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REGULATION OF AMINO ACID TRANSPORT IN CHICK EMBRYO HEART CELLS

III. FORMAL IDENTIFICATION OF THE A MEDIATION AS AN ADAPTIVE TRANSPORT SYSTEM

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SUMMARY

An amino acid transport system, subject to regulation involving a repression–derepression mechanism by its substrate molecules, is operative in chick embryo heart cells (Gazzola, G. C., Franchi, R., Saibene, V., Ronchi, P. and Guidotti, G. G. (1972) *Biochim. Biophys. Acta* 266, 407–421). The formal identification of this system with the A mediation has been achieved by studies yielding the following results:

1. The system which exhibits adaptive control is Na^+ dependent, whereas its regulation (derepression upon incubation of cardiac cells in amino acid-free medium, repression in the presence of pertinent amino acid) is largely Na^+ independent.

2. Among the four transport systems (A, ASC, L, Ly^+) individually discriminated from interfering systems by relatively specific model substrates, only the A mediation showed derepression when cardiac cells were incubated in amino acid-free medium.

3. Among the four model amino acids tested (*i.e.* α-(methylamino)isobutyric acid, *b*(±)-2-aminobicyclo(2,2,1)heptane-2-carboxylic acid, 4-amino-1-guanylpiperidine-4-carboxylic acid, α,α-diethylglycine) only α-(methylamino)isobutyric acid, developed as specific substrate of System A, inhibited the increase in transport activity of cardiac cells incubated under conditions otherwise derepressive.

4. The addition of transport-specific amino acids during incubation under conditions of inhibited protein synthesis increased the rate of degradation of protein components of System A (as estimated by measurements of transport activity). This had no effect on the rate for System ASC and apparently decreased it for Systems L and Ly^+.

5. Experiments, showing that adaptive control of transport activity by the A mediation is operative in such mammalian tissues as newborn mouse and rat heart cells, suggest that this regulation is an intrinsic property of muscle tissue.

Abbreviations: MeAIB, α-(methylamino)isobutyric acid; BCH, 2-aminobicyclo(2,2,1) heptane-2-carboxylic acid; GPA, 4-amino-1-guanylpiperidine-4-carboxylic acid.

INTRODUCTION

The transport of amino acids across the cell membrane is performed by specific systems of mediation acting on discrete groups of substrate molecules[1]. As reported previously[2], a time-dependent adaptive system for the transport of a group of neutral amino acids is operative in chick embryo heart cells and other muscle tissues. The regulation of this system involves a repression–derepression mechanism by its substrate molecules acting at the transcription level coupled to a subsidiary mechanism affecting breakdown (or inactivation) of transport proteins[3].

Studies of competition for entry and of substrate dependence for adaptive control anticipated[2] that the amino acids involved in this regulation corresponded to those assigned to the A mediation by Christensen and Oxender[1,4]. However, no attempts were made to attain a formal identification of this system as an adaptive transport system. This identification has been the purpose of the present study. We investigated: (a) the Na^+ dependence of the transport system subject to adaptive regulation; (b) the changes in activity with time for the principal systems of mediation after discrimination from interfering systems by transport-specific substrates; (c) the repressive properties of model amino acids known to enter the cell by a definite transport system; (d) the rate of degradation of protein components for the various transport systems.

The results of these experiments indicate that the A mediation is the sole transport system which undergoes derepression of its activity in the absence of specific substrate amino acids and exhibits increased degradation of its protein components in the presence of substrate molecules for which it is competent.

MATERIALS AND METHODS

The sources for most of the materials used are listed in the preceding papers of this series[2,3]. α-(Methylamino)isobutyric acid (MeAIB), 2-aminobicyclo(2,2,1) heptane-2-carboxylic acid (BCH; isomeric form $b(\pm)$), 4-amino-1-guanylpiperidine-4-carboxylic acid (GPA) and α,α-diethylglycine were a gift from Dr Halvor N. Christensen (University of Michigan, Mich., U.S.A.).

The procedures of cell isolation from chick embryo hearts by collagenase treatment have been described previously in detail[5–7]. The same procedures have been adopted to prepare isolated cells from newborn mouse and rat hearts. Incubations were carried out in silicone-treated glass vessels at 37.5 °C, under continuous mild stirring[6] in an atmosphere of O_2–CO_2 (95:5, v/v). The basic incubation medium was Krebs–Ringer bicarbonate buffer containing 8 mM glucose; choline chloride and choline bicarbonate replaced NaCl and $NaHCO_3$ when Na^+-free medium was used. Additions (amino acids, inhibitors, *etc.*) and washing procedures of the cells before changes in medium composition are specified in Results.

Initial rates of amino acid uptake were measured by transferring samples of cell suspension into flasks containing the desired medium with the appropriate additions (labelled amino acids, inhibitors) and incubating the flasks at 37.5 °C for 5 min in a Dubnoff metabolic shaker[2].

The means for determining intracellular accumulation of the tracer amino acid and for evaluating the proper corrections to be introduced were as described

by Guidotti *et al.*[6,7]. The procedures for studying [^{14}C]leucine incorporation into protein have been described previously[3].

RESULTS

Na$^+$ dependence

Fig. 1 shows that the activity of amino acid transport, as measured by α-aminoisobutyric acid uptake in experiments of 5 min duration (initial velocity), increases

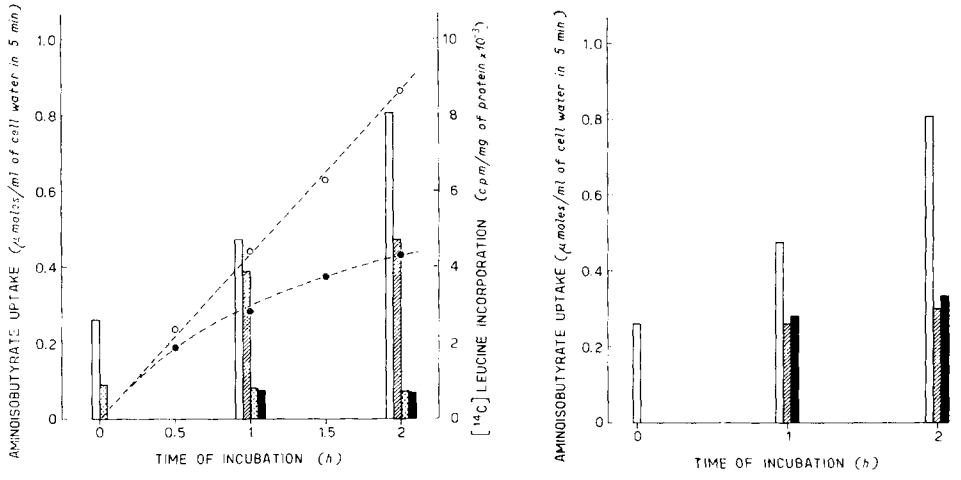

Fig. 1. Uptake of α-aminoisobutyric acid and incorporation of leucine into protein of chick embryo heart cells incubated in the presence and absence of Na^+. Cardiac cell suspensions were incubated for 2 h in Krebs–Ringer bicarbonate buffer (143 mequiv of Na^+/l) or in an Na^+-free medium (Krebs–Ringer bicarbonate buffer in which choline chloride and choline bicarbonate replaced NaCl and $NaHCO_3$) both supplemented with 8 mM glucose. Incubation was at 37.5 °C in an atmosphere of O_2–CO_2 (95:5, v/v). α-Aminoisobutyrate uptake (initial velocity) was measured on samples of cell suspension (10^7 cells) which were washed with Krebs–Ringer bicarbonate buffer or Na^+-free medium, transferred into flasks containing the same media supplemented with 0.1 mM α-amino[1-^{14}C]isobutyric acid and incubated for 5 min at 37.5 °C. When amino acid incorporation into protein was studied, the incubation medium contained 0.1 mM L-[U-^{14}C]leucine. The procedures for protein purification and radioactivity measurements have been described[3]. Legend: open bars, incubation in Krebs–Ringer bicarbonate buffer, α-aminoisobutyric acid uptake in Krebs–Ringer bicarbonate buffer; cross-hatched bars, incubation in Na^+-free medium, α-aminoisobutyric acid uptake in Krebs–Ringer bicarbonate buffer; spotted bars, incubation in Krebs–Ringer bicarbonate buffer, α-aminoisobutyric acid uptake in Na^+-free medium; solid bars, incubation in Na^+-free medium, α-aminoisobutyric acid uptake in Na^+-free medium; ○, leucine incorporation into protein, incubation in Krebs–Ringer bicarbonate buffer; ●, leucine incorporation into protein, incubation in Na^+-free medium.

Fig. 2. Uptake of α-aminoisobutyric acid by isolated cardiac cells: effect of the addition of alanine on changes in transport activity during incubation in the presence and absence of Na^+. Incubations and assay of α-aminoisobutyric uptake were as described in the legend of Fig. 1 except that L-alanine (5 mM, final concentration) was added to the medium during the incubation in some experiments. Legend: open bars, incubation in Krebs–Ringer bicarbonate buffer, α-aminoisobutyric acid uptake in Krebs–Ringer bicarbonate buffer; cross-hatched bars, incubation in Krebs–Ringer bicarbonate buffer *plus* alanine, α-aminoisobutyric acid uptake in Krebs–Ringer bicarbonate buffer; solid bars, incubation in Na^+-free medium *plus* alanine, α-aminoisobutyric acid uptake in Krebs–Ringer bicarbonate buffer.

with time when isolated chick embryo heart cells are incubated in Krebs–Ringer bicarbonate buffer (*cf.* ref. 2). As expected for a preferential substrate of the Na^+-dependent transport system A[1], the initial rate of entry of α-aminoisobutyric acid into the cell decreased sharply when measured in Na^+-free medium. Under these conditions the residual α-aminoisobutyric acid-mediated uptake, which is likely to occur by the Na^+-independent transport system L[2], decreased slightly (as initial velocity) with time whether Na^+ was present during the incubation period (2 h) or not. The replacement of Na^+ by choline in the medium during this period lowered but did not abolish the increase with time of transport activity when measured by α-aminoisobutyric acid uptake in the presence of Na^+. The decreasing rate of [^{14}C]leucine incorporation into protein of cardiac cells incubated in the absence of Na^+ (Fig. 1) suggests that the rate of change in transport activity under these circumstances reflects the progressive decline of protein synthesis (*cf.* ref. 2). The addition of L-alanine (5 mM) during the incubation period (2 h) prevented almost completely the increase with time of transport activity (as measured by α-aminoisobutyric acid uptake in the presence of Na^+, after removal of added alanine, *cf.* ref. 2) either in a Na^+-containing or in Na^+-free medium (Fig. 2). The slightly lower inhibition of transport activity observed under the latter condition is likely to depend on the slower rate of penetration of alanine into the cell and the consequent decreased accumulation of the amino acid in the Na^+-free medium.

Changes in activity of specific transport systems

MeAIB, *b*(±)-BCH and GPA have been used as model substrates with specificity for Systems A, L and Ly^+, respectively[8–11], in order, to discriminate between the various transport systems when changes with time of their individual activity were investigated. The adopted procedure consisted in the determination of transport activity in experiments of 5 min duration (initial velocity) at the beginning and at the end of a 2-h incubation of isolated cardiac cells in Krebs–Ringer bicarbonate. Activity was measured by the uptake of appropriate ^{14}C-labelled amino acids (L-alanine, L-serine, L-phenylalanine, L-lysine) in the absence and in the presence of the unlabelled transport-specific model substrates.

Table I shows that: (a) transport activity increases with time when assayed by alanine or serine, which are known to enter the cell by Systems A[4], ASC[8] and, less efficiently, by System L[4]; the addition of sufficient MeAIB and BCH (during the measurement of transport activity) to prevent alanine or serine uptake by Systems A and L decreases their initial rate of entry by about 70% (alanine) and 60% (serine), respectively; the residual uptake which is likely to occur by the ASC system, for which both amino acids are good substrates[8], does not change (as initial velocity) during the incubation period; (b) when assayed by phenylalanine, which is known to enter the cell by System L (preferentially)[4] and less efficiently by Systems A[1,4] and Ly^+ (with Na^+ as cosubstrate)[12], transport activity does not change with time; the addition of MeAIB and GPA in excess (during the measurement of transport activity) to prevent phenylalanine uptake by Systems A and Ly^+ does not alter appreciably the absolute value of initial entry rate for the latter amino acid at the beginning of the experiment, but reveals a definite decrement of transport activity as the incubation proceeds; the increasing transport activity by the A mediation, through which a progressively larger fraction of phenylalanine can be taken up, is

TABLE I

AMINO ACID UPTAKE BY SPECIFIC SYSTEMS OF MEDIATION IN ISOLATED CARDIAC CELLS: CHANGES IN TRANSPORT ACTIVITY WITH TIME

Cardiac cell suspensions were incubated for 2 h in Krebs–Ringer bicarbonate buffer containing 8 mM glucose. Incubation was at 37.5 °C in an atmosphere of O_2–CO_2 (95:5, v/v). Amino acid uptake (initial velocity) was measured by transferring samples of cell suspension (10^7 cells) into flasks containing Krebs–Ringer buffer supplemented with 8 mM glucose, the ^{14}C-labelled amino acid (0.1 mM, final concn) under study and the unlabelled transport-specific model substrate(s) at 5 mM final concentration, and incubating for 5 min at 37.5 °C. The values are means of 3 separate determinations.

Labelled amino acid	*Discriminating substrate(s)*	*Uptake (μmoles/ml of cell water in 5 min)*	
		Incubation time (h):	
		0	*2*
L-Alanine	None	1.15	1.99
	MeAIB, *b* (±)-BCH	0.34	0.35
L-Serine	None	1.16	1.90
	MeAIB, *b* (±)-BCH	0.46	0.44
L-Phenylalanine	None	0.22	0.21
	MeAIB, GPA	0.20	0.12
L-Lysine	None	0.70	0.42
	b (±)-BCH	0.62	0.40

likely to mask the decrease of amino acid uptake by the L system in the absence of discriminating substrates; (c) when assayed by lysine, which is known to enter the cell by System Ly^+ (ref. 13) and less efficiently by System L[12,14], transport activity decreases with time (*cf.* ref. 2); the addition of BCH in excess (during the measurement of transport activity) to minimize lysine uptake by System L without interfering with the capacity of System Ly^+ (*cf.* ref. 9), decreases slightly the initial rate of entry of the latter amino acid (*cf.* ref. 12), the decrement with time being comparable to that observed in the absence of added BCH.

Repressive properties of transport-specific substrates

As previously reported[2], isolated chick embryo heart cells incubated in Krebs–Ringer bicarbonate buffer showed a rapid increase with time of transport activity for a group of neutral amino acids, including α-aminoisobutyric acid, a preferential substrate for Systems A[1]. The increase in activity was prevented by the addition to the incubation medium of several amino acids (glycine, alanine, serine, proline, methionine) known to enter the cell, either preferentially using the A system (glycine, proline) or an alternative (alanine, serine, methionine) transport system[1,8]. This repressive effect (as a result of a repression–derepression mechanism by amino acids acting at the transcription level coupled to breakdown control of transport proteins[3]) has now been studied with much more transport-specific substrates, added to the medium during the incubation period (3 h). Transport activity was measured at the beginning and at the end of incubation, after appropriate washings

to remove added model substrates, by α-aminoisobutryic acid uptake in 5-min experiments (initial velocity).

Table II shows that the expected increase of transport activity was completely abolished when MeAIB, a model substrate for transport system A[1,8], was present in the medium during the incubation. On the contrary, no repressive effects were

TABLE II

UPTAKE OF α-AMINOISOBUTYRIC ACID BY ISOLATED CARDIAC CELLS: CHANGES IN TRANSPORT ACTIVITY UPON INCUBATION IN THE PRESENCE OF TRANSPORT-SPECIFIC MODEL AMINO ACIDS

Cardiac cell suspensions were incubated for 3 h in Krebs–Ringer bicarbonate buffer containing 8 mM glucose, in the presence and absence of individual model amino acids at 5 mM concn. Incubation was at 37.5 °C in an atmosphere of O_2–CO_2 (95:5, v/v). α-Aminoisobutyrate uptake (initial velocity) was measured at the beginning and at the end of incubation on samples of cell suspension (10^7 cells) which were washed with Krebs–Ringer buffer (to remove the model amino acid when present in the incubation medium), transferred into flasks containing the same medium supplemented with 0.1 mM α-amino-[1-^{14}C]isobutyric acid and incubated for 5 min at 37.5 °C. The values are means of 3 separate determinations.

Model amino acid present during incubation	*Aminoisobutyrate uptake (μmoles/ml cell water in 5 min)*		
	Incubation time (h):		*Difference*
	0	*3*	*(%)*
None	0.27	0.87	+222
α-(Methylamino)isobutyric acid	0.21	0.23	+ 10
b(±)-2-Aminobicyclo(2,2,1) heptane-2-carboxylic acid	0.26	0.97	+273
4-Amino-1-guanylpiperidine-4-carboxylic acid	0.25	0.81	+224
α,α-Diethylglycine	0.29	1.09	+275

observed upon addition of *b*(±) BCH or GPA, which were developed as transport-specific amino acid analogues for Systems L[10,15] and Ly$^+$ (refs 11 and 16). α,α-Diethylglycine, an atypical substrate for System L (Christensen, H. N., personal communication), which enters the cell by a system unusually susceptible to the inhibitory action of almost all neutrall amino acids[9,17], was also ineffective. The fact that the increase of transport activity is prevented in the presence of the non-utilizable analogue MeAIB confirms previous results[2] indicating that the mechanism for adaptive regulation utilizes the intact amino acid molecule rather than metabolic derivatives.

Comparable results have been obtained when the biological model used consisted of isolated cells prepared from newborn mouse and rat hearts (Table III).

Kinetics of transport protein degradation

The availability of rather specific transport substrates by which the operation of interfering systems can be minimized has allowed us to investigate seperately the rate of degradation of protein components of transport Systems A, ASL, L and

TABLE III

UPTAKE OF α-AMINOISOBUTYRIC ACID BY CELLS ISOLATED FROM NEWBORN MOUSE AND RAT HEARTS: CHANGES IN TRANSPORT ACTIVITY UPON INCUBATION IN THE PRESENCE OF TRANSPORT-SPECIFIC MODEL AMINO ACIDS

Incubation of cell suspensions and measurements of aminoisobutyrate uptake were as described in Table II. Since intracellular water volumes for these mammalian cardiac cells were not determined, uptake data are referred as μmoles per g of cell protein.

Model amino acid present during incubation	*Aminoisobutyrate uptake (μmoles/g cell protein in 5 min)*			
	Newborn mouse heart cells		*Newborn rat heart cells*	
	Incubation time (h):		*Incubation time (h):*	
	0	*3*	*0*	*3*
None	0.37	1.17	0.95	1.94
α-(Methylamino)isobutyric acid	0.42	0.49	0.87	0.69
b(±)-2-Aminobicyclo(2,2,1)-heptane-2-carboxylic acid	0.40	1.14	0.96	1.90
4-Amino-1-guanylpiperidine-4-carboxylic acid	0.38	0.97	0.91	1.84
α,α-Diethylglycine	0.42	1.37	0.99	2.03

Ly^+. An estimation of this rate has been obtained by measurements of transport activity as a function of time under conditions of inhibited protein synthesis on the assumption that transport activity is proportional to the amount of transport proteins present in the system at any time (*cf.* ref. 3).

Cardiac cells were incubated in the presence of cycloheximide (at a concentration sufficient to suppress protein synthesis almost completely[3]) with or without added amino acids. Transport activity was determined every 30–45 min (for 150–180 min), after removal of added compounds, by labelled amino acid uptake in 5-min experiments (initial velocity) in the presence of discriminating substrates.

Fig. 3 shows the following. (a) The activity of the A transport system decreases as a single exponential with a half-life of approx. 190 min when cells are incubated in the absence of added transport-specific amino acids; in the presence of alanine (5 mM), transport activity of System A decreases much faster with time (half-life of approx. 60 min). These results confirm those reported previously[3]. A preliminary enhancement of transport activity by this system, as obtained by preincubating the cells in amino acid-free medium, has been required in this experiment to obtain initial rates of α-aminoisobutyric acid uptake which were compatible with their correct measurement. The low rate of penetration of α-aminoisobutyric acid into cycloheximide-treated cardiac cells freshly removed from the embryo and not first incubated *in vitro* (*cf.* ref. 2) did not provide reproducible results in our hands. (b) Transport activity of System ASC appears under the usual definition (see Discussion) to decrease as a single exponential with a half-life of approx. 300 min; the addition of 5 mM alanine, a substrate for this system, during the incubation does not alter the rate of change in activity for this mediation. (c) Transport activities

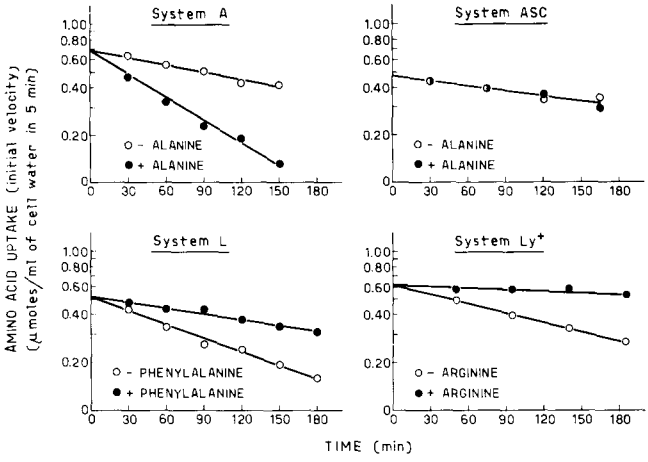

Fig. 3. Changes in amino acid transport activity (Systems A, ASC, L and Ly^+) of cardiac cells incubated under conditions of inhibited protein synthesis in the absence and in the presence of substrate amino acids. Cell suspensions, after a 90-min preincubation in an amino acid-free Krebs–Ringer buffer (System A) or without preincubation (Systems ASC, L and Ly^+), were incubated for 30 min in Krebs–Ringer bicarbonate buffer supplemented with cycloheximide (2 μg/ml) after which they were divided in two equal samples. Samples were incubated for an additional 150–180 min in the same cycloheximide-containing medium in the absence or in the presence of 5 mM alanine (Systems A and ASC), 5 mM phenylalanine (System L) and 5 mM arginine (System Ly+). Incubations were at 37.5 °C in an atmosphere of O_2–CO_2 (95:5, v/v). Amino acid transport activity was measured every 30–45 min on aliquots of cell suspension (10^7 cells) which were washed with Krebs–Ringer bicarbonate buffer (to remove the amino acid when present in the incubation medium), transferred into flasks containing the same medium supplemented with 0.1 mM labelled amino acid and 5 mM discriminating substrate(s) as specified below, and incubated for 5 min at 37.5 °C. System A; α-amino[^{14}C]isobutyric acid uptake in the presence of BCH; System ASC; L-[^{14}C]serine uptake in the presence of MeAIB and BCH; System L. L-[^{14}C]leucine uptake in the presence of MeAIB and GPA; System Ly^+, L-[^{14}C]-lysine uptake in the presence of BCH. The data are plotted as a function of time; note logarithmic scale of the ordinate.

of Systems L and Ly^+ decrease as single exponentials with half-lives of approx. 110 min. (System L) and 150 min (System Ly^+); in the presence of pertinent substrates (5 mM phenylalanine for System L, 5 mM arginine for System Ly^+) transport activity of both systems decrease much slower with time (half-life of approx. 260 min for System L and of more than 800 min for System Ly^+).

DISCUSSION

The foregoing results definitely prove that, among the four systems examined and found operative in chick embryo heart cells for the transport of neutral and basic amino acids, the sole Na^+-dependent A mediation exhibits adaptive control involving a repression–derepression mechanism by its substrate molecules (Tables I and II). Moreover, an increased degradation of transport proteins in the presence of pertinent amino acids has been observed only for this system of mediation (Fig. 3). These results reinforce the concept that the adaptive regulation described for transport system A involves coupled mechanisms acting at two different sites: repression–dere-

pression at gene transcription and control of transport-protein breakdown (or inactivation) at the cell membrane[3]. Typical substrates of the A mediation are the naturally occurring amino acids glycine, L-alanine, L-serine, L-proline and the analogues α-aminoisobutyric acid and MeAIB; the natural L-methionine and the model cycloleucine, though displaying a substantial uptake by the A mediation, are preferential substrates of System L (*cf.* ref. 2).

Alanine and serine are also good substrates of the System ASC[8]. Within the limits of our inhibition analysis for the assignment o the transport activity operationally defined by these substrates (Table I), the ASC system does not show adaptive regulation. In this context, the demonstration that a MeAIB-un-inhibitable component of alanine and serine uptake behaves independently of the A system as far as adaptive regulation is concerned, is likely to provide a further criterion (beyond differences in stereospecificity, sensitivity to pH and metabolic inhibitors[8]) for the formal characterization of the ASC system and may be taken as evidence for its occurrence in the cardiac cell. The previously reported lack of time-dependent increase of transport activity using L-threonine[2], which is known to be a good substrate of the ASC system in Ehrlich ascites tumour cells[8] and in avian erythrocytes[18], favours the same interpretation. Lack of adaptive regulation (Table I, Fig. 3), distinct differences in transport V for the various amino acid substrates[8] and ubiquitous distribution in tissues of higher organisms[1,8] suggest that the ASC system is constitutive. The tendency toward constancy of transport V among the various substrates of the A mediation (typically absent in erythrocytes) indicates a common rate-limiting reaction[8], which appears particularly adequate for a system subject to adaptive regulation by the group of amino acids transported by it.

Na^+ independence and failure to prevent derepression of its transport activity by BCH (and particularly by *b*-isomers whose *b*(−) form is the most specific model substrate for System L so far recognized[10]) (Table II) indicate that the L mediation does not participate in the described regulation. The fact that transport system Ly^+ could also be excluded from the same regulation (Table I) renders negligible the possibility that neutral amino acids [capable of exchange with cationic amino acids in the presence of extracellular Na^+ (refs 12, 19)] may utilize this system for repressive control of their transport activity. The slower decline in transport activity of Systems L and Ly^+ in the presence of specific substrate molecules under conditions of inhibited protein synthesis (Fig. 3) does not allow unequivocal explanations. Pre-existing protein components of these systems might be protected by pertinent substrates during operation. Alternatively, the occurrence of an active exchange diffusion[1,20–22] of the tracer with pre-accumulated amino acid (whose intracellular concentration is likely to increase during the incubation) might simulate an apparent decrease of the rate of transport protein inactivation. Studies are in progress to elucidate this point.

Results presented in Table III, showing that an adaptive control of transport activity by the A mediation is operative in mammalian tissues (newborn mouse and rat heart cells), indicate that this regulation of amino acid transport is an intrinsic property of muscle tissue and emphasize its broad biological significance.

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