Effect of Ionophore A23187 on Basal and Insulin-stimulated Sugar Transport by Rat Soleus Muscle

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SUMMARY

The ionophore A23187 (10 μg/ml) did not affect the uptake of D-[U-^{14}C]xylose by rat soleus muscle incubated under basal conditions. When muscles were incubated in a Ca^{2+}/Mg^{2+}-free (CMF) medium, A23187 promoted the efflux of intracellular Mg^{2+} and the efflux of ^{45}Ca from preloaded muscles. Under these conditions, A23187 inhibited insulin-stimulated sugar transport without affecting ^{125}I-insulin binding by the muscle. A23187 induced a slight fall in muscle ATP (16–18%); this does not appear to be responsible for the inhibitory effect of the ionophore on sugar transport. The inhibitory effect of A23187 was completely abolished when the CMF medium was supplemented with Mg^{2+} and partially reversed by Mn^{2+} or Zn^{2+}; supplementation with Ca^{2+} did not reverse the inhibitory effect of the ionophore. These results suggest that insulin stimulates muscle sugar transport through a mechanism that involves intracellular Mg^{2+}. DIABETES *31*:846–850, October 1982.

According to a current theory, insulin and many other factors that activate muscle sugar transport do so by increasing the cytoplasmic Ca^{2+} level.[1–3] Because it has not yet been possible to measure directly the concentration of free cytoplasmic Ca^{2+} in muscle, this has been inferred from the measurement of ^{45}Ca efflux from preloaded cells,[4,5] the development of tension in soleus muscle[4] and, in adipocytes, by the activation of Ca^{2+}-dependent metabolic reactions.[6] Similarly, the inhibition of insulin-stimulated sugar transport by the omission of Ca^{2+} from the medium, or by the addition of La^{3+} and Ni^{2+}, ions that antagonize Ca^{2+},[1] and the inhibition of adipose tissue pyruvate dehydrogenase by ruthenium red,[7] have suggested a role for Ca^{2+} in insulin action. However, as discussed by Czech,[8] there are indications that insulin action may not involve intracellular Ca^{2+} levels.

Another approach to this question is afforded by the ability of the ionophore, A23187, to promote the movement of Ca^{2+} across biologic membranes.[9] According to the theory that Ca^{2+} activates sugar transport, A23187 should stimulate basal sugar transport by promoting the uptake of external Ca^{2+}. Ca^{2+}-dependent A23187-stimulated sugar transport has been reported in thymocytes,[10] cultured chick embryo breast muscle,[11] avian erythrocytes,[12] and rat heart and diaphragm.[13] Conversely, A23187 does not affect sugar transport in rat soleus muscle[14] or adipocytes.[15,16]

There is a second way in which A23187 can be used to modify intracellular divalent cation concentrations. If muscles are incubated in a medium containing neither Ca^{2+} nor Mg^{2+}, it can be expected that the ionophore should promote the efflux of intracellular Ca^{2+} and Mg^{2+}, thereby antagonizing any tendency to increase the concentration of these two cations in the cytoplasmic water. In this paper, we report that under these conditions A23187 inhibits insulin-stimulated xylose uptake in soleus muscle. The inhibitory effect of A23187 is abolished when the medium is supplemented with Mg^{2+}, but is not affected by the addition of Ca^{2+}. These studies point to a role of Mg^{2+} in the process of insulin-stimulated sugar transport in soleus muscle.

METHODS

Soleus muscles weighing approximately 30 mg were obtained from Sprague-Dawley rats (70–90 g) fed ad libitum; muscles were incubated at 37°C under an atmosphere of O_2-CO_2 (95:5, vol/vol). The basic ("bicarbonate") medium contained NaCl (118 mM), KCl (4.8 mM), $CaCl_2$ (2.6 mM), $MgSO_4$ (1.2 mM); KH_2PO_4 (1.2 mM), and $NaHCO_3$ (25 mM); prior to use the medium was gassed with O_2-CO_2. In many of the experiments described below, the basic medium was modified by the omission of $CaCl_2$ and $MgSO_4$; this is referred to as "Ca^{2+}/Mg^{2+}-free" ("CMF") medium.

Xylose uptake was determined using the method of Korbl

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et al.[17] In this procedure the muscles are first preincubated under the test conditions; the uptake of D-[U-^{14}C]xylose (final concentration 10 mM, specific activity 0.03 μCi/μmol) is then measured over a 5-min period at 37°C, using D-[1-^{3}H]sorbitol (10 mM, specific activity 0.1 μCi/μmol) as the extracellular marker. ^{125}I-insulin binding was determined using a procedure modified from that previously described[18] in that the period of incubation with ^{125}I-insulin at 25°C was increased from 30 min to 90 min. For the determination of ATP, muscles were homogenized in 5% trichloroacetic acid and the extract thus obtained was assayed using luciferase in an LKB-Wallac bioluminometer, Model 1250. The release of Mg^{2+} from soleus muscles into CMF medium was determined using a Varian Techtron Atomic Absorption Spectrophotometer, Model 1000.

MATERIALS

D-[U-^{14}C]xylose and [^{45}Ca]$CaCl_2$ were from the Radiochemical Centre, Amersham. D-[1-^{3}H]sorbitol was from New England Nuclear Corp. (Boston). Beef insulin, twice recrystallized, was from the Commonwealth Serum Laboratories, Melbourne. This was iodinated with sodium [^{125}I]iodide (Radiochemical Centre) as described previously.[18] Luciferase was from LKB-Wallac, Turku, Finland. A23187 and ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetra acetic acid (EGTA) were obtained from Calbiochem-Behring (Australia) Pty. Ltd., Sydney. Stock solutions of A23187 (5 mg/ml) were prepared in dimethyl sulfoxide and stored for up to 2 mo at −20°C. An amount of dimethyl sulfoxide, equal to that added with the A23187, was added to the control incubations.

STATISTICS

To minimize the effect of biologic variation between individual animals, wherever possible the experiments were designed using paired controls. One muscle from each pair was incubated under test conditions, while the second served as the control. The results of these experiments were analyzed for statistical significance using Student's *t* test as applied to paired samples. Where it was not possible to use paired controls, muscles taken from littermates were distributed randomly among the experimental groups and the results subjected to statistical analysis using the standard Student's *t* test.

RESULTS

Preliminary experiments (not shown here) indicated that the uptake of ^{45}Ca by soleus muscle was stimulated maximally by 10 μg A23187/ml. At this concentration, ^{45}Ca uptake was stimulated by approximately 100%. Xylose uptake was measured after muscles were preincubated for 60 min in the presence and absence of A23187 (10 μg/ml). In accord with the observation of Grinstein and Erlij,[14] there was no effect of the ionophore on sugar transport (control, 3.7 ± 0.5 μmol/g/h versus A23187, 3.5 ± 0.3 μmol/g/h, N = 6). Accordingly, we proceeded to examine the effects of A23187 on sugar transport when muscles were incubated in CMF medium.

The experiments presented in Table 1 were undertaken to confirm that A23187 would promote the efflux of Ca^{2+} and Mg^{2+} into a CMF medium. As predicted, A23187 (10 μg/ml) stimulated the release of Mg^{2+} from soleus muscle. From

TABLE 1
Effect of A23187 on the efflux of Ca^{2+} and Mg^{2+} into Ca^{2+}/Mg^{2+}-free medium

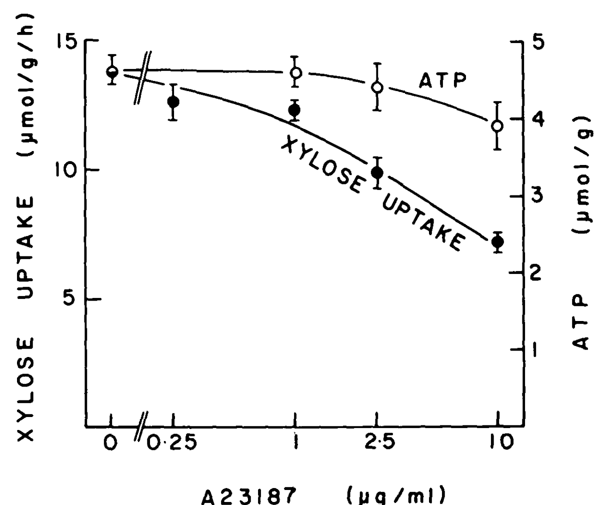
	Mg^{2+} efflux (μmol/g)	^{45}Ca efflux (dpm × 10^{-3}/g)
Control	0.39 ± 0.03 (10)	390 ± 30 (5)
A23187	0.86 ± 0.05 (10)	534 ± 35 (5)
	$P < 0.001$	$P < 0.05$

Mg^{2+} efflux: Soleus muscles were incubated for 30 min at 37°C in 5 ml of CMF medium, then rinsed in CMF medium and incubated a further 30 min in 0.5 ml of CMF medium ± A23187 (10 μg/ml). Mg^{2+} released into the medium was determined using atomic absorption spectrophotometry. ^{45}Ca efflux: Soleus muscles were incubated for 60 min at 30°C in 1 ml of bicarbonate medium containing 2 μCi of ^{45}Ca, then washed twice, each for 30 min at 37°C in 5 ml of CMF medium. Finally, the muscles were incubated for 40 min at 37°C in 0.5 ml of CMF medium ± A23187 (10 μg/ml); the release of ^{45}Ca into the medium during this final incubation was determined. Values are means ± SEM. Numbers of determinations are in parentheses.

previous experiments, the magnitude of the observed Mg^{2+} efflux suggests that this is largely intracellular Mg^{2+}.[19] The efflux of intracellular Ca^{2+} could not be determined by measuring Ca^{2+} directly with atomic absorption spectrophotometry. Instead, we measured the efflux of ^{45}Ca from preloaded muscles following the procedures of Clausen;[4] this, too, was stimulated by A23187.

We have observed that A23187 does not affect basal xylose uptake when muscles are incubated in CMF medium[20] and that insulin-stimulated xylose uptake is not affected by the omission of Ca^{2+} and Mg^{2+} from the incubation medium (unpublished results). Soleus muscles were incubated in CMF medium containing A23187 (0.25–10 μg/ml) for 30 min and then exposed to insulin (0.1 U/ml) for 10 min. Insulin-stimulated xylose uptake was inhibited by A23187 at concentrations of 2.5 μg/ml ($P < 0.005$) and 10 μg/ml ($P < 0.001$) (Figure 1). The inhibitory effect of A23187 (10 μg/ml) was evident 15 min ($P < 0.005$) after exposure to the ionophore and increased with more prolonged exposure (Figure 2).

FIGURE 1. Effect of A23187 on insulin-stimulated xylose uptake and ATP. Soleus muscles were preincubated for 40 min at 37°C in CMF medium in the presence or absence of A23187. Insulin (0.1 U/ml) was added 10 min before the end of the incubation period. Values are means ± SEM of 7 determinations.



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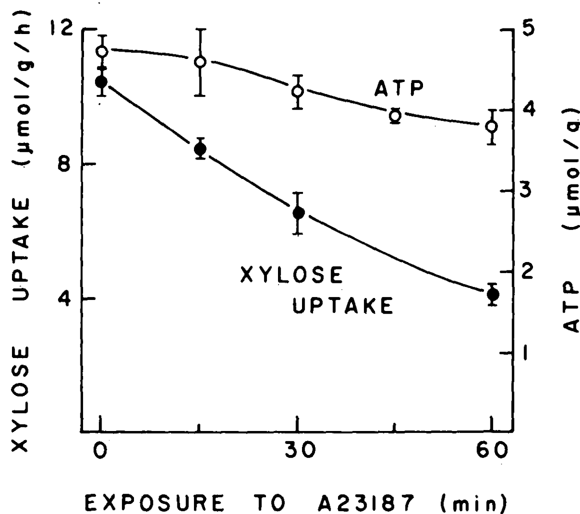


FIGURE 2. Inhibitory effect of A23187 on insulin-stimulated xylose uptake: time course. Soleus muscle pairs were incubated for 60 min at 37°C in CMF medium containing 0.1 U insulin/ml. At various times during this period A23187 (10 μg/ml) was added. Zero time values were measured in the absence of A23187. Values are means ± SEM of 5 determinations.

Previous experiments have shown that the stimulatory effect of insulin on muscle sugar transport is depressed by agents that lower the ATP content of the muscle.[19,21–23] Because A23187 is known to uncouple oxidative-phosphorylation in isolated mitochondria,[9] we asked whether the effects of A23187 shown in Figures 1 and 2 could have been due to the lowering of muscle ATP levels. ATP was measured in soleus muscles after exposure for 40 min to various concentrations of A23187 (Figure 1) or to A23187 (10 μg/ml) for up to 60 min (Figure 2). In both experiments, A23187 was found to lower ATP. This effect was slight in comparison with the effect of those agents previously studied;[19,21–23] it was observed only at the highest concentration of A23187 used (10 μg/ml) (Figure 1), and then only after at least 30-min exposure to the ionophore (Figure 2). The binding of ^{125}I-insulin by soleus muscle is also ATP-dependent.[18,24] Significantly, there was no effect on the binding of ^{125}I-insulin to soleus muscles after incubation for 30 min in the presence of A23187 (10 μg/ml) [control, 0.84 ± 0.13 ng ^{125}I-insulin bound/g muscle (N = 10) versus A23187, 0.83 ± 0.16 (N = 10)]. In this experiment, A23187 lowered muscle ATP from 3.8 ± 0.5 μmol/g (N = 10) to 3.1 μmol/g (N = 10), $P < 0.02$.

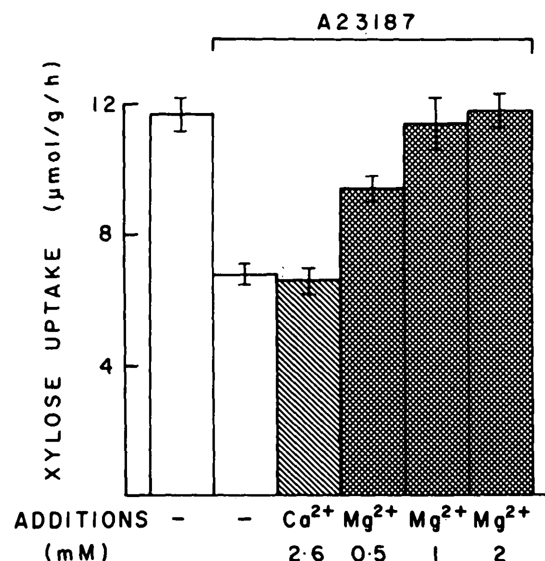
To determine whether the inhibitory effect of A23187 on insulin-stimulated sugar transport was due to the efflux of Ca^{2+} or of Mg^{2+}, muscles were exposed to A23187 (10 μg/ml) for 30 min in a series of CMF media supplemented with Ca^{2+} or Mg^{2+} (Figure 3). The presence of Ca^{2+} (2.6 mM) in the incubation media did not counter the inhibitory effect of A23187; however, this effect was progressively eliminated when the medium was supplemented with Mg^{2+} (0.5–2 mM). Thus far we have assumed that the Ca^{2+} concentration in CMF medium is lower than the concentration of free Ca^{2+} in the intracellular water. This may not be so; traces of Ca^{2+} present as contaminants in the other salts used could be sufficient to prevent any net efflux of intracellular Ca^{2+} under the influence of A23187. To eliminate this possibility, the experiment shown in Figure 3 was repeated using CMF medium supplemented with EGTA (0.1 mM) to eliminate Ca^{2+} from the external medium. The results of this experiment were identical with those presented in Figure 3.

Because A23187 can form complexes with a range of divalent cations, we looked at the ability of other divalent cations to antagonize the inhibitory effect of the ionophore on insulin-stimulated sugar transport. In these experiments, the muscles were first incubated for 30 min in CMF medium containing insulin and either $CoCl_2$, $CuCl_2$, $FeCl_2$, $MnCl_2$, or $ZnSO_4$ (0.1–1 mM); they were then exposed to A23187 (10 μg/ml) for 30 min. The inhibitory effect of A23187 was unaffected by the presence of Co^{2+}, Cu^{2+}, or Fe^{2+} (data not shown). The effect of A23187 was antagonized by Mn^{2+} and by Zn^{2+}; accordingly, the effects of these two cations, along with that of Mg^{2+}, were examined over a wider range of concentrations (Figure 4). The effect of Mg^{2+} did not become apparent until the concentration exceeded 0.1 mM and was maximal by 1 mM; Mg^{2+} effectively restored xylose uptake in A23187-treated muscle to the control value. Optimal concentrations of Mn^{2+} and Zn^{2+} were less effective than Mg^{2+}; however, the effect of these two cations could still be seen at low concentrations (0.1 mM and below) where there was no effect of Mg^{2+}. The effect of A23187 on the ATP content of muscles incubated in CMF media supplemented with Mg^{2+} (1.1 mM), Ca^{2+} (2.6 mM), Mn^{2+} (1.0 mM), and Zn^{2+} (0.1 mM) is shown in Table 2. A23187 still lowered ATP in the CMF-Mg^{2+} medium, but there was no effect of A23187 on muscle ATP when the medium was supplemented with either Ca^{2+}, Mn^{2+}, or Zn^{2+}.

DISCUSSION

According to the theory that sugar transport is stimulated by an increase in cytoplasmic free Ca^{2+}, it could be expected that the ionophore, A23187, would promote a Ca^{2+}-dependent activation of sugar transport. Whereas this has been demonstrated in certain tissues,[10–13] conflicting results have been reported in muscle. Thus, A23187 stimulated sugar

FIGURE 3. Effect of Ca^{2+} and Mg^{2+} on the inhibition of insulin-stimulated xylose uptake by A23187. Soleus muscles were incubated for 60 min at 37°C in CMF medium containing insulin (0.1 U/ml) and, where shown, $CaCl_2$ (2.6 mM) or $MgSO_4$ (0.5, 1.0, or 2.0 mM). Where indicated, A23187 (10 μg/ml) was added 30 min before the end of the incubation period. Values are means ± SEM of 5–7 determinations.



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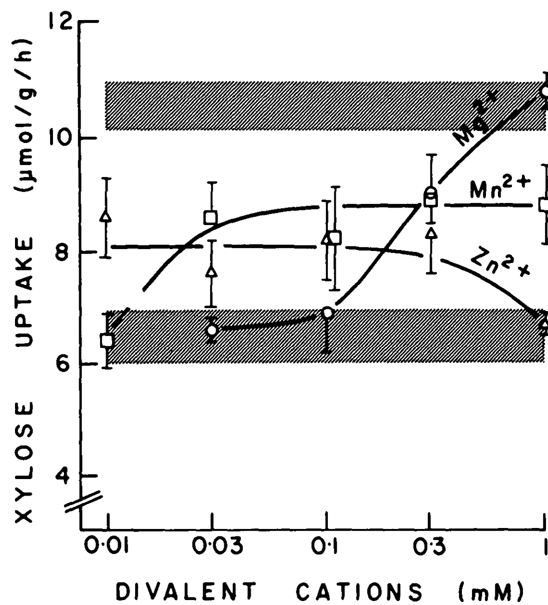

FIGURE 4. Effect of Mg^{2+}, Mn^{2+}, and Zn^{2+} on the inhibition of insulin-stimulated xylose uptake by A23187. Soleus muscles were incubated for 30 min at 37°C in CMF medium containing insulin (0.1 U/ml) and either $MgSO_4$, $MnCl_2$, or $ZnSO_4$ (0.01–1 mM); A23187 (10 μg/ml) was added and the incubation continued for a further 30 min. Values are means ± SEM of 5 determinations. The shaded areas indicate xylose uptake in muscles incubated with insulin in the absence (upper area) and presence (lower area) of A23187.

transport in rat heart and diaphragm[13] and in cultured chick embryo breast muscle,[11] but not in rat soleus muscle.[14] Clausen[25] found that A23187 promoted the Ca^{2+}-dependent increase in 3-O-methyl-glucose efflux from rat soleus muscle; however, as this effect was only partially suppressed by phlorizin or cytochalasin B, it was not certain that stereospecific sugar transport had been activated.

The experiments presented in this paper employed a different approach to examine the putative role of intracellular Ca^{2+} in the regulation of muscle sugar transport. If sugar transport is stimulated by an increase in cytoplasmic free Ca^{2+}, this effect should be antagonized under conditions where A23187 acts to promote Ca^{2+} efflux. We have used a combination of A23187 and CMF medium (±EGTA) to induce the efflux of intracellular Ca^{2+}. Under these conditions, A23187 inhibited insulin-stimulated xylose uptake.

Although A23187 is often referred to as a "Ca^{2+}-ionophore," this is not strictly correct. Its action encompasses a range of other divalent cations as well. If, as assumed, the inhibitory effect of A23187 on insulin action is due to the efflux of some intracellular divalent cation, this effect should be eliminated when efflux is countered by the presence of that cation in the incubation medium. The inhibitory effect of A23187 was undiminished when the CMF medium was supplemented with Ca^{2+}, but was abolished when the medium was supplemented with Mg^{2+} and to a lesser extent with Mn^{2+} and Zn^{2+}. From this we may conclude that the inhibitory effect of the ionophore is related to its effect on intracellular Mg^{2+} rather than intracellular Ca^{2+}.

TABLE 2
Effect of A23187 on muscle ATP: influence of divalent cations

Additions to CMF medium	ATP (μmol/g) −A23187	ATP (μmol/g) +A23187	
—	4.0 ± 0.2	3.6 ± 0.2	$P < 0.02$
Mg^{2+}	4.1 ± 0.2	3.8 ± 0.2	$P < 0.01$
Ca^{2+}	4.2 ± 0.1	4.1 ± 0.1	
Mn^{2+}	3.8 ± 0.3	3.8 ± 0.2	
Zn^{2+}	3.2 ± 0.2	3.2 ± 0.2	

Soleus muscles were incubated for 60 min at 37°C in CMF medium containing (where indicated) $MgSO_4$ (1.1 mM), $CaCl_2$ (2.6 mM), $MnCl_2$ (1 mM), or $ZnSO_4$ (0.1 mM); A23187 (10 μg/ml) was added to one muscle from each pair 30 min before the end of the incubation period. Values are means ± SEM of 5–7 determinations.

Because insulin-stimulated sugar transport in soleus muscle is an ATP-dependent process[21,24] it is appropriate to ask whether the inhibitory effect of A23187 is mediated through the lowering of muscle ATP levels. We do not believe that this is so. First, if we compare A23187 with the other ATP-depleting agents that have been studied,[19,21–23] it is clear that, in comparison with its effect on sugar transport, the effect of the ionophore on ATP levels (see Figures 1 and 2) is considerably smaller than the effects of those other agents. We have recently established that there is a linear correlation between insulin-stimulated xylose uptake and ATP content in anaerobic soleus muscle.[24] Using this as a guide, it can be calculated that a fall in ATP similar to that induced by A23187 (i.e., 16–18%) would lower insulin-stimulated xylose uptake by only 11–13%; this compares with an actual value of 50–60%. Second, ^{125}I-insulin binding by soleus muscle, which is also ATP-dependent,[18,24] is not affected by exposure to A23187 under conditions similar to those where insulin-stimulated xylose uptake is inhibited. Third, when the medium is supplemented with Mg^{2+}, the inhibitory effect of A23187 on insulin action is abolished, but the ionophore still lowers muscle ATP. Conversely, Ca^{2+} counters the effect of A23187 on ATP levels but not on sugar transport. For these various reasons, we conclude that the inhibitory effect of A23187 on insulin-stimulated sugar transport is mediated through some mechanism other than its rather modest effect on muscle ATP levels.

Because this effect is eliminated by the presence of Mg^{2+} in the incubation medium, but not by Ca^{2+}, it must be related to the efflux of intracellular Mg^{2+} rather than Ca^{2+}. This suggests that the stimulatory effect of insulin on muscle sugar transport somehow involves intracellular Mg^{2+}. This is in accord with earlier observations that Mg^{2+} was specifically required for insulin-stimulated glucose uptake by rat diaphragm[26] and soleus muscle.[27] Lostroh and Krahl[28] found that insulin promotes the accumulation of Mg^{2+} in uterine muscle, and suggested that Mg^{2+} may play some role in insulin action.

Rubin has proposed that a number of coordinated processes in chick embryo fibroblasts, including glucose transport, are regulated by the availability of magnesium for transphosphorylation reactions.[29,30] Using procedures similar to those described above, we have demonstrated that intracellular Mg^{2+} is also involved in the stimulatory effects of hyperosmolarity[31] and cooling[20] on soleus muscle sugar transport. This suggests that Mg^{2+} may have a more general role in the regulation of muscle sugar transport by insulin and other insulinlike agents. The overall range of insulinlike agents whose action is mediated through this mechanism has yet to be determined.

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