THE ABILITY OF INSULIN TO ALTER THE STABLE CALCIUM POOLS OF ISOLATED ADIPOCYTE SUBCELLULAR FRACTIONS

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Received April 30, 1976

SUMMARY. The calcium contents of adipocyte plasma membranes, mitochondria and microsomes (endoplasmic reticulum) were measured by atomic absorption. Ruthenium red (RR) and EDTA were used to selectively modify post-homogenization artifacts. Values obtained in the presence of RR appear to approximate the in vivo calcium content for mitochondria. Measurements in the presence of EDTA represented a highly stable pool of calcium for all organelles. Insulin (100 microunits/ml) treatment of adipocytes prior to fractionation caused a conversion of a significant portion of the stable calcium pool to an exchangeable form in mitochondria without altering the total mitochondrial calcium content. Insulin did not affect the calcium content of the plasma membranes. The hormone did significantly increase the calcium content of the microsomes, which probably reflected the increased ability of microsomes isolated from insulin-treated adipocytes to bind and accumulate calcium.

Calcium has been proposed as the possible second messenger for insulin action (1,2). Indirect evidence supporting this has included the identification of intracellular enzymes and enzyme systems which are both insulin and calcium-sensitive (3-7). $^{45}Ca^{2+}$ flux studies with intact cells are difficult to interpret since intracellular calcium is highly compartmentalized (8). Therefore, evaluation of calcium metabolism in subcellular fractions has become necessary. Such studies have identified insulin-induced alterations in $^{45}Ca^{2+}$ binding to adipocyte plasma membranes (9) and microsomes (10) and in energy-dependent calcium uptake into endoplasmic reticulum vesicles (11). Calcium exists in at least two forms in cellular organelles, including ionized pools and complexed or stable pools (8). This is the case for adipocyte plasma membranes (9, 12) and microsomes (10).

The present study documents the ability of insulin treatment of adipocytes to alter the stable calcium pools of adipocyte endoplasmic reticulum and mitochondria without affecting the stable calcium pool of the plasma membranes.

Materials and Methods. Male Wistar rats (125 gm) and chemicals were obtained from standard sources. Reagents were prepared in deionized and final-filtered water with constant monitoring of resistance. Bovine serum albumin (BSA) fraction V had no insulin-like activity. Porcine insulin and desoctapeptide insulin were gifts from Drs. M. Root and R. Chance, Eli Lilly.

Isolated adipocytes were prepared by the method of Rodbell (13) using 0.5 mg collagenase/ml of modified Krebs-Ringer phosphate buffer, pH 7.4 at 37° containing 11.1 mM D-glucose, 30 mg BSA/ml and 1.3 mM calcium. The cells were washed three times, resuspended and preincubated at 37° in two equal aliquots for 10 min. in modified Krebs-Ringer bicarbonate buffer, pH 7.4, equilbrated with 95% O_2, 5% CO_2, containing the same concentrations of glucose, BSA and calcium as the Krebs-Ringer phosphate buffer. Following preincubation, one aliquot of cells received a final concentration of 100 microunits insulin/ml (using stock insulin dissolved in 0.1N HCl and diluted with 0.1% BSA) or a molar equivalent of desoctopeptide insulin dissolved in 0.1% BSA while the other aliquot received an equal volume of 0.1% BSA. These were incubated for 10 min. at 37°. Both aliquots were washed one time and homogenized at 4° in 0.25 M sucrose buffered with 10 mM Tris-HCl, pH 7.4, containing insulin (or desoctapeptide insulin) or 0.1% BSA at the concentration above. The plasma membrane, mitochondrial and microsomal fractions were isolated from both sets of cells in parallel by a modification (14) of the method of McKeel and Jarett (15) except that EDTA was omitted from the standard procedure. Ruthenium Red (RR) (5 μM) and EDTA (1.0 mM), where indicated, were added immediately prior to homogenizing the cells and at all steps thereafter during isolation of the fractions.

The calcium contents of the subcellular fractions were determined by atomic absorption as previously described (9). Protein concentration was determined by the method of Lowry et al (16).

Results and Discussion.

The stable calcium pool of subcellular fractions from control cells:

The calcium content of highly enriched subcellular fractions from control preparations was measured by atomic absorption (Table 1). The plasma membranes contained the highest concentration of calcium (32.5±0.8 n mol/mg protein). The mitochondria contained one-third less and the microsomes 45% less. These values may not reflect the in vivo calcium content of the organelles due to posthomogenization changes including calcium elution from and active calcium uptake into organelles (17). EDTA and ruthenium red (RR) were used to selectively modify and

Table 1

Calcium content of subcellular fractions from adipocytes.

The calcium content of isolated mitochondria, microsomes, and plasma membranes were determined under conditions in which the standard homogenization and isolation media contained either no additions, 1 mM EDTA or 5 μM ruthenium red as outlined in "Materials and Methods." The values are shown ±SE and the number in parentheses indicates the number of preparations assayed. The degrees of significance were determined using the t test for unpaired samples comparing the indicated result to that found with no additions. * $p < .02$, ** $p < .01$, *** $p \ll .001$.

	No Additions	+EDTA	+Ruthenium Red
	n mol calcium/mg protein		
Mitochondria	21.1±1.9 (17)	13.7±2.1 * (8)	36.7±3.0 *** (11)
Microsomes	17.9±2.1 (17)	10.1±1.8 ** (7)	20.6±2.0 (12)
Plasma Membranes	32.5±0.8 (16)	6.5±1.2 *** (7)	22.7±1.0 *** (11)

control these changes. EDTA chelates free calcium in the media (preventing posthomogenization uptake) and elutes the more loosely complexed calcium from the organelles leaving a highly stable pool of calcium. Consistent with this, EDTA significantly lowered (at least $P < .02$) the measured calcium content of all three organelles.

Ruthenium red at 5 uM completely blocks calcium uptake into mitochondria isolated from adipocytes (11) and other cells (18) and inhibits calcium efflux from isolated mitochondria (19, unpublished). Therefore the calcium content of mitochondria isolated in the presence or RR presumably approximates the *in vivo* calcium content of this organelle, reflecting both the stable and exchangeable calcium pools. The calcium content of 36.7±3.0 determined in the presence of RR (Table 1) was double the value found in the absence of the agent and

agreed closely with the value measured by Severson et al (20) for adipocyte mitochondria isolated in the presence of RR and EGTA.

Ruthenium red did not alter the calcium content of endoplasmic reticulum (Table 1). This is consistent with the findings that RR had no direct effect on the binding (10, unpublished) or active uptake $^{45}Ca^{2+}$ by endoplasmic reticulum. In contrast, the addition of RR at the time of homogenization and throughout the isolation procedure resulted in a significant ($P \ll .001$) decrease in the calcium content of the plasma membranes (Table 1). This value must reflect loss of complexed calcium from the membrane since previous studies have shown that there is no exchangeable or uncomplexed calcium present on the plasma membranes as isolated (21).

Effect of insulin on the stable calcium content of the subcellular fractions: Insulin had different effects on the calcium content of each of the subcellular organelles. The mitochondria from insulin-treated cells contained 25% less calcium than paired controls (Table 2). Insulin produced a similar absolute decrease in calcium content when EDTA was added at the time of homogenization. Strikingly, there was no difference between the insulin-treated and control mitochondrial values when RR was used. These data are visually represented in fig. 1. Insulin did not alte the total calcium content of the mitochondria measured in the presence of RR which includes both the exchangeable or free calcium and the stable or complexed calcium pools. In contrast insulin did decrease the highly stable pool as reflected by the EDTA values. Thus insulin caused a conversion of a portion of the stable pool of calcium to an exchangeable form without altering the total calcium content of the mitochondria. The exchangeable pool is visualized as the difference between EDTA and RR values. The values obtained with no additions illustrate the persistance of the insulin effect despite probable post-homogenization artifacts and supports the above contention. This alter-

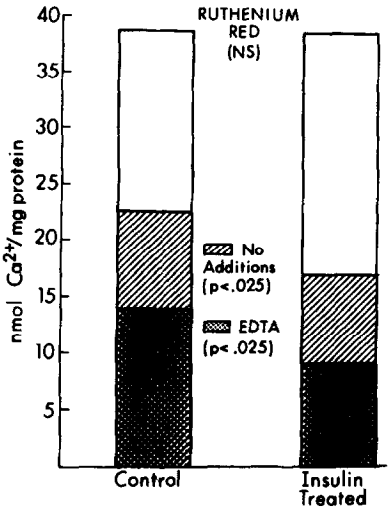

Figure 1 - Graphic representation of the effect of insulin on mitochondrial calcium. The data represented were obtained from Table 2.

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ation of calcium within the mitochondria could be responsible for the insulin activation of pyruvate dehydrogenase, since increases in ionized calcium activate this enzyme system (7). Severson et al (20) concluded that calcium did not account for the insulin activation of pyruvate dehydrogenase since insulin treatment of adipocytes did not increase the incorporation of extra-cellular $^{45}Ca^{2+}$ into the mitochondria. The present findings are not inconsistent with their data and provide a mechanism by which calcium could mediate the insulin activation of pyruvate dehydrogenase.

Insulin treatment of adipocytes caused a 32% increase in the calcium content of microsomes isolated in the absence of EDTA, but had no effect of the highly stable pool measured in the presence of EDTA (Table 2). The values found in the absence of EDTA probably reflected the increased capacity of endoplasmic reticulum recovered from insulin-treated cells to bind (10) and to accumulate calcium (11).

Insulin had no effect on the calcium content of plasma membranes

Table 2

Effect of insulin on the calcium content of subcellular fractions from adipocytes.

Control and insulin-treated cells and their subcellular fractions were prepared in a paired fashion and calcium concentrations assayed as outlined in "Materials and Methods." Where indicated the homogenization and isolation media contained either no additions, 1 mM EDTA or 5 μM RR. Column n indicates the number of paired preparations assayed and the p values were determined by the paired t test.

	Control	Insulin	n	p
	n mol calcium/mg protein			
Mitochondria				
No additions	22.6±1.9	16.9±2.1	12	.025
+EDTA	13.7±2.1	9.2±0.1	8	.025
+Ruthenium Red	38.7±3.6	38.5±3.6	8	NS
Microsomes				
No additions	17.4±2.6	22.9±3.8	10	.01
+EDTA	10.1±1.8	11.4±1.8	7	NS
Plasma Membranes				
No additions	32.8±1.1	33.2±1.8	10	NS
+EDTA	6.5±1.2	7.1±1.2	7	NS

isolated either in the presence or absence of EDTA. This is in contrast to the previous demonstration that isolated plasma membranes from insulin-treated cells have an increased ability to bind $^{45}Ca^{2+}$ (9). This further supports our previous data indicating that the pool of calcium involved in $^{45}Ca^{2+}$ binding is independent of the calcium pools measured by atomic absorption (9, 21). Neither desoctapeptide insulin nor BSA produced any changes in the calcium content of the plasma membrane, mitochondria or endoplasmic reticulum.

These studies support three major points: 1) Two pools of calcium are associated with each subcellular organelle studied, an

exchangeable or ionized pool and a stable or complexed pool. 2) A second messenger system other than calcium appears necessary to produce the insulin-induced alterations in the calcium content of two major intra cellular organelles, the mitochondria and endoplasmic reticulum. 3) Alterations in calcium content of subcellular organelles could play an important role in the final effector system involved in the mechanism of insulin action.

Acknowledgements: The authors thank Mr. and Mrs. Robert Smith for their excellent assistance. This work was supported by U.S. P.H.S. Grant AM 11892, N.I.H. Postdoctoral Fellowship IF22AM01471 (J.M.M.) and N.I.H. Training Grant GM00897 (D.E.B.).

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