding of ouabain in dementia [8], the transport of electrolytes in Bartter's syndrome [9, 10], and physicochemical changes in RBC membranes in diabetes mellitus [11, 12], schizophrenia [13], Down's syndrome [14], and Duchenne muscular dystrophy [15, 16]. The hope that the membranes of RBCs are representative of membranes in general, or of less accessible membranes, has been justified occasionally. Thus, the changes in RBC insulin receptors found in obese pregnant and non-pregnant subjects [6, 7] are similar to the changes seen in other peripheral tissues from these and other insulin-resistant subjects. Similarities also exist between the uptake systems for amino acids [17], serotonin [18], thyroid hormones [19–21], γ-aminobutyric acid [22], and possibly glucose [23] in RBCs or platelets and the CNS.

Tyr-MIF-1 (Tyr-Pro-Leu-Gly-amide) is a recently isolated peptide found in the CNS [24–27] with anti-opiate and anti-depressant properties [27–30]. It has specific, high-affinity binding sites in the brain [31] and a saturable transport system across the blood–brain barrier [32]. Abnormalities in Tyr-MIF-1 concentrations, transport, or binding have been noted or postulated to occur in hypertension [33], aging [34], hypopituitarism [35], and hyperleucinemia [36]. Other N-Tyr-Pro containing peptides are derived from hemoglobin and are termed hemorphins [37]. In the present study, we describe a saturable uptake system in RBCs for Tyr-MIF-1 and Tyr-Pro peptides.

METHODS

Peptides and chemicals. Tyr-MIF-1 was iodinated as previously described [26] with chloramine T and purified on a column of Sephadex G-25. The purity of the ^{125}I-Tyr-MIF-1 (ITMIF) was confirmed with HPLC and by antibody binding. Specific activity was 100 Ci/mmol. All peptides were the gift of either Dr. D. H. Coy or Dr. A. Horvath or were purchased from Bachem (Torrance, CA, U.S.A. or Bubendorf, Switzerland). Theophylline, acetazolamide, ouabain, and reserpine were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.).

RBC preparation. Male ICR mice (17–22 g), purchased from Harlan Sprague Dawley (Indianapolis, IN, U.S.A.), were anesthetized with pentobarbital (65 mg/kg, i.p.), and then decapitated; the whole blood was collected in polypropylene tubes containing 1000 units of heparin sulfate. Human blood was collected from three Caucasian males, 25 to 35 years old. RBCs were processed by standard techniques [38]. Briefly, the blood was centrifuged at 4° for 10 min at 2000 *g*. The plasma, buffy coat, and top 10% of the RBCs were removed. The remaining RBCs were washed 4 times by resuspension in 10–

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† Abbreviations: RBCs, red blood cells; and ITMIF, ^{125}I-Tyr-MIF-1.

15 times their volume of buffer (130 mM NaCl, 5 mM glucose, 50 mM Trizma (pH 7.5), 5 mM KCl, 2 mM $MgCl_2$, and 0.1 mM EDTA) and centrifuged as described above. All peptides and chemicals studied were also dissolved in buffer.

Studies were initiated by mixing the RBCs and test substances together in glass scintillation vials (total volume: 1.5 mL) and incubating at 37° in a metabolic shaker bath at a hematocrit of 20%. All studies (except those investigating the effect of time of storage) used freshly prepared RBCs. At the end of the incubation period, three aliquots of 0.5 mL of the RBC suspension were transferred to 12 × 75 mm borosilicate tubes and centrifuged at 2000 *g* for 10 min at 4°. A portion (0.05 mL) of the cell-free supernatant (*S*) was collected for counting and most of the remaining supernatant discarded. The RBC pellet and remaining supernatant were resuspended, 0.05 mL was removed for counting (R_{mix}), and the hematocrit (*H*) of the resuspended mixture was determined. The cpm per 0.05 mL of RBC (*R*) were then determined by the equation:

$$R = [R_{mix} - S(1 - H)]/H. \quad (1)$$

Nonsaturable uptake was determined by incubation of RBCs in the presence of an excess of Tyr-MIF-1 (10 mmol/L). Transport (*T*) in mol/L of RBCs was determined by the equation:

$$T = R(10^3 \text{ mL/L})(M) \quad (2)$$

where *M* is the number of mol/cpm of the incubation fluid.

Time curve. Human or mouse RBCs were incubated as described above with 2.25 nM ITMIF with or without 10 mM non-radioactive Tyr-MIF-1. Cells were harvested after 15 min, 30 min, 1 hr, 2 hr, 3 hr, 4 hr, and 8 hr of incubation. A zero time value was estimated by the mixing of RBCs and ITMIF together at 4° and immediate centrifugation of the mixture. Specific transport was taken to be the difference between total uptake (cells incubated with only ITMIF) and nonsaturable uptake (cells incubated with 10 mM Tyr-MIF-1). To distinguish between transport and membrane binding, RBCs incubated for 2 hr were washed and lysed either mechanically or with distilled water, centrifuged at 150,000 *g* at 4° for 1 hr, and the supernatant and residue counted. The counts obtained from cells lysed in 30% trifluoroacetic acid (TFA) after 10 min, 30 min, and 2 hr of incubation were tested by HPLC. The effect of temperature on uptake in mouse RBCs was determined by incubation of cells at 37°, 21°, and 0° for 2 hr. The effect of storage time on mouse RBCs was determined by comparison of results obtained with freshly prepared cells with results obtained from cells that had been stored for 24 hr, 72 hr, and 96 hr at 4° at a hematocrit of 40%. The incubation time was 2 hr.

Saturation curve. Mouse RBCs were incubated for 30 min and human RBCs for 4 hr with 2.25 nM ITMIF and an increasing amount of Tyr-MIF-1. The maximum uptake rate (V_{max}) and the amount of non-radioactive material required to achieve 50% of that rate (K_m) were determined with the ALLFIT program (Biomedical Computing Technology Information Center, Vanderbilt Medical Center, Nashville, TN) [39]. The effect of 1 mM Tyr-MIF-1 on the hematocrit was also determined.

Peptides, amino acids, metabolic agents, ions, and glucose. The following peptides, amino acids, and metabolic agents were incubated separately at a concentration of 0.5 mM with mouse RBCs for 2 hr: Tyr-MIF-1, Tyr-Pro-Leu, Tyr-Pro, tyrosine, iodotyrosine, MIF-1, Met-enkephalin, β-casomorphin, morphiceptin, Arg^0-Met-enkephalin, D-Ala^2-Met-enkephalin, D-Tyr^1-Met-enkephalin, Leu-enkephalin (dynorphin 1-5), dynorphin 1-8, dynorphin 1-6, dynorphin 1-4, kyotorphin, dermorphin, each of the essential amino acids, furosemide, theophylline, ouabain, and acetazolamide. The effect of 0.5 mM histidine on the kinetic parameters describing Tyr-MIF-1 transport over 2 hr was determined. The amino acid leucine was used at concentrations of 0.005, 0.5 and 50 mM. Urethane (ethyl carbamate) was tested at 0.5 mM *in vitro* and was also used i.p. in a group of mice as an anesthetic. The role of potassium, magnesium and sodium ions, and glucose on transport was determined by the use of buffers that did not contain these substances. Osmolarity was maintained by substitution of NaCl for KCl, $MgCl_2$, or glucose and by substitution of glucose for NaCl. The results were expressed as the percent inhibition (%*I*) of uptake relative to that induced by 0.5 mM Tyr-MIF-1 with the equation:

$$\%I = \frac{R_{NO} - R_{EXP}}{R_{NO} - R_{TMIF}}(100) \quad (3)$$

where R_{NO} is the cpm/0.05 mL of RBC with no excess Tyr-MIF-1, R_{TMIF} has 0.5 mM Tyr-MIF-1 added, and R_{EXP} has 0.5 mM inhibitor added.

Degradation of ^{125}I-Tyr-MIF-1 by RBC cytosol and membranes. Washed RBCs were lysed in 2 mL of distilled water and the membranes and cytosol separated by centrifugation. The membranes were washed twice and resuspended in 2 mL of distilled water. One milliliter of the cytosol or 1 mL of the suspended membranes was incubated with 2.5×10^6 cpm of ^{125}I-Tyr-MIF-1 at 37° for 2 hr. At the end of the incubation period, 1 mL of 30% TFA was added to both samples which were then centrifuged and the supernatants lyophilized. These were then submitted to HPLC.

Immunoreactive content of Tyr-MIF-1 in RBC cytosol. Studies were done both to determine the concentration of endogenous Tyr-MIF-1 in RBCs and also to determine the characteristics by HPLC of that immunoreactivity.

Two groups of three mice each were anesthetized with pentobarbital, and their RBCs were collected and washed as described above. The hematocrit was determined and 1 mL of these washed RBCs was added to 2 mL of distilled water. Lysed cells were centrifuged at 5000 *g* for 10 min, and 1 mL of 30% TFA was added to the supernatant. This material was lyophilized and immunoreactivity measured by a previously described radioimmunoassay [25, 26]. This process was repeated with mice given 0.5 mg Tyr-MIF-1 i.p. 10 min before decapitation and in

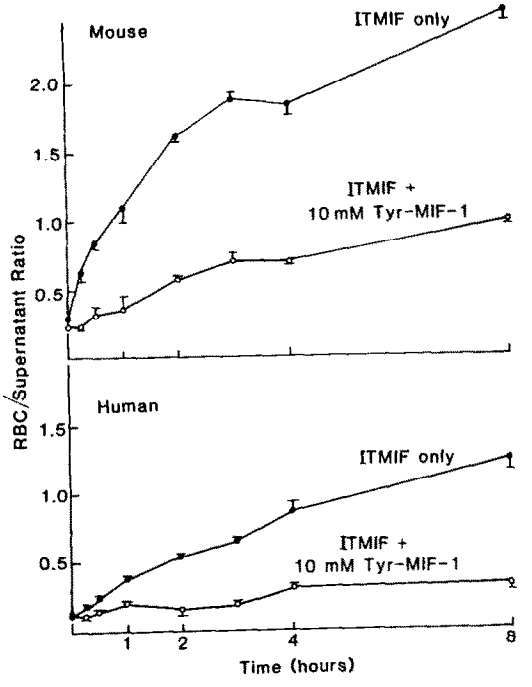

Fig. 1. RBC/supernatant ratios of ITMIF (^{125}I-Tyr-MIF-1) for human and mouse cells in the presence (nonsaturable transport) and absence (total transport) of a 10 mM concentration of non-radioactive Tyr-MIF-1 in the incubation solution. Each point is the mean ± SE of three samples, each done in triplicate.

mice anesthetized with urethane. Results are expressed as nanomoles of immunoreactivity per liter of packed RBCs.

RBCs obtained from about seventy mice were processed as above, and the reconstituted lyophilized material was submitted to HPLC. The immunoreactive material in the HPLC fractions was then determined by radioimmunoassay. Results are expressed as picomoles of immunoreactivity per fraction.

Statistics. Means are expressed with their standard errors. Analysis of variance (ANOVA) was used to test for effect and, when more than two means were to be compared, was followed by Tukey's multiple range test (TMRT).

RESULTS

Time curve. Figure 1 shows the RBC/supernatant ratio vs time in cells incubated with or without 10 mM Tyr-MIF-1. Specific uptake of ITMIF (Fig. 2) into mouse or human RBCs approached an asymptote with time, with mouse RBCs reaching their maximum after 2 hr and human RBCs after 4 hr. When RBCs were lysed either mechanically or with distilled water and centrifuged at 150,000 *g* for 1 hr, 98% of the radioactivity was found in the lysate, showing that ITMIF was transported into the cells and associated with the cytosol. About 58.5, 45.0 and 20.0% of the total radioactivity collected from lysed cells eluted in the positions of the iodinated peptides as determined by HPLC at 10 min, 30 min, and 2 hr respectively. By comparison, 90–99% of the radioactivity in the supernatant eluted as peptide.

Uptake was temperature dependent, being 2.29 ± 0.05 nmol/L RBC at 37°, 1.77 ± 0.09 nmol/L RBC at 21°, and undetectable at 0° after 2 hr of incubation: $F(2,6) = 439$, $P < 0.00005$. Uptake values at all temperatures were found to be statistically different from one another by TMRT ($P < 0.001$). A linear relationship existed between storage time and uptake ($y = -0.0036x + 2.25$, $N = 4$, $r = 0.992$, $P < 0.01$), where x is storage time in hours and y is uptake in nanomoles per liter RBC after 2 hr of incubation, with statistical differences occurring among the transport rates: $F(3,21) = 6.07$, $P < 0.01$.

Saturation curve. Figure 3 shows the saturation curves obtained. The V_{max} was 278 ± 10 (μmol/L RBC/30 min) and the K_m was 695 ± 88 (μmol/L supernatant) for mouse RBCs, while the V_{max} was 17.5 ± 0.7 (μmol/L RBC/4 hr) and the K_m was 37.8 ± 6.9 (μmol/L supernatant) for human RBCs. Tyr-MIF-1 had no significant effect on RBC volume as assessed by hematocrit: 0.433 ± 0.002 (N = 9, no Tyr-MIF-1) vs 0.436 ± 0.001 (N = 10, 1 mM Tyr-MIF-1).

Peptides, amino acids, metabolic agents, ions, and glucose. Table 1 shows the results for the peptide competitors, and Table 2 the results for the metabolic agents with statistical comparison to both 0.5 mM Tyr-MIF-1 (defined as 100% inhibition) and ITMIF (defined as 0% inhibition). The ANOVA for peptide competition was highly significant [$F(21,194) = 146$, $P < 0.001$], as was the ANOVA for the metabolic agents [$F(7,61) = 267$, $P < 0.001$]. Of the metabolic agents, only urethane caused significant inhibition, and it did so even when given i.p. as the anesthetic. All the other metabolic agents had uptakes similar to that found in the absence of non-radioactive Tyr-MIF-1.

The ANOVA for Tyr-MIF-1 and the amino acids was statistically significant [$F(12,91) = 27.5$, $P < 0.001$] primarily because of the inhibition induced by Tyr-MIF-1 (results included in Table 1). However, TMRT showed that histidine and valine also caused statistically significant inhibition ($P < 0.05$). A saturation curve performed in the presence or absence of 0.5 mM, histidine showed that histidine acted as a competitive inhibitor, increasing K_m (71 vs 186 μmol/L supernatant) but not affecting V_{max} (44.1 vs 39.2 μmol/L RBC/2 hr).

The ANOVA for the various components of the buffer was significant [$F(4,37) = 17.1$, $P < 0.001$], and TMRT showed that only the absence of $MgCl_2$ was associated with a significant decrease in uptake, with a reduction from 2.11 ± 0.07 to 1.47 ± 0.04 ($P < 0.05$) nmol/L RBC after a 2-hr incubation.

Degradation of ^{125}I-Tyr-MIF-1 by RBC cytosol and membranes. After incubation with cytosol, about 88% of the radioactivity eluted in the position of iodine or iodotyrosine. By contrast, after incubation with the membranes, about 94% of the radioactivity eluted as peptide, primarily as I-Tyr-Pro, and 99% of the radioactivity of a control (peptide added to TFA and subsequently processed like the samples) eluted as peptide, primarily as ^{125}I-Tyr-MIF-1.

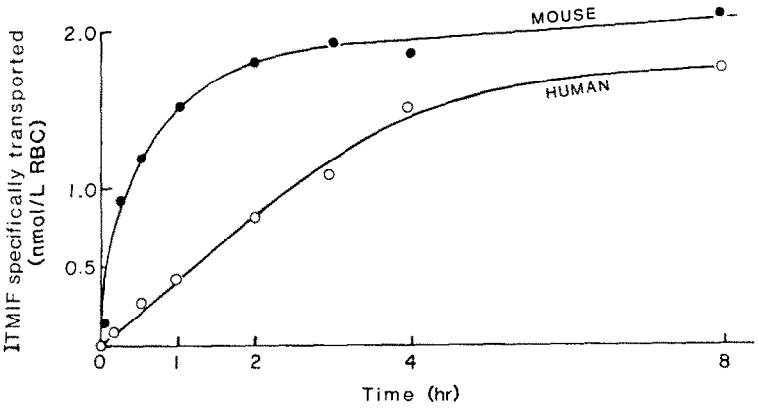

Fig. 2. Uptake of iodinated Tyr-MIF-1 into human and mouse RBCs as a function of time. The nonsaturable component of uptake has been subtracted. Each point represents an N of 3 done in triplicate with standard errors of about 2% of the mean.

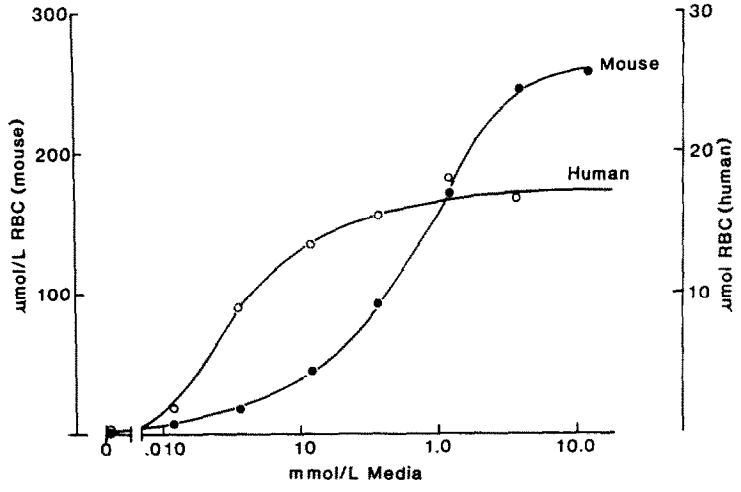

Fig. 3. Uptake of Tyr-MIF-1 into human and mouse RBCs as a function of concentration.

Immunoreactive content of Tyr-MIF-1 in RBC cytosol. In the first part of this study, 0.244 ± 0.075 nmol/L RBC of Tyr-MIF-1-like immunoreactivity was found in mice anesthetized with pentobarbital, 0.455 ± 0.234 nmol/L RBC in mice pretreated with i.p. Tyr-MIF-1, and 0.104 ± 0.014 nmol/L RBC in mice anesthetized with urethane.

In the second part of this study, the largest peak (33.1%) of endogenous immunoreactive material eluted in the position of Tyr-MIF-1 on HPLC (Fig. 4). Smaller peaks of 23.5 and 21.4% each eluted at the beginning and end. Little or no material eluted at the positions of Tyr-Pro or Tyr-Pro-Leu.

DISCUSSION

The results show that saturable, specific, temperature-dependent, and non-energy-dependent uptake of peptides containing the N terminus sequence Tyr-Pro (N-Tyr-Pro) occurs in mouse RBCs. Slower, less avid, but saturable uptake also occurred in human RBCs. This is the first demonstration of RBC transport for this class of peptides, although it has been reported that other peptides are bound by platelets [40] and RBCs [6, 7]. The system appears not to be dependent on the Na^+–K^+-ATPase pump or on cyclic AMP, since the rate of uptake was unaffected by ouabain, theophylline, or the absence of sodium or potassium. The lack of effect of furosemide and acetazolamide also suggests that this system, unlike the brain-to-blood transport system for Tyr-MIF-1 [41], is not dependent on sodium or carbonic anhydrase. Omission of glucose from the buffer did not affect transport, showing that the uptake is probably not energy dependent. This suggests that uptake occurs by carrier-mediated facilitated diffusion. Magnesium, however, does appear to play a role, since buffer lacking this element resulted in a statistically significant reduction in the rate of uptake.

The supernatant derived from cells lysed either

Table 1. Inhibitory effects of various peptides, fragments of Tyr-MIF-1 (Tyr-Pro-Leu-Gly-amide), and amino acids on the uptake of ^{125}Tyr-MIF-1 into mouse RBCs

Substance	% Inhibition
ITMIF only	0.0 ± 2.4*
Tyr-MIF-1†	100.0 ± 3.1‡
Tyr-Pro-Leu†	105.4 ± 1.4‡
Tyr-Pro†	106.0 ± 2.3‡
β-Casomorphin†	103.0 ± 3.4‡
Morphiceptin†	87.1 ± 2.3‡
MIF-1	18.3 ± 1.8*
Kyotorphin (Tyr-Arg)	4.1 ± 4.3*
Met-Enkephalin	31.6 ± 3.9*‡
Arg^0-Met-Enkephalin	55.9 ± 5.3*‡
D-Ala^2-Met-Enkephalin	40.4 ± 1.7*‡
D-Tyr^1-Met-Enkephalin	27.9 ± 4.2*‡
Leu-Enkephalin	6.7 ± 4.0*
Dynorphin 1-4	21.3 ± 4.4*‡
Dynorphin 1-6	31.5 ± 3.4*‡
Dynorphin 1-8	63.1 ± 3.2*‡
Dermorphin	11.5 ± 3.8*
Tyrosine	−4.1 ± 3.8*
Iodotyrosine	−17.6 ± 4.6*
Histidine	48.8 ± 4.4*‡
Valine	25.9 ± 3.8*‡
Methionine	20.6 ± 4.8*
Isoleucine	13.2 ± 3.9*
Threonine	11.2 ± 3.4*
Lysine	3.3 ± 3.7*
Arginine	2.9 ± 3.5*
Tryptophan	2.6 ± 6.4*
Phenylalanine	1.0 ± 4.5*
Leucine (0.005 mM)	8.6 ± 3.9*
Leucine (0.5 mM)	3.8 ± 6.6*
Leucine (50.0 mM)	98.4 ± 1.7‡
D-Leucine (0.5 mM)	2.1 ± 5.7*

All compounds were tested at a concentration of 0.5 mM unless otherwise specified. Percent inhibition is defined relative to Tyr-MIF-1 (100%) and ^{125}I-Tyr-MIF-1 (ITMIF) (0%).

* Inhibition was significantly ($P < 0.05$) less than that produced by 0.5 mM Tyr-MIF-1.

† Indicates a peptide with N-Tyr-Pro.

‡ Statistically significant inhibition ($P < 0.05$).

Table 2. Inhibitory effects of metabolic agents on the uptake of ^{125}I-Tyr-MIF-1 into mouse RBCs

Substance	% Inhibition
ITMIF only	0.00 ± 2.86
Tyr-MIF-1	100.00 ± 1.39*
Furosemide	3.10 ± 3.09
Acetazolamide	2.33 ± 2.89
Ouabain	2.33 ± 2.89
Urethane	60.00 ± 2.62*
Urethane (i.p.)	86.72 ± 0.83*
Theophylline	1.55 ± 2.86

All compounds (except ITMIF and urethane i.p.) were tested at a concentration of 0.5 mM *in vitro*. Urethane (i.p.) was given to effect (anesthesia), which required approximately 40 mg/mouse. Percent inhibition is defined relative to Tyr-MIF-1 (100%) and ITMIF (0%).

* Statistically significant inhibition ($P < 0.05$).

mechanically or osmotically and centrifuged at high speed contained 98% of the sequestered material. This indicates that the peptide was internalized and not simply bound to surface receptors. The uptake decreased about 4% for each 24 hr that the RBCs were stored and was totally inhibited at low temperatures. Chromatography with HPLC confirmed that radioactively labeled Tyr-Pro containing peptides entered RBCs. A large amount of immunoreactivity that eluted at the position of Tyr-MIF-1 on HPLC was also found inside RBCs. The accumulation of the amino acid tyrosine inside the cell probably represents catabolism of the peptides after entry, since the cytosol of the RBC but not its membranes degraded radioactively iodinated peptide to iodide and iodotyrosine. Catabolism outside the cell with subsequent saturable transport of iodide or iodotyrosine is not a feasible explanation, since neither tyrosine nor iodotyrosine inhibited uptake and since HPLC of the incubation fluid showed that over 90% of the radioactivity eluted as peptide. Therefore, the saturable uptake of radioactivity by the RBCs represented transport of peptides, not degradative products. Although degradation occurred after internalization, the RBCs nevertheless contained a substantial amount of immunoreactive Tyr-MIF-1.

Of the fifteen opiate peptides or peptides closely related to Tyr-MIF-1 that were tested, only the four that contained Tyr-Pro (Tyr-Pro-Leu, Tyr-Pro, β-casomorphin, and morphiceptin) caused inhibition that was statistically indistinguishable from that of Tyr-MIF-1. This suggests that the sequence N-Tyr-Pro is the preferred sequence for transport. Four other peptides caused no inhibition at all (MIF-1, Tyr-Arg, Leu-enkephalin, and dermorphin). The lack of inhibition by MIF-1 indicates the importance of an N-terminal tyrosine, while the lack of inhibition by Tyr-Arg shows that arginine cannot substitute for proline in the second position. Although the N-terminal tyrosine is important for inhibition, the inhibition by Arg^0-Met-enkephalin (55.9%) suggests that this is not an absolute requirement. Also, the substitution of a D-tyrosine for the N-terminal L-tyrosine in Met-enkephalin did not result in abolition of inhibitory activity. Seven other peptides caused some inhibition that was significantly ($P < 0.05$) less than that produced by Tyr-MIF-1. Nevertheless, their ability to inhibit uptake, and perhaps also to be transported, shows that there are structural considerations in addition to the presence or absence of Tyr-Pro.

In general, amino acids tested at a concentration of 0.5 mM did not affect the uptake of ITMIF by RBCs. This concentration exceeds that in the blood of normal adults for essential amino acids [42] and, therefore, makes it unlikely that amino acids affect uptake under normal conditions. Histidine and, to a lesser extent, valine did cause statistically significant inhibition of transport at this concentration, although they were less potent than Tyr-MIF-1. Kinetic analysis showed that histidine acts as a competitive inhibitor of the Tyr-Pro system, which suggests that this system may take up histidine. Such systems that take up both peptides and amino acids are unusual and none of the amino acid systems described for the

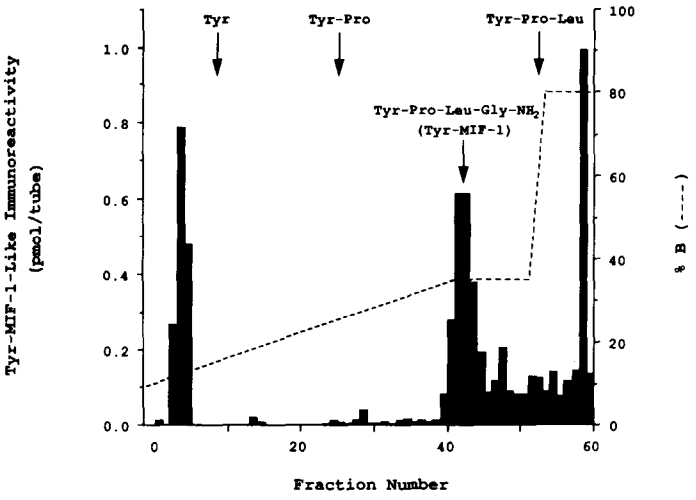

Fig. 4. HPLC characterization of the endogenous Tyr-MIF-1-like immunoreactivity found in the cytosol of mouse RBCs. Solution A contained 0.1% TFA in water, and Solution B contained 0.1% TFA in methanol.

RBC take up histidine and valine exclusively. It is possible, therefore, that histidine may act as a structural analog of the dipeptide Tyr-Pro in this system.

The concentration of ITMIF used in the incubation mixture was 2.25 nM in all studies except, of course, the saturation studies. This approximates the concentration of immunoreactive Tyr-MIF-1 in rodent plasma (1.67 to 3.24 nM) which varies with age [34] and suggests that the transport observed here may occur physiologically, as it appears to do for amino acids [43].

This is further suggested by the finding of the presence of endogenous Tyr-MIF-1-like immunoreactivity in RBCs and by the finding that the concentration of immunoreactivity inside the RBC increased when mice were pretreated with Tyr-MIF-1 and decreased when mice were anesthetized with urethane, which is shown here to inhibit this transport system. Thus, *in vivo* modification of transport rate corresponds to the concentration inside the RBC of Tyr-MIF-1-like immunoreactivity. This makes it likely that the uptake system functions in the intact animal.

Uptake of substances by RBCs has been suggested to play an active role in "buffering" blood levels against fluctuations and may participate in delivery of the substances to organs [44, 45]. Such regulation may be particularly important for opiate peptides derived from hemoglobin, the hemorphins [37], since these peptides contain an N-Tyr-Pro and so may be taken up by the system described here. This uptake system could also be a significant disposal mechanism for peptides that contain N-Tyr-Pro. Thus, RBCs may be a source of production, a site of uptake, and/or a site of degradation. RBCs may also take up other peptides, including other opiates such as β-endorphin, which has been found in high concentrations inside RBCs [46, 47]. Peptides, including opiates, have been shown to be capable of regulating the sodium [48, 49] and calcium [50, 51] transport systems in RBCs.

The system described here for the RBCs appears similar to the receptor system found in brain tissue that binds Tyr-MIF-1 and casomorphins such as morphiceptin [52]. Both the brain receptor and RBC uptake system are sensitive to the Tyr-Pro compounds, less sensitive to the enkephalins, the amino acid tyrosine, and MIF-1, and less rigorous in their requirement for an N-terminal L-tyrosine. This RBC uptake system, as well as the binding in the brain, can be distinguished from the system described for the brain-to-blood transport of Tyr-MIF-1 and the enkephalins [41]. The brain-to-blood transport system is much more exacting in its requirement for an N-terminal L-tyrosine, is not inhibited by Tyr-Pro, Tyr-Pro-Leu, or morphiceptin, and is much more sensitive to inhibition by the enkephalins, acetazolamide, and furosemide, but is not sensitive to inhibition by urethane or histidine. Both transport systems, however, can be inhibited by branched chain amino acids, β-casomorphin, and dynorphin 1-8. The similarities among these systems raise the possiblity that RBCs may be useful in the study of the binding site and the transport system found in the CNS.

The branched chain amino acids valine and leucine inhibited uptake, whereas isoleucine did not. It has been suggested that inhibition of the brain-to-blood transport system for Tyr-MIF-1 and the enkephalins may be a mechanism by which high concentrations of the branched chain amino acids are involved in such diseases as maple syrup urine disease and CNS dysfunction [36]. The results found here suggest that RBC uptake may also be inhibited in these conditions and raise the possibility that this system may have some use as a reflection of this disease process. The doses of leucine and valine that we found to have inhibitory activity are similar to the plasma concentrations found in maple syrup urine disease [42, 53].

Thus, the finding that RBC uptake is sensitive to branched chain amino acids known to cause CNS dysfunction, and the knowledge that the RBC can

be a source of Tyr-Pro containing peptides, raise the possibility that the system may be useful in investigations relating to the transport and binding of tyrosinated peptides in the CNS. The evidence that this uptake system is responsive *in vivo* also raises questions as to its physiological role.

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