Biochimica et Biophysica Acta 821 (1985) 30–36
Elsevier

BBA 72808

Stimulation of glucose transport in skeletal muscle by the sodium ionophore monensin

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(Received May 29th, 1985)

Key words: Glucose transport; Sodium ionophore; Ionophore; Monensin; Ca^{2+} dependence; Na^+-Ca^{2+} exchange; (Mouse diaphragm)

The tissue/medium distribution of the nonmetabolized glucose analog 3-*O*-methyl-D-glucose was measured in mouse diaphragm muscle and related to changes in ^{45}Ca influx, Na^+ content and Na^+-pump activity. In the presence of external Ca^{2+} the sodium ionophore monensin greatly increased cellular Na^+ content (and decreased K^+ content) although ^{86}Rb uptake, reflecting Na^+-pump activity was increased. Concomitantly, ^{45}Ca influx was stimulated, presumably through activation of Na^+-Ca^{2+} exchange. In parallel to the rise in Ca^{2+} influx sugar transport was also increased. Sugar transport was also increased by monensin in the nominal absence of external Ca^{2+}, when Ca^{2+} influx was minimal. To test if monensin releases Ca^{2+} from intracellular storage sites in the absence of external Ca^{2+}, the ionophore was added to medium perfusing rat hind limb preparations and the total Ca content of muscle mitochondria was determined. When Ca^{2+} was present in the perfusate, monensin increased the mitochondrial Ca content. In the absence of Ca^{2+}, the mitochondrial Ca content was lower and was further depressed by monensin, suggesting that elevation of internal Na^+ by monensin may increase mitochondrial Ca^{2+} loss via activation of Na^+-Ca^{2+} exchange across the mitochondrial membrane. The above results are consistent with the effect of monensin on sugar transport being due to alterations in Ca^{2+} distribution. They support the earlier conclusion that regulation of sugar transport in muscle is Ca^{2+} dependent.

Introduction

The membrane transport of glucose and related monosaccharides in muscle and some other types of tissue is rate limiting for overall glucose utilization and is regulated by hormonal and metabolic factors. Alterations in glucose transport caused by these physiological factors and by various other experimental interventions are associated with changes in fluxes and intracellular distribution of Ca^{2+}, leading to the suggestion that glucose transport in muscle is controlled by the level of cytoplasmic Ca^{2+} available for binding to a regulatory site. (see Refs. 1 and 2 for review)

Earlier experiments have shown that glucose transport in muscle is stimulated when internal Na^+ levels are raised through Na^+-pump inhibition using cardiac glycosides or omission of external K^+. It has been suggested that glucose transport stimulation under these conditions is related to accelerated Ca^{2+} influx via Na^+-Ca^{2+} exchange [3]. A possible direct link between the (Na^+ + K^+)-ATPase and sugar transport regulation was, however, not strictly excluded by these experiments. The use of the Na^+-specific ionophore

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Abbreviation: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

monensin provides an alternative means for the manipulation of internal Na^+ levels which does not entail inhibition of the sodium pump.

The results presented here show that monensin in fact stimulates the Na^+ pump but nevertheless accelerates hexose transport, Na^+ influx and Ca^{2+} influx. In the absence of external Ca^{2+}, the stimulatory effect of monensin is consistent with the release of Ca^{2+} from intracellular storage sites. Some of these results have been reported earlier in abstract form [4,5].

Materials and Methods

Materials. Radiolabelled compounds were from New England Nuclear (Boston, MA) and from Amersham Corp. (Oakville, Ontario). Bovine serum albumin, fraction V, and fatty acid-free bovine serum albumin were from Sigma Chemical Co. (St. Louis, MO). All other chemicals and reagents were of the highest commercial quality.

Assays of hexose and Ca^{2+} uptake. Locally bred female Swiss-Webster mice, weighing about 25 g were used. They were killed by cervical dislocation and 'intact' hemidiaphragms were isolated as described by Kono and Colowick [6]. For hexose transport determinations, the tissues were incubated for 30 min at 37°C with gentle shaking in 4.0 ml Krebs-Henseleit bicarbonate medium (pH 7.4) equilibrated with 95% O_2/5% CO_2. This medium contained 1.25 mM Ca^{2+}, 4 mM sodium pyruvate, 0.8% bovine serum albumin and a mixture of ^{14}C-labelled and unlabelled 3-*O*-methyl-D-glucose (total concentration, 5.0 mM) and tracer amounts [^{3}H]inulin serving as extracellular marker. Other additions and modifications were as indicated. Incubation with sugar and inulin was preceded by a 20 min of preincubation in the presence of all other additions. For measurements of calcium uptake the same procedure was followed substituting ^{45}Ca for 3-*O*-methyl-D-glucose.

A paired experimental design was used. A small volume (12 μl) of feshly prepared monensin solution in dimethylsulfoxide was added to one hemidiaphragm and the same volume of solvent alone to the contralateral hemidiaphragm. After incubation the muscles and incubation media were analysed for radioactivity by double-label liquid scintillation counting and the Na^+ and K^+ content was determined by emission flame photometry. The procedures for incubation and sample analysis have been described in detail previously [7]. Results for intracellular Na^+ content are expressed in mmol/l intracellular water. The rates of sugar and ^{45}Ca transport were calculated by first determining the mean paired differences ($\pm$S.E.), i.e. the increments or decrements observed between treated and contralateral control muscles and then adding these values to the mean of control values obtained in a large number of experiments. Results are expressed as rates (μmol/l intracellular water per min). All data on sugar and ion distribution are corrected for the inulin space determined in the same tissue sample.

Na^+ pump activity. Na^+-pump activity was estimated from ^{86}Rb uptake. This was measured following Ku et al. [8]. The tissues were first incubated for 30 min in 4 ml K^+-free Krebs-Henseleit bicarbonate buffer to elevate cellular Na^+ levels in a uniform manner. They were then incubated at 37°C for 10 min in 4 ml of the same buffer but containing 2 mM RbCl and tracer amounts ^{86}Rb; $5 \cdot 10^{-4}$ M ouabain was added to one of each pair of hemidiaphragms. The tissues were briefly washed in ice-cold medium containing unlabelled RbCl, blotted, weighed and digested with a commercial tissue solubilizer before liquid scintillation counting. The term Rb uptake refers to the ouabain-sensitive portion of total Rb uptake, i.e. the mean paired difference in uptake with and without ouabain. Such paired experiments were done at several monensin concentrations and the results are expressed in mmol/l intracellular water per min.

Hind limb perfusion. Male Sprague-Dawley rats, weighing 300 g and starved for 48 h before the experiments, were used and the hind limbs perfused by the procedure of Jefferson et al. [9]. Krebs-Henseleit bicarbonate buffer (pH 7.4) was supplemented with 5 mM D-glucose, 0.15 mM sodium pyruvate and 4% fatty acid-free bovine serum albumin and contained 1.25 mM Ca^{2+} where indicated. This medium was infused into the abdominal aorta at a rate of 10 to 12 ml/min and the effluent collected from the vena cava. After about 20 min of preperfusion to wash out the blood and to equilibrate the preparation, recirculating perfusion was continued for 30 min in the

presence of monensin or other additions, as shown. Samples of hind limb muscle were taken and extracted as described for the hemidiaphragms. The remaining hind limb muscle mass was used for the preparation of mitochondria following the procedure of Dow [10]. To minimize changes in mitochondrial Ca^{2+} levels during isolation, these steps were performed in a medium excluding Na^+ and EDTA and containing Ruthenium red [11]. The total calcium content in extracts of whole muscle and of isolated mitochondria was determined by atomic absorption spectrometry and is expressed as nmol/mg tissue wet wt. or nmol/mg mitochondrial protein. Protein was determined by the method of Lowry et al. [12].

Statistical evaluation was done by Student's *t*-test. Where applicable the probability values refer to the mean paired difference between treated and control tissue.

Results

In order to study membrane transport without interference from subsequent metabolism, the non-metabolized glucose analog 3-*O*-methyl-D-glucose was used [13]. As has been discussed previously [7], the relatively long incubation period needed to obtain valid transport data from incubated isolated muscles, such as the diaphragm, precludes determinations of initial undirectional influx rates. Consequently, the net influx determined after 30 min of incubation underestimates true influx and the values shown are, therefore, only a semi-quantitative measure of activity of the sugar transport system. Nevertheless, they closely reflect changes in transport caused by a particular treatment.

The effect of monensin on the function of the sodium pump is shown in Fig. 1. Rubidium uptake was decreased to a minor extent (to 86.5% of control) by 10^{-9} M monensin. At higher concentrations there was a highly significant increase in Rb^+ uptake, to 165.3 and 160.2% of control at 10^{-6} and 10^{-5} M monensin, respectively.

The effect of monensin on the intracellular Na^+ content and on the influx of ^{45}Ca is shown in Fig. 2. There was a highly significant, dose-dependent and almost parallel increase in both of these parameters. Thus, as the ionophore raises internal

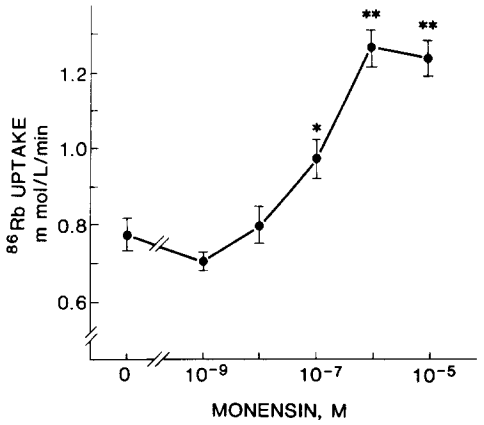

Fig. 1. Effect of monensin on ^{86}Rb uptake in mouse diaphragm. Paired hemidiaphragms were incubated with and without $5 \cdot 10^{-4}$ M ouabain and ^{86}Rb uptake was determined as described in Methods. The mean paired difference (±S.E.), representing ouabain sensitive Rb uptake is plotted as a function of the monensin concentration. The data are means of 11 to 17 pairs, 23 for control and are expressed in mmol/l intracellular water per min. * $P < 0.05$; ** $P < 0.01$ for difference from control uptake.

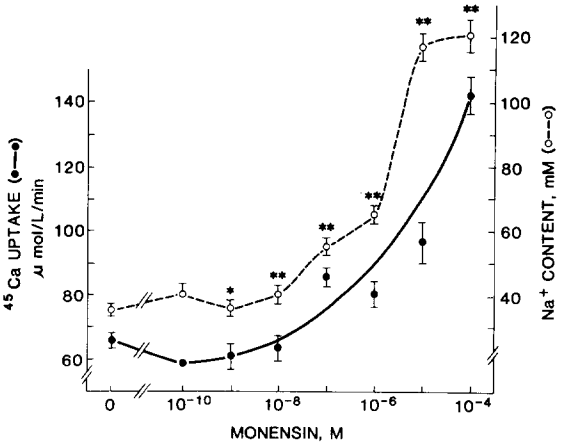

Fig. 2. Effect of monensin on ^{45}Ca influx and cellular levels of Na^+ in mouse diaphragm. Muscles were incubated with and without monensin in a paired design, as described in Materials and Methods. The mean paired differences (±S.E.) in ^{45}Ca uptake were added to mean control uptake and expressed as transport rates. Data for ^{45}Ca uptake are from 9 to 16 paired experiments in the presence of 1.25 mM Ca^{2+}. Results for Na^+ content are pooled from a total of 16 to 38 paired experiments with and without external Ca^{2+}. Data are expressed per litre of intracellular water. * $P < 0.05$; ** $P < 0.01$ for difference from respective controls.

Na^+ content, Ca^{2+} influx is also stimulated, presumably because of enhanced Na^+-Ca^{2+} exchange. The data on ^{45}Ca influx are from experiments in the presence of 1.25 mM external Ca^{2+}. Monensin-stimulated Na^+ influx was the same in the presence and absence of Ca^{2+} and the data shown are the pooled means from experiments with and without Ca^{2+} in the medium. Changes in tissue K^+ content were reciprocal to those of Na^+ (not shown).

Fig. 3 shows the effect of monensin on the influx of 3-*O*-methyl-D-glucose in the presence and absence of external Ca^{2+}. There was a highly significant increase in hexose uptake. In the presence of Ca^{2+}, this rise was dose dependent at lower monensin concentrations but tended to flatten and decrease somewhat at higher ionophore concentrations. In the nominal absence of external Ca^{2+}, control 3-*O*-methyl-D-glucose uptake was somewhat higher but the response to monensin was almost parallel to that in the presence of Ca^{2+}.

To determine if this effect of monensin may be due to contamination by small amounts of Ca^{2+}, such as leaking from the cut edges of the tissue, 3-methylglucose uptake was determined in the presence of 0.5 mM EGTA. 10^{-6} M monensin increased transport under these conditions to 70.4 $\pm$ 4.0 (9) μmol/l per min and 10^{-7} M monensin to 76.9 $\pm$ 4.6 (7) μmol/l per min, compared to the control value of 32.0 $\pm$ 3.8 (16). These effects are not significantly different from those in the absence of EGTA (Fig. 3). Addition of EGTA alone to Ca^{2+}-free medium also had no significant effect. Determination of total Ca^{2+} showed that the Ca^{2+} content of nominally Ca^{2+}-free medium, initially $0.8 \cdot 10^{-6}$ M, rose after incubation with the tissue to $1.2 \cdot 10^{-6}$ M in the absence of EGTA and to $2.2 \cdot 10^{-6}$ M in its presence. Addition of 10^{-6} M monensin further increased total Ca^{2+} in the medium to $2.6 \cdot 10^{-6}$ M. Of course, in the presence of 0.5 mM EGTA virtually all of this Ca^{2+} should be bound to the chelator.

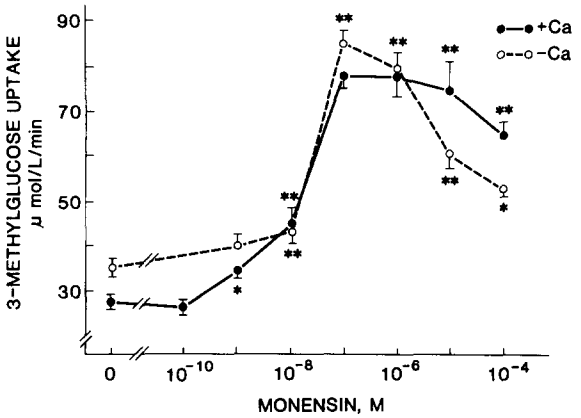

Fig. 3. Effect of monensin on the transport of 3-*O*-methyl-D-glucose in mouse diaphragms. Muscles were incubated and data calculated as shown in Fig. 2. Tissues were incubated in medium with 1.25 mM Ca^{2+} (●———●) or in Ca^{2+}-free medium (O- - - - - -O). The data are mean paired differences ($\pm$S.E.) for 7 to 13 pairs with 1.25 mM Ca^{2+} and 5 to 9 pairs without added Ca^{2+}, and are given in mmol/l intracellular water per min. * $P < 0.05$; ** $P < 0.01$ for difference from respective controls.

When 3-*O*-methyl-D-glucose transport was stimulated by insulin monensin had little additional effect, presumably because the maximal capacity of the hexose transport system was being approached. In paired experiments in the presence of 0.25 milli unit/ml of insulin, 10^{-7} M monensin increased 3-*O*-methyl-D-glucose transport by only 7.0 $\pm$ 1.3 (5) μmol/l per min which is significant ($P < 0.05$) but much less than the increase of 50.8 $\pm$ 1.8 (7) observed in the absence of insulin (see Fig. 3).

The calcium channel antagonist D600 (methoxyverapamil) was shown previously to antagonize the increase in sugar transport in resting muscle caused by several stimulatory agents, including insulin [14]. Compared to the basal control rate, the increase in transport caused by 10^{-6} M monensin was the same with and without 1 μM D600, 44.5 $\pm$ 3.84(4) and 50.8 $\pm$ 2.5(11) μmol/l per min, respectively. However, D600 was shown to increase basal transport of 3-*O*-methyl-D-glucose [14], and a paired comparison of D600 alone to D600 combined with monensin is more meaningful: the stimulatory effect of 10^{-6} M monensin was 28.3 $\pm$ 1.2(4) μmol/l per min which is significantly ($P < 0.01$) less than in the absence of D600, (see above). Na^+ or K^+ were not affected by the drug.

The possible effect of monensin to release Ca^{2+} from internal stores needs to be evaluated as it could explain the stimulatory effect on sugar transport in the absence of external Ca^{2+} (Fig. 3). Monensin was added to the medium perfusing the

TABLE I

TOTAL CALCIUM CONTENT OF WHOLE TISSUE AND OF ISOLATED MITOCHONDRIA FROM PERFUSED RAT HIND LIMB MUSCLES

Rat hind limbs were perfused as described in Materials and Methods and calcium content was determined in samples of whole muscle and in mitochondria isolated from the same muscles. $+Ca^{2+}$ and $-Ca^{2+}$ refer to the presence of 1.25 mM Ca^{2+} in the perfusion medium. The data are means ± S.E. (number of determinations).
Units: for whole muscle nmol/mg tissue wet wt.; for mitochondria nmol/mg protein.

	Ca content; conditions of perfusion		
	control	1 μM monensin	6 μM A23187
Whole muscle			
$+Ca^{2+}$	1.70 ± 0.13(4)	2.18 ± 0.04(4) [a]	2.23 ± 0.15(4) [a]
$-Ca^{2+}$	1.24 ± 0.12(4) [a]	1.06 ± 0.04(5)	1.13 ± 0.09(6)
Mitochondria			
$+Ca^{2+}$	4.7 ± 0.5(4)	6.9 ± 1.3(4) [a]	5.0 ± 0.5(4)
$-Ca^{2+}$	4.1 ± 0.2(4)	3.2 ± 0.1(4) [b]	2.6 ± 0.1(6) [b]

[a] $P < 0.05$ compared to control with Ca^{2+}.
[b] $P < 0.01$ compared to Ca^{2+}-free control.

isolated rat kind limb preparation and the total calcium content of intact muscle samples and of mitochondria was determined. Table I shows that in the presence of 1.25 mM external Ca^{2+}, 10^{-6} M monensin increased total tissue calcium content by about 28%. For comparison, the addition of 6 μM A23187, a calcium ionophore had a similar effect. In the nominal absence of external Ca^{2+}, the control total calcium content was lower than in the presence of Ca^{2+} and neither monensin nor A23187 had any further significant effect. In mitochondria isolated from hind limb muscle perfused with 1.25 mM Ca^{2+} and monensin, the calcium content was increased by 47% but A23187 had no significant effect. When hind limb perfusion was done in the absence of Ca^{2+}, control levels of calcium in the mitochondria were somewhat lower (−13%) and both monensin and A23187 further decreased calcium content to 78% and 63%, respectively, of the Ca^{2+}-free control level. These data are consistent with an effect of monensin to increase tissue uptake and mitochondrial accumulation of Ca^{2+} if the ion was present in the external medium. Conversely, in the absence of external Ca^{2+}, the data suggest an increased loss of Ca^{2+} from the mitochondria.

Discussion

Monensin is an acidic carboxylic ionophore which has been shown to form lipid-soluble neutral complexes with monovalent cations, thus mediating their transfer across biological membranes [15]. Because of these chemical properties and because of its strong preference for Na^+ monensin causes rapid Na^+ influx into cells. This is largely balanced by K^+ efflux, but some H^+ efflux also takes place. The biological effects of monensin are related to the direct and indirect consequences of the resulting increase in internal Na^+. A high internal Na^+ concentration is known to stimulate Na^+-Ca^{2+} exchange in muscle and nerve cells [16], thus leading to increased influx and/or decreased efflux of Ca^{2+} and consequently to a rise in cytoplasmic Ca^{2+} and in total cellular calcium content. Among the biological effects that have been linked to this sequence of events are a transient inotropic effect, followed by a rise in resting tension, in the myocardium [17] and stimulation of several types of secretory cells whose activity is controlled by the level of cytoplasmic Ca^{2+}. Monensin has also been shown to stimulate the release of Ca^{2+} from intracellular storage sites [18]; this may be due in part to stimulation of mitochondrial Na^+-Ca^{2+} exchange which is the main pathway for Ca^{2+} release from muscle mitochondria [19]. A moderate depression in tissue ATP content was also observed which may be related to increased futile cycling of Na^+, K^+, and Ca^{2+} across cellular membranes, i.e. to increased activity of the sodium and calcium pumps as a

homeostatic response to increased passive influx.

In the present study monensin was used as a tool to increase cellular Na^+ content without inhibiting the sodium pump, as would occur with cardiac glycosides. The purpose of this work was to obtain additional data on the nature of the link between raised Na^+ content and increased hexose transport across the sarcolemma; this has been earlier attributed [3] to an increase in cytoplasmic Ca^{2+}, leading to its greater availability for binding to a specific transport regulating site.

The data show, firstly, that effective concentrations of monensin indeed significantly stimulate ouabain-sensitive Rb^+ uptake and thus increase the activity of the sodium pump. Secondly, the same concentrations of monensin cause a simultaneous rise in the influx of Na^+, Ca^{2+} and 3-*O*-methyl-D-glucose into mouse diaphragm muscle. A qualitatively identical pattern of response was found in isolated cardiac myocytes [20]. Thus, the effect of monensin to increase 3-*O*-methyl-D-glucose transport in skeletal and cardiac muscle in the presence of external Ca^{2+} seems to be associated with Ca^{2+} influx into the cells and is in that respect similar to the stimulatory effect of cardiac glycosides in resting skeletal and cardiac muscle which we have described previously [21].

The effect of monensin to stimulate 3-*O*-methyl-D-glucose transport also in the absence of external Ca^{2+} indicates that the ionophore may act as well by a different mechanism, perhaps through releasing Ca^{2+} from internal sources. The results with EGTA indicate that stimulation of hexose transport by monensin in a Ca^{2+}-free medium may not be attributed to residual Ca^{2+} in the medium.

To test the possibility that monensin causes a net release of mitochondrial Ca^{2+} we have measured the total calcium content in whole muscle and in mitochondria isolated from rat hind limbs perfused or without monensin. For comparison, the divalent cation ionophore A23187 was also tested. As expected, monensin increased the calcium content of whole muscle in the presence of external Ca^{2+} but had no effect in its absence. A23187 behaved similarly. For determination of mitochondrial calcium content it was necessary to modify the classical procedures for mitochondrial isolation which do not preserve the original, in vivo, calcium content of the mitochondria. To minimize any gain or loss of Ca^{2+} during homogenization and centrifugation we have used isolation and suspension media [11] which include Ruthenium red, an inhibitor of mitochondrial Ca^{2+} uptake, and exclude Na^+ and EDTA, agents promoting mitochondrial Ca^{2+} loss. This approach to minimizing mitochondrial loss or gain of Ca^{2+} during the isolation procedure has been used before [22] and is at present the only one to minimize, if not totally prevent such undesirable Ca^{2+} exchanges.

The data in Table I show that mitochondrial calcium was significantly increased when monensin was present in the Ca^{2+}-containing hind limb perfusion medium. This is to be expected, as the mitochondria will tend to accumulate the excess Ca^{2+} which has entered the cell. In contrast, in the absence of Ca^{2+} from the perfusion medium monensin caused a significant drop in mitochondrial calcium content; this would be consistent with activation of mitochondrial Na^+-Ca^{2+} exchange by the increased level of cytoplasmic Na^+ induced by monensin. It is unlikely that these changes are due to the action of monensin or A23187 during mitochondrial isolation. These agents have ample time to act under favourable conditions prior to homogenization, whereas during the mitochondrial isolation step the medium is expressly designed to prevent mitochondrial Ca^{2+} influx of efflux via Na^+-Ca^{2+} exchange and, moreover, the ionophores are greatly diluted.

We have shown previously that the divalent cation ionophore A23187 also stimulates hexose transport in cardiac and skeletal muscle [23] and in avian erythrocytes [24]. The present data reveal that this agent also decreased mitochondrial calcium content after Ca^{2+}-free perfusion but did not increase it significantly after perfusion in the presence of Ca^{2+}. The failure of mitochondria to accumulate Ca^{2+} in the presence of A23187, despite massive Ca^{2+} influx into the cells may be due to a greater action of A23187 to increase the Ca^{2+} permeability of the mitochonddrial membrane, thus blunting net Ca^{2+} uptake.

The above results indirectly support the notion that the stimulation of 3-*O*-methyl-D-glucose transport by monensin in the absence of external Ca^{2+} may also be related to alterations in Ca^{2+} distribution, namely to the release of Ca^{2+} from

the mitochondria. We have recently reported [11] that stimulation of hexose transport in muscle by Li^+ in a Ca^{2+}-free medium also involves mitochondrial Ca^{2+} release. Effects of monensin on Ca^{2+} release from other internal sites, such as the sarcoplasmic reticulum, remain as yet unexplored. A more direct approach to this question is provided by experiments with avian erythrocytes which do not possess a Na^+-Ca^{2+} exchange mechanism. In pigeon red cells monensin stimulates 3-*O*-methyl-D-glucose transport independently of the presence or absence of Ca^{2+} in the medium [5] and this effect is accompanied by increased Ca^{2+} efflux from the cells into Ca^{2+}-free medium, indicating greater availability of cytoplasmic Ca^{2+} for exit. Mitochondria appear to be the most likely source of this stored calcium.

It may be concluded that the results with monensin provide additional evidence which is consistent with a role of Ca^{2+} in the control of hexose transport activity in muscle and certain other cell types.

Acknowledgement

We thank Mrs. B. Cook for excellent technical assistance. This work was supported by grants from the Medical Research Council of Canada and the Manitoba Heart Foundation. I.B. is a Career Investigator of the MRC.

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