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DISSOCIATION OF INSULIN'S EFFECTS ON CELL METABOLISM AND ON SUBCELLULAR CALCIUM TRANSPORT SYSTEMS OF 3T3-L1 ADIPOCYTES

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SUMMARY. Exposure of 3T3-L1 adipocytes to 7.0 nM insulin for 120 min at 37°C induced a progressive increase in cell accumulation of ^{3}H-D-glucose and the incorporation of its ^{3}H into lipid. Membrane vesicles prepared from these same metabolically responsive cells actively accumulated Ca^{2+}. Insulin treatment of 3T3-L1 cells had no effects on the N_3-insensitive or N_3-sensitive Ca^{2+} accumulation or on the passive Ca^{2+} efflux from the membrane vesicles. Ca^{2+} concentration studies showed no changes in K_m or V_{max} of the calcium uptake. Thus, insulin's effects on 3T3-L1 adipocyte metabolism appear to be independent of stable alterations in these systems.

It has been postulated that the effects of insulin are mediated by the redistribution of subcellular Ca^{2+} (1). Several enzymes, most of which are cytoplasmic, are regulated by insulin and are sensitive to changes in Ca^{2+} concentration (2-8). Moreover, insulin treatment of some cell types has been reported to affect both the Ca^{2+} fluxes in intact cells (9-12) and the Ca^{2+} handling by subcellular fractions prepared from these hormone-treated cells (13-15). The means by which insulin alters subcellular calcium remains unknown.

An important candidate for the regulation of subcellular Ca^{2+} is the N_3-insensitive Ca^{2+} transport system present in membrane vesicles from a variety of cells including fat (16), liver (17) and cultured cells (18-19). The characteristics of this calcium uptake are similar to those of the energy-

ABBREVIATIONS

N_3, azide; MIX, 3-isobutyl-1-methyl-xanthine; BSA, bovine serum albumin; KRP, Krebs Ringer Phosphate; cpm, counts per minute; EGTA, ethyleneglycol-bis-(β-amino-ethyl ether) N,N'-tetraacetic acid.

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dependent Ca^{2+} uptake system in the sarcoplasmic reticulum in muscle (20). Insulin has been reported to alter this Ca^{2+} transport in non-muscle cells; however, the observed effects have been contradictory: The N_3-insensitive Ca^{2+} pump of adipocytes was increased by insulin (13) while that of hepatocytes was decreased (14) or unchanged (15).

We have examined the effects of insulin on metabolism and on subcellular Ca^{2+} transport using 3T3-L1 adipocytes. These cells are a subclone of mouse 3T3 fibroblasts that progressively acquire the characteristics of mature adipocytes in culture (21) and develop a sensitivity to physiologic concentrations of insulin (22-24). In response to 7.0 nM insulin for 10-120 min, 3T3-L1 adipocytes demonstrated an increase in ^{3}H-D-glucose accumulation and a stimulation of its ^{3}H incorporation into total lipid. A non-mitochondrial Ca^{2+} transport system has been studied in homogenates of these same hormone-sensitive cells and found to be unchanged by insulin treatment.

MATERIALS AND METHODS. BSA (Fraction V) used in the protein assay was obtained from Calbiochem while that used in the KRP buffer was from Reheis Chemicals. $^{45}CaCl_2$ (25 mCi/mg), ^{3}H-3-D-glucose (10 Ci/mmol) and Omnifluor were purchased from New England Nuclear. NA and PCS liquid scintillation cocktails were from Beckman Instruments, Inc. and Amersham Corp., respectively. All other chemicals were of reagent grade quality and from standard sources. All solutions were prepared with water deionized by a double-chambered, mixed bed ion-exchange system and filtered through a 0.12 μm pore size filter from Continental Water. A small Ca^{2+} contamination in all reagents was monitored by atomic absorption and included in all calculations of experimental data. Insulin was from Eli Lily and Co.

Cell Culture. 3T3-L1 preadipocytes (American Type Culture Collection) were grown as described (21). Differentiation into adipocyte-like cells was induced by treatment with 0.25 μM dexamethasone and 0.5 mM MIX for 2 days (25) followed by 10 μg insulin/ml for 5 days (26). 3T3-L1 adipocytes were then maintained in medium without added hormones for at least 2 days. The medium was changed twenty-four hours prior to use of the cells.

^{3}H-Glucose Accumulation and Incorporation of ^{3}H Into Lipid. These two metabolic processes were assessed using a technique developed by Moody et al. (27). Cell monolayers were gently washed 3 times with 10 ml of KRP-1% BSA buffer, pH 7.4, and then incubated with and without 7.0 nM insulin in KRP-1% BSA buffer, pH 7.4, containing 0.25 mM D-glucose plus a tracer of ^{3}H-3-D-glucose at 37°C for the time specified. The reaction was terminated by decanting the incubation mixture and washing the monolayers twice with cold KRP-1% BSA, pH 7.4, and twice with cold 10 mM Tris buffer, pH 7.4. Cells were scraped off with a rubber policeman and ruptured at 4°C with 15 strokes in a Dounce homogenizer using a tight-fitting pestle. Aliquots of the total homogenate were added to each of the PCS and Omnifluor liquid scintillation cock-

tails and counted for ^{3}H. The amount of radioisotope found in the PCS samples represented the total ^{3}H-D-glucose taken into the cells while that found in the Omnifluor samples was the ^{3}H incorporated into the total lipid fraction.

Ca^{2+} Uptake. The total homogenate was centrifuged at 600 x g for 10 min at 4°C to remove nuclei, cell debris and unbroken cells. The uptake of Ca^{2+} by membrane vesicles found in the supernatant fraction was studied using ^{45}Ca and a rapid Millipore filtration method (16). The standard uptake assay was performed at 37°C for 10 min with an incubation mixture at pH 6.8 containing 0.10 M KCl, 0.05 M imidazole, 5mM $MgCl_2$ and ATP, 10 mM NaN_3, 10 mM K oxalate and an added Ca^{2+} of a 5 μM plus a tracer of $^{45}CaCl_2$. The assay was initiated by the addition of about 20 μg protein and the uptake was terminated by Millipore filtration of 250 μl aliquots of the incubation mixture. The filters were immediately washed with three 5 ml portions of cold 0.25 M sucrose and then air-dried. $^{45}Ca^{2+}$ was measured by liquid scintillation counting. Non-specific binding of Ca^{2+} was accounted for with control tubes containing no protein. The observed cpm were converted to nmol Ca^{2+} by comparison with standard aliquots of the incubation mixture dried on filters and counted. Uptake was expressed in terms of nmol Ca^{2+}/mg protein in the 600 x g supernatant fraction.

Studies of Insulin Effects. Paired cell monolayers were washed and incubated with or without 7.0 nM insulin for 10 to 120 minutes. ^{3}H-glucose accumulation and incorporation of ^{3}H into lipids were assessed as described above. Another aliquot of the homogenate was used to measure Ca^{2+} uptake. Thus, in each study of insulin's effect on Ca^{2+} uptake, the metabolic response of the cells to insulin was measured.

Ca^{2+} Efflux. Efflux was assessed in a manner similar to Ca^{2+} transport. However, membrane vesicles were preloaded with Ca^{2+} and $^{45}Ca^{2+}$ by incubating in the same assay medium but omitting the oxalate. EGTA (1 mM) was then added to prevent further accumulation of $^{45}Ca^{2+}$. The amount of Ca^{2+} remaining within the vesicles was determined by Millipore filtration and liquid scintillation counting. Efflux was calculated as the percent Ca^{2+} remaining inside the vesicles compared to the total Ca^{2+} present before adding EGTA.

Protein Measurement. Protein was measured by the method of Lowry et al. (28) using BSA as the standard.

Statistics. P values were determined using a paired studdent's T test.

RESULTS AND DISCUSSION

As illustrated in Figure 1, insulin enhanced the accumulation of ^{3}H-D-glucose (circles) into 3T3-L1 adipocytes as well as incorporation of its ^{3}H into total lipid (squares) over a 120 min incubation period. The hormone effect on ^{3}H incorporation into lipid tended to be slightly greater but was not significantly different from the insulin effect on sugar accumulation ($p > 0.10$).

Membrane vesicles in the 600 x g supernatant fractions prepared from these same hormone-sensitive 3T3-L1 adipocytes accumulated Ca^{2+}. Under the standard assay conditions, the Ca^{2+} uptake was energy-dependent as indicated by a 98% decrease in the absence of ATP. The omission of oxalate, which acted as a

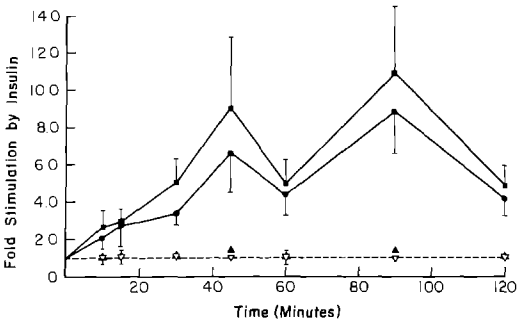

Figure 1 - Insulin's Effects on 3T3-L1 Adipocyte Glucose Metabolism and Subcellular Ca^{2+} Transport.

Cell accumulation of ^{3}H-D-glucose (circles), its incorporation of ^{3}H into lipid (squares) and Ca^{2+} uptake (open inverted triangles) were measured as described in the methods section. Passive Ca^{2+} binding (closed triangles) was determined by assaying for uptake in the absence of ATP and oxalate. Fold stimulation by insulin was the ratio of cpm/mg protein for hormone-treated cells compared to cpm/mg protein for control cells. Values are the means $\pm$ standard error of 3 or 4 experiments, each done in duplicate.

trapping agent for the Ca^{2+} within the vesicles (29), resulted in a 92% decrease in transport. This stimulation by oxalate suggested that Ca^{2+} was translocated into the membrane vesicles and was not just bound to their exterior. The amount of mitochondrial Ca^{2+} transport, as judged by the N_3-sensitive Ca^{2+} uptake, was about 14% of the total activity.

Insulin treatment of the differentiated cells over a course of 120 min had no effects on the N_3-insensitive Ca^{2+} uptake or on the passive Ca^{2+} binding measured by omitting ATP and oxalate from the assay (Fig. 1). The N_3-sensitive Ca^{2+} transport was similar for membrane vesicles from untreated and hormone-treated cells (1.7 $\pm$ 0.3 and 2.1 $\pm$ 0.2 nmol Ca^{2+}/mg protein/10 min, respectively, $p > 0.05$). Moreover, the addition of insulin directly to the vesicles prior to assay did not alter the uptake activity.

Ca^{2+} uptake and efflux by the membrane vesicles was unaffected by treatment with 7.0 nM insulin for 10 min. Ca^{2+} uptake was linear up to 30 min (Figure 2, panel A). Hormone exposure did not alter the uptake activity. Ca^{2+} efflux by membrane vesicles from insulin-treated and control cells were also not different (Figure 2, panel B). Although not shown, insulin treatment

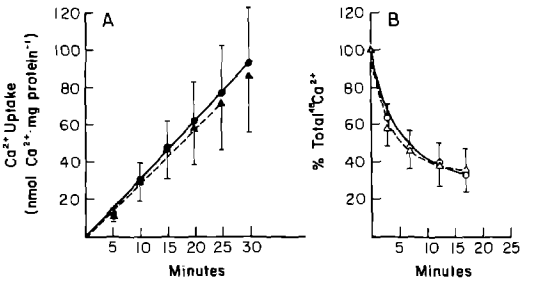

Figure 2 - Insulin's Effects on Ca^{2+} Uptake and Efflux from 3T3-L1 Adipocyte Membrane Vesicles.
Cells were treated with (triangles) and without (circles) 7.0 nM insulin for 10 min at 37°C. Ca^{2+} transport (panel A, closed symbols) and efflux (panel B, open symbols) were assayed as given in the methods section. Values are the mean ± standard error of 3 paired experiments, each done in duplicate samples.

for 90 min rather than 10 min produced no significant changes in Ca^{2+} uptake or efflux. The patterns were similar to those shown in Fig. 2.

The kinetic parameters of the 3T3-L1 adipocyte Ca^{2+} transport system were also unaffected by insulin treatment for 10 or 90 min. Figure 3 demonstrates that the Ca^{2+} uptake by membrane vesicles from untreated (open circles) and hormone-treated (closed triangles) cells for 90 min were substrate-dependent and saturable. Their transport activities were similar at each of the Ca^{2+} concentrations ($p > 0.10$). The Eadie-Hofstee plots of their Ca^{2+} dose response were linear, suggesting a single class of Ca^{2+} transport sites. This was further supported by a calculated Hill coefficient of 0.9. Analysis of three paired experiments by Eadie-Hofstee kinetics yielded an apparent K_m of 18.3 ± 0.3 μM total Ca^{2+} for control cells and 19.2 ± 0.5 μM for insulin-treated cells ($p > 0.05$). On the basis of ionized Ca^{2+} (30), both had a K_m of 3.9 ± 0.1 μM. The V_{max} were 9.2 ± 1.1 and 9.4 ± 2.0 nmol Ca^{2+}/mg protein/min for untreated and hormone-treated 3T3-L1 adipocytes, respectively ($p > 0.60$). Moreover, their Ca^{2+} uptake activities were similar when transport was assayed in the presence of excess Ca^{2+} and EGTA (450 and 500 μM, respectively) to minimize any possible differences in Ca^{2+} contamination (8.4 ± 0.8 and 8.2 ± 1.5 nmol Ca^{2+}/mg protein/min, respectively, $p > 0.70$).

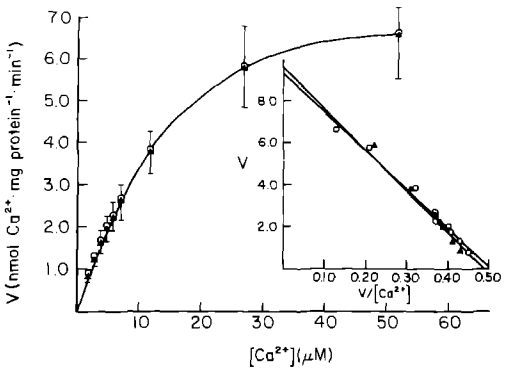

Figure 3 - Insulin's Effects on the Ca^{2+} Dose Response of Ca^{2+} Transport by 3T3-L1 Adipocyte Membrane Vesicles.

Cells were treated with (closed triangles) and without (open circles) 7.0 nM insulin for 90 min at 37°C. Ca^{2+} uptake was measured as described in the methods section; and the total Ca^{2+} concentration was varied from 2 to 52 uM. The inset contains the Eadie-Hofstee plots of the presented data. The lines were drawn by linear regression analysis. Values are the means ± standard error of 3 paired experiments performed in duplicate samples.

Shorter exposure to insulin (10 min) also resulted in no alterations in K_m or V_{max}. The results of these experiments were as shown in Figure 3.

In summary insulin produced no stable changes in the subcellular calcium uptake system which we have studied in 3T3-L1 cells. This finding cannot be attributed to hormonal unresponsiveness of the cells since responses to insulin were documented in each experiment using ^{3}H-D-glucose. The data do not exclude effects of insulin on calcium handling by the plasma membranes (31, 32), since calcium uptake in our system was decreased only slightly by digitonin (Bruns, D.E. Unpublished). However, the results suggest that insulin's effect on microsomal calcium uptake in adipocytes is unimportant in the general mechanism of the hormone's action.

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REFERENCES

1. McDonald, J.M., Bruns, D.E. and Jarett, L. (1976) Biochem. Biophys. Res. Commun. 71:114-121.
2. Wade, D.R., Chalmers, T.M. and Hales,C.N. (1970) Biochim. Biophys. Acta 218:496-507.
3. Mangiello, V. and Vaughan, M. (1973) J. Biol. Chem. 248:7164-7170.
4. Taylor, S.I., Mukherjee, C. and Jungas, R.L. (1973) J. Biol. Chem. 248: 73-81.
5. Randle, P.J. and Denton, R.M. (1973) Symp. Soc. Exp. Biol. 27:401-428.
6. Kissebah, A.H., Vydelingum, N., Tulloch, B.R., Hope-Gill, H. and Fraser, T.R. (1974) Horm. Metab. Res. 6:247-255.
7. Chiang, P.K. and Sacktor, B. (1975) J. Biol. Chem. 250:3399-3408.
8. Kono, T., Robinson, F.W. and Sarver, J.A. (1975) J. Biol. Chem. 250: 7826-7835.
9. Alm, B., Efendic, S. and Low, H. (1970) Horm. Metab. Res. 2:142-146.
10. Werner, S., Hall, K. and Low, H. (1974) Horm. Metab. Res. 6:319-325.
11. Hope-Gill, H., Kissebah, A., Tulloch, B., Clarke, P., Vydelingum, N. and Fraser, T.R. (1975) Horm. Metab. Res. 7:194-196.
12. Kissebah, A.H., Clarke, P., Vydelingum, N., Hope-Gill, H., Tulloch, B. and Fraser, T.R. (1975) Europ. J. Clin. Invest. 5:339-349.
13. McDonald, J.M., Bruns, D.E. and Jarett, L. (1978) J. Biol. Chem. 253: 3504-3508.
14. Waltenbaugh, A.A. and Friedman, N. (1978) Biochem. Biophys. Res. Commun. 82:603-608.
15. Taylor, W.M., Bygrave, F.L., Blackmore, P.F. and Exton, J.H. (1979) FEBS Letters 104:31-34.
16. Bruns, D.E., McDonald, J.M. and Jarett,L. (1976) J. Biol. Chem. 251: 7191-7197.
17. Moore, L., Chen, T., Knapp, H.R. and Landon, E.J. (1975) J. Biol. Chem. 250:4562-4568.
18. Moore, L. and Pastan, I. (1976) J. Cell Physiol. 91:289-296.
19. Moore, L. and Pastan, I. (1977) J. Biol. Chem. 252:6304-6309.
20. MacLennan, D.H. and Holland, R.P. (1975) Ann. Rev. Biophys. Bioeng. 4: 377-402.
21. Green, H. and Meuth, M. (1974) Cell 3:127-133.
22. Rubin, C.S., Lai, E. and Rosen, O.M. (1977) J. Biol. Chem. 252:3554-3557.
23. Rosen, O.M., Smith, C.J., Fung, C. and Rubin, C.S. (1978) J. Biol. Chem. 253:7579-7583.
24. Karlsson, F.A., Grunfeld, C., Kahn, C.R. and Roth, J. (1979) Endo. 104: 1383-1392.
25. Rubin, C.S., Hirsch, A., Fung, C. and Rosen, O.M. (1978) J. Biol. Chem. 253:7570-7578.
26. Green, H. and Kehinde, O. (1975) Cell 5:19-27.
27. Moody, A.J., Stan, M.A. and Stan, M. (1973) Horm. Metab. Res. 6:12-16.
28. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193:265-275.
29. Martonosi, A. and Feretos, R. (1964) J. Biol. Chem. 239:648-658.
30. Katz, A.M., Repke, D.I., Upshaw, J.E. and Polascik, M.A. (1970) Biochem. Biophsy. Acta 205:473-490.
31. McDonald, J.M., Bruns, D.E. and Jarett, L. (1975) Proc. Nat. Acad. Sci. USA 73:1542-1546.
32. Pershadsingh, H.A. and McDonald, J.M. (1979) Nature 281:495-497.