PHOSPHOLIPASE C AND MECHANISMS OF ACTION OF INSULIN AND CORTISOL ON GLUCOSE ENTRY INTO FREE ADIPOSE CELLS *

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Received October 11, 1965

Suspensions of free adipose cells, prepared by the collagenase method of Rodbell (1964), exhibit many of the metabolic functions previously described for fat pads. Such cells remained extremely sensitive to low concentrations of insulin (Rodbell, 1964) and of glucocorticoids (Table 1 below, and Fain et al, 1965) insofar as glucose and lipid metabolism is concerned. These cells also exhibited extreme surface sensitivity, adhering to, and lysing upon contact with, glass surfaces, necessitating use of plastic or siliconized laboratory ware (Rodbell, 1964, and present observations). One explanation for this physical behavior is that the crude bacterial collagenase preparations, used to liberate cells from epididymal fat pads, may also have removed an outer protein membrane from the cell surface, thereby exposing a lipid or lipoprotein layer (Weiss, 1963). Since such cells remained sensitive to the effects of insulin and cortisol, it is possible that sites of actions of these hormones are at an inner, lipid or lipoprotein layer of the cell boundary membranes. To examine this possibility, free adipose cells have been treated with phospholipase C (phosphatidylcholine-cholinephosphohydrolase, EC 3.1.4.3), and the ability of such

* Supported by grant AM-05475 from the National Institutes of Health, Bethesda, Md.

cells to take up and metabolize glucose compared with cells also treated with insulin and cortisol. Low concentrations of phospholipase C mimicked the action of insulin in stimulating glucose uptake and metabolism by free adipose cells. Higher concentrations of phospholipase C imitated the effect of cortisol in inhibiting glucose entry and metabolism. The effects of insulin and cortisol could be elicited only with "intact" adipose cells. A theory of the mechanism of action of insulin is discussed in the light of the present results.

Experimental

Free rat adipose cell suspensions were prepared as described by Rodbell (1964), except that 0.05% gelatin replaced albumin, collagenase treatment was for 1.5 h, and glucose was eliminated from incubation and wash fluids. Final cell suspensions contained 1 - 1.5 mg of protein per ml, cell protein being determined by a biuret procedure (Layne, 1957) following extraction of 1 ml of cell suspension with the solvent mixture of Dole (1956) and washing isolated protein with 95% ethanol. Such cells required no "protective protein", e.g., serum albumin (Rodbell, 1964), during metabolic studies, although bovine serum albumin, to a degree directly proportional to its concentration, increased the level of glucose metabolism[1].

In a final volume of 1 ml of modified Krebs-Ringer bicarbonate buffer, pH 7.4, 0.5 ml of cell suspension and 3.75 μmoles of U-^{14}C-glucose (450,000 cpm) were incubated with shaking at 37° in an atmosphere of 5% carbon dioxide in oxygen for 2 h (experiments with insulin

[1] Blecher, M., unpublished observations.

and/or PHL-C[2]) or 4 h (experiments with cortisol). Where present, PHL-C (partially purified alpha toxin of Cl. welchii, 3.04 U per mg, lot 6421, Worthington Biochem. Corp., Freehold, N.J.), insulin (25.4 U per mg, lot 2054, Mann Research Labs., New York) and cortisol (Chemed, Odenton, Md.) were added in buffer solution. Incubations were ended by injection of 0.1 ml of 2 N sulfuric acid into the incubation mixture through a serum cap seal, and the liberated carbon dioxide trapped in 0.25 ml of Hyamine-10X (Rohm and Haas, Philadelphia) previously injected into a hanging glass center well. The total lipid fraction was isolated from cell as previously described (Rodbell, 1964). Radioactivities were determined with a Nuclear-Chicago liquid scintillation spectrometer.

During 4 h of incubation, 4.80 $\pm$ 0.24 (S.E.$_m$) µmoles of glucose carbon were oxidized to carbon dioxide and 5.24 $\pm$ 0.28 µmoles converted to total lipids, per mg of cell protein. These parameters of glucose metabolism were linear during 4 h of incubation.

To demonstrate hydrolytic activity of PHL-C on adipose cell phosphatides, triplicate acidified aqueous extracts of incubation mixtures (remaining after extraction of lipids) were pooled, concentrated by rotary evaporation, then subjected to paper chromatography (Ansell and Spanner, 1961) to isolate product phosphoryl amines. With 1 and 10 µg of PHL-C, sufficient to affect glucose metabolism (Fig. 1), phosphoryl ethanolamine and phosphoryl serine were identified; with 0.1 µg of enzyme, which had no significant effect upon glucose metabolism, only traces of phosphoryl serine were detectable.

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[2]PHL-C, phospholipase C (phosphatidylcholine-cholinephosphohydrolase, EC 3.1.4.3).

Results and Discussion

In confirmation of a previous report (Rodbell, 1964), insulin stimulated glucose metabolism by free adipose cells (Fig. 1). At

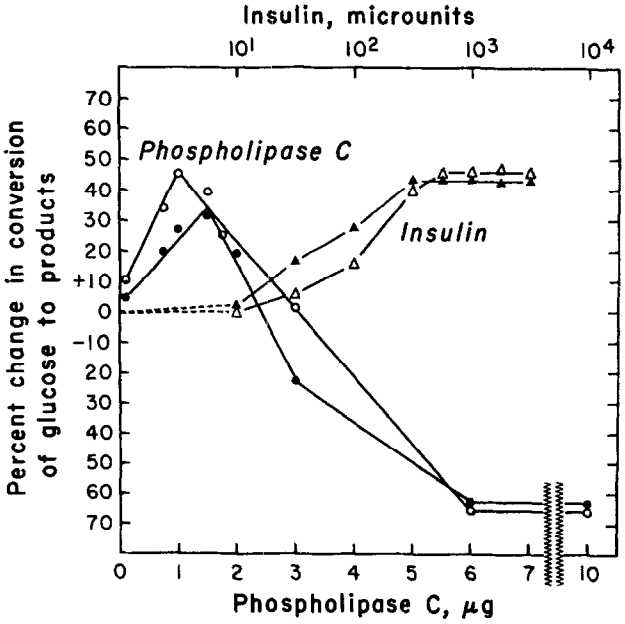

Fig. 1. Changes in the conversion of U-^{14}C glucose to carbon dioxide (closed symbols) and to total lipids (open symbols) due to insulin (triangles) and to phospholipase C (circles). See text for conditions.

concentrations of insulin up to about 500 μU per ml a log dose relationship existed; insulin exerted no additional effect beyond this concentration. Physiological concentrations (10^{-7}M) of cortisol inhibited glucose metabolism of free adipose cells during 4 h of incubation (Table 1, line 1).

PHL-C, concurrently with, or as a result of, its hydrolytic action upon cell membrane phosphatides, exhibited a biphasic action upon glucose metabolism in free adipose cells, stimulating at lower (< 3 μg) and inhibiting at higher (> 3 μg) concentrations of the en-

zyme (Fig. 1 and Table 1). Since both parameters of glucose metabolism, i.e., oxidation to carbon dioxide and conversion to lipids, were stimulated by appropriate concentrations of PHL-C, it is likely that the primary effect of the enzyme was exerted at an early point in glucose metabolism; parallel direct assays for glucose uptake by free adipose cells indicated that this, indeed, was the case. The inhibitory effects of high concentrations of PHL-C were likely due to destruction of membrane integrity of large portions of the cell population; leakage of cell protein into the medium under these conditions has been observed[3].

TABLE 1

Effects of Phospholipase C and Cortisol

on Glucose Metabolism by Adipose Cells

Additions	μmoles glucose carbon converted to product per mg cell protein in 4 h.			
	Carbon dioxide		Total lipids	
	Without cortisol	With cortisol	Without cortisol	With cortisol
Basal	4.06	2.30	4.45	2.05
+Phospholipase C, μg				
0.1	3.92	2.11	4.39	1.81
0.2	4.07	2.06	4.12	1.97
1.0	5.34	3.76	6.20	4.03
1.5	5.48	4.27	7.08	5.18
2.0	5.62	4.73	6.78	5.66
3.0	3.23	3.44	4.66	4.05
10.0	1.63	1.33	1.49	1.31

See text for conditions. Cortisol, 4.6×10^{-7} M.

[3] Rodbell, M., personal communication.

Free adipose cells, in which glucose entry was stimulated by 1 to 3 μg of PHL-C, still responded to the inhibitory effects of cortisol, although the net effect on glucose entry remained a positive one (Table 1). In the presence of non-effective amounts of the enzyme (0.1 to 0.2 μg), the usual inhibitory effect of cortisol was evident. However, in the presence of large amounts of PHL-C (10 μg), cortisol was without significant effect. Thus, an "intact" cell boundary or membrane was required for the inhibitory action of cortisol.

Free adipose cells, in which glucose entry was stimulated by PHL-C, still remained sensitive to the effects of insulin (Table 2, exp. 1). Since the effects of the enzyme plus hormone were not

TABLE 2

Effects of Phospholipase C and Insulin on Glucose Metabolism by Adipose Cells

Experiment	Additions	μmoles glucose carbon converted to product per mg cell protein in 2 h	
		Carbon dioxide	Total lipids
1	Basal	2.85	3.14
	+Insulin, 1.0 mU	6.13	7.00
	+PHL-C, 0.67 μg	3.99	4.79
	+Insulin + PHL-C	6.16	7.12
2	Basal	2.87	3.23
	+Insulin, 1.0 mU	4.16	4.54
	+PHL-C, 6.0 μg	1.09	1.13
	+Insulin + PHL-C	1.32	1.33

See text for conditions

strictly additive under these conditions, it is likely that the cells had attained the maximum stimulation possible. However, in the pres-

ence of large amounts of PHL-C (6 μg), insulin stimulated glucose entry to only a very small extent. Thus, as with cortisol, an "intact" cell membrane was required for insulin action.

Although no mechanisms of action of glucocorticoids on glucose entry into adipose cells have been, or are presently, proposed, many theories have been propounded to account for the action of insulin in stimulating extracellular to intracellular transfer of glucose molecules (Krahl, 1961, review). Among these theories, one appears related to the present results: it was proposed that insulin interacts with lipoprotein in the cell boundary membrane, thereby changing the entire charge distribution in the membrane and allowing intermolecular rearrangements in the membrane and attached structures, thereby facilitating access of glucose (and certain other water-soluble molecules) to the intracellular space (Krahl, 1961). This theory receives support from one interpretation of the present results, viz., that the stimulation of glucose entry caused by PHL-C was due to alterations in the charge distribution in the cell membrane brought about by limited hydrolysis of the polar phosphatides of membrane lipoprotein; extensive hydrolysis of these phosphatides by high concentrations of PHL-C destroyed sites of action of insulin, and perhaps of cortisol as well. Thus, insulin and PHL-C, by different primary actions, may both have altered the charge distribution, thereby invoking the facilitative mechanism for glucose entry. This may explain why an effect of insulin was superimposable upon the stimulatory effect of the enzyme (Table 2, exp. 1); under the experimental conditions, a certain number of the sites of action of insulin remained available despite the action of PHL-C.

Acknowledgement: The author is indebted to Miss Joan Carr for dedicated assistance in the performance of these studies.

References

Ansell, G.B., and Spanner, S., Biochem. J. 79, 176 (1961).
Dole, V.P., J. Clin. Invest. 35, 150 (1956).
Fain, J.N., Kovacec, V.P., and Scow, R.O., J. Biol. Chem. 240, 3522 (1965).
Krahl, M.E., The action of insulin on cells, Academic Press, N.Y., 1961.
Layne, E., in S.P. Colowick and N.O. Kaplan, eds., Methods in enzymology, vol. 3, Academic Press, N.Y., 1957, p. 450.
Rodbell, M., J. Biol. Chem. 239, 375 (1964).
Weiss, L., in D.J. Bell and J.K. Grant, eds., The structure and function of the membranes and surfaces of cells, Biochem. Soc. Sympos. no. 22, Cambridge Univ. Press, London, 1963, p. 32.