The Effect of Insulin on the Washout of [^{45}Ca]Calcium from Adipocytes and Soleus Muscle of the Rat

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Insulin stimulates the washout of ^{45}Ca from preloaded isolated fat-cells, whole epididymal fat-pads and isolated soleus muscles of the rat. This effect occurs within 10 min after the addition of the hormone, and it can be detected at concentrations down to those measured in rat plasma. When K^+ is omitted from the washout medium, the effect on soleus muscles is more pronounced and increases with the time of exposure.

In whole epididymal fat-pads of the rat, insulin was found to stimulate the washout of ^{45}Ca (Clausen, 1969b). Preliminary studies indicated that isolated fat-cells showed a similar response (Martin *et al.*, 1973), and in both liver plasma membranes (Marinetti *et al.*, 1972) and 'ghosts' of fat-cells insulin was found to diminish the binding of Ca^{2+} (Kissebah *et al.*, 1975). In a hyperosmolar milieu it could be demonstrated that insulin elicits a marked rise in the resting tension of rat soleus muscles (Clausen *et al.*, 1974, 1975), and it was shown that, in cultured chick myoblasts, insulin stimulates the release of ^{45}Ca (Schudt *et al.*, 1976). All of these observations indicated that the hormone might exert a hitherto poorly characterized action on some cellular calcium pool, eventually leading to a mobilization of Ca^{2+} ions. The present paper describes the results of experiments designed to detect and quantify possible effects of insulin on the exchange of ^{45}Ca in intact fat-cells and muscles of the rat.

Experimental

Materials

^{45}Ca (360 mCi/mmol) was obtained from the Isotope Laboratory, Danish Atomic Energy Commission, Risø, DK 4000 Roskilde, Denmark. Monocomponent pig insulin (lot no. MC-S 823081, 25 units/mg) was a gift from the Novo Research Laboratories, Copenhagen, Denmark. Bovine serum albumin was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and used after 24 h of dialysis against water in the cold.

Methods

Experiments with adipocytes. The techniques for the preparation of isolated fat-cells, the incubation media and the procedures for sampling of cells, as

well as counting of ^{45}Ca radioactivity, were described previously (Martin *et al.*, 1975).

The washout of ^{45}Ca from whole epididymal fat-pads was measured as described elsewhere (Clausen, 1970), and the fraction of ^{45}Ca released from the tissue per min was calculated as in analogous experiments with 3- O -[^{14}C]methylglucose (Clausen, 1969a).

Experiments with soleus muscles. Soleus muscles (wet wt. 25–35 mg) were prepared from young fed rats (60–70 g) as described by Kohn & Clausen (1971) and incubated in Krebs–Ringer bicarbonate buffer (Cohen, 1951). After loading with ^{45}Ca for 60 min, the washout of radioactivity into unlabelled medium was followed for 200 min, and the fraction of ^{45}Ca released per min calculated as earlier reported (Clausen *et al.*, 1975).

Results and Discussion

We have shown (Martin *et al.*, 1975) that the washout of ^{45}Ca from isolated fat-cells displays a very rapid early phase lasting less than 1 min, followed by a much slower phase which probably represents release of ^{45}Ca from intracellular pools. Table 1 shows the effect of adding insulin during the period 10–20 min after the onset of washout. Insulin clearly accelerates the release of ^{45}Ca from the cells, and the effect can be detected at concentrations down to 0.01 munit/ml, well within the physiological range (Blackshear & Alberti, 1974). When added during the later, slow phase of washout (60 min after the onset of washing), insulin produced no significant change in the fractional loss of ^{45}Ca .

In whole epididymal fat-pads, the washout of ^{45}Ca is much slower, and from around 60 min after the onset of washout the fractional loss reaches an almost constant value (Clausen, 1970). Table 2 shows that during this slow phase of washout, insulin at concentrations ranging from 0.05 to 10 munits/ml increased the rate coefficient by 10–18% in the period from 10 to 20 min after its addition. At the higher

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Table 1. *Effect of insulin on the washout of ^{45}Ca from fat-cells*

Isolated fat-cells (1 ml packed volume) were incubated with shaking for 1 h at 37°C in 6 ml of Krebs–Ringer bicarbonate buffer containing 1% bovine serum albumin and 1.27 mM- ^{45}Ca (5 $\mu\text{Ci}/\text{ml}$). At the end of this period the suspension was centrifuged (600 g) and the medium was aspirated off. The cells were washed with 10 ml of the same buffer without ^{45}Ca and resuspended in 6 ml of Krebs–Ringer bicarbonate buffer again with no ^{45}Ca . The cells were incubated with shaking for 10 min, divided into two equal portions, centrifuged, and the medium was aspirated off. Fresh medium was added to one portion of cells and fresh medium containing insulin to the other. Samples were taken immediately and after 10 min incubation. Cells and medium were separated by centrifugation through dinonyl phthalate (Gliemann *et al.*, 1972) and their ^{45}Ca radioactivity was determined. Care was taken to ensure that the cells were evenly suspended before sampling, and that media were thoroughly gassed with O_2/CO_2 (19:1). The fraction of cellular ^{45}Ca released into the medium was calculated and the results expressed as the mean of three observations $\pm$ S.E.M.

Experimental conditions	Fraction of ^{45}Ca released/10 min	Change in relation to controls (%)	P
Control	0.65 $\pm$ 0.02		
Insulin (0.01 munit/ml)	0.84 $\pm$ 0.03	+29	<0.01
Control	0.60 $\pm$ 0.03		
Insulin (0.1 munit/ml)	0.75 $\pm$ 0.01	+25	<0.02
Insulin (1 munit/ml)	0.72 $\pm$ 0.01	+20	<0.05
Control	0.62 $\pm$ 0.01		
Insulin (0.1 munit/ml)	0.80 $\pm$ 0.01	+29	<0.01
Control	0.71 $\pm$ 0.02		
Insulin (1 munit/ml)	0.87 $\pm$ 0.01	+23	<0.01
Control	0.41 $\pm$ 0.04		
Insulin (10 munits/ml)	0.65 $\pm$ 0.02	+59	<0.01

Table 2. *Effects of insulin, Ca^{2+} and ZnCl_2 on the washout of ^{45}Ca from adipose tissue*

Whole epididymal fat-pads were prepared from fed male Wistar rats weighing 100–120 g and loaded with ^{45}Ca by incubation for 60 min in Krebs–Ringer bicarbonate buffer containing 1% dialysed bovine serum albumin and 1.27 mM- ^{45}Ca (2 $\mu\text{Ci}/\text{ml}$). They were then transferred through a series of counting vials containing 2 ml of unlabelled buffer without or with Ca (1.27 mM). At the end of washout, the radioactivity retained in the tissue and the amount released during each washout period was determined, and the fractional loss of ^{45}Ca calculated as previously described (Clausen, 1969a, 1970). The fraction of ^{45}Ca lost in the interval from 100 to 110 min after the onset of washout is given, $\pm$ S.E.M. Insulin and ZnCl_2 , when added, were present from 90 min after the onset of washout. The number of observations is given in parentheses.

Experimental conditions	Fraction of ^{45}Ca released/10 min	Change in relation to controls (%)	P
(a) Krebs–Ringer bicarbonate buffer in which CaCl_2 had been replaced by an equi-osmolar amount of NaCl			
Control	0.199 $\pm$ 0.004 (5)		
Insulin (0.05 munit/ml)	0.218 $\pm$ 0.004 (5)	+10.0	<0.01
Control	0.191 $\pm$ 0.007 (13)		
Insulin (0.1 munit/ml)	0.217 $\pm$ 0.008 (13)	+13.5	<0.025
Control	0.214 $\pm$ 0.008 (10)		
Insulin (1 munit/ml)	0.251 $\pm$ 0.015 (10)	+17.5	<0.05
Control	0.180 $\pm$ 0.007 (13)		
Insulin (10 munits/ml)	0.212 $\pm$ 0.007 (12)	+17.7	<0.01
Control	0.191 $\pm$ 0.006 (7)		
ZnCl_2 (10 μM)	0.187 $\pm$ 0.006 (7)	-2.1	>0.10
(b) Krebs–Ringer bicarbonate buffer containing 1.27 mM-Ca			
Control	0.200 $\pm$ 0.212 (4)		
Insulin (1 munit/ml)	0.240 $\pm$ 0.003 (5)	+19.8	<0.01

Table 3. *Effects of insulin, albumin and ZnCl_2 on the washout of ^{45}Ca from soleus muscles*

Isolated rat soleus muscles (tendons resected) were loaded by incubation for 60 min in Krebs–Ringer bicarbonate buffer containing 1.27 mM- ^{45}Ca ($2\mu\text{Ci/ml}$). 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 per min was determined in the time interval from 20 to 40 min after the addition of the compounds indicated and compared with the control values obtained within the same experiment. Results are means $\pm$ S.E.M.; the number of observations is given in parentheses.

Experimental conditions	Fraction of ^{45}Ca released/20 min	Change in relation to controls (%)	P
(a) Krebs–Ringer bicarbonate buffer (180–200 min after onset of washout)			
Control	0.102 ± 0.006 (9)		
Insulin (5 munits/ml)	0.118 ± 0.006 (9)	+15.8	<0.10
Control	0.091 ± 0.003 (9)		
Insulin (100 munits/ml)	0.113 ± 0.003 (12)	+24.1	<0.001
Control	0.094 ± 0.004 (6)		
Serum albumin (0.005%)	0.094 ± 0.005 (6)	+0.2	>0.95
(b) Krebs–Ringer bicarbonate buffer in which all K^+ was replaced by Na^+ (110–130 min after onset of washout)			
Control	0.102 ± 0.004 (4)		
Insulin (1 munit/ml)	0.112 ± 0.002 (4)	+10.4	<0.05
Control	0.095 ± 0.005 (7)		
Insulin (5 munits/ml)	0.118 ± 0.004 (7)	+23.9	<0.005
Control	0.119 ± 0.006 (9)		
Insulin (100 munits/ml)	0.159 ± 0.006 (9)	+33.5	<0.001
Control	0.122 ± 0.011 (5)		
ZnCl_2 ($10\mu\text{M}$)	0.102 ± 0.006 (5)	–16.0	>0.10

concentrations (1–10 munits/ml), the effect was also apparent during the first 10 min of exposure to insulin. The time course of the effect of insulin on 3-O-methylglucose washout shows a similar dependence on insulin concentration (Clausen, 1969a). Since ZnCl_2 ($1\mu\text{M}$) did not induce any significant change in the rate of ^{45}Ca washout, it seems unlikely that the effect of insulin is an outcome of the Zn present in the insulin preparation. As in earlier studies (Clausen, 1970), most of the experiments with fat-pads were performed with a buffer from which Ca^{2+} had been omitted. However, as shown in Table 2, the inclusion of Ca^{2+} (1.27 mM) in the efflux medium does not produce any change in the rate of ^{45}Ca washout or its response to insulin.

In view of the fact that in excitable tissues the washout of ^{45}Ca is clearly stimulated by the presence of Ca^{2+} in the bathing fluid (Reuter & Seitz, 1968; Blaustein & Hodgkin, 1969), it seems surprising that in epididymal fat-pads Ca^{2+} has no effect on the fractional loss of ^{45}Ca . However, other experiments showed that immediately after the addition of Ca^{2+} to the washout medium, the fractional loss of ^{45}Ca showed a marked transient rise, followed by a return

to approximately the same steady-state value (Clausen, 1969b). In another non-excitabile tissue, the rat pancreas, a similar response to the addition of Ca^{2+} was observed. Once the steady state was re-established, the fractional loss of ^{45}Ca was only 18% faster than that measured in the medium without added Ca^{2+} (Case & Clausen, 1973). These results indicate that ^{45}Ca is transported out of the cells by a mechanism which is similar to that described by Schatzmann (1973) in erythrocytes, where wide variations in the extracellular Ca^{2+} concentration had no effect on the rate of Ca^{2+} efflux.

The observation that insulin stimulated the washout of ^{45}Ca from fat-cells appears to disagree with the results of Kissebah *et al.* (1975), who showed that the rise in ^{45}Ca release induced by adrenaline was suppressed by insulin. Lipolytic hormones stimulate the washout of ^{45}Ca from adipose tissue (Clausen, 1969b, 1970), and it seems likely that the inhibitory effect of insulin on ^{45}Ca release from adrenaline-stimulated fat-cells is a result of the anti-lipolytic effect of the hormone. Procaine, another anti-lipolytic agent (Hales & Perry, 1970), also suppressed the rise in ^{45}Ca release seen on the addition of adrenaline (Kissebah

Fig. 1. Effect of insulin on the washout of ^{45}Ca from rat soleus muscles

Experimental conditions as described in the legend to Table 3. During the entire washout period, all K^+ in the buffer was replaced by an equimolar amount of Na^+ . Each point represents the mean of nine observations, with bars denoting S.E.M. $\circ$, Control; $\bullet$, +insulin.

et al., 1975). Thus it seems reasonable to assume that insulin itself can induce a release of ^{45}Ca which is not related to lipolytic processes. This conclusion is further supported by the observation that in rat soleus muscles the hormone produces similar effects on the washout of ^{45}Ca (Table 3).

In the standard incubation medium, the effect of insulin on ^{45}Ca release from rat soleus can only be detected in the late phase of the washout, and it is not much larger than that found in the fat-pads. Since the omission of K^+ from the incubation medium has been shown to potentiate the effect of insulin on sugar transport (Kohn & Clausen, 1972), we decided to look for an effect on ^{45}Ca release in such a modified medium. When all K^+ in the buffer was replaced by Na^+ , the effect of insulin on ^{45}Ca release seemed to be more pronounced (Table 3), and also showed a progressive increase with time (Fig. 1). After 100 min of exposure to insulin, the muscles released ^{45}Ca about 55% faster than the controls. Again, ZnCl_2 had no effect, and albumin at a concentration equimolar with 100 munits of insulin/ml did not produce any change either (Table 3). It should be noted that since all the experiments described in the present paper were performed without any metabolizable substrate, the effect of insulin on ^{45}Ca release seems to be independent of the presence of glucose or any other exogenous energy sources in the incubation medium.

The main conclusion that may be drawn from these results is that insulin induces a release of ^{45}Ca from

two of its major target cells. This may either be the result of a release of ^{45}Ca bound to the outer surface of the plasma membrane, an increase in the permeability of the plasma membrane to Ca^{2+} , or an increased rate of exchange of ^{45}Ca between cellular calcium pools and the cytoplasm.

The plasma membrane is capable of binding appreciable amounts of Ca^{2+} (Gent *et al.*, 1964; Sulakhe *et al.*, 1973; McDonald *et al.*, 1976), and since insulin seems to interfere with this process (Marinetti *et al.*, 1972; Kissebah *et al.*, 1975), the increased release of the radioisotope may consist of a rapid depletion of a readily available pool at the cell surface. On the other hand, as shown in the experiments with K^+ -free buffer, the effect of insulin on ^{45}Ca release is not necessarily transient, and recent experiments with myoblasts indicate that the rate of ^{45}Ca efflux remains elevated for at least 1.5 h after the addition of insulin (Schudt *et al.*, 1976). From the data available, it is not possible to determine whether these effects are associated with an increase in the permeability of the plasma membrane to Ca^{2+} , but it seems likely that displacement of bound Ca^{2+} can alter the permeability of the plasma membrane to Ca^{2+} and other compounds (Clausen, 1975).

As pointed out by Ashley *et al.* (1972) and Schatzmann (1973), the efflux of Ca^{2+} is mainly a function of the concentration of free Ca^{2+} ions in the cytoplasm. This in turn is very dependent on the rate of exchange in pools of calcium in mitochondria (Borle, 1973) or endoplasmic reticulum or bound to the surface of membranes. Therefore an increased rate of ^{45}Ca washout from the whole cell or tissue may be the result of accelerated release from any of these pools. If the release is precisely matched with an increased re-uptake of calcium into the cellular pools, the rise in ^{45}Ca release from the whole cell only reflects an increase in the specific radioactivity of the ^{45}Ca in the cytoplasm. However, the observation that insulin can induce a rise in the resting tension of rat soleus muscles indicates that the hormone may elicit a rise in the cytoplasmic Ca^{2+} ion concentration (Clausen *et al.*, 1975). In fat-cells, stimulation of Ca^{2+} uptake is accompanied by an increase in the activity of pyruvate dehydrogenase (Clausen *et al.*, 1974), a mitochondrial enzyme which is activated by a Ca^{2+} -dependent phosphatase (Denton *et al.*, 1972). It has been suggested that the stimulating effect of insulin on pyruvate dehydrogenase is mediated by Ca^{2+} ions (Severson *et al.*, 1974). McDonald *et al.* (1976) showed that insulin produces a relative increase in the exchangeable fraction of mitochondrial calcium as well as a rise in the microsomal calcium content. This provides further indirect evidence that insulin causes a redistribution of calcium within the cell, and it is difficult to envisage how this can take place without a rise in the cytoplasmic concentration of Ca^{2+} ions available for transport.

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