Circadian variations of A-mediated transport in rat-liver plasma membrane vesicles

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Proline *Nutritional regulation* *Prandial and post-prandial phases* *Intestinal absorbtion* *Portal aminoacidemia*

1. INTRODUCTION

Among the 3 transport systems for neutral amino acids known to operate at the hepatic level, only system A has been shown to be responsive to hormonal and nutritional stimuli. The inductive effect of glucagon [1,5], insulin [2,5,8], corticoids [1,9], and catecholamines [1,10] is now well documented. Direct short-term induction of stimulated A-mediated transport by amino acid deprivation and reversal by amino acid supplementation, has been shown in cultures hepatocytes [11]. Hormonal and/or nutritional treatments applied in vivo, induce changes in hepatic A-mediated transport that can be readily demonstrated in vitro at the cellular level [1,12,13]. Rats kept on regular light–dark schedules exhibit distinct rhythms of hormonal secretions and ingest most of their food (70%) during the night. This behavior results in alternate phases of low (light-phase) and high (dark-phase) portal aminoacidemia [14]. Circadian changes in hepatic A-mediated transport can thus be expected [15]. Here, amino acid transport activity was measured in plasma-membrane vesicles from the liver of rats fed ad libitum a low casein diet and killed at different time-points of the light–dark cycle. This technique has the advantage of using natural amino acids as substrate. Proline was chosen as substrate because, more than alanine [16,17], it is preferentially transported through system A in hepatocytes.

2. MATERIALS AND METHODS

Adult male Sprague-Dawley rats (48) were kept under a 12/12 light–dark schedule and fed ad libitum a semisynthetic diet containing 12% casein, 53% starch, 20% sucrose, 8% peanut oil, 2% cellulose, 4% mineral mixture, and 1% vitamin mixture. After 3 weeks, they were killed by decapitation in groups of 6 at regularly spaced time-points: 4 groups during the light-phase (3,6,9 and 12 h exposure to light), 4 groups during the dark-phase (3,6,9 and 12 h exposure to darkness).

2.1. *Preparation of the plasma-membrane-enriched fractions*

Livers were processed essentially according to [18]. Briefly, after homogenization in a Dounce homogeneizer (10 strokes of each of the 2 pestles) in 5 vol. cold buffer containing 0.25 M sucrose, 0.2 mM $CaCl_2$; 10 mM Hepes /KOH (pH 7.5) homogenates were filtered, diluted 10-fold with buffer and made 1 mM with EDTA. Supernatants and fluffy layers from 2 consecutive centrifugations at 1000 × *g* for 10 min, were pooled and centrifuged at 20 000 × *g* for 30 min. The resulting pellet was resuspended in 5 ml buffer containing 1 mM EDTA and layered onto a discontinuous gradient made from equal volumes of 39.5% (w/w) and 20% (w/w) sucrose in 10 mM Hepes/KOH (pH 7.5). The gradient was centrifuged in a SW.25.2 rotor in an L20 Beckman Ultracentrifuge at 22 000 rev./min for 2.5 h. The material at the interface was then collected and diluted with 1.5 vol. EDTA buffer and centrifuged at 32 000 rev./min for 45 min in a 50 Ti rotor. The final pellet was resuspended in 1 ml buffer without EDTA, divided into small aliquots, quickly frozen in liquid nitrogen and stored at − 40°C pending assay. Transport assays as well as determinations of protein and enzyme activities were performed within 1–15 days after preparation. Protein [19] and

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5′-nucleotidase (EC 3.1.3.5) [20] were determined in the original homogenates and in the various subfractions in order to assess recovery and enrichment in the plasma-membrane fractions.

2.2. *Transport assays*

The procedure in [18,21] was followed. The reaction, performed at 25°C, was started by mixing 20 μl membrane preparation with 80 μl buffer (0.25 M sucrose, 0.2 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM Hepes/KOH (pH 7.5) containing 0.2 mM L-[3,4(*n*)–^{3}H]proline (spec. act. reaction mixture 20 Ci/mol), in presence of either 100 mM Na- or K-SCN. Samples, withdrawn at stated time-points, were added to 1 ml cold stop-solution (0.25 M sucrose, 100 mM NaCl, 0.2 mM $CaCl_2$, 10 mM Hepes/KOH (pH 7.5) filtered through nitrocellulose filters (Sartorius 0.45 μm) and washed with 3 ml cold stop-solution. Counting of the dried filters was achieved in a liquid-scintillation counter using a cumene-based scintillation mixture (Aqualuma). Proline transport was determined at each time-point on individual preparations from 6 rats, each assay being done in triplicate and repeated. Significance of experimental differences was assessed by analysis of variance.

3. RESULTS

3.1. *Plasma-membrane preparations*

5′-Nucleotidase was selected as enzyme-marker after it was ascertained that its activity was not impaired by freezing the samples. As shown in table 1, its specific activity in liver remained at comparable levels throughout the whole light–dark cycle. Although enrichment was on the same order (3.3–4.3-fold increase in specific activity) in all plasma-membrane preparations, the yield was more variable and represented from 4.63–8.75% of original homogenate activity, resulting in variable final membrane concentrations. To take this variability into account, active transport was expressed as pmol proline/unit 5′-nucleotidase (μmol P liberated/h).

3.2. *Transport studies*

Proline uptake was measured 10, 60 and 90 s (active uptake) and 60 min after application of the sodium gradient (external > internal), the last point being taken as a measure of equilibrium conditions. Maximal uptake occurred at 60 s. When expressed in terms of overshoot as % of intravesicular proline remaining at 60 min, the magnitude of Na^+-stimulated uptake exhibited clear circadian variations (fig.1). The accumulation ratio was insignificant (100 ± 4%) during the light-phase. During the dark-phase, on the contrary, there was a modest (146 ± 6%) but highly significant ($P < 0.01$) concentrative uptake.

Active proline transport (Na^+–K^+-uptake) determined in conditions close to initial velocity, i.e., 10 s after application of the ionic gradients (fig.2), exhibited circadian variations resembling those presented in fig.1. Rates of transport were particularly low at L_3,L_6 and L_9. They rose before the end of the light-phase, between L_9 and L_{12} to values similar to those observed during darkness. In spite of this acceleration of transport near the shift from light to dark, proline transport during the light-phase remained on the average (5.51 ± 0.54) significantly lower ($P < 0.05$) than during the dark-phase (7.17 ± 0.53). The difference between the 2 periods became highly significant ($P < 0.01$) when both L_{12} and D_{12} were withdrawn from the comparison. It is interesting to note that SEM at

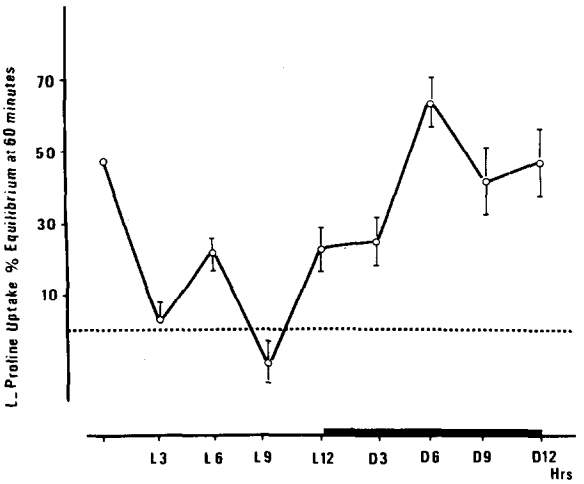

Fig.1. Sodium-gradient stimulated transport of L-proline in rat liver plasma-membrane vesicles from rats killed at different times over the light–dark cycle. Na^+-stimulated proline transport measured at 60 s is expressed as % of proline accumulation at equilibrium (60 min). Each point represents the mean of 6 individual preparations. Vertical bars represent ± SEM.

Table 1
5'-Nucleotidase specific activity in homogenates and plasma-membrane fractions

	Lightness or darkness (h)	Homogenate (spec. act.)	Plasma-membrane fractions		
			Spec. act.	Enrichment (rel. spec. act.)	Recovery (% homogenate)
Rats killed during light-phase	3	5.10 ± 0.24	19.64 ± 1.11	3.8 ± 0.2	4.63 ± 0.87
	6	4.96 ± 0.25	16.97 ± 1.49	3.3 ± 0.3	5.09 ± 0.33
	9	4.25 ± 0.21	15.62 ± 1.14	3.8 ± 0.3	5.84 ± 0.56
	12	5.10 ± 0.40	18.61 ± 1.19	3.7 ± 0.4	7.47 ± 1.34
Rats killed during dark-phase	3	4.36 ± 0.20	18.01 ± 0.59	4.2 ± 0.2	5.59 ± 0.44
	6	4.53 ± 0.33	16.59 ± 1.32	3.7 ± 0.2	5.59 ± 0.16
	9	4.48 ± 0.54	15.37 ± 1.73	3.7 ± 0.2	6.40 ± 0.44
	12	5.53 ± 0.24	20.91 ± 2.0	3.8 ± 0.5	8.75 ± 1.30

Specific activity is expressed as μmol phosphorus . h^{-1} . mg protein $^{-1}$; values are the mean ± SEM from 6 rats

these 2 transitional time-points (L_{12}, 7.52 ± 1.03; D_{12}, 7.04 ± 1.24) were much higher than at other time-points, reflecting the metabolic variability at the turning-points of lighting phases.

Na^+-dependent proline transport, measured at 10 s exhibited saturation kinetics towards increased proline concentration in the extravesicular medium in both light- and dark-phase preparations (fig.3). At 0.2 mM proline, proline transport was inhibited by 25% in the presence of 50 mM leucine while at the same concentration alanine exerted nearly complete inhibition (94%).

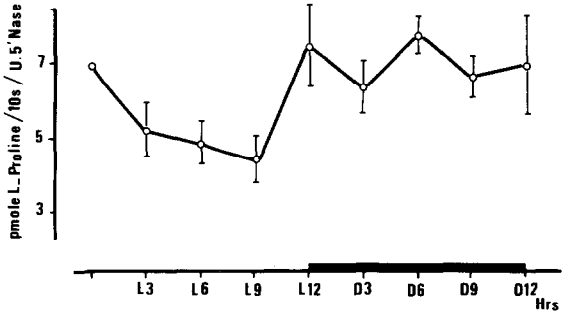

Fig.2. Circadian variations of initial rates of L-proline active transport. Active transport, corresponds to the difference between 10 s uptake in the presence of 100 mM Na-SCN and 100 mM K-SCN; each point corresponds to the mean of 6 individual preps. Vertical bars represent ± SEM.

Fig.4 correlates proline uptake at equilibrium (60 min) with osmolarity of the medium. At constant extravesicular proline concentration (0.2 mM) proline accumulation was in inverse linear relationship with medium osmolarity. The nearly complete

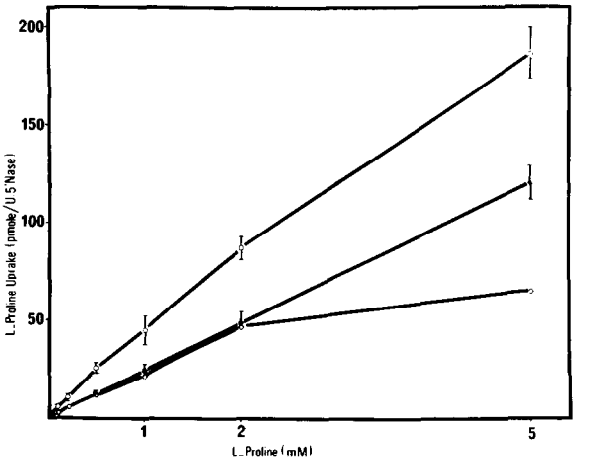

Fig.3. Effect of L-proline concentration on initial rates of L-proline uptake by plasma-membrane vesicles from rats killed at different times: (○—○) uptake in presence of 100 mM Na-SCN; (●—●) uptake in presence of 100 mM K-SCN; (◇—◇) active transport (Na^+–K^+). Each point represents the mean of determinations made on 8 pooled samples from 6 rats (4 from animals killed during light-phase, 4 from animals killed during dark-phase). Vertical bars represent ± SEM.

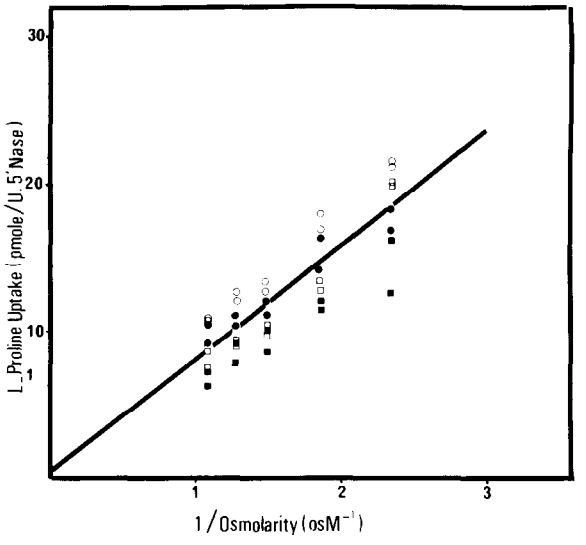

Fig.4. Effect of medium osmolarity on L-proline uptake. Uptake was terminated after 60 min incubation in presence of 100 mM Na-SCN, in medium containing various concentrations of sucrose. Symbols represent the mean of triplicate determinations made on pooled samples from 6 rats killed at L_6 (○), L_9 (△), D_6 (●) and D_9 (▲).

exclusion of proline at infinitely high osmolarity shows that unselective proline adsorption was minimal. Therefore, uptake was truly intravesicular. Our results thus strongly suggest that the hepatic plasma-membrane vesicles did transport proline through a carrier system which exhibited circadian variations. In [16,17] hepatocytes transported proline through both system A (64%) and ASC (36%). Since the latter escapes adaptative regulation [3,6,11], its relative contribution to total Na^+-dependent transport diminishes upon stimulation of A-mediation. ASC-mediated proline transport measured in presence of Li^+ as supporting ion [22] represented 35 ± 6% of total Na^+-dependent transport when measured at L_6 and only 10 ± 3% at D_9. However, it was still 28 ± 6% of total when measured at D_6 in spite of a clear stimulation of total active transport (fig.2).

4. CONCLUSION

Proline uptake by liver plasma-membrane vesicles was found to be saturable, sensitive to stimulation by Na^+ and, to a lesser extent, by Li^+ showing that systems A and ASC were both operative in these preparations as is the case in whole hepatocytes [6,11,17,22]. The values for concentrative accumulation (overshoot) were low compared to those obtained in [18] with alanine in similar preparations. The use of proline instead of alanine may explain in part these differences since the rate of proline transport is half that of alanine in hepatocytes [16,17]. Plasma-membranes vesicles from liver of rats killed at different times in the light–dark cycle exhibited circadian variations in transport activity, reaching 50%. In so far as these plasma-membrane fractions are representative of the in vivo liver membrane-system, our results show that liver amino acid transport activity is subjected to daily variations under strictly physiological conditions. On the strength of what is known about sensitivity of transport systems to hormonal and nutritional stimulation, it is likely that system A is mainly involved. Among the hormones susceptible to play a part in transport activation, glucagon is an unlikely factor since we have shown elsewhere portal glucagonemia to be low and to exhibit insignificant variations in rats fed the low-protein, carbohydrate-rich diet used here [23]. Portal insulinemia, on the contrary exhibits a peak in the middle of the night, but at that time, proline transport has already been elevated for several hours.

Amino acid concentration in the incubating medium has been shown to be an important factor in the regulation of transport activity in cultured hepatocytes [11]. Amino acid concentrations in the extracellular fluid which is the in vivo equivalent of the incubating medium are dependent on those of the portal blood since the 2 compartments are in close continuity. A certain degree of inverse relationship should therefore exist between changes in portal aminoacidemia and induction–desinduction of stimulated transport. However, no clear correlation can be demonstrated between the 2 parameters [14], beyond the fact that induction of stimulated transport does occur during a period of steady low aminoacidemia and desinduction during a period of steady high aminoacidemia. The time necessary for portal aminoacids to significantly alter liver extracellular compartment may explain the lag, especially since the feeding of the low-protein diet entails only moderate changes in portal aminoacidemia [14]. Nutritional manipulations such as increasing protein content of the diet or spacing of the meals, will help clarify the impact of changes

in liver extracellular amino acid concentration on transport activity. Nevertheless, given the high level of integration prevailing in the organism as a whole, nutritional regulation should not be viewed outside the context of a cooperation with hormonal factors.

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