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CHARACTERISTICS OF LEUCINE TRANSPORT BY ISOLATED HEPATOCYTES OF ANTARCTIC FISH AT LOW TEMPERATURES

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Hepatocytes prepared by collagenase perfusion from Antarctic nototheniid fish of genus *Trematomus* are active in uptake of [^{14}C]leucine at 0, 5, and 10°C. The system is saturable with apparent K_m about 1.0 mM. Isoleucine and phenylalanine were major competitors, valine was about one-half as effective, while alanine, glycine and histidine had no effect. Temperature dependency of rates in the 0–10°C range yielded E_a = 65 kJ/mol (Q_{10} = 2.7). The average first-order rate constant at 0°C was 0.1 min^{-1}, one-third the value of 0.3 min^{-1} estimated for clearance of [^{14}C]leucine by liver of these species in vivo. Affinity and specificity agreed well with in vivo data on liver clearance of leucine, both in Antarctic fish at 0°C and in temperate fish acclimated to 10°C and 20°C. The results indicate similar modifications of leucine transport associated with evolutionary cold adaptation and seasonal acclimation in fish.

Introduction

Antarctic fish provide a valuable experimental system for study of fundamental processes in vertebrate tissues at extreme low temperatures. These species are well adapted to their habitat temperature, which is relatively constant year-round at −1.9°C in McMurdo Sound. Cold adaptation is evidenced by rates of respiratory metabolism that are several times the values predicted for that temperature on the basis of data in other fish species [1]. Rates of protein synthesis in vivo similarly are two to three times the predicted levels [2]. Adaptations in a variety of tissue and enzyme systems also have been reported [3–6].

The operation of amino acid transport systems in Antarctic fish is of particular interest because of the well-known effects of temperature on membrane fluidity and function [7]. Studies in temperate fish have indicated that temperature has both qualitative and quantitative effects on amino acid transport. Measurements of [^{14}C]leucine uptake by toadfish liver in vivo at 20°C gave evidence of a saturable, concentrative system of relatively high specificity [8]. When fish were cooled to 10°C, however, uptake fell markedly with an apparent loss of concentrative ability [9,10]. Evidence for recovery of the system was obtained after 2–3 weeks of cold acclimation [11]. Studies of leucine uptake in vivo in the Antarctic species *Trematomus hansoni* at 0°C have shown properties similar to those observed in toadfish at 20°C, although rates are much slower, as expected at the lower temperature [12].

New techniques for preparation of highly active hepatocytes by collagenase perfusion [14,15] offer an alternate approach to the study of temperature effects on transport processes and provide opportunities to compare the behavior of isolated cells with in vivo data. Studies in rat hepatocytes indi-

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cate that at least four systems are involved in transport of neutral α-amino acids, including the A (alanine), L (leucine), ASC (alanine, serine, cysteine), and N (asparagine, glutamine, histidine) systems [15–19]. The uptake of leucine, isoleucine and valine in the rat hepatocyte is mediated by the L-system [20].

The present study examines properties of leucine transport in isolated hepatocytes of two Antarctic notothenid fish species. In addition to their ability to function at low temperatures, these species are unusual in their extreme intolerance to increased temperature. Upper incipient lethal temperature is about +6°C [21]. Hepatocytes maintained in monolayer culture similarly show a very narrow temperature range; optimal rate of protein synthesis is obtained at 7°C, declining significantly at 10°C [22]. The temperature dependency of transport has been examined here in an attempt to assess the role of membrane function in relation to this effect.

Materials and Methods

Animals. Specimens of *Trematomus bernacchii* and *T. hansoni* (95–270 g) were obtained by fishing through the sea ice of McMurdo Sound, Antarctica, with hook-and-line and baited traps. Fish were maintained in running seawater aquaria at −1.5°C and fed with chopped fish every 3–4 days. Fish were lightly anesthetized by exposure to benzocaine at 0.05 g/l prior to cannulation for hepatocyte preparation.

Isolation of hepatocytes. The preparation of hepatocytes by collagenase perfusion was based on the methods of Berry and Friend [13], Seglen [14], and Stanchfield and Yager [23]. All procedures were carried out at 5°C, as described in detail elsewhere [22]. After dispersion the cells were washed twice by gently resuspending and sedimenting in a balanced salt solution (TBS-BSA) containing the following ingredients: 240 mM NaCl, 5 mM KCl, 0.4 mM KH_2PO_4, 0.3 mM Na_2HPO_4, 4 mM $NaHCO_3$, 5.6 mM glucose, 10 mM Hepes buffer (pH 7.8), and 1% bovine serum albumin. Ionic composition was adjusted to conform to *Trematomus* serum [24]. In some cases the washing steps were carried out in a complete culture medium (TCM) consisting of bicarbonate-free Waymouth's medium (Gibco, Grand Island, NY) supplemented with 240 mM NaCl, 25 mM Hepes (pH 7.8), 10 mU/ml bovine insulin, 5.8 μg/ml streptomycin, 63 μg/ml penicillin, and 50 μg/ml gentamycin. Waymouth's medium contains 0.38 mM leucine and other amino acids. All biochemicals were obtained from Sigma Chemical Company (St. Louis, MO). Cells washed in TCM were sedimented and washed once with the amino acid-free medium TBS-BSA immediately before use.

Transport assays. Samples of 100 μl of TBS-BSA containing 0.1 μCi of L-[U-^{14}C]leucine and 0.2 μCi of [G-^{3}H]inulin (New England Nuclear) and other components as indicated were placed in Beckman microfuge tubes and brought to the experimental temperature. Reaction was started by addition of 100 μl of cells at 0.1 g wet weight/ml in TBS-BSA that had been pre-incubated for 10 min at the experimental temperature. Final leucine concentration in control experiments was 0.1 mM; competing amino acids were tested at 1.5 mM. Tubes were run in groups of six and sample order was randomized. Preparation time per group was about 1 min. Transport was terminated by centrifugation for 5 s. The supernatant was collected for determination of isotope ratio of the medium (^{14}C dpm/^{3}H dpm) by double isotope counting in Biofluor (New England Nuclear). The tube walls and pellet surface were quickly rinsed twice with ice-cold TBS and the walls wiped dry. Pellets were dispersed in 150 μl cold 10% trichloroacetic acid for precipitation of protein and nucleic acids. After centrifugation, 100 μl of the supernatant was counted in Biofluor to obtain [^{3}H]inulin and [^{14}C]leucine recovery in cells. Efficiencies in a Beckman LSC100 scintillation spectrometer were 35% for ^{3}H and 65% for ^{14}C.

Extracellular ^{14}C was calculated by multiplying ^{3}H dpm in the cell sample by the ^{14}C/^{3}H ratio of the medium. Results averaged 650 dpm per 100 μl supernatant; comparison with ^{14}C concentration in the medium (1065 dpm/μl) yielded a value of 0.9 μl for extracellular volume per cell pellet. Intracellular uptake was calculated from total ^{14}C dpm in the cells after subtraction of the extracellular ^{14}C dpm. First-order rate constants (k) were obtained from the intracellular uptake data (A) by graphical and least-squares analysis according to

the equation

$$\ln\left(1 - \left(A/A_{eq}\right)\right) = -kt \qquad (1)$$

Equilibrium uptake (A_{eq}) averaged 2000 ± 200 (8) per 100 µl sample, corresponding to 2.8 µl cell water per cell pellet. Uptake velocity in pmol/µl per min was obtained as the product of k and external leucine concentration in pmol/µl. Statistical analyses were based on the t-test. Average values of K_m and V_{max} were obtained by least-squares fitting of the Michaelis-Menten equation [25]. Arrhenius activation energy was obtained graphically from a plot of log k vs. inverse absolute temperature. Computations were performed on a Hewlett-Packard 2100S computer at Amundsen-Scott Base, and on a Bell and Howell Apple computer.

Results

Fig. 1 illustrates the leucine uptake capacity of the Antarctic cells as a function of temperature at two external leucine concentrations. Elevation of temperature above that of the animal's habitat produced a normal increase in uptake rate; increasing the external leucine concentration (Fig. 1b) led to saturation of uptake. Table I summarizes uptake velocities obtained from the first-order rate constants for eight preparations from *T. bernacchii* (body weight 105 ± 12 g) and *T. hansoni* (body weight 243 ± 38 g). No significant differences between the two species were noted. Cells prepared in balanced salt solution showed slightly greater rates at all temperatures than those prepared in culture medium, although results were significant ($P < 0.05$) only at 5°C (21.3 ± 2.3 vs. 15.7 ± 0.4). Arrhenius activation energy for the 0–10°C range was 65 kJ/mol, or $Q_{10} = 2.7$, with a 95% confidence range of 52–77 kJ/mol.

Table II presents kinetic parameters at the three temperatures of measurement. The values for each experimental series were obtained by least-squares fitting of the Michaelis-Menten equation after inspection of the double reciprocal plot for linearity. Experiments at 0°C showed good reproducibility, however at 5 and 10°C two preparations (not included in Table II) gave significantly elevated values of K_m (4–5 mM).

Competition experiments at the three temperatures (Table III) indicated inhibition of [^{14}C]leucine uptake by 1.5 mM isoleucine and

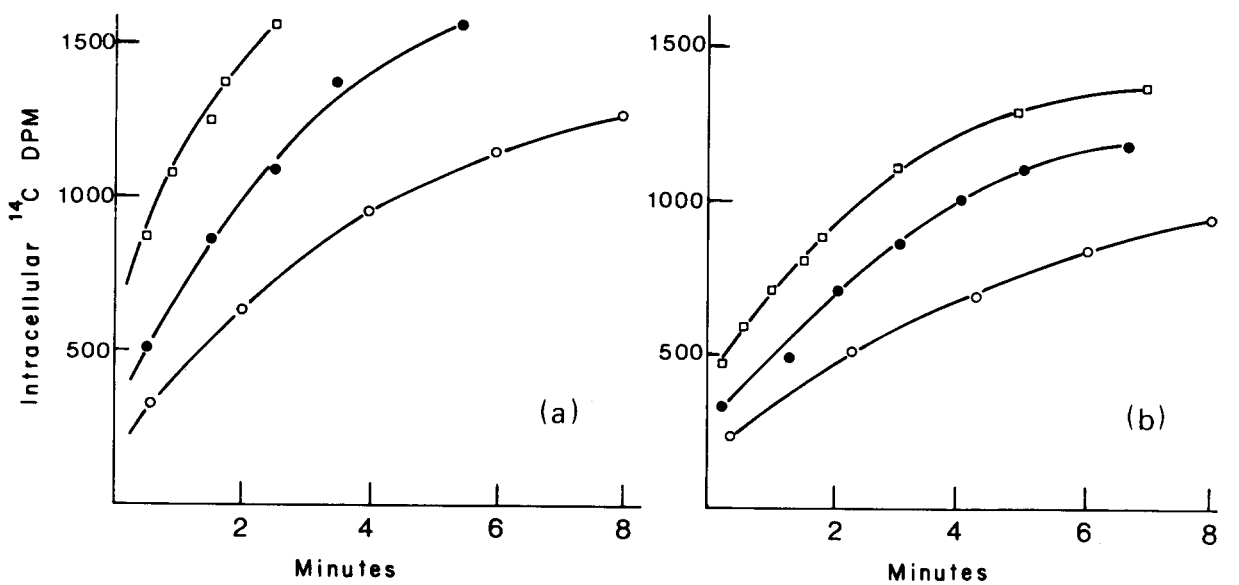

Fig. 1. Time-course of uptake of [^{14}C]leucine into intracellular space of *T. bernacchii* hepatocytes at 0°C (○———○), 5°C (●———●), and 10°C (□———□). (a) External leucine concentration was 0.1 mM. (b) External leucine concentration was 1.6 mM.

TABLE I

TEMPERATURE DEPENDENCY FOR LEUCINE INFLUX BY ISOLATED HEPATOCYTES OF *TREMATOMUS BERNACCHII* AND *T. HANSONI* AT 0.1 mM EXTERNAL LEUCINE CONCENTRATION

Means are presented with standard deviation and number of experiments in parentheses.

Temperature (°C)	Velocity (pmol/μl per min)
0	10.3 ± 1.7(8)
5	19.4 ± 3.4(6)
10	27.3 ± 3.1(5)

TABLE II

KINETIC PARAMETERS OF LEUCINE TRANSPORT AT VARIOUS TEMPERATURES IN ISOLATED HEPATOCYTES OF ANTARCTIC FISH

Means with standard error are presented with number of experiments in parentheses.

Temperature (°C)	K_m (mM)	V_{max} (pmol/μl per min)
0	1.15 ± 0.13(6)	124 ± 24
5	1.05 ± 0.30(4)	213 ± 48
10	0.97 ± 0.11(3)	405 ± 99

TABLE III

EFFECTS OF VARIOUS COMPETING AMINO ACIDS AT 1.5 mM ON THE UPTAKE OF 0.1 mM [^{14}C]LEUCINE

Mean ± standard error (number of experiments) is presented.

Amino acid	Per cent of control rate		
	0°C	5°C	10°C
Alanine	84 ± 3(3)	96 ± 3(6)	97 ± 4(9)
Glycine	98 ± 4(5)	95 ± 7(3)	95 ± 8(6)
Histidine	98 ± 8(3)	86 ± 3(3)	90 ± 4(2)
Valine	58 ± 3(6)	73 ± 3(6)	86 ± 3(6)
Isoleucine	43 ± 2(3)	51 ± 3(5)	64 ± 2(4)
Phenylalanine	41 ± 3(5)	55 ± 5(5)	68 ± 3(6)

phenylalanine at the same degree observed upon addition of unlabeled leucine at this concentration. Valine was a partial inhibitor, whereas alanine, glycine and histidine were without effect. A similar trend was observed at 1.0 mM competitor concentration and 0°C with 59 ± 6 (3) for phenylalanine, 71 ± 6 (3) for valine and 97 ± 8 (2) for histidine. The three inhibitory amino acids all showed a decline in activity ($P < 0.05$) as temperature was increased. This group of preparations showed a similarly diminished effect when unlabeled leucine was added to a concentration of 1.5 mM.

Discussion

The present results provide evidence of a leucine transport system in Antarctic fish hepatocytes of relatively high affinity and specificity. The first-order rate constants for leucine uptake at 0°C averaged one-third the value of 0.3 min^{-1} estimated in vivo from kinetic analysis of leucine clearance by liver [12]. This fraction of in vivo activity is consistent with results for protein synthetic activity of these cells, which ranged from 15 to 50% of in vivo levels [22]. Absolute values for uptake are low, as expected at 0°C; the comparable rate constant for leucine uptake by rat hepatocytes at 37°C is 2.4 min^{-1} [20], or almost 20-times the highest value obtained in the Antarctic preparations.

The leucine affinity data, with K_m averaging 1.0 mM and a range as low as 0.65 mM, compare well with other results in fish liver. Although K_m is not known for Antarctic fish in vivo, half-maximal uptake occurs at an injection dose of 1.5 μmol [12], as in toadfish liver at 20°C [8]. In the latter case, K_m has been estimated at 0.6 mM [8]. Preliminary data on leucine uptake by isolated toadfish hepatocytes (20°C) have yielded the same result (Haschemeyer, A.E.V., unpublished data). Values reported for other systems range from 0.01 to 0.1 mM in mouse 3T3 and hamster ovary cells [25] to 5.5 mM in rat hepatocytes [20].

The similarity in K_m values for the fish liver systems in vivo and in culture provide encouragement for the use of collagenase-prepared hepatocytes as a model for study of transport. There have been few comparative studies of this type, prim-

arily due to the difficulty in obtaining kinetic parameters in vivo (see, for example, Ref. 26). Similarities between sugar transport in isolated rat hepatocytes and the clearance of these substances by liver in vivo have been reported; however, affinity (from K_m) was 3–5-times lower in the isolated cells [27]. This reduction in affinity does not occur in the Antarctic fish hepatocytes at 0°C.

The competition studies showed major effects by isoleucine and phenylalanine, suggesting that they share the same pathway, as in other systems [20,26–28]. Valine, however, was much less effective than in rat hepatocytes [20], and histidine, found to be a major competitor of leucine transport in mammalian cells [29,30] was without effect. Overall, the results for the Antarctic hepatocytes were closest to those obtained in liver of toadfish at its acclimation temperature [8], suggesting that similar characteristics may be associated with adaptation over evolutionary times and with seasonal temperature acclimation. Several studies suggest that membrane fluidity may play a role in both of these adaptations [31–33].

Transport rate increased normally with temperature in most of the preparations studied. The Arrhenius activation energy for this system agrees well with results on temperature dependency of transport in hepatocytes [34] and of liver protein synthesis in fish [35]. Several preparations, however, showed diminished leucine affinity at the higher temperatures with $K_m = 4–5$ mM, similar to that in rat hepatocytes [20]. Competitor inhibition was also reduced with increasing temperature. This may reflect a specific temperature effect on activity of one or more leucine transporters, or a membrane transition of some kind. A general thermal deterioration is also a possibility, although freshly isolated *Trematomus* cells in suspension were found to be active in protein synthesis for several hours at 10°C [22]. As yet, therefore, the present results cannot account for the extremely low upper lethal temperature of these species.

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