Na^+-dependent proline transport in isolated membrane vesicles from the L6 muscle cell line

Stimulation of uptake by intravesicular proline

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Membrane vesicles of L6 myoblasts were prepared in order to study the amino acid transport system A. The role of the membrane in the adaptive response of transport to amino acid-supplementation was assessed. The membranes, prepared by N_2 cavitation, displayed Na^+ (but not K^+)-dependent L-proline uptake. An overshoot of L-[^{3}H]proline uptake was observed after exposure of the vesicles to an inward Na^+ gradient. Isolated membrane vesicles loaded with 50 μM proline displayed countertransport (stimulation of proline uptake). It is concluded that the adaptive decrease of proline uptake observed in amino acid-supplemented cells cannot be accounted for by trans-inhibition of transport.

Membrane transport *Myoblast* *Amino acid transport* *Isolated membrane vesicle* *Muscle cells* *Amino acid-supplementation* *Trans-inhibition*

1. INTRODUCTION

The L6 cell line is a good experimental model for studying transport regulation in muscle cells in vitro [1,2]. Proline enters L6 cells through a sodium-dependent mechanism which is probably equivalent to the transport system A in [3]. In several cell types the activity of this system is regulated by hormones and by the amino acid concentration in the surrounding medium (e.c. [4] for review). We have found that proline uptake into L6 cells is markedly depressed by pre-incubation in media supplemented with amino acids [5]. Attempts to investigate the mechanism of transport depression by supplementation in intact cells [6] suggested that an adaptive response took place, but did not rule out trans-inhibition as an alternative possibility. Trans-inhibition refers to a reduced rate of amino acid entry, resulting from the interaction of intracellularly trapped substrate with the transporter. If indeed system A is subject to trans-inhibition, the phenomenon should be also apparent in isolated membranes. On the other hand, adaptive responses probably require the interplay of other cellular elements in addition to the plasma membrane. Hence, an adaptive response to amino acid supplementation is unlikely to occur in isolated membranes. In order to approach this and other aspects of the process of transport regulation, an isolated membrane preparation is required in which the proline uptake system is preserved. Plasma membranes from cells in culture can be prepared by a variety of procedures, and their usefulness in the study of transport phenomena has been stressed by several investigators [7–10]. As pointed out in [11], nitrogen cavitation is the method of choice to produce large, sealed membrane vesicles, suitable for transport determinations. In addition, by careful selection of the nitrogen pressure, one can minimize lysis of mitochondria, nuclei and other organelles, thus preventing the production of a mixture of membranes which is difficult to resolve. This communication reports the isolation of membrane vesicles from L6 muscle cells and the charac-

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terization of sodium-dependent proline uptake in the isolated vesicles. The effects of proline supplementation were tested in these vesicles. In addition, proline uptake was measured in membrane vesicles derived from amino acid-supplemented and -depleted cells.

2. MATERIALS AND METHODS

2.1. *Cells*

L6 myoblasts were grown in monolayers in 75 cm^2 culture flasks in α-minimal essential medium containing 2% fetal calf serum, at 37°C under an atmosphere of 5% CO_2 and 95% air. Cultures were maintained in continuous passages by trypsinization as in [5].

2.2. *Membrane preparation*

Cell disruption was accomplished by an adaptation of the method in [11]. Cells approaching confluence were incubated in fresh medium 24 h prior to membrane preparation. The cells from 8–10 flasks were then rinsed with phosphate-buffered saline solution, and scraped off with a rubber policeman. All further procedures were done at 4°C. The suspended cells were washed with 200 mM sucrose, 0.2 mM $MgSO_4$, 20 mM Tris–HCl (pH 7.5) by sedimentation at 50 × *g* for 5 min in a bench top centrifuge. About 5×10^6 cells were then suspended in 20 ml of the same buffer and their viability determined by Trypan blue exclusion (in all cases viability at this stage exceeded 90%). The cell suspension was equilibrated with 700 lb/in^2 N_2 in a cavitation chamber (Parr Instruments) with magnetic stirring, for 15 min on ice. After pressure release, the collected lysate (containing intact nuclei but less than 5% intact cells) was made 1 mM with EDTA and centrifuged at 600 × *g* for 15 min, thereby sedimenting intact cells and nuclei. The supernatant (S_{600}) was centrifuged at 10000 × *g* for 15 min and the resultant supernatant (S_{10K}) was finally centrifuged at 40000 × *g* for 60 min to yield a microsomal pellet (P_{40K}). Typical yields were (in mg protein): intact cells 17.6; disrupted ells 17.0; S_{600} = 14.4; S_{10K} = 10.8; P_{10K} = 1.4; P_{40K} = 1.8. Protein was estimated by the method in [12].

2.3. *Transport measurements*

15 μl of a membrane suspension (about 10 mg protein/ml in the sucrose–Tris buffer) were incubated with 15 μl of 5 μM [^{3}H]proline (40 μCi/ml) in 20 mM HEPES (pH 7.5) containing either 200 mM NaCl or 200 mM KCl. After variable time periods, the suspensions were diluted with 3 ml ice-cold isotonic NaCl or KCl, passed through Millipore filters (0.22 μm pore size) and washed twice on the filters. These were then counted by scintillation in Budgetsolve. The radioactivity bound to the filters in the absence of membranes was subtracted from all measurements.

2.4. *Materials*

L-[3,4-^{3}H]Proline (15.6 Ci/mmol) was purchased from New England Nuclear. α-Minimal essential medium and fetal calf serum were from Gibco.

3. RESULTS AND DISCUSSION

Membranes were prepared from L6 cells as outlined in section 2. When the microscopic appearance was analyzed by thin section, a predominant population of membrane-bound vesicles was observed. Enzyme marker determinations showed that the membranes were 8-fold enriched in the plasma membrane marker bis(*p*-nitrophenylphosphatase) relative to the disrupted cell suspension (1.5 and 0.2 $nmol.mg^{-1}.min^{-1}$, respectively). Moreover, the membranes were relatively depleted of the mitochondrial marker cytochrome *C* oxidase (2.2 $nmol.mg^{-1}.min^{-1}$ *vs* 7.0 $nmol.mg^{-1}.min^{-1}$ in the disrupted cell suspension). Fig.1 shows a representative determination of the time course of proline uptake into these membranes. The figure shows that uptake in the presence of sodium was faster than in potassium media. However, the equilibrium concentration of proline inside the microsomes was the same in the presence of either cation. The internal volume of the transporting vesicles was estimated from the amount of proline taken up at equilibrium (60 min). The calculated volume (1.9 μl/mg protein), is similar to those obtained in other vesicular systems [13], and therefore suggests that a significant fraction of the vesicles is engaged in transport. In similar experiments, the concentration of proline varied between 2.5 μM and 2 mM, and uptake was determined after 2 min. Although these are not rigorous determinations of the initial rates of uptake, approximate values of the kinetic parameters could be calculated: The apparent

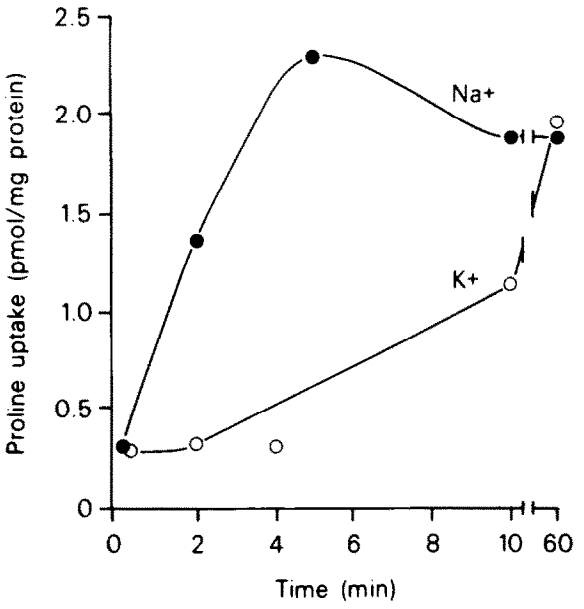

Fig.1. Time course of proline uptake by membrane vesicles from L6 cells. Uptake of [^{3}H]proline (5 μM) was carried out as described in section 2, in the presence of 100 mM NaCl (●) or KCl (○).

K_m was 0.1 mM and V_{max} approximated 75 pmol.mg^{-1}.min^{-1}. In intact L6 cells, K_m is 0.3 mM [5].

In the presence of sodium, a small but significant increase above the equilibrium uptake was observed at short times, which eventually subsided to reach equilibrium (fig.1). This change in concentration of proline was probably driven by the short-lived, inwardly directed sodium gradient. The gradient is most likely dissipated after 8 min, resulting in the equilibration of the intravesicular proline with the external medium. Similar 'overshoots' of sodium-dependent amino acid or glucose uptake have been observed in membranes from other cells [13–20]. Some of these studies have suggested that the overshoot depends both on the chemical sodium gradient and on the electrical gradient. In membranes from L6 cells, no increase in the size of the overshoot was observed when thiocyanate replaced chloride in the outer solution (not shown). This could indicate that the conductivity of the vesicles to both anions was similar, or alternatively, that in these membranes proline uptake is insensitive to the electrical potential.

The membranes obtained in this study constitute a useful system to investigate the mechanism of regulation of amino acid transport observed in vivo. As mentioned in section 1, incubating L6 muscle cells in the presence of proline results in a decrease in their transport capacity for amino acid substrates of system A. It was therefore of interest to determine whether such a response could be observed in the isolated vesicles. For this purpose, isolated membranes were either exposed to amino acid-free media or else loaded wih 50 μM proline for 30 min prior to the determination of proline uptake. Uptake was measured at 45 s and 2 min. The results in table 1 indicate that sodium-dependent transport of [^{3}H]proline was higher in proline-loaded than in amino acid-free vesicles. The fact that trans-inhibition of system A could not be elicited in the isolated membrane system, suggests that this mechanism is unlikely to have caused the decreased proline uptake in proline-supplemented intact cells [5]. In fact, these results indicate that transport system A displays trans-stimulation. This mechanism was probably obscured in amino acid-loaded cells by the adaptive inhibitory response.

Table 1

Effect of proline preloading on [^{3}H]proline uptake

Expt no.	Proline uptake (pmol.min^{-1}.mg protein^{-1})			
	Control vesicles		Loaded vesicles	
	Na^+	K^+	Na^+	K^+
1	0.43 ± 0.1	0.3 ± 0.1	3.2 ± 0.4	0.34 ± 0.1
2	1.6 ± 0.4	1.4 ± 0.2	3.1 ± 0.7	1.6 ± 0.4
3	0.5	0.23	2.5 ± 0.5	0.6 ± 0.3

Membrane vesicles were prepared as described in section 2. They were then incubated for 30 min in the absence (control) or presence (loaded) of 50 μM non-radioactive proline, in 100 mM NaCl, 20 mM HEPES (pH 7.4) at room temperature. The membranes were then diluted 10-fold by exposure to 10 volumes of uptake medium (100 mM NaSCN or KSCN, 10 mM HEPES–Tris (pH 7.5) and 40 μCi/ml [^{3}H]proline). The concentration of proline was adjusted to 5 μM with cold proline in the case of control membranes. Uptake was determined in duplicate for 45 s and 2 min as described in section 2. Protein (30–50 μg) was used per determination. The data are means ± SEM (n = 4) except in control experiment 3, where the mean of two measurements is reported

Finally, we also analyzed whether membranes from amino acid-deprived cells retain the stimulated proline transport observed in intact cells. Results of 3 independent experiments performed in duplicate indicated that proline uptake (measured after 2 min in the presence of 2.5 μM proline) was not significantly lower in the membranes from proline-supplemented cells than in the membranes from deprived cells ($P > 0.05$). Thus, the stimulation of amino acid uptake undergone by the cells in vivo is not preserved in the isolated membrane vesicles. It is conceivable that (cytoplasmic) factors essential to maintain the activation of transport are inactivated or lost during the isolation procedure.

In conclusion, membrane vesicles of L6 myoblasts were prepared in order to study the amino acid transport system A. The membranes displayed Na^+ (but not K^+)-dependent L-proline uptake, and an overshoot of L-proline uptake was observed after exposure of the vesicles to a NaCl gradient. Isolated membrane vesicles loaded with 50 μM proline displayed trans-stimulation. Thus, the adaptive decrease of proline uptake observed in amino acid-supplemented cells cannot be accounted for by trans-inhibition of transport.

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REFERENCES

[1] Logan, W.J. (1978) Muscle and Nerve, May/June 78, 206–212.
[2] Klip, A., Li, G. and Logan, W.J. (1982) Biochim. Biophys. Acta 687, 265–280.
[3] Christensen, H.N., De Cespedes, C., Handlogten, M.E. and Ronquist, G. (1973) Biochim. Biophys. Acta 300, 487–523.
[4] Guidotti, G.G., Borghetti, A.F. and Gazzola, G.C. (1978) Biochim. Biophys. Acta 515, 329–366.
[5] Logan, W.J., Klip, A. and Gagalang, E. (1982) J. Cell Physiol. 112, 229–236.
[6] Klip, A., Logan, W.J. and Gagalang, E. (1982) J. Cell Physiol. 113, 56–66.
[7] Kaback, H.R. (1974) Science 186, 882–892.
[8] Lever, J.E. (1977) J. Supramol. Struct. 6, 103–124.
[9] Sachs, G., Jackson, R.J. and Rabon, E.C. (1980) Am. J. Physiol. 238, G151–G164.
[10] Murer, H. and Kinne, R. (1980) J. Membr. Biol. 55, 81–95.
[11] Hochstadt, J., Quinlan, D.C., Rader, R.L., Li, G. and Dowd, D. (1975) in: Methods in Membrane Biology (Korn, E.D. ed) vol.5, pp.117–162, Plenum, New York.
[12] Bradford, M.M. (1976) Anal. Biochem. 72, 248–252.
[13] Klip, A., Grinstein, S., Marti, T. and Semenza, G. (1979) FEBS Lett. 105, 224–228.
[14] Colombini, M. and Johnstone, R.M. (1974) J. Membr. Biol. 15, 261–276.
[15] Sigrist-Nelson, K., Murer, H. and Hopfer, U. (1975) J. Biol. Chem. 250,5674–5680.
[16] Quinlan, D.C., Parnes, J.R., Shalom, R., Garvey III, T.Q., Isselbacher, K.J. and Hochstadt, J. (1976) Proc. Natl. Acad. Sci. USA 73, 1631–1635.
[17] Hamilton, R.T. and Nilsen-Hamilton, M. (1976) Proc. Natl. Acad. Sci. USA 73, 1907–1911.
[18] Lever, J.E. (1977) Biochemistry 16, 4328–4334.
[19] Kilberg, M.S. and Christensen, H.N. (1980) Membrane Biochemistry 3, 155–168.
[20] Shapiro, M.P. and Heinz, E. (1980) Biochim. Biophys. Acta 600, 898–911.