Role of calcium in serum-stimulation of hexose transport in muscle cells

Amira Klip, Grace Li and William J. Logan

Division of Neurology, Department of Paediatrics, Research Institute, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8, Canada

Received 17 August 1983

Serum stimulates glucose uptake into several cells in culture. In intact muscle, an increase in cytosolic free Ca^{2+} has been proposed to mediate the activation of glucose uptake by hormones and other stimuli [Cell Calcium (1980) 1, 311–325]. We report that hexose (2-deoxy-D-glucose) uptake into L6 muscle cells in culture is enhanced several-fold by fetal calf serum. The increase in uptake is due to stimulation of transmembrane transport, since serum also stimulated uptake of the non-metabolizable hexose 3-*O*-methyl-D-glucose. The role of Ca^{2+} in this stimulation was assessed: (i) stimulation of transport by serum was independent of the presence of extracellular Ca^{2+} during the incubation with serum; (ii) the intracellular levels of free Ca^{2+}, measured by the fluorescence of the novel Ca-indicator quin-2, were identical in serum-stimulated and control cells. It is concluded that hexose transport can increase in muscle cells without concomitant changes in cytoplasmic free Ca^{2+}.

Intracellular calcium *Muscle cell* *Glucose uptake* *Membrane transport* *Serum-activated transport*

1. INTRODUCTION

One of the most conspicuous effects of serum on cells in culture is the stimulation of hexose uptake [1–3]. It has been suggested that such increase in hexose transport mediates the well-known growth-promoting effects of serum on cell cultures [4–7]. The mechanisms responsible for serum activation of either hexose transport or cell growth remain unknown.

In the isolated soleus muscle of the rat, it has been shown that a variety of hormones and conditions that stimulate hexose transport also increase the efflux of Ca^{2+} from the muscle to the medium [8–10]. The latter has been interpreted to represent an increase in the cytoplasmic levels of Ca^{2+}, and the time course of its onset has suggested that intracellular Ca^{2+} mobilization is a prerequisite for the stimulation of hexose transport [10]. However, direct measurements of the cytoplasmic Ca^{2+} levels have not been made under the conditions that lead to stimulation of hexose uptake. Consequently, the possibility that Ca^{2+} mediates this effect remains unproven.

We have shown that the L6 muscle cell line constitutes a viable model for measurements of the initial rate of hexose transport and its regulation in muscle [11]. We here report that serum stimulates hexose uptake into L6 cells in culture, and explore some aspects of the mechanism of this regulation, in particular the possible role of extra- and intracellular Ca^{2+}. The free concentration of the latter was monitored by the novel procedure in [12], based on the fluorescence of the quinoline derivative quin-2, a Ca^{2+}-indicator. This compound is generated intracellularly upon hydrolysis of the parent compound quin-2-tetraacetoxymethyl ester by cytosolic esterases. The cells are hence loaded with the impermeant quin-2 which is unable to leave the cells. The fluorescence emitted by the compound is proportional to the cytosolic levels of free Ca^{2+} and calibration of fluorescence

Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

vs $[Ca^{2+}]$ is done in each individual experiment by determination of the fluorescence maxima (after cell permeabilization to Ca^{2+}) and minima (after quenching with $MnCl_2$).

Results are presented on the dependence of the serum-stimulation of hexose uptake on extracellular Ca^{2+} and on the levels of cytosolic Ca^{2+} in serum-stimulated and control cells.

2. EXPERIMENTAL

2.1. *Transport measurements*

L6 myoblasts were grown in minimal essential medium (MEM) containing 2% fetal calf serum as in [11]. For transport studies the cells were trypsinized, transferred into 1-cm^2 wells (6×10^4 cells/well), and incubated in the same medium at 37°C. After 4 days the cells were confluent, aligned and fusion was clearly recognized. Transport of 2-deoxy-D-glucose (2-D-G) or of 3-*O*-methyl-D-glucose in monolayer cultures or in suspended cells was measured in Krebs buffer as in [11]. In all instances only carrier-mediated uptake is reported which was estimated as the fraction of total uptake sensitive to the alkaloid cytochalasin B [11]. Protein was determined as in [13].

2.2. *Calcium determination*

Free cytosolic Ca^{2+} levels were determined as in [12]. Monolayers of L6 cells incubated with or without serum were trypsinized (as in [11]), washed and loaded with quin-2 by incubation with 20 μM of the parent compound tetraacetoxymethyl ester in MEM for 30 min at 37°C. Removal of the unhydrolysed ester was achieved by 2 successive washes in 1 ml MEM followed by sedimentation in an Eppendorff centrifuge. The cells were resuspended at about 1×10^5 cells/ml in the following medium: 10 mM HEPES-Na (pH 7.3), 10 mM D-glucose, 140 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$ with or without 3 mM $CaCl_2$, and kept in the dark. Fluorescence was determined at 37°C in a Perkin Elmer 650-40 spectrofluorometer, at 339 nm excitation and 495 nm emission wavelengths, using slits of 2 and 15 nm, respectively. Maximum fluorescence (F_{max}) was obtained by permeabilizing the cells to Ca^{2+} with 1 μM ionomycin, and minimum fluorescence (F_{min}) was calculated as in [15] after quenching with 4 mM $MnCl_2$. From these values, the level of cytosolic $[Ca^{2+}]$ in nM was calculated from the observed fluorescence (F) according to the equation:

$$[Ca^{2+}] = \frac{115\ (F - F_{min})}{F_{max} - F}$$

Identical values of F_{max} were obtained by the use of ionomycin or of 0.1% Triton X-100 (as in [12]). Similar values of F_{min} were calculated by the equation $F_{min} = 1/6\ (F_{max} - F_{min}) + F_{min}$ [15,16] as from the fluorescence value in 0.1% Triton, 20 mM Tris (pH 9), 5 mM ethylene glycol tetraacetic acid (as used in [12]). Cell viability (trypan-blue exclusion) was determined at the end of each determination. Only experiments in which viability was >80% under these conditions were judged acceptable for the calculation of cytosolic-free Ca^{2+}. The intracellular concentration of quin-2 was calculated by interpolating the fluorescence value of a suspension of quin-loaded cells in calibration curves of hydrolyzed quin-tetraacetoxymethyl ester, using the value of cell volume calculated in [11].

2.3. *Materials*

Fetal serum and Dulbecco's minimal essential medium (MEM) were purchased from Gibco. 2-Deoxy-D-[^{3}H]glucose and 3-*O*-methyl-D-[^{3}H]glucose were from New England Nuclear. Quin-2-tetraacetoxymethyl ester was obtained from Lancaster Synthesis.

3. RESULTS

3.1. *Serum dependence of 2-D-G uptake*

L6 cells were incubated for 5 h with increasing concentrations of fetal calf serum, followed by the determination of initial rates of 2-D-G uptake. Fig.1A shows that hexose uptake was enhanced progressively by serum concentrations in the range 0–10%. At the latter concentration, the stimulation of transport was 5.6-fold. In several independent similar experiments the stimulation by 10% serum varied between 3- and 6-fold (within each experiment [24 determinations] the reproducibility was >95%).

We have previously shown that in L6 cells transmembrane transport is rate-limiting to the uptake of 2-D-G [11]. In order to further ascertain that the stimulating action of serum was on the

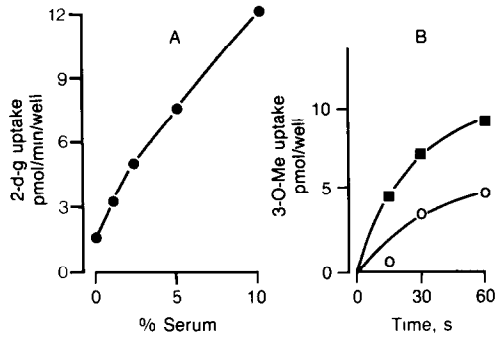

Fig.1. (A) Serum-dependence of 2-deoxy-D-glucose (2-D-G) uptake. L6 cells were grown as described in section 2 and were then exposed for 5 h to the indicated serum concentrations in MEM at 37°C. Measurements followed by initial rates of carrier-mediated uptake of 2-D-G. Each point is the mean of 3 determinations, in 1 experiment representative of 3. The protein content/well was 68 μg. (B) Effect of serum on uptake of 3-*O*-methyl-D-glucose. L6 cells were incubated for 5 h in MEM at 37°C lacking (○) or containing (■) 10% serum. Carrier-mediated uptake of 3-*O*-methyl-D-glucose was then measured for the time periods indicated in the abscissa. Points are the mean of 3 determinations. Protein content/well was 75 μg.

transport of 2-D-G rather than on its subsequent phosphorylation by phosphokinase, studies were also made using the non-phosphorylating hexose 3-*O*-methyl-D-glucose. Fig.1B shows that uptake of this analog is also stimulated by serum. The amount of 3-*O*-methyl-D-glucose taken up by serum-deprived or supplemented cells equilibrated at the same value after 10 min, indicating that the size of intracellular compartment was the same in both cases.

3.2. *Effect of serum on the kinetic parameters of transport*

The kinetic parameters of 2-D-G uptake were determined after a 5-h incubation with or without 10% serum. For these studies, during the transport assay [2-D-G] was varied from 0.5–5 mM, and the data were linearized by the method of Eadie-Hofstee. Least squares analysis yielded values for V_{max} of 161 ±39 pmol.min^{-1}.mg^{-1} (95% confidence interval, n = 13) in the absence of serum and 1260 ± 220 pmol.min^{-1}.mg^{-1} (n = 15) in cells incubated with serum. In addition, the value of K_m also increased: from 0.25 mM without serum to 0.87 mM with serum.

3.3. *Effect of cycloheximide on the stimulation of transport by serum*

Stimulation of transport by serum requires several hours to be fully expressed. This suggests that active protein syntheses may be required to elicit the response. To test this possibility, the incubation with 10% serum was performed in the absence or presence of 5 μg cycloheximide/ml, and the rates of hexose uptake were subsequently compared. In 6 determinations, the antibiotic markedly interfered with the response to serum: uptake of 0.1 mM 2-D-G was 75.97 ± 8.94 pmol.min^{-1}.mg protein^{-1} when serum treatment was performed in the absence of cycloheximide, and 128.75 ± 17.02 pmol.min^{-1}.mg protein^{-1} when serum treatment was in the presence of the drug.

3.4. *Role of Ca^{2+} in the serum effect*

3.4.1. Extracellular calcium

The possibility that Ca^{2+} was required during the incubation with serum for the manifestation of its effect on 2-D-G uptake was tested by performing the 5-h incubation with serum in the presence and absence of Ca^{2+}. For this purpose MEM had to be substituted for Krebs buffer (120 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 20 mM Tris–HCl, pH 7.4) containing 1 mg D-glucose/ml and 10 mg bovine serum albumin/ml. $CaCl_2$, when present, was 1 mM. Because EGTA was found to be deleterious to L6 cells, the solutions without Ca^{2+} are only nominally Ca^{2+}-free.

Table 1 shows that in Ca^{2+}-free buffer, 10% serum was capable of eliciting a 2-fold stimulation of 2-D-G uptake (relative to that of cells incubated in the absence of both Ca^{2+} and serum). In parallel determinations in the presence of Ca^{2+}, the stimulation of 2-D-G uptake by serum was 1.7-fold. The lower stimulation of serum observed in these studies relative to those described above was due to the use of Krebs buffer instead of MEM. However, the lack of effect of Ca^{2+} removal is clear.

It must be pointed out that although Ca^{2+} was missing during the 5-h incubation with serum, it was present during the subsequent determination of transport in Krebs buffer (10 min). Calcium was furnished during the uptake assay to preclude any possible acute effects of Ca^{2+}-removal on the transport mechanism per se. However, similar experiments were also carried out using Ca^{2+}-free

Table 1

Role of Ca^{2+} in the production of the effect of serum

	Hexose uptake (pmol.min^{-1}.mg protein^{-1})[a]			
	Experiment 1		Experiment 2[b]	
	−Ca	+Ca	−Ca	+Ca
−Serum[c]	55 ± 12	82 ± 9	65 ± 5	78 ± 3
+10% Serum	110 ± 1	140 ± 40	200 ± 19	230 ± 6

[a] Carrier-mediated uptake of 0.1 mM 2-deoxy-D-[^{3}H]glucose was determined for 10 min in Krebs buffer as described in section 2; results are the mean ± SE of 3 determinations

[b] Like experiment 1, but without Ca^{2+} in the transport assay solution

[c] 5 h at 37°C

[d] Absence or presence of Ca^{2+} during serum incubation in Krebs buffer supplemented with 1 mg glucose/ml and 10 mg bovine serum albumin/ml

Table 2

Cytosolic Ca^{2+} levels and hexose uptake in serum-treated and untreated L6 cells in suspension[a]

	Cytosolic $[Ca^{2+}]$[b] (nM)	Hexose uptake[c] (pmol.min^{-1}.mg protein^{-1})
−Serum	359 ± 47	129 ± 19
+10% Serum	377 ± 53	333 ± 32

[a] Monolayers of L6 cells were incubated for 5 h in MEM with or without 10% fetal calf serum; the cells were then resuspended by trypsinization as in [11]

[b] Determined by the fluorescence of quin-2 as described in section 2; results are the mean ± SE of 4 determinations

[c] Carrier-mediated uptake of 0.1 mM 2-deoxy-D-[^{3}H]glucose in suspended cells was determined by centrifugation through a phtalate-oil phase as in [11]; results are the mean ± SE of 3 independent experiments performed in duplicate

Krebs buffer during the transport assay. Table 1 shows that omission of Ca^{2+} throughout both the incubation period and the transport determination did not prevent the stimulation of hexose transport by serum.

3.4.2. Cytosolic calcium

In order to measure the levels of cytosolic-free Ca^{2+} by the procedure in [12] it was found convenient to maintain the cells in suspension during the fluorescence determinations. For this purpose, the muscle cells, which normally grow on monolayers attached to a plastic substratum, had to be resuspended by trypsinization. L6 cells remain as a stable suspension for over 1 h after such treatment [11]. It was therefore necessary to test whether the effect of serum on hexose uptake was retained after cell detachment with trypsin. After a 5-h incubation with or without 10% serum, the cells were trypsinized and transport of 2-D-G in suspended cells was determined as in [11]. The results in table 2 show that the rate of cytochalasin B-sensitive uptake of 0.1 mM 2-D-G was 2.6-fold higher in serum-supplemented cells than in the deprived cells. Moreover, when uptake of 2-D-G was assayed after loading the cells with quin-2, serum-supplemented cells still showed a more than 2-fold increase in uptake.

The effect of serum on cytosolic-free Ca^{2+} levels was then assessed under the same conditions: 5 h exposure to serum-containing or -lacking medium, followed by trypsinization and cell resuspension. Loading with quin-2 (see section 2) followed in the absence or presence of 10% serum. All subsequent wash steps were also performed in the absence or presence of serum. Cytosolic $[Ca^{2+}]$ was then measured by the fluorescence procedure described in section 2. Table 2 presents the results from one representative of 3 independent experiments, each performed in quadruplicate. It was observed that the levels of Ca^{2+} in the cytosol of serum-deprived or supplemented cells were identical.

4. DISCUSSION

Intracellular Ca^{2+} ions have been repeatedly invoked (not shown) to underlie the stimulation of hexose transport by 'insulin-like' factors particularly in muscle tissue [8–10]. Hence it is imperative to ascertain a role of this cation in the regulation of transport. Three lines of evidence are considered in this communication which argue against the involvement of Ca^{2+} in the stimulation of hexose transport:

(i) Extracellular Ca^{2+} was not required for the expression of the stimulation of sugar uptake in

muscle cells in culture (table 1). This suggests that if changes in the cytosolic levels of Ca^{2+} were to occur upon serum addition, the source of the cation would have to be intracellular.

(ii) No difference was found in the cytosolic-free Ca^{2+} of serum-treated and untreated cells when the concentration of the cation was directly monitored by the fluorescence of quin-2 (table 2). However, it cannot be ruled out that local changes in free Ca^{2+}, not detected by the fluorophore, occurred in the vicinity of the sugar transporter and regulate its activity.

(iii) Hexose uptake into serum-treated cells remained elevated after loading with quin-2. This Ca^{2+}-indicator is a potent Ca^{2+}-chelator (K_d = 115 nM) which reached concentrations of up to 5 mM inside the cells under our experimental conditions. Hence, if a limited amount of Ca^{2+} entered the cytoplasm in response to exposure to serum, the chelator could prevent the cytosolic concentration of Ca^{2+} from changing significantly. This would be consistent with the failure of serum to increase cytosolic [Ca^{2+}] (table 2). However, if this interpretation is correct, the presence of quin-2 should also prevent the effects of serum on hexose transport, if the latter were Ca^{2+}-mediated. This was, as shown above, not the case pointing to the independence of hexose transport activity from cytoplasmic-free Ca^{2+} levels.

From the above considerations it is concluded that an increase in cytosolic Ca^{2+} does not mediate the activation of hexose uptake by serum. This does not rule out the possibility that the effects of other factors which stimulate hexose uptake, such as insulin, may be mediated by the cation. The mechanisms for activation of sugar transport by insulin and serum are probably different, since the response to the hormone occurs within minutes, whereas the response to serum develops in hours. Moreover, while the action of insulin on sugar uptake into muscle is independent of protein synthesis [14], the development of the response to serum in L6 muscle cells was blocked by cycloheximide. This is also the case for the enhancement of hexose uptake by serum in fibroblasts in culture [2]. Thus, it would be of particular importance to determine whether the action of insulin is brought about by changes in Ca^{2+} concentration. Different mechanisms of regulation of sugar transport may underlie the response to acute and chronic stimuli.

ACKNOWLEDGEMENTS

We are grateful to Dr S. Grinstein for stimulating discussions and revision of this manuscript. This work was supported by the Muscular Dystrophy Association of Canada and by the Medical Research Council of Canada. A.K. is an MRC Scholar.

REFERENCES

[1] Sefton, B.M. and Rubin, H. (1971) Proc. Natl. Acad. Sci. USA 68, 3154–3157.
[2] Kletzien, R.F. and Perdue, J.F. (1974) J. Biol. Chem. 249, 3383–3387.
[3] Bradley, W.E.C. and Culp, L.A. (1974) Exp. Cell Res. 84, 335–350.
[4] Pardee, A.B. (1964) Natl. Cancer Inst. Monogr. 14, 7–20.
[5] Holley, R.W. (1972) Proc. Natl. Acad. Sci. USA 70, 1364–1367.
[6] Balk, S.D., Whitfield, J.F., Youdale, T. and Braun, A.C. (1973) Proc. Natl. Acad. Sci. USA 70, 675–679.
[7] Tupper, J.T., Kaufman, L. and Bodine, P.V. (1980) J. Cell. Physiol. 104, 97–103.
[8] Bihler, I., Charles, P. and Sawh, P.C. (1980) Cell Calcium 1, 327–336.
[9] Sorensen, S.S., Christensen, F. and Clausen, T. (1980) Biochim. Biophys. Acta 602, 433–445.
[10] Clausen, T. (1980) Cell Calcium 1, 311–325.
[11] Klip, A., Logan, W.J. and Li, G. (1982) Biochim. Biophys. Acta 687, 265–280.
[12] Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982) J. Cell. Biol. 94, 325–334.
[13] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[14] Yu, K.T. and Gould, M.K. (1979) Biochem. Biophys. Res. Commun. 87, 9–16.
[15] Hesketh, T.R., Smith, G.A., Moore, J.P., Taylor, M.V. and Metcalfe, J.C. (1983) J. Biol. Chem. 258, 4876–4882.
[16] Rink, T.J., Sanchez, A., Grinstein, S. and Rothstein, A. (1983) Biochim. Biophys. Acta 762, 593–596.