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THE RELATIONSHIP BETWEEN THE TRANSPORT OF GLUCOSE AND CATIONS ACROSS CELL MEMBRANES IN ISOLATED TISSUES

IX. THE ROLE OF CELLULAR CALCIUM IN THE ACTIVATION OF THE GLUCOSE TRANSPORT SYSTEM IN RAT SOLEUS MUSCLE

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SUMMARY

1. The role of cellular Ca^{2+} in the transport of glucose has been investigated by determining the time-course of tension development and the release of ^{45}Ca and 3-*O*-[^{14}C]methylglucose from preloaded rat soleus muscles.

2. Electrical stimulation, 2,4-dinitrophenol (0.05 mM) and hyperosmolarity (200 mM mannitol) were all found to induce a rapid rise in tension and the rate coefficient of ^{45}Ca release, which coincided with an acceleration of 3-*O*-[^{14}C]methylglucose efflux.

3. Caffeine (10 mM) or exposure to K^+-substituted buffer induced a rapid increase in tension and the release of ^{45}Ca, but a much later stimulation of 3-*O*-methylglucose efflux. This delayed response may be related to the fact that both factors induce a pronounced suppression of the effect of various agents known to stimulate sugar transport.

4. Following a washout period of 120 min at 0 °C, the return to 30 °C elicited a prompt transient rise in the rate coefficient for the release of ^{45}Ca and 3-*O*-[^{14}C]methylglucose to levels, respectively, 2.8 and 14.6 times the control levels measured at 30 °C. The magnitude of these peaks appeared to be a function of the duration of the exposure to 0 °C. Cooling also led to a stimulation of the uptake of 3-*O*-[^{14}C]methylglucose, and phlorizin suppressed the rise.

5. It was not possible to detect any significant effect of insulin on basal tension or on the influx or efflux of ^{45}Ca. However, in a hyperosmolar environment, insulin (10–100 munits/ml) induced a marked further rise in tension, indicating that the hormone can elicit a redistribution of cellular Ca^{2+}.

6. It is concluded that a rise in the cytoplasmic concentration of free Ca^{2+} constitutes a part of the mechanism by which the glucose transport system is activated by a variety of stimuli, perhaps also insulin.

INTRODUCTION

It has repeatedly been proposed that Ca^{2+} plays a significant role in the stimulation of sugar transport induced by a variety of factors and conditions [1–10]. A

rise in the cytoplasmic level of Ca^{2+} may act as a trigger in a number of cellular processes (contraction, secretion, enzyme activation, the perception of light, etc.). If this ion should be ascribed an analogous function as an activator for the glucose transport system, factors which induce a rise in the concentration of free Ca^{2+} in the cytoplasm, should produce a stimulation of sugar transport. Furthermore, the rise in Ca^{2+} should precede and probably be related quantitatively to the magnitude of sugar transport acceleration.

The purpose of the present study has been to determine whether these requirements could be met for a number of widely different stimuli for sugar transport in rat soleus muscle.

The amount of free Ca^{2+} in the cytoplasm constitutes only a minute fraction of total cellular calcium, which is mainly retained in the mitochondria, the endoplasmic reticulum and bound to various membranes. It is extremely difficult, therefore, to measure the cytoplasmic level of free Ca^{2+}. This is determined by the relative rates at which cellular pools accumulate and release Ca^{2+} as well as by the net transport across the plasma membrane. Caffeine and metabolic inhibitors, which have been shown to induce a loss of calcium from isolated preparations of endoplasmic reticulum [11], and mitochondria [12] produce a rise in the rate of ^{45}Ca release from preloaded intact tissues [13–16]. This is presumably the outcome of a rise in the cytoplasmic level of isotopic calcium available for efflux across the plasma membrane [14], and it is reasonable to expect that the rate coefficient of ^{45}Ca release to some extent can reflect changes in the cytoplasmic level of free Ca^{2+} [15]. However, apart from the possibility that this parameter may only reflect changes in the permeability of the plasma membrane to calcium, isotopic data alone will not yield reliable information about the concentration of Ca^{2+} in the cytoplasm. In the present study, therefore, the well documented sensitivity of the contractile apparatus to Ca^{2+} has been utilized for the detection of possible increases in the concentration of this ion. An attempt has been made to assess the temporal and quantitative relationships between tension, ^{45}Ca release and 3-*O*-methylglucose transport in rat soleus muscle.

The results indicate that a rise in the cytoplasmic level of Ca^{2+} may be of significance in the activation of the glucose transport system. However, the specific locus of this action has not been defined, and other cellular components seem to modify the sensitivity of the glucose transport system towards Ca^{2+}.

METHODS

Except where otherwise stated, all experiments were performed with soleus muscles isolated from fed Wistar rats weighing 60–70 g. The slightly modified Krebs–Ringer bicarbonate buffer used as the standard incubation medium, the procedures for the isolation and incubation of soleus muscles as well as for the determination of 3-*O*-methylglucose transport were the same as described earlier [17].

Preliminary measurements of ^{45}Ca accumulation in soleus muscles showed that a considerable amount of radioactivity was taken up in the tendons, but if these were carefully resected, the ^{45}Ca radioactivity appeared to be evenly distributed in the remainder of the muscle. Provided that the resection of the tendons was done with a minimum of damage to the muscle fibers, these remained relaxed, and the efflux of 3-*O*-methylglucose was not significantly different from that measured with

the tendons present.

When the tendons were trimmed away, the soleus muscles could no longer be attached to the polyethylene tubes used in previously reported efflux experiments [17], and they were therefore placed in small polyethylene "baskets" attached to the tubes used for gassing with the standard O_2–CO_2 mixture.

The rate of ^{45}Ca release was determined by loading the muscles for 60 min in Krebs–Ringer bicarbonate buffer containing 2 μCi/ml of ^{45}Ca (1.27 mM). The washout of ^{45}Ca radioactivity was followed during transfer through a series of tubes containing 3 ml of unlabelled buffer. At the end of the washout period, the muscles were homogenized in 4 ml 5 % trichloroacetic acid, and after centrifugation for 10 min at 2000 × *g*, 1.5-ml aliquots of the clear supernatant were withdrawn for counting. There was no detectable binding of isotopic calcium to the protein sediment. In order to avoid precipitation of calcium, trichloroacetic acid was also added to the efflux media at a final concentration of 5 %, and 1.5 ml withdrawn for counting. 10 ml of a toluene–Triton X-100 scintillation mixture was used for counting, and when checked by external standardization, the counting efficiencies of the tissue extracts and the efflux media were found to be the same. The amount of ^{45}Ca retained in the tissue at each interval of the efflux period and the fraction of ^{45}Ca lost per min were calculated as described earlier [18].

The tension of the soleus muscles was recorded as described elsewhere (Elbrink et al., unpublished). In brief, the muscles were mounted vertically on plastic holders in tubes containing Krebs–Ringer bicarbonate buffer, which was continuously gassed with a mixture of O_2 : CO_2 (95 : 5, v/v). The proximal tendon was attached to a fixed platinum hook, the distal to an isometric force transducer. Tension was recorded with a Servograph pen recorder (Model REA 310). For the determination of single twitch tension, a Harvard isometric force transducer (Model 373) with an electronic recording module (Model 350) were used. The muscles were stimulated with a Harvard Model 344 stimulator delivering square wave pulses of 0.3–0.5 ms duration and the voltage adjusted to 1.2–1.5 times threshold. The platinum hook of the holder served as one electrode, and another platinum electrode was placed next to the distal end of the muscle.

Before tension changes were recorded, the muscles were allowed to equilibrate for 30 min. The resting tension was routinely set at 1.0 g and could be maintained for several hours.

Chemicals, isotopes and hormones

All chemicals were of analytical grade. 3-*O*-methylglucose, L-glucose and bovine serum albumin (Fraction V) were obtained from Sigma Co. (St. Louis). 3-*O*-[^{14}C]methylglucose (58 Ci/mole), [U-^{14}C]sucrose (600 Ci/mole) and L-[$^{14}C_1$]-glucose (3 Ci/mole) were products of the Radiochemical Centre, Amersham, U.K. ^{45}Ca (200 mCi/g) was purchased from the Danish Atomic Energy Commission, Isotope Laboratory, Risø. Mono-component pork insulin lot No. MC-S 821506 (25 I.U./mg, purified by chromatography) was a gift from the Novo Research Laboratories (Copenhagen).

RESULTS

Fig. 1 illustrates the effects of electrical stimulation on the time-courses of the

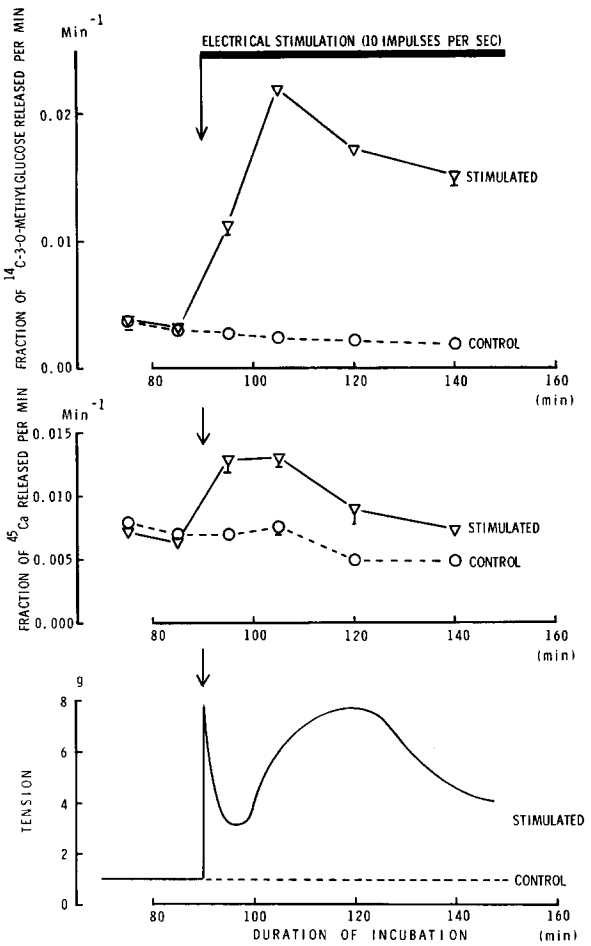

Fig. 1. Effect of electrical stimulation on tension and the release of ^{45}Ca and 3-*O*-[^{14}C]methylglucose from rat soleus muscle. Isolated rat soleus muscles were loaded by incubation for 60 min in Krebs–Ringer bicarbonate buffer containing either 1 mM of 3-*O*-[^{14}C]methylglucose (1 μCi/ml) or 1.27 mM of ^{45}Ca (2 μCi/ml). They were then transferred through a series of tubes containing 3 ml of unlabelled buffer, and at the end of washout, the radioactivity retained in the tissue and the amount released during each period were determined. The fraction of radioactivity released during each interval was calculated as described elsewhere [18]. Each curve represents the mean of 3 observations with bars indicating S.E. where this exceeds the size of the symbols. The lowest curve shows the maximum isometric tension as recorded in parallel experiments with muscles suspended vertically in plastic holders. At the time indicated by the arrow, the muscles were stimulated directly via platinum electrodes at a frequency of 10 impulses per s [17]. For further details, see Materials and Methods.

rate coefficients for the release of ^{45}Ca and 3-*O*-[^{14}C]methylglucose from preloaded soleus muscles. The tension was recorded in a separate series of experiments carried out under closely similar conditions. For comparison, these are plotted on the same time scale. It appears that the onset of the contractions is immediately followed by a gradual increase in the rate coefficient for the release of ^{45}Ca and 3-*O*-[^{14}C]methylglucose. (Although it appears that the rise in the release preceeds the onset of the

contractions, this is merely due to the fact that the rate coefficients are calculated for 10-min periods and the points simply linked with lines). The frequency used (10 impulses per s) was found to give maximal stimulation of 3-*O*-[^{14}C]methylglucose release, and under these conditions, the time-course of tension development indicated that the muscles were fatigued. Other experiments showed that there was a graded stimulation of the washout of 3-*O*-[^{14}C]methylglucose down to a frequency of 1/s. and that this rise could be entirely suppressed by phlorizin (5 mM) [17].

One of the first reports on the stimulating effect of metabolic inhibitors on sugar transport [19] already mentioned that these agents induced contractures in rat diaphragm muscle, and more detailed investigations have shown that curtailment of energy supply may be associated with a transient rise in tension [20]. In earlier studies with rat soleus muscle, the metabolic inhibitors 2,4-dinitrophenol, cyanide and

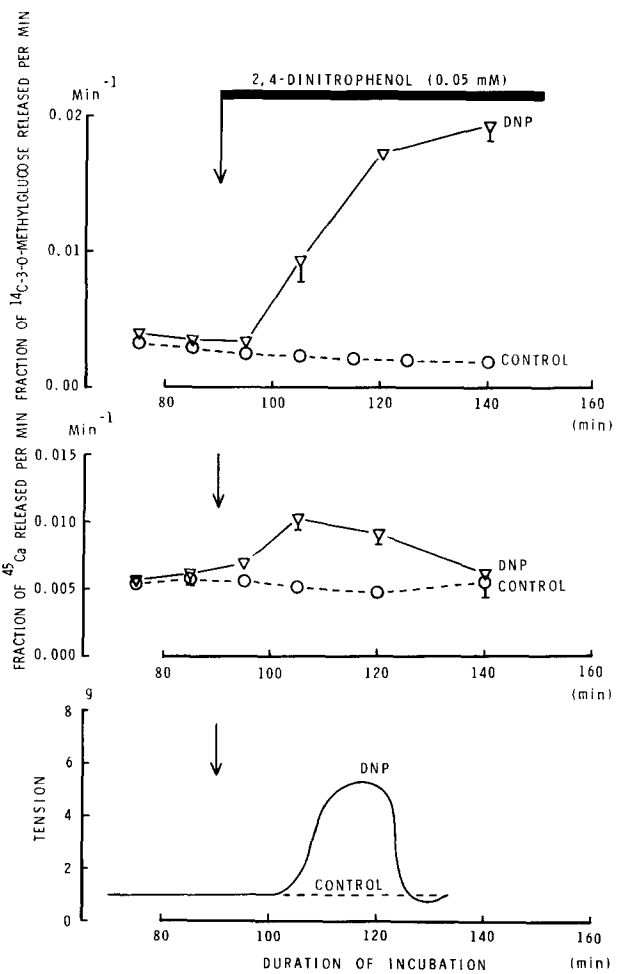

Fig. 2. Effect of 2,4-dinitrophenol (DNP) on tension and the release of ^{45}Ca and 3-*O*-[^{14}C]methylglucose from rat soleus muscle. Experimental conditions as described in the legend to Fig. 1. Each curve represents the mean of 3 observations with bars indicating S.E.

ethacrynic acid were also seen to induce contractions [17]. These compounds induced a phlorizin-suppressible rise in the efflux of 3-*O*-[^{14}C]methylglucose. From Fig. 2 it can be seen that the addition of 2,4-dinitrophenol (0.05 mM) leads to a rise in tension which can be detected in approx. 10 min. The rate coefficient for the release of ^{45}Ca was significantly increased within the first 10-min period of exposure to the inhibitor, but a clear-cut rise in the washout of the labelled sugar was only seen in the second 10-min period and may coincide with the onset of the contracture.

Several investigations have demonstrated that hyperosmolarity can induce a rise in resting tension [21–23] and stimulate the release of ^{45}Ca from preloaded

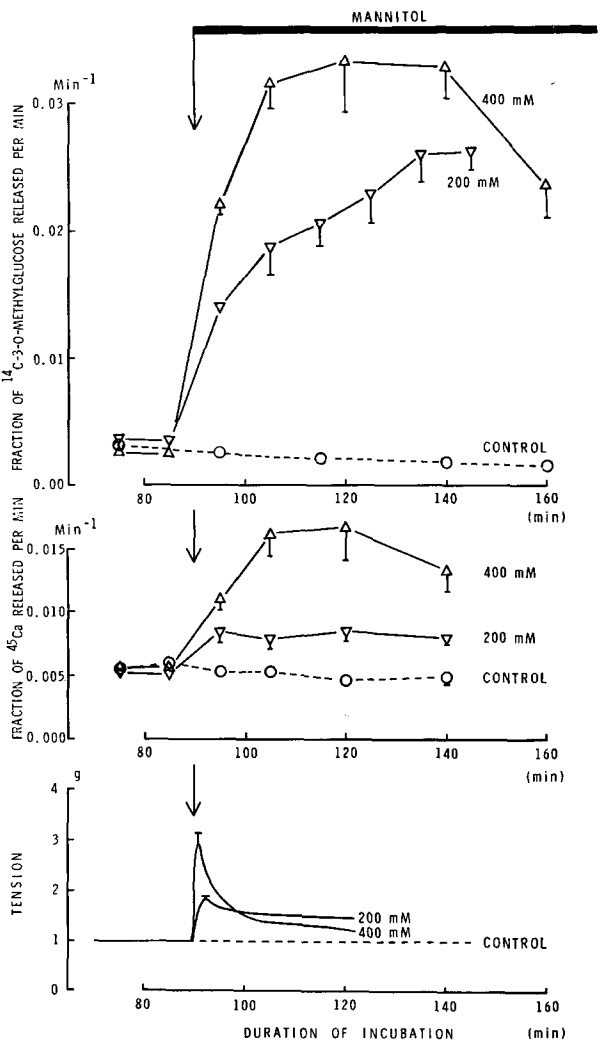

Fig. 3. Effect of hyperosmolarity on tension and the release of ^{45}Ca and 3-*O*-[^{14}C]methylglucose from rat soleus muscle. Experimental conditions as described in the legend to Fig. 1. The osmolarity of the washout medium was augmented by the addition of mannitol at the indicated concentrations. Each curve represents the mean of 3–4 observations with bars indicating S.E.

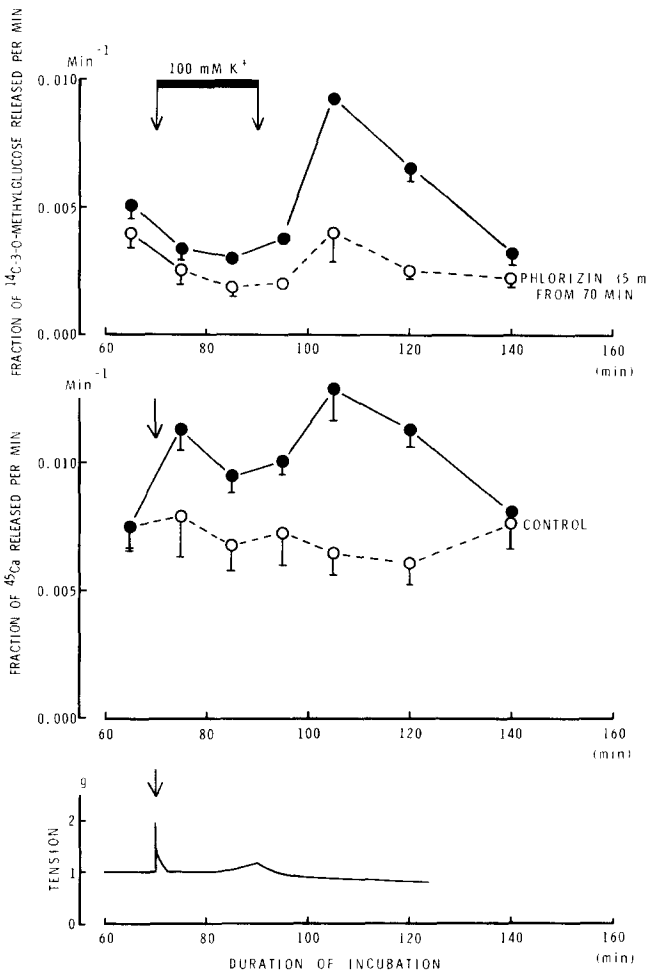

Fig. 4. Effect of K^+-substitution on tension and the release of ^{45}Ca and 3-*O*-[^{14}C]methylglucose from rat soleus muscle. Experimental conditions as described in the legend to Fig. 1. During the interval indicated by the arrows and the black bar, 100 mM of NaCl in the washout medium was replaced by KCl. For one group of muscles, phlorizin (5 mM) was included in the washout medium from the onset of K^+ exposure. Each curve represents the mean of 3 observations with bars indicating S.E.

tissues [16, 24]. This appeared to offer another means of modifying the calcium distribution, which was of particular interest in view of the close similarity between the effects of hyperosmolarity and insulin on sugar transport in muscle and adipose tissue [25, 26]. When the osmolarity was increased by the addition of mannitol (200 or 400 mM), the tension started to rise within the first min. Within the first 10 min of exposure to this buffer the release of both ^{45}Ca and 3-*O*-[^{14}C]methylglucose were significantly augmented (Fig. 3). The increase in all three parameters was concentration dependent, but it should be noted that even when the tension was returning towards the resting level, the washout of labelled calcium and 3-*O*-methylglucose continued to rise above the control level.

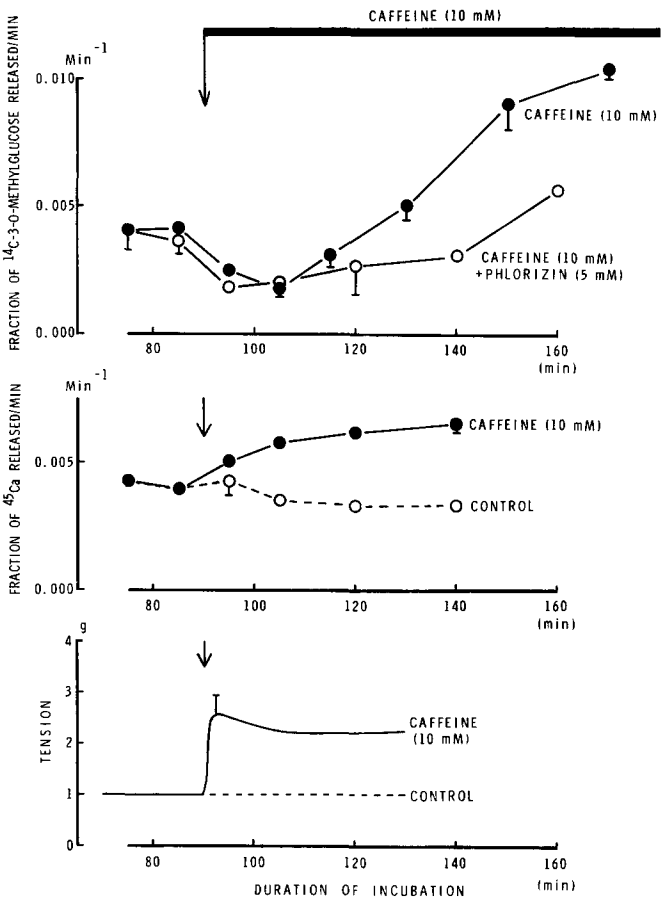

Fig. 5. Effect of caffeine on tension and the release of ^{45}Ca and 3-*O*-[^{14}C]methylglucose from rat soleus muscle. Experimental conditions as described in the legend to Fig. 1. Each curve represents the mean of 4 observations with bars indicating S.E.

It is well established that K^+ depolarization can induce phasic contractures in skeletal muscle. From Fig. 4 it can be seen that when soleus muscles are exposed to a buffer in which 100 mM of the NaCl is replaced by an equivalent amount of KCl, a prompt and short-lasting contracture is induced. Whereas the rate coefficient for the release of ^{45}Ca is increased within the first 10 min, the release of 3-*O*-[^{14}C]methylglucose is not significantly stimulated before the washout is allowed to continue in the standard buffer containing 6 mM K^+. It should be noted that the rise in the rate coefficient for the release of 3-*O*-[^{14}C]methylglucose seen upon the return to normal buffer correlates in time with a renewed rise in the release of isotopic calcium.

Caffeine (10 mM) was found to induce a rapid rise in tension and a gradual increase in the rate coefficient for the release of ^{45}Ca (Fig. 5). This was not associated with any immediate increase in the washout of 3-*O*-[^{14}]methylglucose. It was not until 50 min after the addition of the drug that a phlorizin-sensitive acceleration of sugar efflux could be detected. This, together with the observation that caffeine inhibits

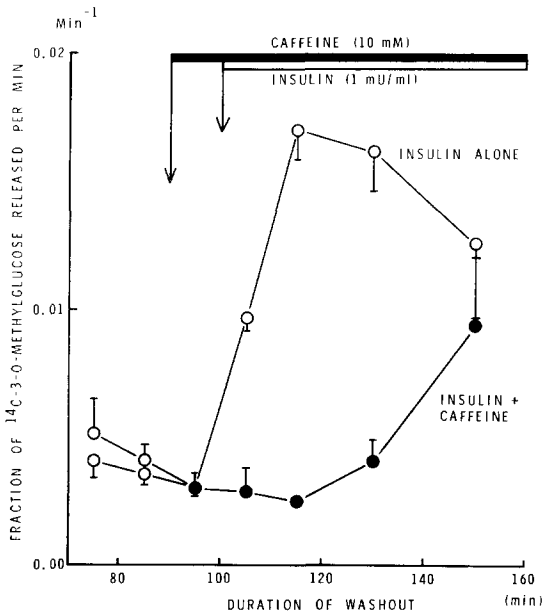

Fig. 6. Effect of caffeine on insulin-stimulated release of 3-*O*-[^{14}C]methylglucose from rat soleus muscle. Experimental conditions as described in the legend to Fig. 1. The effect of insulin (1 mU/ml) was tested in the absence and in the presence of caffeine (10 mM). Each curve represents the mean of 3 observations with bars indicating S.E.

TABLE I

EFFECT OF CAFFEINE, INSULIN OR COOLING ON 3-*O*-METHYLGLUCOSE UPTAKE IN RAT SOLEUS

Isolated soleus muscles were incubated for 60 min in Krebs–Ringer bicarbonate buffer containing either 1 mM 3-*O*-[^{14}C]methylglucose (0.2 μCi/ml) or 1 mM [U-^{14}C]sucrose (0.2 μCi/ml). The results are given as the amount of 3-*O*-methylglucose taken up in the space not available to sucrose with S.E. and the number of observations in parentheses.

Additions or pretreatment	3-*O*-Methylglucose uptake (nmoles/g wet wt per 60 min)	*P*
Control	130±28 (7)	>0.10
Caffeine (10 mM)	92±22 (7)	
Insulin (100 mU/ml)	377±27 (7)	
Caffeine (10 mM) +insulin (100 mU/ml)	140±19 (6)	<0.001
Preincubation for 120 min at 30 °C	76±15 (8)	
Preincubation for 120 min at 0 °C	201±18 (8)	<0.001

glucose metabolism in isolated fat cells [27], urged us to test whether it could modify insulin-stimulated 3-*O*-methylglucose transport. From Fig. 6 it can be seen that when added at the same concentration (10 mM), the stimulating effect of a submaximal concentration of insulin (1 munits/ml) is blocked completely. In the presence of insulin at a supramaximal concentration (100 munits/ml), 5 and 10 mM of caffeine suppressed the rate coefficient of 3-*O*-[^{14}C]methylglucose efflux by 65.3 and 93.2 % respectively. From Table I it can be seen that the drug also reduced the stimulating effect of this concentration of insulin on the uptake of this sugar, but had no effect on non-stimulated uptake as measured in a 60-min incubation period.

Caffeine has been reported to disrupt the integrity of the sarcoplasmic tubules in muscle [28]. However, the spaces available to [U-^{14}C]sucrose or L-[$^{14}C_1$]glucose were not significantly altered by incubation for 60 min in the presence of the drug (data not presented).

In whole epididymal fat pads prepared from fed rats (100–120 g), caffeine (10 mM) was also found to abolish the stimulating effect of insulin (10 munits/ml) on the efflux of 3-*O*-[^{14}C]methylglucose (Clausen, T., unpublished).

Some of the conditions tested above have also been found to stimulate the influx of calcium in muscle [29, 30] and Elbrink et al., in preparation). In order to determine the possible role of extracellular calcium in the stimulation of sugar transport, the experiments were repeated in a buffer from which calcium had been omitted, and EGTA (0.5 mM) added so as to obtain a further reduction in its Ca^{2+} level. From Fig. 7 it can be seen that under these conditions, electrical stimulation (2

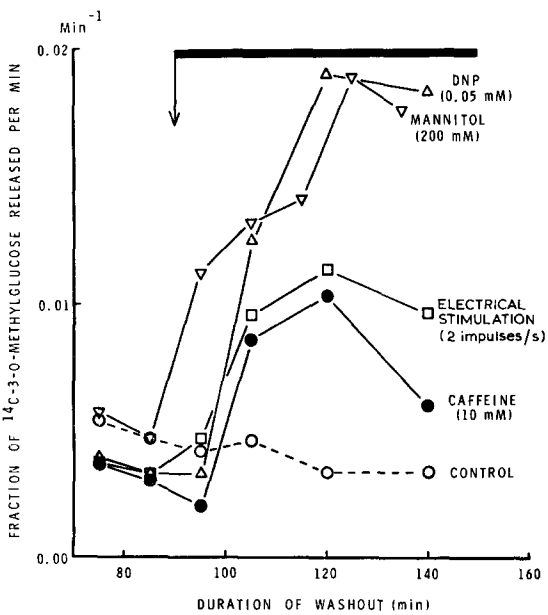

Fig. 7. Effect of Ca omission on the response to various stimuli for 3-*O*-methylglucose transport. Experimental conditions as described in the legend to Fig. 1. The washout of labelled 3-*O*-methylglucose took place in Krebs–Ringer bicarbonate buffer from which calcium had been omitted and EGTA (0.5 mM) added so as to obtain a further lowering of the Ca^{2+} concentration. Each curve represents the mean of 2–4 observations.

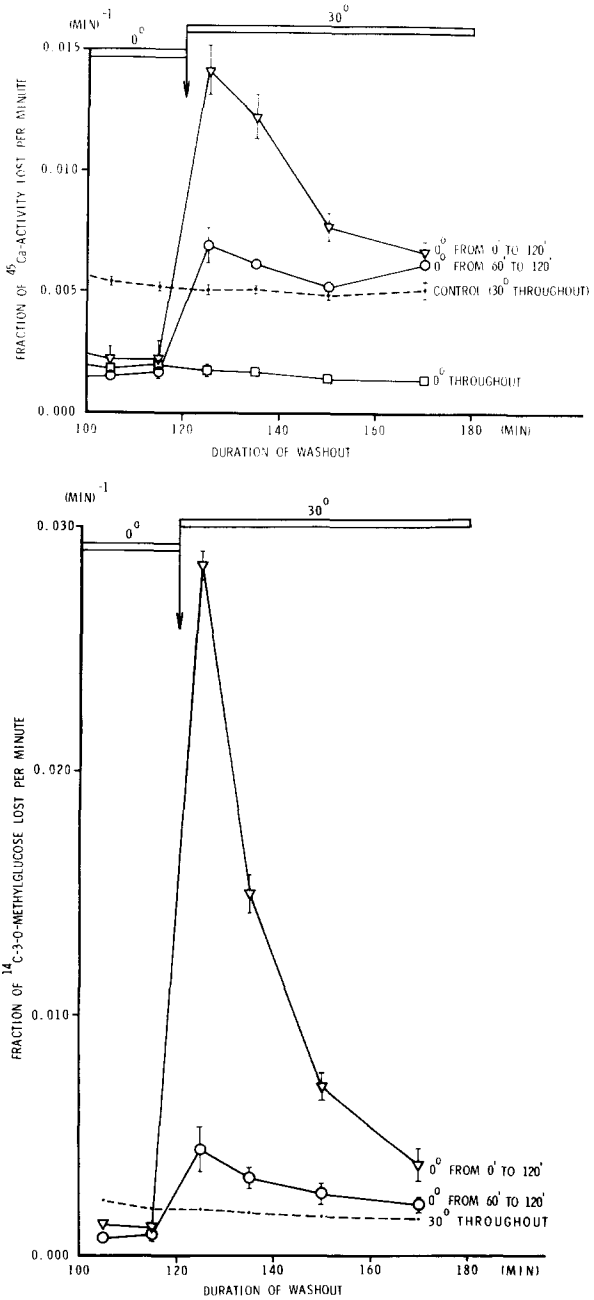

Fig. 8. Effect of cooling an the release of ^{45}Ca and 3-*O*-[^{14}C]methylglucose from rat soleus muscle. Experimental conditions as described in the legend to Fig. 1. All loading took place at 30 °C, but as indicated, the washout of some groups of muscles was performed at 0 °C for 60, 120 or 180 min. From the time indicated by the arrow, the washout was in some cases allowed to continue at 30 °C. Each curve represents the mean of 3–4 observations with bars indicating S.E. (A) The washout of ^{45}Ca. (B) The washout of 3-*O*-[^{14}C]methylglucose.

impulses per s), hyperosmolarity, 2,4-dinitrophenol and caffeine still stimulated the release of 3-*O*-[^{14}C]methylglucose. It should be noted that the effect of caffeine on the washout of labelled sugar was much earlier in onset (cf. with Fig. 4).

In kidney cells, exposure to 4 °C markedly decreases the rate coefficient of ^{45}Ca release, and the return to the standard incubation temperature of 37 °C induces a prompt rise to levels considerably above those of uncooled controls [31]. The magnitude of this rebound efflux was correlated to the duration of cooling. This suggested another means of increasing the Ca^{2+} level in the cytoplasm, and it was therefore determined whether the release of ^{45}Ca and 3-*O*-[^{14}C]methylglucose in rat soleus muscle were affected by exposure to 0 °C with subsequent rewarming. As shown in Figs. 8A and 8B, lowering of the temperature of the efflux medium leads to a decrease in the rate coefficients for the release of ^{45}Ca and 3-*O*-[^{14}C]methylglucose. Within the first 10-min period after the return to the standard incubation temperature of 30 °C, both rate coefficients showed a marked rise. The magnitude of these transient responses depended upon the duration of the exposure to cold, and after 120 min of incubation at 0 °C, the peak values of the rate coefficients for the release of labelled calcium and 3-*O*-methylglucose were, respectively, 2.8 and 14.6 times the control values measured at 30 °C. Phlorizin (5 mM) suppressed the maximum rise in sugar efflux by 70.2 % ($p < 0.001$), indicating that this represents a stimulation of carrier-mediated sugar transport. In agreement with an earlier report [32], cooling was also found to produce a slight increase in tension.

From Table I it can be seen that the stimulating effect of exposure to the cold on sugar transport is not restricted to efflux only, but that the uptake of 3-*O*-[^{14}C]-methylglucose is also increased. When measured during a 60-min incubation period,

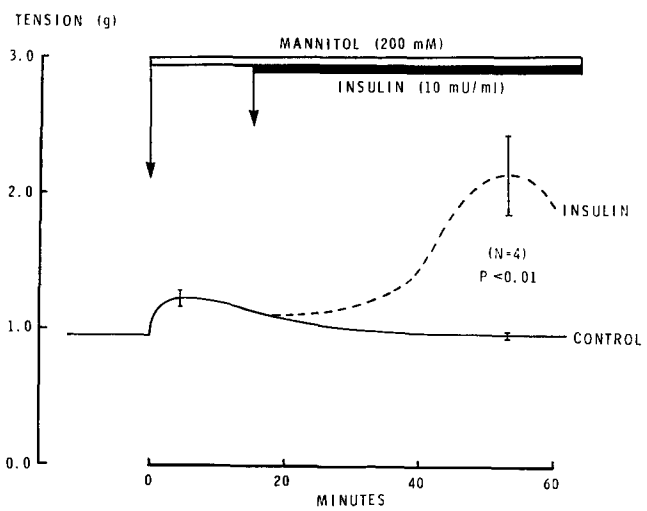

Fig. 9. Effect of hyperosmolarity and insulin on tension in rat soleus muscle. Soleus muscles were prepared from 48 h fasted rats and tension recorded as described in the legend to Fig. 1. The incubation medium was the standard Krebs–Ringer bicarbonate buffer to which 0.1 % dialyzed bovine serum albumin had been added. Two groups of muscles were both exposed to hyperosmolarity by the addition of mannitol (200 mM), one served as a control, and the other was given insulin as indicated. Each curve represents the mean of 4 observations with bars indicating S.E.

the increase is not very marked, presumably due to the transient nature of this phenomenon.

On the basis of some of the relationships between sugar transport, tension and ^{45}Ca washout described above, it appeared reasonable to expect that the major regulator of sugar transport, insulin, might also affect calcium-distribution. However, even in a large series of experiments, it was not possible to detect any significant effect of the hormone on ^{45}Ca uptake, ^{45}Ca release or tension. Only when calcium-exchange and tension were increased by incubation in a hyperosmolar buffer, could it be demonstrated that the addition of insulin induced a quite marked further rise in tension (Fig. 9). This was seen both in fasted and fed animals, and did not depend on the presence of albumin, glucose or other substrates in the incubation medium. Insulin antibody prepared from rabbits completely abolished this effect without inducing any change in the resting tension or that induced in the hyperosmolar medium. A significant effect could often, although not consistently, be demonstrated with concentrations as low as 2.5 munits/ml.

DISCUSSION

The purpose of this study has been to test the hypothesis originally proposed by Holloszy and Narahara [2], that Ca^{2+} might be of importance in the processes by which the glucose transport system is activated. This contention was primarily based on the observation that contractile activity induced by electrical stimulation, K^+-depolarization or caffeine led to an increase in the permeability to 3-*O*-methylglucose in frog sartorius muscle. Further evidence was obtained by the finding that the potentiation of twitch tension by nitrate ions also was associated with an increased uptake of this sugar [3]. It was proposed that this could be related to the inhibitory effect of NO_3^- on the uptake of calcium in the sarcoplasmic reticulum.

In agreement with this evidence, Ilse and Ong [6] found that when 50 mM of NaCl in the incubation medium was replaced by D-mannitol, both the uptake of ^{45}Ca and 3-*O*-methylglucose in rat diaphragm were stimulated by around 50 %. In addition, studies with whole epididymal fat pads showed that 2,4-dinitrophenol, cyanide, epinephrine, adrenocorticotropin and glucagon produced a rapid rise in the rate coefficient for the release of ^{45}Ca from preloaded tissues, indicating that the cytoplasmic level of free Ca^{2+} was increased [4, 5]. The same agents induced a stimulation of 3-*O*-methylglucose efflux which was correlated in time with the accelerated release of labelled Ca^{2+} [18]. Furthermore incubation with ouabain, in K^+-free or Na^+-substituted media was found to promote the retention of labelled calcium [5], and under the same conditions, the transport of 3-*O*-methylglucose and glucose consumption were gradually accelerated [5, 18, 33].

From the present study with rat soleus muscle, it would appear that with some stimuli (electrical stimulation 2,4-dinitrophenol and hyperosmolarity), there is a correlation in time between the rise in 3-*O*-[^{14}C]methylglucose efflux, the increase in tension and the release of ^{45}Ca. The data are compatible with the idea that these factors induce an increase in the cytoplasmic level of free Ca^{2+} ions which precedes or coincides with the rise in sugar permeability.

It is well established that electrical stimulation causes a rise in the cytoplasmic level of Ca^{2+}, the main source of which is the sarcoplasmic reticulum [34].

Inhibition of energy production is likely to interfere with the accumulation of calcium in mitochondria and the sarcoplasmic reticulum, and the binding of calcium to sarcolemma has also been reported to require ATP [35]. In the presence of 2,4-dinitrophenol, therefore, all of these pool might contribute to a rise in the cytoplasmic Ca^{2+} level. This is reflected in the marked increase in the washout of labelled calcium found in earlier [5, 16, 36] as well as the present study. Furthermore, the tension may increase, and depending on the degree of ATP-depletion, a state of rigor may later develop.

The calcium-mobilizing effect of hyperosmolarity has not been characterized in detail, and it is difficult to identify the source of the rise in ^{45}Ca release induced by this condition (Fig. 3). Two pools may be involved, the sarcoplasmic reticulum and the plasma membrane. In sarcoplasmic vesicles isolated from skeletal muscle [37, 38] and smooth muscle [39], both Na^+ and K^+ have been found to impede the accumulation of calcium. In a hyperosmolar environment, the shrinking of the cells will lead to a rise in the cytoplasmic concentrations of Na^+ and K^+, and this might favour a redistribution of Ca^{2+} from the sarcoplasmic reticulum into the cytoplasm. In plasma membranes isolated from erythrocytes, the binding of calcium is diminished by increasing the ionic strength [40, 41], and in artificial lipid membranes, the adsorption of calcium is decreased by increasing the concentration of NaCl or KCl [42]. Thus it seems reasonable to assume that an increase in the ionic strength of the cytoplasm may induce a release of calcium from the inner surface of the plasma membrane which could contribute to the rise in the cytoplasmic level of Ca^{2+}.

Cold exposure and rewarming has not to our knowledge been taken into consideration as a factor which might stimulate sugar transport, and for the present purpose, it was selected because this procedure suggested itself as a convenient means of inducing a graded increase in the cytoplasmic level of Ca^{2+} [31]. Like kidney cells, soleus muscles were found to show a large rise in the rate coefficient for the release of ^{45}Ca when returned to the standard incubation temperature of 30 °C. This marked rebound phenomenon which depended on the duration of the cooling is compatible with the idea that the concentration of free Ca^{2+} available for transport out of the cells had been increased during the exposure to 0 °C. The fact that 3-*O*-methylglucose efflux showed a concomitant rise provides a further argument for the hypothesis that calcium is involved in the stimulation of the glucose transport system. Apart from the theoretical significance of this observation, it would appear that the widely used practice of chilling tissues before incubation is undesirable in studies of calcium and sugar transport.

Potassium contractures have earlier been shown to lead to an increase in the rate of 3-*O*-methylglucose uptake in frog sartorius muscle (as measured after the return to the standard incubation medium with normal K^+-content) [2]. K^+-substitution has otherwise been shown to inhibit basal glucose uptake [43, 44] sugar transport [43, 45], and to suppress the stimulating effect of various agents on sugar transport in rat muscle [33, 45, 46]. In the present experiments, K^+-substitution was found to induce an immediate and transient rise in tension, which was associated with an increase in the washout of ^{45}Ca. The efflux of 3-*O*-[^{14}C]methylglucose, however, was not increased before the muscles were returned to the standard incubation medium containing 6 mM of K^+, because in a K^+-rich buffer sugar transport appears to be relatively resistant to stimulation. These experiments can therefore not

allow any quantitative evaluation of the potential role of Ca^{2+} in stimulating sugar transport.

The importance of following the time-course of changes in the permeability is further illustrated by the experiments with caffeine. In frog sartorius muscle, this drug was found to stimulate the uptake of 3-*O*-methylglucose [2] and D-xylose [47]. This effect could be detected after 15 min of exposure, but increased progressively with the duration of incubation [2]. In rat soleus muscle, caffeine produced no significant change in the amount of 3-*O*-[^{14}C]methylglucose taken up within 60 min (Table I), and in the efflux experiments, the rate coefficient for the release of the labelled sugar was not increased before 50 min after the addition of caffeine. The initial effect of caffeine on sugar transport rather seemed to be inhibitory, as reflected in the slight decrease in basal rate of 3-*O*-methylglucose release and the very marked suppression of the stimulating effect of insulin on both influx and efflux. Like in the experiments with K^+-rich medium, it was possible to obtain a clear dissociation in time between the rise in tension and ^{45}Ca release on one hand and the acceleration of 3-*O*-[^{14}C]methylglucose on the other. In both cases, this appears to be the result of interference with the mechanisms leading to activation of the sugar transport system.

The effect of insulin on tension indicates that under certain conditions, the hormone can induce a redistribution of calcium leading to a rise in the cytoplasmic concentration of Ca^{2+}. In soleus muscle, it was not possible to detect any significant effect of insulin on the influx or efflux of ^{45}Ca, but in epididymal fat pads [4] and isolated fat cells [10, 48], insulin was found to accelerate the release of ^{45}Ca. The recent reports that insulin can induce displacement of ^{45}Ca bound to artificial lipid membranes [49] and diminish the binding of ^{45}Ca to plasma membranes isolated from the liver [50], point to the possibility that a significant action of this hormone could consist in a displacement of calcium bound to plasma membrane. At present, it can only be proposed as a working hypothesis [10] that under normal conditions, this leads to a slight localized rise in the cytoplasmic concentration of Ca^{2+}, and the possible significance of such an effect for the action of insulin on sugar transport cannot yet be assessed.

The data reported in the literature together with the present results seem compatible with the view that a rise in the cytoplasmic level of Ca^{2+} can directly or indirectly induce a stimulation of the glucose transport system.

A lowering of the cellular ATP has been proposed as a signal that might elicit stimulation of sugar transport [51, 52].

It cannot be excluded that the factors investigated in the present study may induce a localized decrease in the ATP level. At present, the relative roles of ATP and Ca^{2+} in the activation of the glucose transport system are difficult to determine without further information about their concentrations and specific actions on cellular elements near or at the plasma membrane.

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