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

X. EFFECT OF GLUCOSE TRANSPORT STIMULI ON THE EFFLUX OF ISOTOPICALLY LABELLED CALCIUM AND 3-*O*-METHYLGLUCOSE FROM SOLEUS MUSCLES AND EPIDIDYMAL FAT PADS OF THE RAT

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Summary

(1) The relationship between Ca^{2+} and sugar transport has been studied by comparing the washout of ^{45}Ca and 3-*O*-[^{14}C]methylglucose from preloaded isolated rat soleus muscles and whole epididymal fat pads.

(2) In soleus muscle, nine different agents with well established stimulating effects on glucose transport were all found to produce a marked increase in 3-*O*-[^{14}C]methylglucose washout, which in each instance was preceded by or coincided with a rise in the washout of ^{45}Ca.

(3) Trypsin, 2,4-dinitrophenol, *p*-chloromercuriphenylsulfonic acid, H_2O_2 and hyperosmolarity all produced dose-dependent stimulation of the washout of ^{45}Ca and 3-*O*-[^3H]methylglucose. Regression analysis showed a highly significant correlation between the increases in the two parameters ($P < 0.001$).

(4) Depolarization and Na^+ influx induced by veratrine were found to be associated with a marked rise in ^{45}Ca release followed by stimulation of 3-*O*-[^{14}C]methylglucose washout.

(5) In epididymal fat pads, six different agents known to stimulate glucose transport were found to produce a highly significant ($P < 0.001$) increase in the washout of ^{45}Ca and 3-*O*-[^{14}C]methylglucose.

(6) It is concluded that in the major targets for insulin action, activation of the glucose transport system can be elicited by a rise in cytoplasmic Ca^{2+} concentration brought about by mobilization of Ca^{2+} from endogenous cellular pools.

Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

Introduction

Studies performed with a wide variety of tissues and cell types have suggested that calcium plays a significant role in the function of the glucose transport system. Hence, if Ca influx was stimulated using the ionophore A23187, the uptake of sugars was found to be augmented in thymocytes [1,2], pigeon red cells [3], heart atria [4], cultured myoblasts [5] and the exocrine pancreas [6].

In experiments where the cytoplasmic Ca^{2+} level was increased by inducing a mobilization of calcium from cellular pools, a considerable stimulation of 3-*O*-methylglucose transport was found in muscle [7], thymocytes [1,2] and epididymal fat pads [8]. These effects were shown to be independent of the presence of calcium in the extracellular milieu [7], and it seems reasonable to assume that the activation of the glucose transport system can be elicited by a rise in the cytoplasmic Ca^{2+} level. From this it could be expected that not only agents known to mobilize Ca^{2+} into the cytoplasm would stimulate sugar transport, but furthermore, that most if not all of the many substances known to stimulate sugar transport might act via an influence on the cellular distribution of Ca^{2+}.

The present study was undertaken with the purpose of exploring this possibility in soleus muscle and epididymal adipose tissue of the rat. Particular emphasis was laid on the comparison of the relative time course and magnitude of the changes in the efflux of ^{45}Ca and isotopically labelled 3-*O*-methylglucose.

For all of the 11 different agents tested, the stimulation of sugar transport was found to be preceded by or to coincide with a rise in the washout of ^{45}Ca from the preloaded tissues. Part of the data have been presented earlier in an abbreviated form [9].

Methods

Experiments with soleus muscles

The procedures for the preparation and incubation of isolated rat soleus muscles have been described in detail elsewhere [10]. Muscles weighing 30–40 mg were obtained from fed Wistar rats in the weight range 60–70 g and incubated in Krebs-Ringer bicarbonate buffer containing 1.27 mM Ca^{2+}. All experiments were performed at 30°C, and in order to ensure adequate oxygenation, the muscles were kept agitated by continuous aeration with a mixture of 95% O_2/5% CO_2. The majority of the washout experiments were performed using a standard technique described earlier [10]. The muscles were loaded with isotopically labelled calcium or 3-*O*-methylglucose for 60 min and thereafter washed out in a series of tubes containing unlabelled buffer without or with the additions indicated. The fractional loss of isotope was determined using intervals of from 1 to 10 min duration. As in an earlier study [7], the washout of ^{45}Ca and 3-*O*-[^{14}C]methylglucose was determined in separate series of experiments, but in order to allow a closer comparison, some experiments were performed using combined loading with ^{45}Ca and 3-*O*-[^{3}H]methylglucose. When the channels of the liquid scintillation spectrometer were set for the

separation of ^{45}Ca and ^{3}H activity, the fractional loss of both isotopes could be measured in the same muscle.

In some experiments, the washout was performed using a thermostatically controlled polyethylene chamber (volume 2 ml) in which the muscles were continuously perifused at a flow rate of 2 ml/min. Following the standard loading period of 60 min, the muscle was introduced into the chamber, where it was supported by a nylon grid so as to allow ready access of the perfusion medium with a minimum of damage to the fibers. Using a LKB 2115 peristaltic pump, the chamber could be perfused with buffer from a reservoir which was continuously equilibrated with a mixture of 95% O_2/5% CO_2. In order to obtain more thorough washing of the muscle, the same gas mixture was injected at a carefully adjusted rate into the tube connecting the pump with the chamber. The compounds tested were injected closer to the entry of the chamber, thus allowing almost immediate access to the muscle. Using a fraction collector, samples of the buffer leaving the chamber could be obtained at short intervals of time (down to 30 s). The time-lag for passage of buffer from the chamber to the fraction collector was 7 s. With four chambers in parallel, the system allowed the simultaneous measurement of washout from two pairs of muscles.

Experiments with epididymal fat pads

These were performed essentially as described in earlier reports [11,12]. Whole epididymal fat pads were obtained from fed Wistar rats weighing 110—120 g and, following a wash, loaded for 60 min in polyethylene counting vials containing 3 ml of buffer with ^{45}Ca or 3-*O*-[^{14}C]methylglucose (1 mM). Following 60 min of loading, the fat pads were washed out in a series of counting vials containing 2 ml of buffer. At the end of washout, the radioactivity remaining in the tissue was determined, and by counting the radioactivity released into the washout vials, the fractional loss could be calculated as described earlier [11]. All fat pad experiments were performed at 37° C and the Krebs-Ringer bicarbonate buffer used contained 1% of dialyzed bovine serum albumin.

Chemicals, hormones and isotopes

All chemicals used were of analytical grade. Bovine serum albumin was used after dialysis against distilled water for 24 h at 4° C, followed by neutralization with NaOH. Albumin, *p*-chloromercuribenzoic acid, *p*-chloromercuriphenylsulfonic acid, 2,4-dinitrophenol, trypsin and veratrine were all obtained from Sigma Co., St. Louis. Adrenaline was a product of Rhone-Poulenc (Paris). ^{45}Ca (1000 Ci/mol) was obtained from the Danish Atomic Energy Commission Isotope Laboratory, Risø, Denmark. 3-*O*-[^{3}H]Methylglucose (2—5 Ci/mol) and 3-*O*-[^{14}C]methylglucose (50 Ci/mol) were products of The Radiochemical Centre, Amersham, U.K.

Results

Fig. 1 shows the time-course of the effects of veratrine on the washout of ^{45}Ca and 3-*O*-[^{14}C]methylglucose from preloaded rat soleus muscles. This

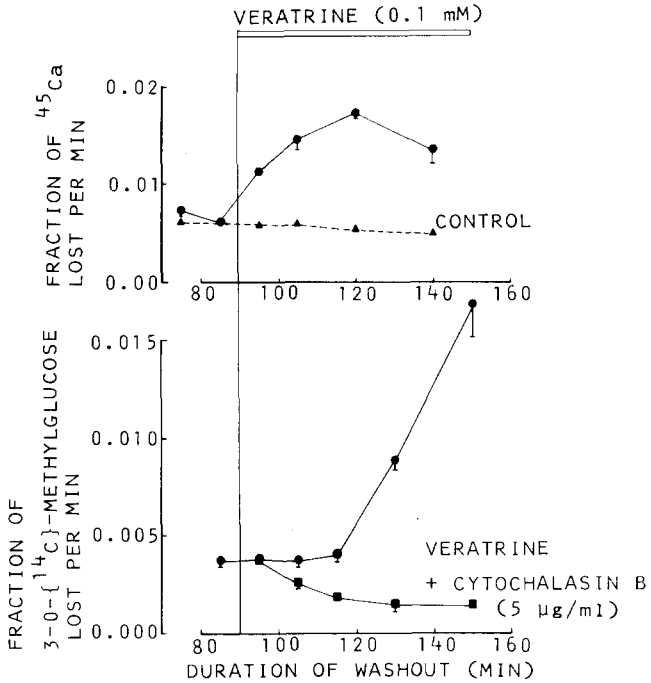

Fig. 1. Effect of veratrine on the release of ^{45}Ca and 3-*O*-[^{14}C]methylglucose from rat soleus muscle. Isolated rat soleus muscles were loaded by incubation for 60 min at 30° C in Krebs-Ringer bicarbonate buffer containing either 1 mM 3-*O*-[^{14}C]methylglucose (2 µCi/ml) or 1.27 mM ^{45}Ca (2 µCi/ml). They were then transferred through a series of tubes containing 3 ml of unlabeled buffer without or with the additions indicated. At the end of washout, the radioactivity retained in the tissue and the amount released were determined. The fraction of radioactivity released during each interval was calculated as described elsewhere [11]. Each curve represents the mean of three observations with the vertical bars indicating the S.E. where this exceeds the size of the symbols.

compound was selected because it is known to induce depolarization, Na^+ influx, release of Ca^{2+} into the cytoplasm and contraction in muscle [13]. It appears that within the first 10 min of exposure, there is a significant rise in the washout of ^{45}Ca, and it was noted that the muscles underwent contraction indicating that the cytoplasmic concentration of Ca^{2+} was elevated. The washout of 3-*O*-[^{14}C]methylglucose was only clearly increased about 40 min after the addition of veratrine. This could not be ascribed to non-specific cell damage as indicated by the fact that the rise was totally abolished by cytochalasin B (5 µg/ml).

In the following, a series of agents known to stimulate sugar transport were tested for possible effects on ^{45}Ca washout. These experiments were performed in two different ways; either simply by transferring the muscles through a series of tubes at 1—2 min intervals or, as described in Methods, by using a fraction collector for the repeated sampling of the effluent from a small polyethylene chamber in which the muscle was placed after loading with isotopic Ca or 3-*O*-methylglucose. Both methods provided more detailed information of the time-lags involved in the action of the agents tested.

Several studies have shown that the exposure to hyperosmolar incubation

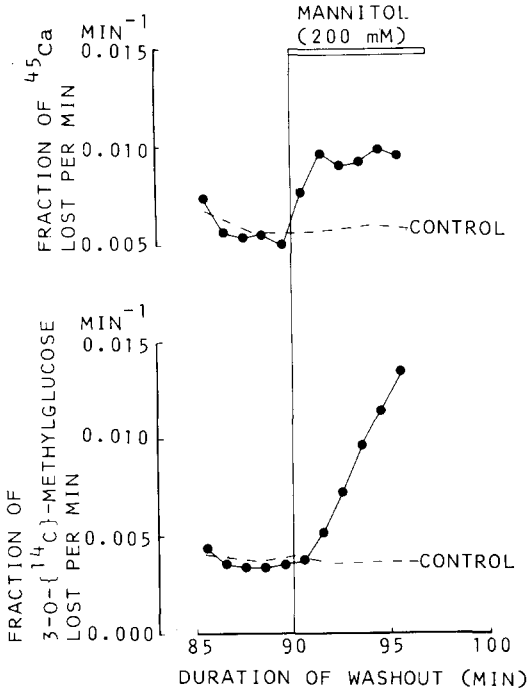

Fig. 2. Effect of hyperosmolarity on the washout of ^{45}Ca and 3-*O*-[^{14}C]methylglucose from rat soleus muscle. Experimental conditions as described in the legend to Fig. 1. The washout medium was made hyperosmolar by addition of mannitol at the indicated concentration. Each curve represents the mean of two to three observations.

media leads to a marked stimulation of sugar transport [7,12]. More recently, it was reported that in the rat soleus muscle, hyperosmolarity induces a rise in resting tension which can be detected within seconds, indicating that there is a rapid rise in the cytoplasmic concentration of Ca^{2+} [14]. From Fig. 2 (upper panel), it can be seen that within the first minute of exposure to buffer made hyperosmolar by the addition of mannitol (200 mM), the fractional loss of ^{45}Ca is markedly stimulated. From the lower panel of the same figure it can be seen that the washout of 3-*O*-[^{14}C]methylglucose is not increased before during the second minute of exposure and that there is a continued rise in the fractional loss of the sugar even when the fractional loss of ^{45}Ca has reached a relatively constant level.

Trypsin, another agent known to stimulate sugar transport in muscle [15], and adipocytes [16] were found to induce a large and rapid rise in the fractional loss of ^{45}Ca (Fig. 3, upper panel). Whereas this was clearly detectable within the first minute of exposure, the efflux of 3-*O*-[^{14}C]methylglucose did not show any statistically significant increase before during the fourth minute of exposure to trypsin (Fig. 3, lower panel).

Also for 2,4-dinitrophenol, it was possible to detect an early stimulation of ^{45}Ca efflux followed by a rise in 3-*O*-[^{14}C]methylglucose efflux (Fig. 4, upper and lower panel, respectively). The stimulating effect of other metabolic poisons, salicylate (5 mM), and CN^- (2 mM), on 3-*O*-[^{14}C]methylglucose efflux

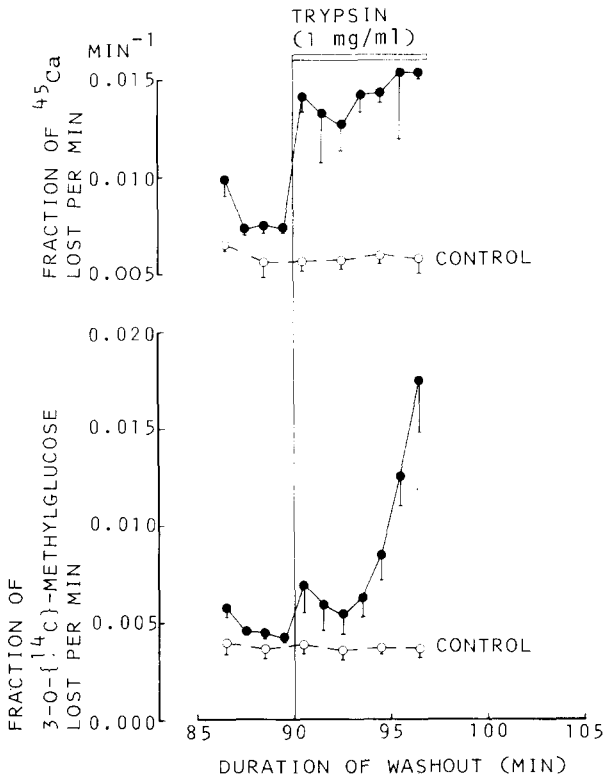

Fig. 3. Effect of trypsin on the washout of ^{45}Ca and 3-*O*-[^{14}C]methylglucose from rat soleus muscle. Experimental conditions as described in the legend to Fig. 1. Each curve represents the mean of four observations with vertical bars indicating S.E.

was also found to be preceded by an acceleration of ^{45}Ca washout (see Tables I and II).

p-Chloromercuribenzoic acid, *p*-chloromercuriphenylsulfonic acid and H_2O_2, which are well characterized stimuli for glucose transport in adipocytes [17,18], were studied in several series of experiments (see also Tables I and II and Fig. 7). All of these agents gave a clear-cut stimulation of both ^{45}Ca and 3-*O*-[^{14}C]methylglucose efflux, but neither repeated washing experiments with sampling at 1—2 min intervals, nor perifusion experiments allowed us to detect a significant time-lag between the two effects. As can be seen from Fig. 5, *p*-chloromercuribenzoic acid (0.5 mM) induces an almost simultaneous and continuous rise in the release of ^{45}Ca and 3-*O*-[^{14}C]methylglucose into the perifusing buffer. Also with H_2O_2 (5 mM), the increases in the fractional loss of the two isotopes appeared to coincide, but here the rate of 3-*O*-[^{14}C]methylglucose efflux continued to rise even after the fractional loss of ^{45}Ca had reached a relatively constant level (Fig. 6). At a concentration of 1 mM, H_2O_2 gave a somewhat smaller, albeit highly significant stimulation of both ^{45}Ca and 3-*O*-[^{14}C]methylglucose efflux (Table I).

It should be noted that, as for veratrine, all of the stimulating effects on 3-*O*-[^{14}C]methylglucose efflux described above were considerably or com-

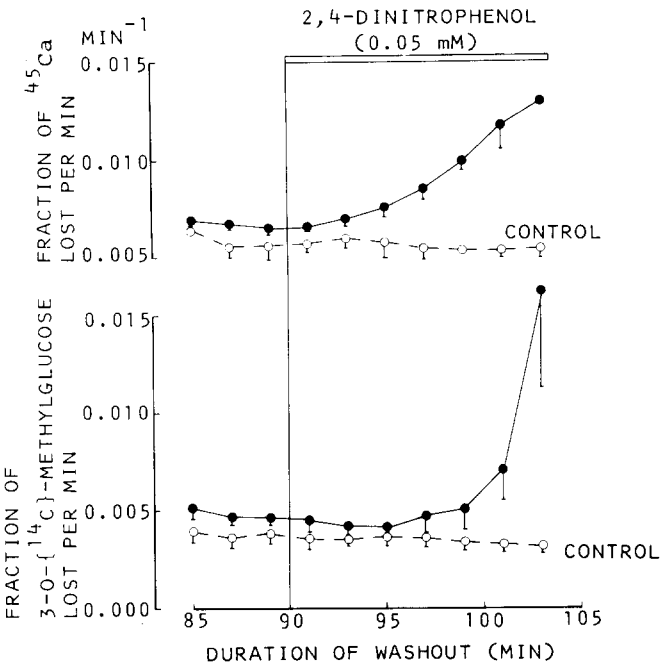

Fig. 4. Effect of 2,4-dinitrophenol on the release of ^{45}Ca and 3-*O*-[^{14}C]methylglucose from rat soleus muscle. Experimental conditions as described in the legend to Fig. 1. Each curve represents the mean of four observations with vertical bars indicating S.E.

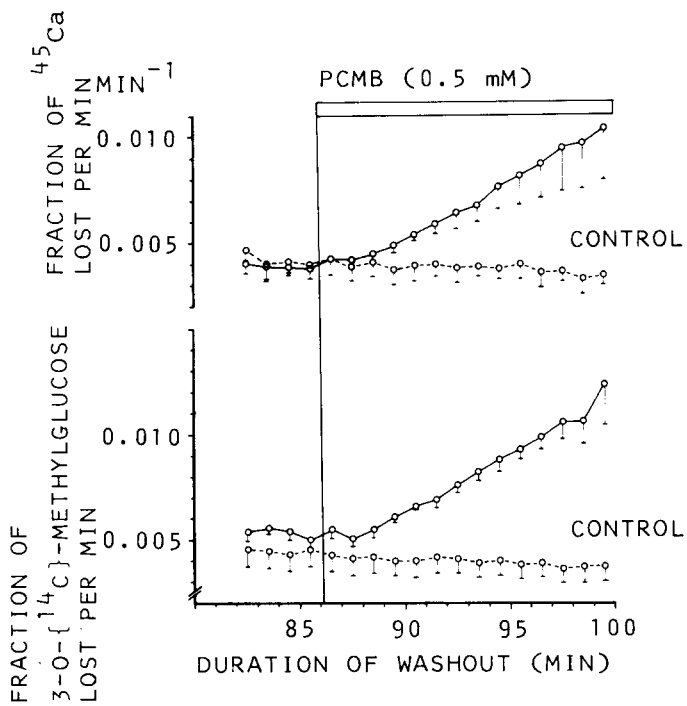

Fig. 5. Effect of *p*-chloromercuribenzoic acid (PCMB) on the release of ^{45}Ca and 3-*O*-[^{14}C]methylglucose from rat soleus muscle. Following the standard loading period described in the legend to Fig. 1, the muscle was introduced into a thermostatically controlled polyethylene chamber in which it was continuously perifused. Using a fraction collector, samples of the buffer leaving the chamber could be obtained at short intervals of time. The fractional loss of radioactivity during each interval of time was calculated as described earlier [11]. Each curve represents the mean of three observations with vertical bars indicating S.E.

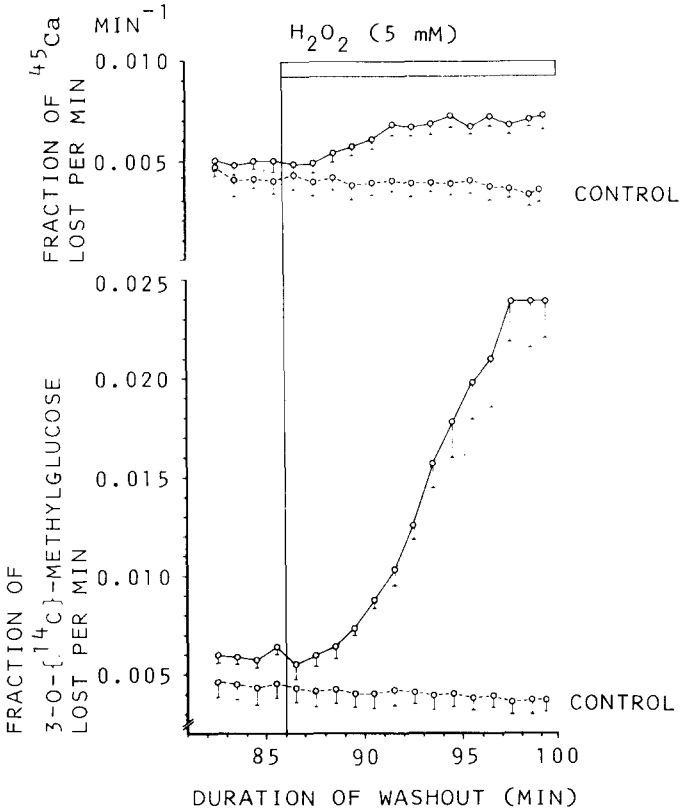

Fig. 6. Effect of H_2O_2 on the efflux of ^{45}Ca and 3-*O*-[^{14}C]methylglucose from rat soleus muscle. Experimental conditions as described in the legend to Fig. 5. Each curve represents the mean of three observations with vertical bars indicating S.E.

pletely suppressed by cytochalasin B (5 μg/ml), which did not affect the rise in ^{45}Ca efflux. Furthermore, since essentially similar results were obtained using a Ca^{2+}-free washout medium containing 0.5 mM EGTA, extracellular calcium seemed to be of minor importance for the immediate action of these stimuli (see also Fig. 7 in Ref. 7).

In spite of the differences in the time course and the relative size of the effects, all of the agents tested here and in an earlier study [7] induced a rise in ^{45}Ca efflux which generally preceded or coincided with the stimulation of 3-*O*-methylglucose efflux. In order to illustrate the general nature of this relationship, the maximum rises in the fractional loss of isotopic calcium and sugar were determined and compiled in Table I.

A similar set of data obtained in parallel experiments with whole epididymal fat pads are presented in Table II. As in the experiments with soleus muscles, it is evident that when the different agents are compared, there is no strict correlation between the relative increases in the fractional loss of ^{45}Ca and 3-*O*-[^{14}C]methylglucose. In the fat pad, some agents (mannitol and adrenaline) produce a relatively modest rise in ^{45}Ca efflux, whereas others (H_2O_2 and CN^-) elicit pronounced effects.

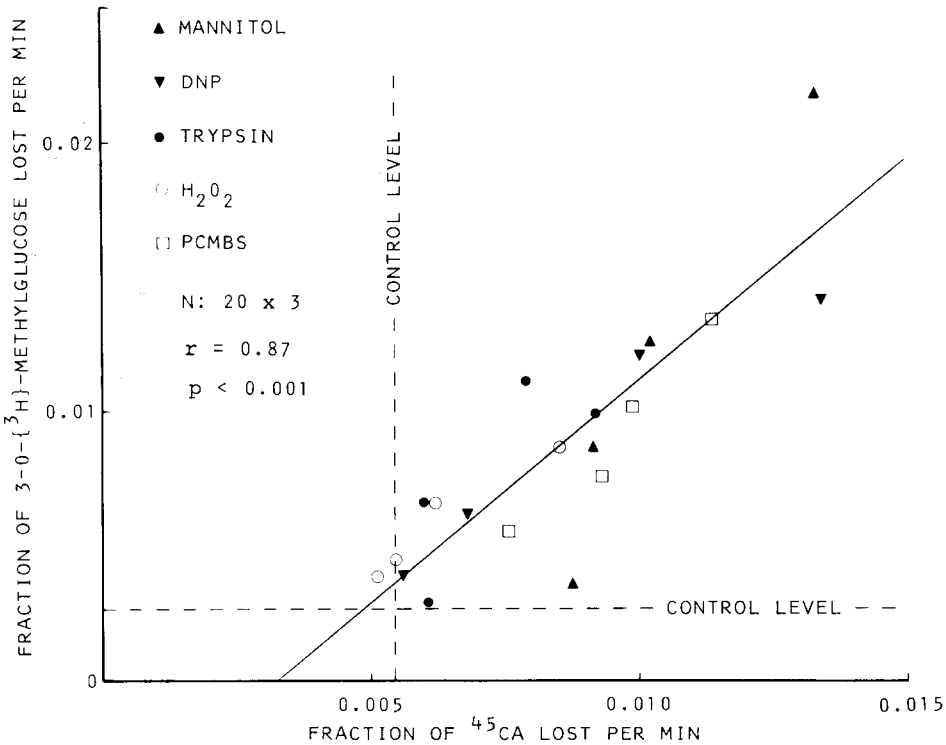

Fig. 7. Relationship between the fractional loss of ^{45}Ca and 3-*O*-[^{3}H]methylglucose induced by glucose-transport stimuli. Rat soleus muscles were loaded by incubation for 60 min in buffer containing 1.27 mM ^{45}Ca (5 μCi/ml) and 1 mM 3-*O*-[^{3}H]methylglucose (10 μCi/ml). The fractional loss of the two isotopes was determined as described in the legend to Fig. 1. Following 90 min of washout, the following agents were added: ●, trypsin (1—500 μg/ml); ○, H_2O_2 (0.1—5.0 mM); ▲, mannitol (100—400 mM); , 2,4-dinitrophenol (DNP) (0.005—0.5 mM); □, *p*-chloromercuriphenylsulfonic acid (PCMBS) (0.5—5.0 mM). The fractional loss of ^{45}Ca as determined within the first 10 min after the addition of these compounds is plotted against the fractional loss of 3-*O*-[^{3}H]methylglucose measured from 30 to 40 min after the onset of stimulation in the same individual muscles. Each point represents the mean of three observations. The full-drawn line is constructed by the method of least squares. The two dashed lines represents the control levels for the fractional loss of ^{45}Ca and 3-*O*-[^{3}H]methylglucose, respectively.

Although the present data only allow us to suggest a qualitative relationship between the mobilisation of isotopic calcium and the stimulation of sugar transport, it seemed justifiable to assess whether each agent, when tested at a series of concentrations, would possibly yield a correlation between the rise in the fractional loss of ^{45}Ca and 3-*O*-[^{3}H]methylglucose. Experiments performed with dual labelling allowed us to obtain a direct comparison of the increase in the two parameters for each individual muscle. For a number of agents (mannitol (100—400 mM), H_2O_2 (0.1—5.0 mM), trypsin (1—500 μg/ml), 2,4-dinitrophenol (0.005—0.5 mM), *p*-chloromercuriphenylsulfonic acid (0.5—5.0 mM)), there was a dose-dependent increase in the fractional loss of both ^{45}Ca and ^{3}H activity. When the increase in the fractional loss of ^{45}Ca measured during the first 10 min period after the addition of the stimulating agents was compared with the subsequent washout of 3-*O*-[^{3}H]methylglucose, a highly significant correlation was obtained. Thus, for the next three washout

TABLE I

EFFECT OF VARIOUS GLUCOSE TRANSPORT STIMULI ON THE WASHOUT OF ^{45}Ca AND 3-*O*-[^{14}C]METHYLGLUCOSE FROM RAT SOLEUS MUSCLES

Experimental conditions as described in the legend to Fig. 1. The maximum values reached following the addition of each agent are given as mean ± S.E. with the number of observations in parentheses. All values presented are significantly higher than the unstimulated control level ($P < 0.01$). PCMB, *p*-chloromercuribenzoic acid; PCMBS, *p*-chloromercuriphenylsulfonic acid; DNP, 2,4-dinitrophenol.

Stimulating agent (added 90 min after the onset of washout)	^{45}Ca washout (fraction of ^{45}Ca activity lost per min)	3-*O*-[^{14}C]methylglucose washout (fraction of ^{14}C activity lost per min)
Controls	0.0053 ± 0.0002 (17)	0.0036 ± 0.0004 (13)
Trypsin (0.1 mg/ml)	0.0090 ± 0.0007 (7)	0.0222 ± 0.0028 (6)
Mannitol (200 mM)	0.0092 + 0.0008 (13)	0.0134 ± 0.0015 (9)
H_2O_2 (1 mM)	0.0067 ± 0.0002 (7)	0.0102 ± 0.0008 (10)
H_2O_2 (5 mM)	0.0095 ± 0.0005 (8)	0.0204 ± 0.0027 (5)
PCMB (0.1 mM)	0.0111 ± 0.0005 (8)	0.0098 ± 0.0008 (9)
PCMBS (1.0 mM)	0.0107 ± 0.0006 (6)	0.0078 ± 0.0006 (5)
DNP (0.05 mM)	0.0136 ± 0.0011 (8)	0.0193 ± 0.0014 (16)
Veratrine (0.1 mM)	0.0174 ± 0.0004 (5)	0.0143 ± 0.0018 (9)
Salicylate (5.0 mM)	0.0118 ± 0.0008 (3)	0.0140 ± 0.0010 (3)
Caffeine (10 mM)	0.0065 ± 0.0003 (3)	0.0098 ± 0.0011 (5)

periods the regression lines for the correlation between the two parameters had *P* values below 0.005, 0.001 and 0.001, respectively (Fig. 7). It should be noted that these lines intersect the control levels at points which are not significantly different from the intercept of the dashed lines indicating control levels for the fractional losses of ^{45}Ca and 3-*O*-[^{3}H]methylglucose.

TABLE II

EFFECT OF VARIOUS GLUCOSE TRANSPORT STIMULI ON THE WASHOUT OF ^{45}Ca AND 3-*O*-[^{14}C]METHYLGLUCOSE FROM WHOLE EPIDIDYMAL FAT PADS

Fat pads were obtained from 110—120 g fed male Wistar rats and loaded with either ^{45}Ca or 3-*O*-[^{14}C]-methylglucose for 60 min. They were then washed in a series of vials containing non-radioactive buffer and the fractional loss of each isotope determined as described earlier [11]. The maximum values reached following the addition of each agent are given as mean ± S.E. with the number of observations in parentheses. All values presented are significantly higher than the unstimulated control level ($P < 0.005$). PMCB, *p*-chloromercuribenzoic acid.

Stimulating agent (added 90 min after the onset of washout)	^{45}Ca washout (fraction of ^{45}Ca activity lost per min)	3-*O*-[^{14}C]methylglucose washout (fraction of ^{14}C activity lost per min)
Controls	0.021 ± 0.001 (8)	0.012 ± 0.001 (9)
Trypsin (0.1 mg/ml)	0.037 ± 0.002 (6)	0.028 ± 0.004 (7)
Mannitol (450 mM)	0.032 ± 0.004 (3)	0.042 ± 0.005 (3)
H_2O_2 (1 mM)	0.036 ± 0.004 (3)	0.021 ± 0.002 (3)
H_2O_2 (5 mM)	0.063 ± 0.004 (3)	0.037 ± 0.001 (3)
PCMB (0.5 mM)	0.040 ± 0.005 (3)	0.048 ± 0.003 (3)
NaCN (2 mM)	0.035 ± 0.001 (5)	0.022 ± 0.003 (3)
Adrenalin (10^{-5} M)	0.030 ± 0.001 (5)	0.028 ± 0.003 (8)

Discussion

A wide variety of agents have been shown to mimic the action of insulin in stimulating the transport of glucose and other sugars across the plasma membrane in muscle and adipocytes. This offers several possibilities for testing the proposal [19—21] that Ca^{2+} is involved as an obligatory step in the activation of the glucose-transport system. If this were the case, it should be possible to detect a rise in the cytoplasmic Ca^{2+} level seen by the glucose-transport system, no matter what agent is used as a stimulus. A further requirement is that this rise should precede or coincide with the stimulation of glucose transport, and finally, for each individual stimulus there is reason to expect a positive correlation between the amount of Ca^{2+} made available and the response of the glucose-transport system. Even though all of the agents tested in the present study appear to act according to these criteria, there is considerable variation with respect to the time-lag of action as well as the relative magnitude of the changes in the washout of ^{45}Ca and 3-*O*-[^{14}C]methylglucose. Thus, veratrine was found to induce an early and very large increase in ^{45}Ca release followed by a late and progressive stimulation of 3-*O*-[^{14}C]methylglucose efflux. Also in the fat-pad experiments, the time course and the absolute magnitude of the rise in the same two parameters showed considerable variation with different stimulating agents (see Table II). This variability has several explanations.

First, although it has been demonstrated that the rate of ^{45}Ca efflux depends on the concentration of free Ca^{2+} available for transport in the cytoplasm [22,23], the time course of Ca^{2+} mobilization is likely to depend on the nature, localization and size of the cellular calcium pool from which it originates. For instance, whereas 2,4-dinitrophenol and H_2O_2 have been shown to induce Ca^{2+} release from mitochondria [24,25], caffeine and veratrine are, rather, mobilizing Ca^{2+} from the sarcoplasmic reticulum and trypsin from the plasma membrane [26].

Second, in addition to their effect on various cellular calcium pools, the different agents tested may influence the transport processes by which Ca^{2+} gains access to the washout medium. For instance, ATP depletion induced by metabolic poisons can interfere with the function of Ca^{2+}-activated transport ATPase in the plasma membrane. Furthermore, changes in the cytoplasmic Na^+ and K^+ concentrations can alter the conditions for clearing cytoplasmic Ca^{2+} via the Na^+-Ca^{2+} exchange mechanism [27].

Third, some of the agents shown to induce Ca^{2+} mobilization may at the same time have an intrinsic inhibitory action on the glucose-transport system. Thus, it was earlier demonstrated that caffeine can, to a considerable extent, suppress the stimulating effect of insulin on 3-*O*-[^{14}C]methylglucose efflux [7,28]. Furthermore, ATP depletion induced by 2,4-dinitrophenol or other metabolic poisons was shown to interfere with the action of insulin on glucose transport in fat cells [29] and soleus muscles [30].

For these reasons, the patterns of ^{45}Ca release and sugar-transport stimulation are bound to show variations dependent on the nature of the agents used. However, for an individual agent, it can be expected that provided there is a dose-dependent mobilization of Ca^{2+} into the cytoplasm, the stimulation

of glucose transport will show progressive increase with the rise in the cytoplasmic Ca^{2+} level. Although it should again be recalled that the fractional loss of ^{45}Ca from the tissue only represents an indirect reflection of the free Ca^{2+} level in the cytoplasm, this expectation seems to be fulfilled by the demonstration of highly significant correlations between the stimulation of ^{45}Ca release and 3-*O*-[^{3}H]methylglucose washout (see Fig. 7).

It seems resonable to conclude that even though Ca^{2+} is not necessarily essential for the activation of the glucose-transport system, the appearance of Ca^{2+} at the inner surface of the plasma membrane seems to be a common feature of the action of a wide variety of glucose-transport stimuli. This study has been restricted to such 'insulin-like' substances, and it is evident that their Ca^{2+}-mobilizing action is earlier and more pronounced than that of insulin (see Refs. 7 and 31). This is surprising in view of the pronounced effect of this hormone on glucose transport. On the other hand, the data indicate that certain insulin-like substances elicit a large rise in sugar transport in spite of a modest change in ^{45}Ca efflux. The 'efficiency' of Ca^{2+} in eliciting an activation of the glucose-transport system is likely to depend on the conditions in the microenvironment of the plasma membrane, and very small amounts of calcium may be adequate, provided they are made available in the right locus [32]. Since the 'clearing' of calcium from the cytoplasm is an energy-requiring process, it seems reasonable to assume that natural stimuli for the glucose transport would act with a minimum of rise in cytoplasmic Ca^{2+} concentration. This may be part of the explanation for the relatively small size of the calcium mobilization induced by insulin.

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