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Extracellular creatine regulates creatine transport in rat and human muscle cells

(transport regulation/L6 cells)

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ABSTRACT **Muscle cells do not synthesize creatine; they take up exogenous creatine by specific Na^+-dependent plasma membrane transporters. We found that extracellular creatine regulates the level of expression of these creatine transporters in L6 rat muscle cells. L6 myoblasts maintained for 24 hr in medium containing 1 mM creatine exhibited 1/3rd of the creatine transport activity of cells maintained for 24 hr in medium without creatine. Down-regulation of creatine transport was partially reversed when creatine-fed L6 cells were incubated for 24 hr in medium lacking creatine. Down-regulation of creatine transport occurred independently of amino acid and glucose transport. Furthermore, the down-regulation of creatine transporters by extracellular creatine was slowed by inhibitors of protein synthesis. These results suggest that creatine induces the expression of a protein that functionally inactivates the creatine transporters. Regulation of creatine transport by extracellular creatine also was observed in L6 myotubes and in cultures of human myoblasts and myotubes. Hence, the activity of creatine transport represents another site for the regulation of creatine homeostasis.**

Transport of nutrients into mammalian cells is mediated by specific membrane-bound carrier systems (1–7), several of which are regulated by the concentration of the corresponding nutrient in the external medium. Cells incubated in medium depleted of amino acids, glucose, or K^+ increase their capacity to transport the missing solute (1–7). Moreover, solute transport can regulate various cellular functions. For example, amino acid-dependent gluconeogenesis is regulated by extracellular alanine (3).

Phosphocreatine (*P*-Cr) is an important source of chemical energy in heart, brain, skeletal muscle (8), and macrophages (9), but neither muscle cells (8) nor macrophages (9, 10) synthesize creatine. These cells take up creatine from plasma by means of a specific uphill transport process that requires extracellular Na^+ (11, 12). There is little information regarding the mechanism(s) by which cells regulate their intracellular creatine stores. The only previously identified site for regulation occurs at the level of creatine biosynthesis in the liver, pancreas, and kidney where dietary creatine serves as an end-product repressor for the first step in *de novo* creatine biosynthesis (8); i.e., the formation of guanidinoacetate from arginine and glycine.

We show here that the concentration of creatine in the external medium specifically affected the rate of creatine transport in the L6 rat muscle cell line by a protein synthesis-dependent process. Regulation of creatine uptake also was observed in L6 cells that had differentiated into myotubes as well as in human myoblasts and myotubes.

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METHODS

Cell Lines. L6 myoblasts (13) were grown in 75-cm^2 flasks at 37°C in Eagle's minimal essential medium (MEM) with 15% fetal bovine serum under an atmosphere of 5% CO_2/95% air. Cells grown in flasks were trypsinized and plated at 5×10^4 cells per 12-mm coverslip or 15×10^4 cells per 35-mm Petri dish for 20 hr before use. In experiments using myotubes, L6 cells were plated at 5×10^4 cells per 12-mm coverslip in MEM with 15% fetal bovine serum for 24 hr before incubating the cells in MEM with 5% horse serum. After 4–8 days, the cells fused (>3 nuclei per cell) into myotubes; the degree of fusion (>70%) was determined on monolayers fixed and stained with Giemsa stain. Human muscle cells were cloned and prepared as described (14).

Creatine Uptake Studies. Three or four coverslip cultures per time point were incubated in MEM or phosphate-buffered saline (PBS) containing 5 mM glucose, 2% dialyzed fetal bovine serum, and supplemented with 0.5 μCi of [1-^{14}C]creatine per ml (1 Ci = 37 GBq) (final creatine concentration, 45 μM). At each time point, coverslips were washed (successively in six beakers each containing 100 ml of ice-cold PBS), air-dried, and counted in an LKB liquid scintillation counter. Creatine uptake, expressed as pmol of creatine per μg of cell protein, was calculated from the known specific activity of creatine in the medium and cell-associated [^{14}C]creatine. Na^+-independent uptake of creatine was measured in cells maintained in Na^+-free medium in which choline was substituted for Na^+ as described (11). K_m and V_{max} values of creatine uptake were calculated from the data in Fig. 3 by the method of Lineweaver and Burk.

Miscellaneous Methods and Materials. Cell viability (>95%) was determined by trypan blue exclusion (15). Protein determinations of cells were carried out as described (16). ATP and *P*-Cr were measured in L6 cells maintained in 35-mm Petri dishes as described (9). All media and sera were obtained from GIBCO. Dialyzed fetal bovine serum was prepared by dialysis vs. 0.15 M NaCl as described (10). All materials and chemicals were obtained from commercial sources. [1-^{14}C]creatine was from either Amersham (23–29 μCi/mmol) or ICN (11 μCi/mmol). α-[*methyl*-^{3}H]Aminoisobutyric acid (AiB) (10–25 Ci/mmol) was from New England Nuclear. Purity of the radiolabeled creatine was >95%, determined by thin-layer chromatography as described (9). The data given represent the average of triplicate samples whose values fall within 10% of one another.

RESULTS

Creatine Uptake into L6 Myoblasts Is Affected by the Extracellular Creatine Concentration. [1-^{14}C]Creatine uptake in L6 myoblasts was linear for at least 2 hr (Fig. 1). Less than 5% of the creatine taken up was phosphorylated to *P*-Cr

Abbreviations: *P*-Cr, phosphocreatine; AiB, aminoisobutyric acid.

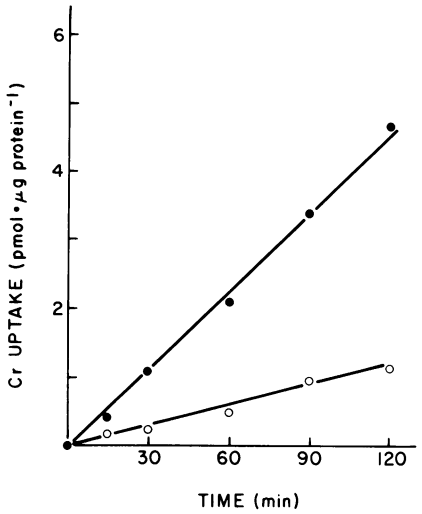

FIG. 1. [^{14}C]Creatine uptake in creatine-starved and creatine-fed L6 cells. L6 cells were maintained on coverslips in the absence (●) or presence (○) of 5 mM creatine in MEM supplemented with 15% dialyzed fetal bovine serum. After 24 hr, cells were washed and incubated for various times in 0.5 ml of PBS supplemented with 2% dialyzed fetal bovine serum, 5.5 mM glucose, and 45 μM [^{14}C]creatine (0.5 μCi/ml). The uptake of [^{14}C]creatine was measured as described.

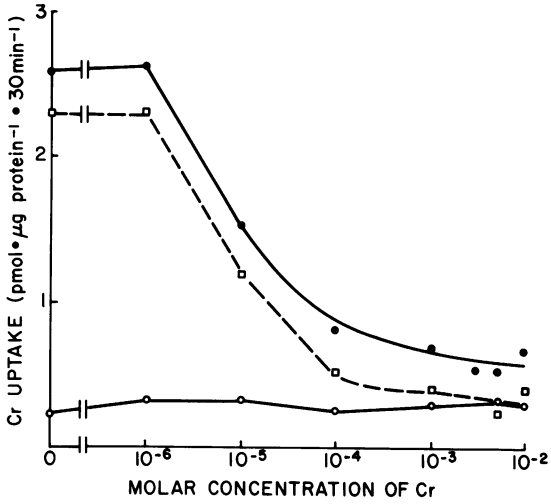

FIG. 2. Creatine concentration required to down-regulate [^{14}C]creatine uptake. L6 cells were maintained in medium containing the indicated concentrations of creatine for 24 hr. At that time, the cells were washed and [^{14}C]creatine uptake was measured as described in Fig. 1. ●, Total [^{14}C]creatine taken up by L6 cells; ○, [^{14}C]Creatine uptake measured in the absence of Na^+. □, Na^+-dependent component of transport calculated by subtracting the difference between total and Na^+-independent components of creatine uptake (11).

(Table 1). Except for nonenzymatic conversion of creatine to creatinine (which accounts for <2% of creation) and *P*-Cr, there are no other known metabolic products of creatine. Thus, all intracellular [^{14}C]creatine measured in these experiments reflects creatine uptake without significant metabolism of creatine to other products.

To examine the effect of extracellular creatine on creatine transport, L6 myoblasts were maintained in either MEM containing 15% dialyzed fetal bovine serum (creatine-starved) or in MEM containing 15% dialyzed fetal bovine serum and 5 mM creatine (creatine-fed). After 24 hr, both the creatine-starved and creatine-fed cells were washed with PBS and creatine transport activity was measured. Creatine transport in creatine-fed cells was linear, but at a rate that was 30% of that observed in creatine-starved cells (Fig. 1). When creatine-fed cells were transferred to medium lacking creatine for 24 hr, the rate of creatine transport increased 2- to 3-fold above that observed in cells maintained continuously in the presence of 5 mM creatine (data not shown). These results indicate that cells maintained in medium containing a high creatine concentration exhibit down-regulation of creatine transport activity and that this effect is partially reversed when the cells are maintained for an additional 24 hr in medium lacking creatine.

The Na^+-Dependent Component of Creatine Transport Is Affected by Extracellular Creatine Concentration. Creatine transport in L6 cells can be divided into two components: a saturable, high-capacity, high-velocity process that is Na^+ dependent, and a low-capacity, low-velocity process that is Na^+ independent. To determine the concentration of extracellular creatine that elicits down-regulation of creatine transport, L6 cells maintained for 24 hr in medium containing various concentrations of creatine were assayed for total and Na^+-independent creatine uptake (Fig. 2). Na^+-dependent creatine uptake was calculated as the difference between these two rates. Na^+-dependent creatine transport was down-regulated by >1 μM extracellular creatine with 50% inhibition occurring in the range of physiological plasma creatine—i.e., between 20 and 30 μM extracellular creatine (Fig. 2). The Na^+-independent component of creatine uptake did not change significantly in response to extracellular creatine concentration.

Two control experiments confirmed that creatine-fed cells do not release sufficient unlabeled creatine to decrease the specific activity of extracellular labeled creatine used in uptake measurements. First, creatine-fed L6 myoblasts were washed and incubated for 30 min in 0.5, 1, or 1.5 ml of fresh PBS containing the same final [^{14}C]creatine concentration. The rates of creatine uptake were then measured and found to be identical (1.1 pmol per μg per 30 min) in all samples. Second, L6 cells showed the same percentage down-regulation of their creatine transport capacity when creatine uptake was measured with 17 μM [^{14}C]creatine or 34 μM [^{14}C]creatine.

Regulation of creatine uptake was not restricted to L6 myoblasts. L6 cells that had differentiated to myotubes,

Table 1. ATP and *P*-Cr content and the rate of creatine uptake in different cell types

	Extracellular creatine	ATP, nmol per mg of protein	*P*-Cr, nmol per mg of protein	Creatine uptake, pmol per μg of protein per 30 min
L6 myoblasts	<10 μM	21	0.1	1.3
	5 mM	24	0.4	0.4
L6 myotubes	<10 μM	ND	ND	3.2
	5 mM	ND	ND	1.6
Human myoblasts	<10 μM	22	41	0.8
	5 mM	ND	ND	0.3

L6 and human myoblasts and myotubes were prepared as described and maintained in 35-mm Petri dishes in MEM containing 15% dialyzed fetal bovine serum for 24 hr in the presence of <10 μM creatine or 5 mM creatine. ND, not done.

cloned human skeletal myoblasts, and myotubes maintained in 5 mM creatine for 24 hr demonstrated a >50% reduction in [^{14}C]creatine uptake compared to cells maintained in very low concentrations of creatine (Table 1).

Extracellular Creatine Concentration Did Not Alter Creatine Efflux. Differences in creatine accumulation between creatine-fed and creatine-starved cells may have reflected differences in creatine efflux. To examine this possibility, creatine-starved cells were incubated for 24 hr in medium containing [^{14}C]creatine and unlabeled creatine at final total creatine concentrations of 15, 45, and 2150 μM. After 24 hr, the radiolabeled medium was removed, and the cells were washed and further incubated in medium lacking creatine. Thirty and 60 min thereafter, the amounts of radiolabeled creatine in both the cells and the medium were measured. The rates of creatine release from the cells were equal under all three conditions of creatine loading; ≈3.3% of the total intracellular creatine effluxed per hr (Table 2). Furthermore, cells preincubated in medium containing 1.0 μCi of [^{14}C]creatine and unlabeled creatine at a final concentration of 2150 μM were chased in medium containing the same concentration of unlabeled creatine (2150 μM) or in medium containing no extracellular creatine. Once again, ≈4% of intracellular creatine was released from the cells per hr (data not shown).

Three conclusions can be drawn from these experiments. First, extracellular creatine had no appreciable effect on creatine efflux. Second, creatine efflux can occur against a concentration gradient. Third, release of unlabeled intracellular creatine from creatine-fed cells does not significantly alter the amount of creatine taken up. We calculate that the amount of creatine released in 30 min from L6 cells incubated for 24 hr in medium containing 2150 μM creatine is <1.8 nmol (compared with 45 nmol of [^{14}C]creatine used in creatine uptake experiments).

Kinetics of Creatine Transport in Creatine-Fed and Creatine-Starved L6 Cells. Creatine-fed and creatine-starved cells exhibited similar K_m values (60 and 40 μM, respectively) for the Na^+-dependent component of creatine uptake (Fig. 3). However, V_{max} values in creatine-starved cells were ≈3 times greater than the V_{max} values of creatine-fed cells (Fig. 3, legend). Control experiments demonstrated that Na^+-independent uptake of creatine was similar in both creatine-fed and creatine-starved cells, further confirming that only the Na^+-dependent component of transport responds to extracellular creatine (Fig. 3). These results indicate that preincubation of cells in high creatine concentration had no effect on the affinity of creatine for its carrier protein; they suggest that creatine regulates either the number or turnover of creatine transporters.

Table 2. Creatine efflux in L6 cells preincubated with various concentrations of extracellular creatine

Extracellular creatine, μM	Intracellular creatine, pmol per μg of protein	Creatine efflux	
		pmol per μg of protein per hr	% released per hr
9.0	4.3	0.13	3.1
45	12	0.38	3.2
150	22	0.79	3.6
2150	215	6.0	2.8

L6 cells were prepared on coverslips as described. Cells were incubated for 24 hr in 0.5 ml of MEM containing 5% dialyzed fetal bovine serum, 0.5 μCi or 1.0 μCi of [^{14}C]creatine per ml, and sufficient unlabeled creatine to bring the final creatine concentration in the medium to the level indicated. After 24 hr, the cells were washed and maintained at 37°C for 3 hr in PBS supplemented with 2% dialyzed fetal bovine serum. At 30- to 60-min intervals, the medium was removed and the coverslips were washed in ice-cold PBS, and the total radioactivity in the cells and in the chase medium (for each coverslip) was determined. Efflux was calculated by subtracting the amount of radioactivity in the cells at 3 hr from values measured at 0 hr. Efflux is also expressed as percentage intracellular creatine released per hr. In parallel experiments, L6 cells preincubated in 2150 μM creatine for 24 hr down-regulated creatine uptake by >70% compared to cells preincubated in the absence of extracellular creatine.

Creatine Must Enter Cells to Exert Its Activity. L6 myoblasts were incubated for 8 hr in Na^+-free medium (choline buffer) in the presence or absence of 5 mM creatine. The cells were then incubated for 30 min in PBS containing 5 mM glucose and 17 μM [^{14}C]creatine. The rates of creatine transport were equal in cells preincubated in choline buffer (0.25 pmol per μg of protein per 30 min) and choline buffer supplemented with 5 mM creatine (0.24 pmol per μg per 30 min). In contrast, myoblasts incubated for 8 hr in PBS supplemented with 5 mM creatine exhibited a 60% reduction of creatine transport activity (0.18 pmol per μg per 30 min) as compared to myoblasts maintained in the absence of creatine (0.41 pmol per μg per 30 min). Control experiments demonstrated that myoblasts maintained in choline buffer containing 5 mM creatine for 8 hr took up 12 pmol per μg of creatine compared to myoblasts maintained in PBS containing 5 mM creatine, which took up 73 pmol per μg of creatine. These data suggest that creatine must enter the cell to exert its regulatory activity on creatine transport.

Extracellular Creatine Has No Effect on the Uptake of Other Nutrients. To determine whether Na^+-dependent transport systems in L6 cells respond to changes in extracellular creatine concentration, we measured Na^+-dependent transport activity occurring through the "A" amino acid transport system using [^{14}C]AiB as a probe. No differences were observed in AiB uptake in creatine-starved or creatine-fed L6 cells (Fig. 4*A*). As expected, AiB uptake increased ≈7-fold in L6 cells maintained for 24 hr in the absence of nonessential amino acids (PBS supplemented with 5.5 mM glucose), compared with cells maintained in amino acid replete medium (MEM) (Fig. 4*A*). The absence of

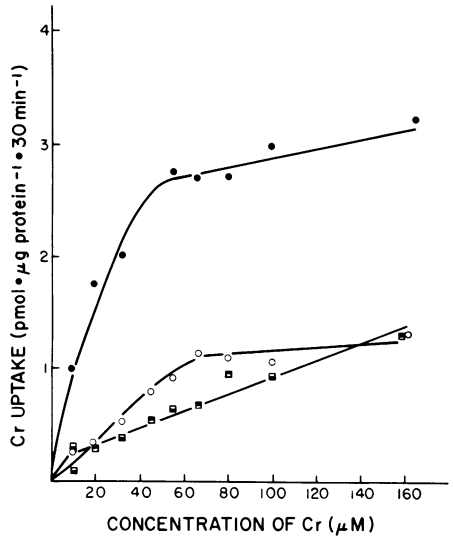

FIG. 3. Kinetic analyses of creatine uptake. L6 cells were maintained on coverslips in the presence (○) or absence (●) of 5 mM creatine as described. After 24 hr, the cells were washed several times with PBS (or Na^+-free medium) and incubated in the corresponding medium supplemented with 2% dialyzed fetal bovine serum and a mixture of [^{14}C]creatine and nonradiolabeled creatine to yield the desired creatine concentration. Na^+-dependent uptake was calculated as described in Fig. 2. The average K_m values of three separate experiments for creatine-fed (○) and creatine-starved (●) L6 cells were 60 and 40 μM, respectively. The average V_{max} values for creatine-fed and creatine-starved L6 cells were 3.5 and 9.1 pmol per μg per 30 min, respectively. ■ and □, Na^+-independent uptake for both creatine-fed and creatine-starved L6 cells, respectively.

amino acids in the medium did not affect the uptake of creatine in L6 cells (Fig. 4*B*). These results are consistent with those reported by Christensen *et al.* (17), which show that creatine does not inhibit amino acid uptake into rat diaphragm. The effect of creatine starvation on glucose uptake was measured by using 3-*O*-[^{14}C]methylglucose. Glucose uptake was 0.4 pmol per μg of protein per 30 min in both creatine-starved and creatine-fed cells (data not shown). These experiments indicate that amino acid, glucose, and creatine transport are regulated independently of one another in these cells.

***P*-Cr Does Not Regulate Creatine Transport.** L6 myoblasts maintained for 24 hr in medium containing 45 μM creatine accumulate 0.1 nmol of *P*-Cr and 5 nmol of creatine per mg of protein. L6 myoblasts maintained in medium containing 2 mM extracellular [^{14}C]creatine accumulate $\approx$1.0 nmol of *P*-Cr and >16 nmol of creatine per mg of protein. Thus, a very small percentage of creatine is phosphorylated to *P*-Cr even though creatine-starved and creatine-replete L6 myoblasts contain similar amounts of ATP (24 and 20 nmol per mg of protein, respectively). In contrast to L6 myoblasts, *P*-Cr is the major creatine metabolite in normal human skeletal muscle and cultured human muscle cells (ref. 8; Table 1). Both L6 cells and human muscle cells exhibit creatine-mediated regulation of creatine transport. These findings suggest that intracellular stores of *P*-Cr do not play a significant role in regulating creatine transport in creatine-fed cells.

To confirm that creatine and not *P*-Cr regulates creatine transport, we examined creatine transport in two situations in which *P*-Cr stores were depleted. First, L6 cells were incubated in medium containing 50 mM 2-deoxyglucose for 6 hr. This treatment depleted *P*-Cr stores by >75% but had no effect on the rate of creatine uptake (9, 11) or on the capacity of creatine to down-regulate creatine transporters in L6 cells. The rate of creatine transport in L6 cells incubated for 6 hr in medium containing 50 mM 2-deoxyglucose and 5 mM creatine was 50% of that observed in L6 cells maintained for 6 hr in the same medium without creatine. Second, L6 cells maintained in the absence of glucose for 6 hr contained <25% of *P*-Cr compared to cells maintained in glucose-replete medium. Under glucose-free conditions, 5 mM extracellular creatine down-regulated creatine transport >50% in 6 hr. Thus, the intracellular concentration of *P*-Cr did not correlate with the ability of extracellular creatine to down-regulate creatine transport.

We also tested the ability of several analogues of creatine to down-regulate creatine transport. D- or L-ornithine, creatinine, glycine, and *P*-Cr (all at 5 mM) had no effect on this process. Both 5 mM guanidinoacetate and 5 mM guanidinopropionate, which compete for transport with creatine in muscle (8) and macrophages (11), down-regulated creatine transport activity by >50% in 24 hr (data not shown). Since both guanidinoacetate and guanidinopropionate are poor substrates for phosphorylation by creatine kinase (18), these results are consistent with the idea that the phosphorylation of creatine or its analogues is not required for down-regulation of creatine transport.

Time Course of Creatine Transport Regulation by Extracellular Creatine. L6 cells placed in medium containing 5 mM creatine down-regulated creatine transport by 50% within 3–6 hr (Fig. 5). By 24 hr, creatine transport was inhibited maximally (70–80%). In contrast, up-regulation of creatine transport in the absence of extracellular creatine occurred slowly; the rate of creatine transport was not increased when creatine-fed cells were incubated for 8 hr in medium lacking extracellular creatine. After 24 hr in creatine-free medium (reversing conditions), the rate of creatine transport increased 3-fold from that observed in L6 cells maintained continuously in the presence of 5 mM creatine (data not shown).

Down-Regulation of Creatine Transporters Is Slowed in the Absence of Protein Synthesis. Creatine-starved L6 myoblasts incubated for 6 hr with 10 μg of cycloheximide per ml exhibited no change in the rate of creatine transport compared to creatine-starved L6 cells maintained in the absence

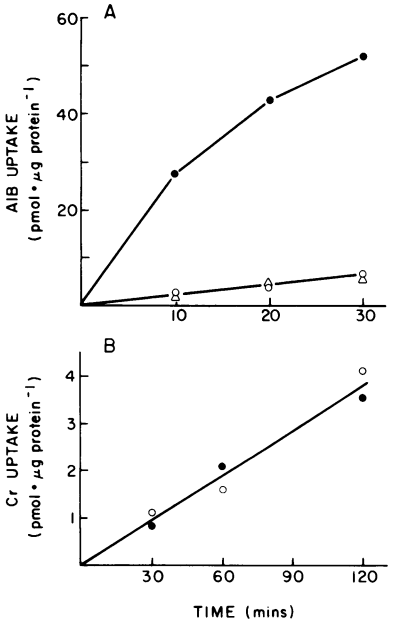

FIG. 4. (*A*) [^{14}C]AiB uptake in L6 cells. L6 cells were maintained in MEM supplemented with 15% dialyzed fetal bovine serum in the absence (○) or presence (△) of 5 mM creatine, or in PBS supplemented with 15% dialyzed fetal bovine serum (●). After 24 hr, the cells were washed in PBS and [^{14}C]AiB uptake was measured as described. (*B*) [^{14}C]creatine uptake in L6 cells maintained in the absence of amino acids. L6 cells were maintained in either PBS (containing 5.5 mM glucose) (●) or MEM supplemented with 15% dialyzed fetal bovine serum (○). After 24 hr, [^{14}C]creatine uptake was measured as described.

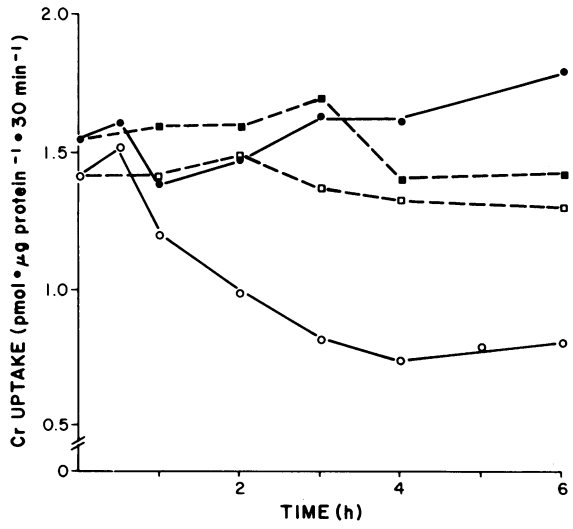

FIG. 5. Time course of down-regulation of creatine transport. L6 cells were maintained for 24 hr in MEM supplemented with 15% dialyzed fetal bovine serum. At that time, all cultures were transferred to PBS supplemented with 5.5 mM glucose and 15% dialyzed fetal bovine serum. Some cultures received 5 mM creatine (○), 10 μg of cycloheximide per ml (■), 5 mM creatine and 10 μg of cycloheximide per ml (□), or no additions (●). At the indicated time intervals, all cells were extensively washed and [^{14}C]creatine uptake was measured as described. Cycloheximide (10 μg/ml) used in these experiments did not affect cell viability and inhibited [^{35}S]methionine incorporation into perchloric acid-insoluble materials by >95% after a 1-hr incubation (data not shown).

of drug (Fig. 5). In contrast, cycloheximide prevented the creatine-induced down-regulation of creatine transport.

Incubation of creatine-starved cells for 24 hr in 1.0 μg of cycloheximide per ml caused only a 30% reduction (average of five experiments) in creatine uptake activity compared to cells maintained in the absence of drug. Creatine transport activities for cells maintained in the absence or presence of cycloheximide were 0.9 and 0.63 pmol per μg of protein per 30 min, respectively. Cells maintained for 24 hr in 5 mM creatine exhibited a 75% reduction in the rate of creatine transport, and this reduction in creatine transport was partially blocked in cells treated with cycloheximide and creatine (0.16 vs. 0.4 pmol per μg of protein per 30 min, respectively). These results, together with those in Fig. 5, indicate that creatine transporters turn over at a relatively slow rate; they suggest that down-regulation of creatine transport by extracellular creatine requires protein synthesis.

The Regulation of Creatine Transport Does Not Occur by a "Trans-Inhibition" Process. While the above results suggest that newly synthesized proteins are involved in down-regulation by extracellular creatine, it was possible that an increased intracellular creatine concentration also might cause a specific transport block by interacting with the cytoplasmic side of the transport molecule [a process called trans-inhibition (1)]. To examine this possibility, L6 cells were preincubated for 1 hr in the presence of low (34 μM) or very high (50 mM) concentrations of extracellular creatine. The cells were then washed in PBS and the rates of creatine transport were measured in the presence of 34 μM radiolabeled creatine. The rates of creatine transport were similar under both preincubation conditions, even though cells preincubated in 50 mM extracellular creatine accumulated 20-fold more intracellular creatine than cells preincubated in 34 μM creatine (>85 pmol/μg and <1.2 pmol/μg, respectively; Table 3). In contrast, creatine transport was down-regulated by >60% in L6 cells maintained for 8 hr in 5 mM extracellular creatine. These cells accumulated ≈70 pmol of intracellular creatine per μg during the 8-hr incubation (data not shown). These results demonstrate that the physical presence of a large store of intracellular creatine does not directly decrease creatine transport.

DISCUSSION

Primary and established muscle cell lines regulate their rates of Na^+-dependent creatine transport in response to a physiological range of creatine concentrations (10–200 μM; Figs. 1 and 2). Similar regulation of creatine transport has been observed in a variety of primary and transformed macrophages (unpublished data), suggesting that this type of regulation is a general property of creatine-utilizing cells.

Table 3. A brief increase in intracellular creatine did not affect the rate of creatine transport

Extracellular creatine, mM	Rate of creatine uptake,* pmol/μg	Intracellular creatine,† pmol/μg
0	0.46	1.2
5	0.52	22
50	0.43	85

*L6 myoblasts were prepared on coverslips and incubated for 1 hr in the presence of various concentrations of creatine in PBS supplemented with 2% dialyzed fetal bovine serum and 5.5 mM glucose. The cells were extensively washed and further incubated for 30 min in PBS supplemented with 5.5 mM glucose, 2% dialyzed fetal bovine serum, and 34 μM radiolabeled creatine as described.

†The amount of intracellular creatine was determined by incubating L6 cells with [^{14}C]creatine for 1 hr in PBS supplemented with 0–50 mM unlabeled creatine. The intracellular creatine content was calculated from the amount of [^{14}C]creatine in the cells and the specific activity of [^{14}C]creatine in the medium. The actual amounts of radiolabel measured were 8000 cpm per coverslip culture for cells incubated in 34 μM creatine and 600 cpm per coverslip culture for cells incubated in 50 mM creatine.

Down-regulation of creatine transport is accompanied by a decrease in V_{max} by a factor of 2.7 and no change in K_m values of Na^+-dependent creatine transport. Our kinetic data cannot distinguish between physical depletion of creatine transporters from the plasma membrane and alteration in their rates of turnover. While sodium is cotransported with creatine, it is unlikely that any changes in intracellular sodium occur in the presence of high extracellular creatine. If such changes in intracellular Na^+ do occur, their magnitude is too small to alter AiB uptake (Fig. 4*A*).

Inhibition by cycloheximide of creatine-mediated down-regulation of creatine transporters suggests that high concentrations of intracellular creatine induce the synthesis of a protein that either inhibits creatine transporters or functionally removes them from the plasma membrane. Such a mechanism is consistent with our kinetic studies, which revealed a decrease in V_{max} values of creatine transport in creatine-fed L6 cells compared to creatine-starved cells.

System A amino acid transport, a Na^+-dependent process, is regulated in a variety of cells (4, 5, 19, 20) by a mechanism referred to as adaptive regulation, which involves the synthesis of transporter and regulator proteins. Several groups (4, 20) showed that puromycin and cycloheximide block the decline of amino acid transport that normally occurs after the addition of AiB to serum-starved cells. This is similar to what we have found with respect to creatine transport.

Since muscle cells must rely on exogenous creatine, their ability to stabilize creatine flow may represent an important pathway for regulating creatine homeostasis and controlling their phosphagen content. Unregulated creatine uptake might lead to depletion of ATP stores as the creatine is phosphorylated to *P*-Cr by creatine kinase, thereby creating an unfavorable metabolic situation. Down-regulation of creatine transporters would prevent the accumulation of excessive concentrations of intracellular creatine.

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1. Kilberg, M. S., Barber, E. F. & Handlogten, M. E. (1985) *Curr. Top. Cell. Regul.* **25**, 133–163.
2. Guidotti, G. G., Borghetti, A. F. & Gazzola, G. C. (1978) *Biochim. Biophys. Acta* **515**, 329–336.
3. Exton, J. H., Mallette, L. E., Jefferson, L. S., Wong, E. H. A., Friedmann, N., Miller, T. B., Jr., & Park, C. R. (1970) *Recent Prog. Horm. Res.* **26**, 411–453.
4. Gazzola, G. C., Dall'Asta, V. & Guidotti, G. G. (1981) *J. Biol. Chem.* **256**, 3191–3198.
5. Logan, W. J., Klip, A. & Gagalang, E. (1982) *J. Cell. Physiol.* **112**, 229–236.
6. Boerner, P. & Saier, M. H., Jr. (1985) *J. Cell. Physiol.* **122**, 316–322.
7. Lienhard, G. E. (1983) *Trends Biochem. Sci.* **8**, 125–127.
8. Walker, J. B. (1979) *Adv. Enzymol.* **50**, 177–242.
9. Loike, J. D., Kozler, V. F. & Silverstein, S. C. (1979) *J. Biol. Chem.* **254**, 9558–9564.
10. Loike, J. D., Kozler, V. F. & Silverstein, S. C. (1984) *J. Exp. Med.* **159**, 746–757.
11. Loike, J. D., Somes, M. & Silverstein, S. C. (1986) *Am. J. Physiol.* **251**, C128–C135.
12. Daly, M. & Siefter, S. (1980) *Arch. Biochem. Biophys.* **203**, 317–324.
13. Yaffe, D. (1968) *Proc. Natl. Acad. Sci. USA* **61**, 477–483.
14. Miranda, A. F., Babiss, L. & Fisher, P. B. (1986) *Methods Enzymol.* **119**, 619–628.
15. Boyse, E. A., Old, L. J. & Chouroulinkov, J. (1964) *Methods Med. Res.* **10**, 39–47.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
17. Christensen, H. N., Cushing, M. K. & Streicher, J. A. (1949) *Arch. Biochem.* **23**, 106–110.
18. Kenyon, G. L. & Reed, G. H. (1983) *Adv. Enzymol.* **54**, 367–426.
19. Kilberg, M. S., Han, H.-P., Barber, E. F. & Chiles, T. C. (1985) *J. Cell. Physiol.* **122**, 290–298.
20. Handlogten, M. E. & Kilberg, M. S. (1984) *J. Biol. Chem.* **259**, 3519–3525.