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Calmodulin-dependent modulation of pH sensitivity of the amino acid transport system L in human placental choriocarcinoma cells

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Abstract

The JAR human placental choriocarcinoma cells express the amino acid transport system L. The activity of this system is Na^+-independent and is stimulated by acidic extracellular pH. Treatment of cells with the calmodulin antagonist CGS 9343B results in a marked stimulation of the system L activity. At a CGS 9343B concentration of 50 μM, the stimulation of activity measured at pH 7.5 is about 75–100%. This effect is not blocked by cycloheximide, actinomycin D, colchicine or cytochalasin D suggesting that the stimulation is not due to de novo synthesis of the carrier protein or recruitment of the carrier protein from an intracellular pool. The stimulatory effect of CGS 9343B is reproducible with other calmodulin antagonists. Treatment with CGS 9343B significantly modifies pH sensitivity of the system. The stimulatory effect of H^+ is markedly reduced in treated cells compared to control cells. The stimulation of activity at pH 5.5 vs. pH 7.5 is 55% in control cells but only 8% in treated cells. Similarly, the stimulatory effect of CGS 9343B is reduced by H^+. The stimulation of activity seen with 50 μM CGS 9343B is 80% at pH 8.0, but only 26% at pH 5.5. In addition, H^+ and CGS 9343B affect the kinetic parameters of system L in a similar manner, the stimulation in both cases being primarily due to an increase in the maximal velocity. The apparent competitive nature between the effects of H^+ and CGS 9343B is also observed with other calmodulin antagonists. These results show that the transport function and pH sensitivity of the amino acid transport system L in placental choriocarcinoma cells are modulated by calmodulin by processes which do not involve de novo synthesis nor recruitment of the carrier protein.

Key words: Leucine transport; System L; Calmodulin antagonist; pH sensitivity; Choriocarcinoma cell; Placenta

1. Introduction

Several transport systems participate in the transfer of neutral amino acids across the plasma membrane of mammalian cells [1–3]. Among these transport systems, system L exhibits certain unique characteristics. This system shows preference for branched chain and aromatic amino acids, many of which are nutritionally essential. Unlike most other amino acid transport systems, the activity of system L is not influenced by Na^+. Other special features of this system include *trans*-stimulation and H^+-dependent modulation. In several cell types, the activity of system L has been shown to be significantly stimulated by lowering of pH below 7.5 [4–7]. This phenomenon, together with the notable Na^+-independence of system L, has led to the suggestion that H^+ may be involved in the energization of this system [4,8]. In fact, evidence has been presented in recent years that, at least in certain cell types, a transmembrane H^+ gradient is indeed the driving force for system L [9].

System L is expressed in human placenta where it is likely to participate in the transfer of amino acids from mother to fetus [10,11]. The characteristics of this transport system in the placenta have been studied in detail using purified brush border membrane vesicles or intact villous tissue derived from normal term human placentas [12–15]. Recently, we have provided evidence for expression of system L in the JAR human

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Abbreviations: CGS, CGS 9343B or 1,3-dihydro-1-[1-(4-methyl-4*H*, 6*H*-pyrrolo[1,2-*a*]-[4,1]benzoxapin-4-yl)-methyl)-4-piperindinyl]-2*H*-benzimidozol-2-one maleate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; AIB, α-aminoisobutyric acid; BCH, 2-*endo*-aminobicyclo[2,2,1]heptane-2-carboxylic acid; SDS, sodium dodecyl sulfate; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

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placental choriocarcinoma cell line [16]. This cell line has proved to be very useful in investigations involving regulatory aspects of this transport system. The activity of system L in JAR cells is stimulated by activation of protein kinase C, unaffected by agents which elevate intracellular levels of cAMP, and stimulated by agents which interfere with calmodulin-dependent processes [16]. The present study was undertaken to investigate the calmodulin-dependent regulation of the activity of system L in JAR cells in detail. The results of the study show that the stimulation of system L activity by calmodulin antagonists does not involve synthesis nor recruitment of new carriers. Stimulation of system L activity by calmodulin antagonists is also accompanied by changes in one of the distinctive characteristics of the transport system, namely its pH sensitivity. After treatment with calmodulin antagonists, system L in JAR cells becomes significantly less responsive to changes in extracellular pH.

2. Materials and methods

2.1. Materials

L-[4,5(n)-^{3}H]Leucine (specific radioactivity, 60 Ci/mmol), [2(n)-^{3}H]taurine (specific radioactivity, 25.6 Ci/mmol), and L-[3-^{3}H]alanine (specific radioactivity, 76.9 Ci/mmol) were purchased from Du Pont-New England Nuclear (Boston, MA, USA). The JAR human placental choriocarcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). Unlabeled amino acids, calmidazolium, cycloheximide, actinomycin D, cytochalasin D and dimethyl sulfoxide were from Sigma (St. Louis, MO, USA). Colchicine was obtained from Aldrich (Milwaukee, WI, USA) and W-7 from Research Biochemicals (Natick, MA). CGS 9343B (CGS) was a gift from Ciba-Geigy (Summit, NJ, USA). RPMI-1640 (with L-glutamine), fetal bovine serum and penicillin (10 000 units/ml)/streptomycin (10 000 μg/ml) were purchased from Gibco (Grand Island, NY, USA).

2.2. Cell culture

JAR cells were routinely cultured in 75 cm^2 Corning culture flasks with RPMI medium, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were maintained at 37°C and 5% CO_2. Subconfluent cultures were treated with phosphate-buffered saline containing 0.1% trypsin and 0.25 mM EDTA. Subcultures were started from the released and washed cells. For all experiments, the cells were seeded in 35-mm disposable Petri dishes (Falcon) at a density of $1.5 \cdot 10^6$ cells per dish and allowed to grow to confluence. The medium was replaced the next day (2nd day) with fresh medium containing the desired substances for pretreatment of the monolayers. The uptake measurements were performed using confluent monolayers on the 3rd day.

2.3. Cell treatment

Stock solutions of CGS, W-7, calmidazolium, cycloheximide, actinomycin D, colchicine and cytochalasin D were prepared in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in culture medium during treatment was 0.15–0.75%. Control cells were treated with respective concentrations of dimethyl sulfoxide in each experiment. Preincubations were performed using desired concentrations of the compounds for 4 or 16 h. After treatment, the monolayers were washed once with the uptake buffer before initiation of uptake measurement.

2.4. Uptake measurement

This was done at room temperature (21–22°C) as described previously [16]. Uptake of leucine was measured in the absence of Na^+. The composition of uptake buffer for these measurements was 25 mM Hepes-Tris (pH 8.0) or 25 mM Mes-Tris (pH 5.5), 140 mM choline chloride, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose. Uptake buffers of differing pH over the pH range of 5.5–8.0 were prepared by mixing the above-described two buffers, thereby changing the ratios of Hepes, Mes, and Tris in the buffer. Uptake of alanine and taurine was measured in the presence of NaCl. The composition of uptake buffer for these experiments was 25 mM Hepes-Tris (pH 7.5), 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose. All uptake measurements were made with a 1-min incubation to approximate initial uptake rates. Uptake was terminated by aspiration of the medium, followed by quick washing (four times) of the monolayers with respective uptake buffers. There was no significant difference in the uptake whether the washing was done at 4°C (ice-cold buffer) or at room temperature and therefore this step was routinely carried out at room temperature. The cells were then solubilized with 1 ml of 0.2 M NaOH/1% SDS and the radioactivity associated with the cells was counted by liquid scintillation spectrometry.

For each experiment, the samples for protein measurement were prepared as described earlier [17] and the determination of protein in these samples was done according to the method of Lowry et al. [18].

2.5. Statistics

Each experimental point shown represents the mean ± S.E. of three to eight (mostly four) independent

measurements. The kinetic constants were calculated by linear regression of the Eadie-Hofstee plot and confirmed by nonlinear regression methods using the computer software *Fig. P* version 6.0 (Biosoft, Cambridge, UK). The calculated parameters are shown with their S.E. values

3. Results

3.1. Specific nature of the stimulatory effect of CGS on system L activity

CGS is a very specific calmodulin antagonist which selectively blocks calmodulin-dependent processes and has no effect on protein kinase C [19]. This compound, under the name Zaldaride, is currently in clinical trials as an intestinal calmodulin inhibitor in the therapy of secretory diarrhea [19–21]. Our previous studies have shown that treatment of JAR cells with this calmodulin antagonist markedly stimulated the activity of system L [16]. The effect was noticeable within 1 h of treatment and the stimulation persisted and the magnitude of stimulation remained unaltered at least up to 20 h of treatment. Therefore, most experiments in the present study were performed with a treatment period of 16 h. First, the specificity of the action of CGS on system L was investigated. JAR cells were treated with 50 μM CGS for 16 h, following which the activities of system L and two other amino acid transport systems (β and ASC) were measured. System L activity was determined with leucine as the representative substrate by using a Na^+-free uptake medium. Activities of β and ASC systems were determined with taurine and alanine as the representative substrates respectively by using a NaCl-containing uptake medium. As shown in Fig. 1, treatment of cells with CGS stimulated system L activity markedly, confirming our previous findings [16]. In contrast, the same treatment resulted in a marked inhibition of the activity of the β system. Under identical conditions, the activity of system ASC remained unaffected. These results demonstrate that the stimulatory effect of CGS on system L is not without specificity.

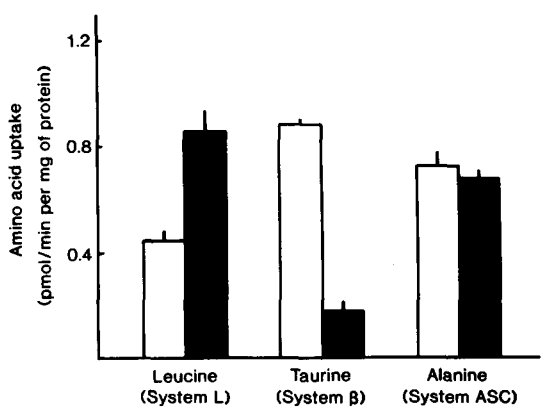

Fig. 1. Effect of CGS on the uptake of leucine, taurine, and alanine in JAR cells. Confluent monolayer cultures of JAR cells were treated with (filled bars) or without (open bars) 50 μM CGS for 16 h. After this treatment, uptake of L-[^{3}H]leucine (10 nM), [^{3}H]taurine (50 nM), and L-[^{3}H]alanine (15 nM) was measured using a 1-min incubation. Uptake of taurine and alanine was measured from an uptake medium (pH 7.5) containing 140 mM NaCl whereas uptake of leucine was measured from an uptake medium (pH 7.5) containing 140 mM choline chloride instead of NaCl. Values are means ± S.E. of four determinations.

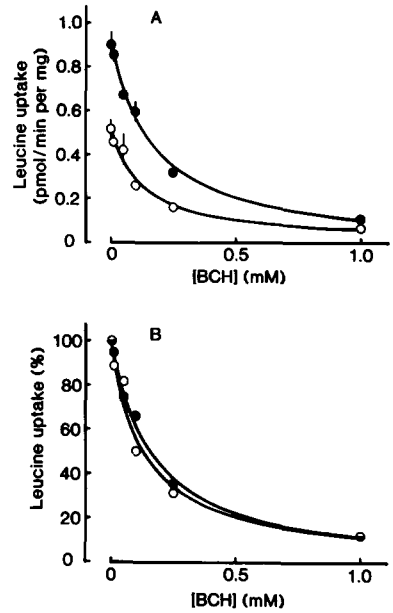

Fig. 2. Dose-response relationship for the inhibition of leucine uptake by BCH in control and in CGS-treated JAR cells. Confluent monolayer cultures of JAR cells were treated for 16 h in the absence (○) or presence (●) of 50 μM CGS. Following the treatment, uptake of L-[^{3}H]leucine (10 nM) was measured using a 1-min incubation in the absence or presence of increasing concentrations (0.01–1 mM) of BCH from a Na^+-free uptake medium (pH 7.5). Results are given either as the actual uptake values (A) or as percent of the respective control uptake values measured in the absence of BCH (B). Values are means ± S.E. of four determinations.

To further confirm that the increase in leucine uptake after CGS treatment was indeed due to stimulation of system L activity, we studied the effects of BCH on leucine uptake in control and in CGS-treated cells (Fig. 2A). BCH is a specific substrate for system L and, as expected, competed with leucine for the uptake process very effectively in control cells. At a concentration of 1 mM, BCH blocked the uptake of leucine by ≈ 90%, suggesting that the uptake of leucine in control cells occurs almost exclusively via system L. Treatment of cells with CGS stimulated leucine uptake. As seen in control cells, leucine uptake in CGS-treated cells was also markedly inhibitable by BCH. The inhibition rates of leucine uptake by increasing concentrations of BCH were in fact indistinguishable in control and in CGS-

Table 1
Inhibition of the control and CGS-stimulated [^{3}H]leucine transport by selective amino acids in JAR cells

Amino acid	Leucine uptake (pmol/min per mg of protein)			
	control	%	CGS	%
None	0.48±0.01	100	0.86±0.03	100
BCH	0.16±0.02	33	0.29±0.03	34
Leucine	0.12±0.01	25	0.23±0.01	27
Tryptophan	0.11±0.01	23	0.15±0.02	17
Phenylalanine	0.10±0.01	21	0.13±0.02	15
Proline	0.48±0.01	100	0.87±0.05	101
AIB	0.48±0.01	100	0.87±0.03	101

After 16 h treatment without (control) or with 50 μM CGS, uptake of radiolabeled leucine (10 nM) from a Na^+-free uptake medium (pH 7.5) was measured in confluent monolayer cultures of JAR cells by using a 1-min incubation. Concentration of unlabeled amino acids was 250 μM. Values are means±S.E. of four determinations.

treated cells (Fig. 2B). This means that stimulation of system L by CGS was (within the errors of the measurement) exclusively responsible for the observed increase in leucine uptake after CGS treatment. This conclusion is further supported by the findings that leucine uptake in control and in CGS-treated cells was markedly inhibitable by the system L-specific substrates BCH, tryptophan and phenylalanine and was unaffected by proline and AIB which are not recognized as substrates by system L (Table 1). When the potencies of these different amino acids to inhibit the uptake of radiolabeled leucine in control and in CGS-treated cells were compared, a high degree of correlation ($r^2 = 0.99$) was evident, supporting the conclusion that CGS treatment increases leucine uptake by stimulating the activity of system L.

3.2. Mechanism of CGS-induced stimulation of system L

We then investigated the possible mechanisms responsible for the stimulation of system L by CGS. Theoretically, there are several possible mechanisms by which CGS treatment can cause the stimulation. It can be due to CGS-induced increase in the carrier density in the plasma membrane either by de novo synthesis of new carrier protein or by recruitment of carrier protein into the plasma membrane from an intracellular pool. Alternatively, posttranslational modification of the carrier protein induced by CGS treatment might modulate the transport activity of system L. To determine whether de novo synthesis of carrier protein is involved in the process, we compared the effects of CGS on system L activity after treating the cells with CGS in the absence and in the presence of actinomycin D, an inhibitor of transcription, or cycloheximide, an inhibitor of translation. The experimental conditions for these treatments were chosen such that actinomycin D blocked > 95% of the incorporation of uridine and leucine into trichloroacetic acid-insoluble cellular components and that cycloheximide blocked > 95% of the incorporation of leucine into trichloroacetic acid-insoluble cellular components [22]. Under these conditions in which transcriptional and translational processes were effectively blocked, the stimulatory effect of CGS on system L remained intact (Table 2), strongly suggesting that de novo synthesis of the carrier protein is not responsible for CGS-induced stimulation of system L. To determine the possible involvement of carrier recruitment in the phenomenon, we employed colchicine and cytochalasin D as blockers of the function of microtubules and/or microfilaments. When treatment of cells with CGS was made in the presence of these blockers of carrier recruitment, the stimulatory effect of CGS on system L remained largely unaffected (Table 2). Surprisingly, colchicine and cytochalasin D themselves had a stimulatory effect on system L in the absence of CGS. Colchicine increased leucine uptake by about 35% and cytochalasin D increased the uptake by about 60%. The apparent decrease in the CGS-induced stimulation in the presence of colchicine and cytochalasin D was actually due to this increase in system L activity caused by these agents themselves. Cycloheximide and actinomycin D themselves also caused about 10% stimulation of leucine uptake. These results demonstrate that neither de novo synthesis nor recruitment is responsible for the stimulatory effect of CGS on system L activity. CGS is a specific and potent calmodulin antagonist and thus has the potential to affect cellular processes involving phosphorylation/dephosphorylation of proteins by influencing the activities of calmodulin-dependent protein kinases and/or phosphoprotein phosphatases. Therefore, it appears that calmodulin-dependent stimulation of system L is likely due to calmodulin-induced changes in the phosphorylation state of either the

Table 2
Effects of cycloheximide, actinomycin D, colchicine and cytochalasin D on the CGS-induced stimulation of leucine uptake in JAR cells

	Leucine uptake (pmol/min per mg protein)		
	−CGS	+CGS	%
Control	0.43±0.02	0.77±0.03	179
Cycloheximide	0.47±0.02	0.73±0.01	155
Actinomycin D	0.48±0.01	0.70±0.02	146
Colchicine	0.58±0.05	0.79±0.04	136
Cytochalasin D	0.68±0.01	0.90±0.04	132

Confluent monolayer cultures of the JAR cells were treated for 16 h with culture medium (control), plus cycloheximide (40 μM) or plus actinomycin D (0.032 μg/ml), or plus colchicine (20 μM) or plus cytochalasin D (20 μM) in the absence (−CGS) or presence of 50 μM CGS (+CGS). After the treatment, uptake of [^{3}H]leucine (10 nM) was measured with a 1-min incubation in a Na^+-free uptake medium (pH 7.5). Uptake in the presence of CGS was calculated as percent of the respective control value measured in the absence of CGS. Values are means±S.E. of four determinations.

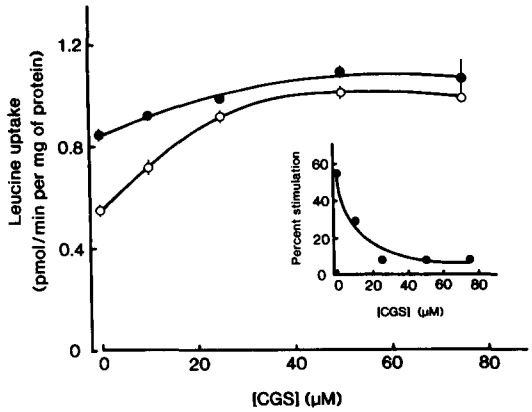

Fig. 3. Dose-response relationship for the attenuation by CGS of the acid pH-induced stimulation of leucine uptake in JAR cells. Confluent monolayer cultures of JAR cells were treated for 16 h in the absence or presence of increasing concentrations of CGS (0–75 μM). Following the treatment, uptake of L-[^{3}H]leucine (10 nM) was measured using a 1-min incubation from a Na^+-free uptake medium of either pH 7.5 (○) or pH 5.5 (●). Values are means ± S.E. of four determinations. (Inset) Results are given as percent stimulation of uptake seen at pH 5.5 compared to pH 7.5 at each concentration of CGS.

carrier protein itself or other protein(s) which might be involved in the modulation of system L activity.

3.3. pH sensitivity of system L in control and in CGS-treated cells

Several studies have demonstrated that the activity of system L is influenced by pH of the extracellular medium [4–7]. The activity is significantly stimulated when the medium is acidified. This phenomenon is observed in several cell types, including JAR cells. Because treatment of JAR cells with the calmodulin antagonist CGS also stimulates the activity of system L, an effect similar to that of an acidic extracellular pH, we investigated whether there is any change in the pH sensitivity of system L as a result of CGS treatment. Fig. 3 describes the effects of increasing concentrations of CGS on the H^+-induced activation of system L. In this experiment, system L activity was measured at two different extracellular pH, 7.5 and 5.5, after treating the cells with different concentrations of CGS. In non-treated cells, the activity of system L was 55% greater at pH 5.5 than at pH 7.5, confirming the H^+ effect. CGS treatment increased the activity at both pH values, but the effect was much greater at pH 7.5 than at 5.5. In addition, the H^+-dependent stimulation was found to be progressively decreased as the cells were treated with increasing concentrations of CGS (Fig. 3, inset).

We then investigated the effect of H^+ on the stimulatory effect of CGS. In this experiment, the activity of system L was measured at different extracellular pH values (range, 5.5–8.0) in control and in CGS-treated cells. The results given in Fig. 4 show that an increase in the extracellular H^+ concentration enhanced the activity of system L in control cells, confirming previous findings. The activity measured at pH 5.5 was about 65% greater than the activity at pH 8.0. This pH effect was significantly altered after CGS treatment. The activity at pH 8.0 was about 80% greater in CGS-treated cells than in non-treated cells. Interestingly, the stimulatory effect of acidic extracellular pH is decreased to an appreciable extent by CGS treatment. In CGS-treated cells, stimulation of system L at pH 5.5 versus pH 8.0 was still noticeable but to a much less extent than in control cells (16% in treated cells versus 67% in control cells). The stimulatory effect of CGS was found to be progressively decreased as the H^+ concentration at which the activity of system L was measured increased (Fig. 4, inset). The CGS-induced enhancement of the activity was 80% at pH 8.0, but this effect was reduced to about 25% at pH 5.5.

In order to compare the effects of CGS and H^+ on the kinetic parameters of system L, cells were treated with or without CGS and the activity of system L was determined at pH 5.5 and at pH 7.5 with varying concentrations of leucine (range, 10–250 μM). Uptake measurements were made in the presence of a fixed concentration (10 nM) of radiolabeled leucine. The H^+ effect was analyzed by comparing the activities of system L measured at pH 7.5 and pH 5.5 in cells treated in the absence of CGS. The CGS effect was analyzed by comparing the activities measured at pH 7.5 in cells

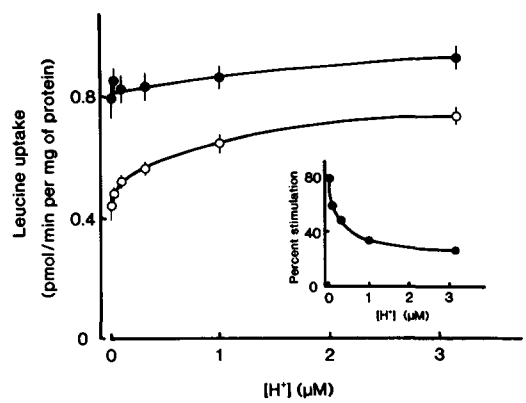

Fig. 4. Influence of H^+ concentration in the uptake medium on leucine uptake in control and CGS-treated JAR cells. Confluent monolayer cultures of JAR cells were treated for 16 h in the absence (○) or presence (●) of 50 μM CGS. Following the treatment, uptake of L-[^{3}H]leucine (10 nM) was measured using a 1-min incubation from a Na^+-free uptake medium. pH of the uptake medium was varied over a range of 8.0–5.5, corresponding to a H^+ concentration range of 0.01–3.16 μM. Values are means ± S.E. of three to eight determinations. (Inset) Results are given as percent stimulation of uptake seen in CGS-treated cells compared to control cells at each H^+ concentration.

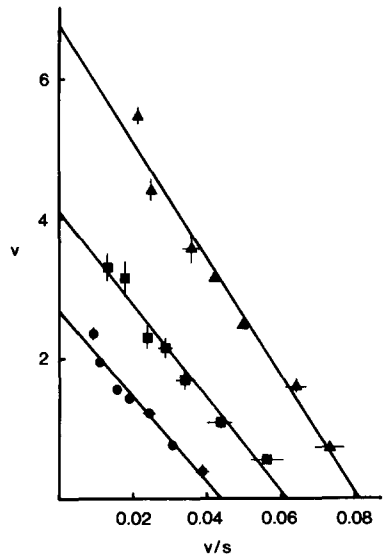

Fig. 5. Influence of CGS treatment and an acidic pH on the kinetics of leucine uptake in JAR cells. Confluent monolayer cultures of JAR cells were treated for 16 h in the absence (●, ■) or presence (▲) of 50 μM CGS. Following the treatment, uptake of leucine was measured over a concentration range of 10–250 μM using a 1-min incubation from a Na^+-free uptake medium of either pH 7.5 (●, ▲) or pH 5.5 (■). Concentration of L-[^{3}H]leucine was maintained at 10 nM and total concentration of leucine was altered by adding appropriate concentrations of unlabeled L-leucine. Uptake of radiolabel measured in the presence of 5 mM unlabeled leucine was used to determine the non-mediated component. This value was subtracted from total uptake to calculate mediated uptake which was used in the kinetic analysis. Results are given as Eadie-Hofstee plots (v/s versus v). v, uptake in nmol/min per mg of protein; s, leucine concentration in μM. Values are means ± S.E. of four determinations.

which were treated in the absence and in the presence of CGS. Kinetic parameters were determined by using values for mediated uptake. The non-mediated component of leucine uptake was determined from the uptake of radiolabel in the presence of an excess amount (5 mM) of unlabeled leucine and was subtracted from the total uptake to determine mediated uptake. Contribution to the total uptake by this non-mediated component was ≈ 5% at 10 μM leucine and ≈ 15% at 250 μM leucine. The non-mediated component was not affected by H^+ and by CGS treatment (results not shown). The mediated uptake rates were hyperbolically related to leucine concentration and the relationship obeyed Michaelis-Menten kinetics describing a single mediated process in control and in CGS-treated cells. This was the case whether the activity was measured at pH 7.5 or at 5.5. The data are given as Eadie-Hofstee plots in Fig. 5. The plots were linear in all cases ($r^2 > 0.96$). The values for the kinetic parameters, K_t and V_{max}, determined from these plots are given in Table 3. Both CGS and H^+ affected the activity of system L primarily by increasing the V_{max}. Both had only a small effect on the K_t.

Table 3
Influence of CGS and uptake medium pH on the kinetic parameters of leucine uptake in JAR cells

Kinetic parameter	Experimental condition		
	−CGS pH 7.5	+CGS pH 7.5	−CGS pH 5.5
K_t (μM)	61.3 ± 5.7	83.3 ± 6.3	66.2 ± 5.5
V_{max} (nmol/min per mg)	2.7 ± 0.1	6.8 ± 0.3	4.1. ± 0.2

The kinetic parameters were calculated from the experimental data given in Fig. 5. Values are means ± S.E.

Most experiments described thus far in this paper relating to the stimulatory effects of CGS and H^+ were made at a leucine concentration of 10 nM, a value at least three orders of magnitude less than the K_t value. To determine whether CGS and H^+ are capable of stimulating system L at leucine concentrations close to the K_t value, we studied the influence of CGS and H^+ on leucine uptake at a concentration of 60 μM (see Table 3). Fig. 6 shows that system L was activated by CGS and H^+ even at this leucine concentration. Uptake of leucine was about 50% greater at pH 5.5 than at pH 7.5, redocumenting the H^+ effect. Similarly, leucine uptake was stimulated by CGS treatment, both at pH 7.5 and at pH 5.5, confirming the CGS effect. Again, the apparent competitive nature of the H^+ and CGS effects was evident. H^+ considerably reduced the stimulatory effect of CGS, the stimulation being 120% at pH 7.5 and 55% at pH 5.5. In a similar manner, CGS reduced the stimulatory effect of H^+. When uptake values at pH 7.5 and at pH 5.5 were compared, the H^+-dependent stimulation was 50% in control cells and 8% in CGS-treated cells.

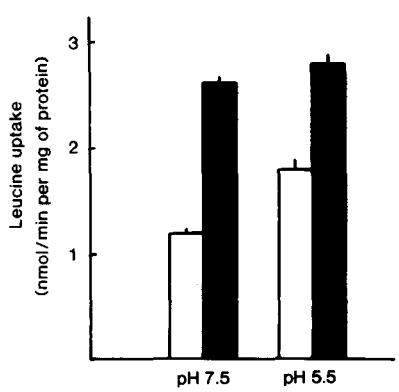

Fig. 6. Influence of CGS on the acid pH-induced stimulation of leucine uptake in JAR cells measured at a leucine concentration approximately equal to the K_t value. Confluent monolayer cultures of JAR cells were treated for 16 h in the absence (open bars) or presence (filled bars) of 50 μM CGS. Following the treatment, uptake of leucine was measured at a concentration of 60 μM using a 1-min incubation from a Na^+-free uptake medium of either pH 7.5 or pH 5.5. Values are means ± S.E. of four determinations.

Table 4
Effects of calmodulin antagonists on the pH influence of leucine uptake in JAR cells

Calmodulin antagonist	Leucine uptake (pmol/min per mg of protein)		
	pH 8.0	pH 5.5	%
Control	0.48±0.01	0.71±0.03	148
CGS	0.78±0.02	0.88±0.03	113
W-7	0.66±0.02	0.80±0.04	121
Calmidazolium	0.56±0.01	0.74±0.02	132

Confluent monolayer cultures of JAR cells were treated for 4 h with culture medium in the absence (control) or presence of 75 μM CGS, 100 μM W-7 or 15 μM calmidazolium. After the treatment, uptake of [^{3}H]leucine (10 nM) was measured with a 1-min incubation in a Na^+-free uptake medium of either pH 8.0 or pH 5.5. Uptake at pH 5.5 was calculated as percent of the respective uptake value measured at pH 8.0. Values are means ± S.E. of four determinations.

3.4. Effects of other calmodulin antagonists on the pH sensitivity of system L

The effects of two additional calmodulin antagonists, W-7 and calmidazolium, on system L activity were studied and compared with the effects of CGS (Table 4). As seen with CGS, the activity of system L was found to be stimulated to a significant extent by W-7 (37%) and by calmidazolium (17%). Similarly, these antagonists were also able to decrease the pH sensitivity of system L as did CGS. These studies showing that not only CGS but also other calmodulin antagonists possess the ability to stimulate system L and to decrease its H^+-induced activation strongly suggest that calmodulin-dependent cellular processes are involved in the modulation of system L activity and its pH sensitivity.

4. Discussion

There are several studies reporting on the regulation of the amino acid transport system L. The activity of system L in MDCK cells is stimulated to a significant extent by insulin, but inhibited by glucocorticoids [23]. Activation of protein kinase C by phorbol esters enhances system L activity in Chang liver cells [24]. Interestingly, phorbol esters do not affect system L in fibroblasts [25], suggesting that the regulation of system L may be tissue-specific. Previous studies from our laboratory have shown that system L in the JAR placental choriocarcinoma cell line is stimulated by phorbol esters, unaffected by cholera toxin and forskolin which elevate cellular levels of cAMP, and stimulated by agents which block calmodulin-dependent processes [16]. The results of the present investigation considerably enhance our understanding of the regulation of system L activity by calmodulin antagonists.

The data presented in this paper demonstrate that the stimulation of the activity of system L by calmodulin antagonists does not involve de novo synthesis nor recruitment of new carrier proteins. The stimulation is noticeable within 1 h and the effect is not blocked by inhibitors of transcriptional and translational processes. The experimental conditions employed here with cycloheximide and actinomycin D are appropriate to block de novo synthesis of proteins in JAR cells [22]. These studies lead to the conclusion that de novo synthesis of system L protein is not responsible for CGS-induced stimulation of system L activity. In Balb/3T3 mouse cells, treatment with cycloheximide to block cellular protein synthesis results in a marked stimulation of system L activity [26] and this stimulation has been ascribed to the *trans*-stimulatory effect of intracellular amino acids which accumulate inside the cells due to blockade of protein synthesis. Such a phenomenon does not occur in JAR cells, because system L in these cells does not possess the *trans*-stimulation characteristic. Experimental conditions which are expected to deplete intracellular amino acids do not have any noticeable effect on system L activity, measured as the uptake of radiolabeled leucine, in control as well as CGS-treated cells [16]. In addition, preloading of the cells with the system L-specific amino acids such as tyrosine, tryptophan, phenylalanine and leucine does not enhance the uptake of radiolabeled leucine (data not shown). The inability of system L to be stimulated by substrates on the *trans*-side is not unprecedented. Segel et al. [27] has reported that system L in chronic leukemic lymphocytes does not possess the property of *trans*-stimulation. In addition, the stimulation of leucine uptake in JAR cells by treatment with cycloheximide per se is small unlike the robust stimulation observed in Ehrlich tumor cells. Cycloheximide is known to activate protein kinase C [28,29]. Since the activity of system L in the JAR cells is enhanced by activators of protein kinase C [16], it is possible that the small stimulatory effect of cycloheximide seen in JAR cells is actually due to activation of protein kinase C. The CGS-induced stimulation of system L is also not the result of recruitment of new carrier proteins into the plasma membrane from an intracellular pool because suppression of microtubular/microfilament function by colchicine and cytochalasin D does not prevent the CGS effect. Interestingly, treatment of the cells with these agents itself leads to a significant stimulation of system L activity, suggesting that these compounds may themselves have calmodulin antagonistic activity.

Lack of involvement of de novo synthesis and recruitment of new carrier proteins in mediating the CGS effect suggests that the phenomenon might involve posttranslational modification of proteins. It is well known that calmodulin participates in protein phosphorylation via specific protein kinases and also in

protein dephosphorylation via specific phosphoprotein phosphatases. Therefore, we postulate that the stimulation of system L activity by CGS involves alteration in the phosphorylation state of either the carrier protein itself or other protein(s) which might modulate the function of the carrier. This hypothesis however remains to be tested.

One of the unique characteristics of system L is its pH sensitivity. H^+ stimulates the activity of system L. Treatment of cells with calmodulin antagonists causes a significant change in this characteristic. The stimulatory effect of H^+ is reduced by treatment with calmodulin antagonists. Similarly, H^+ decreases the stimulatory effect of calmodulin antagonists. In addition, H^+ and calmodulin antagonists affect the kinetic parameters of system L in a similar manner, the stimulation in both cases being primarily due to an increase in the maximal velocity. Even though the exact mechanism by which H^+ enhances system L activity in JAR cells is not known, the present study demonstrating the competitive nature between the stimulatory effects of H^+ and calmodulin antagonists indicates that these two processes are related.

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