ON THE MECHANISM OF ACTION OF PHOSPHOLIPASE A AND INSULIN ON GLUCOSE ENTRY INTO FREE ADIPOSE CELLS *

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Recent reports (Blecher, 1965; Rodbell, 1966) showed that low concentrations of phospholipase C (PHL-C) acted as did insulin in stimulating glucose utilization by free adipose cells, prepared by treatment of tissue with a bacterial collagenase. Higher concentrations of the enzyme imitated cortisol in inhibiting glucose utilization, and such cells also failed to respond to either insulin or cortisol (Blecher, 1965). Neither amount of Cl. welchii PHL-C caused detectable hydrolysis of cell phosphatidyl choline, the primary substrate of this enzyme (Ansell and Hawthorne, 1964), although trace amounts of phosphoryl-serine and -ethanolamine were detected; no correlation could be made between formation of these trace hydrolytic products and effects of PHL-C on glucose utilization (Blecher, 1965). It was suggested (Blecher, 1965) that the action of PHL-C on plasma membrane lipoproteins (the basement membrane is lacking in such cells - Rodbell, 1966) might alter the charge distribution in a manner which invokes the facilitative mechanism for glucose entry, a mechanism which has been also postulated for insulin (Krahl, 1961). Rodbell (1966) has proposed a mechanism for these effects of PHL-C which may be mutually inclusive with the above: PHL-C, or insulin, converts cell boundary lipoproteins from laminar to micellar forms, thereby creating interstices which permit the carrier-mediated passage of glucose and amino acids into the adipose cell.

These postulates receive support from the present experiments which demonstrate that another lipolytic enzyme, phospholipase A [PHL-A, phosphatide acyl hydrolase (E.C. 3.1.1.4) of snake venom], affects glu-

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cose utilization by free adipose cells in a manner similar to that of PHL-C, and the data suggest that it does so by stimulating a facilitative mechanism in the cell boundary membrane.

Experimental

Rat adipose cell suspensions were prepared as previously described (Blecher, 1965), except that tissue was obtained from younger (5-7 weeks) rats, collagenase (from Cl. histolyticum, Worthington Biochemical Corp. lots 6597 and 65104) treatment was reduced to 1 h, and washed cell suspensions were used at protein concentrations of 0.5 to 1.0 mg per ml.

PHL-A activities of lyophilized venoms from Naja Naja (Ross Allen lot 91365 and Koch-Light 7317) were more effective than that of Crotalus adamanteus (Koch-Light lot 7291) in all parameters tested. Concentrated solutions of these venoms in 0.154 M NaCl remained stable at 4° for at least 2 weeks. Contaminating enzymes, e.g., proteases, were removed from PHL-A preparations by heating 2 mg per ml solutions at pH 5.5 (0.01 M citrate buffer) at 100° for 5 or 10 min.; denatured protein was centrifuged off, and the supernatant fluid adjusted to pH 7.4

PHL-A activity was assayed by a turbidimetric procedure at 750 mμ and 37° using egg yolk suspensions (Marinetti, 1965); as determined in a Gilford multiple sample absorbance recorder, lysophosphatide production, i.e., decrease in absorbancy, was linear for 10 min at enzyme concentrations up to 10 μg per ml. A unit of PHL-A activity caused a decrease in $A_{750\ m\mu}$ of 0.1 units per min; the preparation of Naja Naja venom used in the following experiments had a specific PHL-A activity of about 88 units per mg protein.

The composition of basal incubation mediums, incubation conditions, and isolation of radioactive CO_2 and total lipids were as previously described (Blecher, 1965). Glucose was assayed in aliquots of incubation mediums by a glucose oxidase procedure (Blecher and Glassman, 1962), following removal (floatation) of cells by centrifugation in plastic tubes.

To test for production of lysophosphatides and disappearance of their parent compounds after incubation with PHL-A, acidified incubation mixtures were subjected to previously described procedures (Abramson and Blecher, 1965) for the isolation of phospholipids; phospholipids were separated and identified by thin layer chromatography (Abramson and Blecher, 1964; Skipski et al, 1964).

Results and Discussion

PHL-A, at concentrations up to 6 μg per ml, stimulated glucose uptake and conversion of U-^{14}C-glucose to $^{14}CO_2$ and ^{14}C-total lipids in adipose cells (Fig. 1). Above this concentration, PHL-A inhibited all three parameters of glucose utilization, maximum inhibition being obtained at about 20 μg per ml. These effects of PHL-A were similar in direction and extent to those seen with PHL-C (Blecher, 1965; Rodbell, 1966); furthermore, these stimulatory effects of PHL-A, and of PHL-C, were similar to those of insulin (Rodbell, 1964; Blecher, 1965).

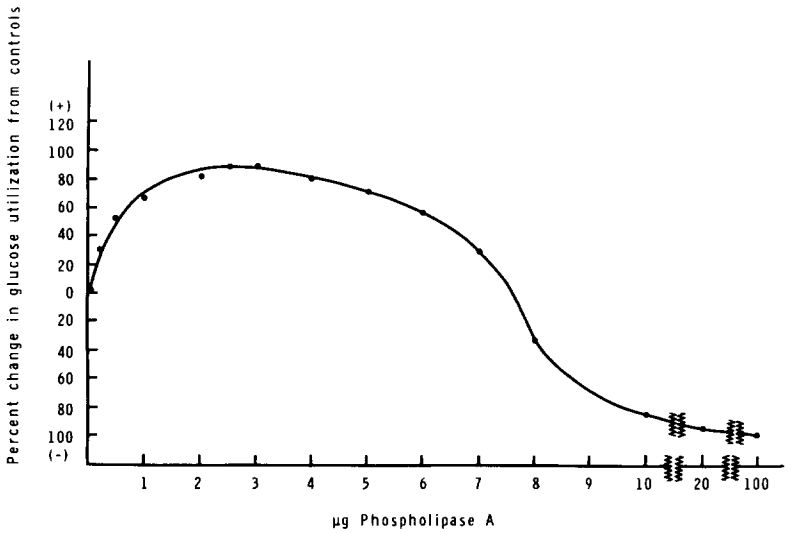

Fig. 1. Changes in the utilization of glucose by adipose cells due to PHL-A. Glucose utilization is expressed as μmoles of U-^{14}C-glucose carbon converted to $^{14}CO_2$ per mg cell protein in 2 h. PHL-A was present throughout the incubation period. Incubation conditions were as previously described (Blecher, 1965). Similar data were obtained when formation of ^{14}C-total lipids or glucose uptake were the parameters plotted.

Evidence that the aforementioned effects of Naja Naja venom preparations were due to their content of PHL-A was obtained by heating venoms under conditions known to remove heat-labile contaminating proteases but not to impair appreciably PHL-A activity (Hughes, 1935; Hayaishi, 1955; Condrea et al, 1965). Heating venom for 5 min at pH 5.5 and 100° precipitated about 14% of its protein but left unimpaired the ability of the enzyme preparation to stimulate glucose utilization by adipose cells; heating for 10 min reduced this ability by only 15%. As judged by the turbidimetric assay with egg yolk phospholipoprotein,

heat treatment for 5 or 10 min reduced the specific PHL-A activity about 30%.

Following incubation of adipose cells for 2 h with ineffective (0.01 μg), stimulatory (2.5 μg) and inhibitory (10 μg) amounts of PHL-A, phosphatides were isolated from pooled triplicate incubation mixtures. Total lipids amounted to about 56 mg per mg cell protein, with phospholipids representing about 1.5% of the total. Among cell phospholipids, phosphatidyl ethanolamine, phosphatidyl serine (largest amount), phosphatidyl choline, sphingomyelin and trace amounts of lysophosphatidyl choline were identified in each case, including controls; PHL-A failed to increase noticeably the amount of lysophosphatide or to decrease the amounts of PHL-A substrates in these cells.

Low concentrations of insulin stimulated glucose utilization in adipose cells; maximum effects of the hormone were observed at about 0.5 mU per ml (Rodbell, 1964; Blecher, 1965). Three μg of Cl. perfringens PHL-C with 1.0 mU of insulin was reported to decrease the effect of the hormone (Rodbell, 1966). We have observed, however, that 3 μg of Cl. welchii PHL-C did not inhibit the effect of 1.0 mU of insulin, but rather, failed to stimulate glucose metabolism beyond the maximum obtainable with insulin alone. The present observations with PHL-A plus insulin are similar to those previously reported for PHL-C plus insulin (Blecher, 1965). That is, as seen in Table 1, adipose cells in which glucose utilization was stimulated by PHL-A

TABLE 1

Effects of Phospholipase A and Insulin on Glucose Utilization by Adipose Cells

Additions	μmoles glucose carbon converted to product per mg cell protein in 2 h				μmoles glucose taken up per mg cell protein in 2 h	
	Carbon dioxide		Total lipids			
	- In	+ In	- In	+ In	- In	+ In
1. Basal	2.39	4.72	2.37	6.46	1.49	3.94
2. PHL-A, 0.02 μg	2.43	4.53	2.41	6.26	1.53	3.63
3. PHL-A, 2.5 μg	4.53	4.90	5.54	6.96	2.94	4.14
4. PHL-A, 20.0 μg	0.14	0.12	0.16	0.16	0.56	0.80

See text for conditions. Insulin, where present (+ In), 1.0 mU per ml.

(line 3) still remained sensitive to the stimulatory effects of insulin; however, the effects of the enzyme plus hormone were only slightly greater than those of insulin alone, suggesting saturation of the glucose entry system. In the presence of high, inhibitory concentrations of PHL-A (line 4), insulin stimulated glucose entry to only an insignificant extent and failed to affect the other two parameters of glucose metabolism.

In order to determine whether or not the stimulatory effects of PHL-A on glucose utilization by adipose cells reflected an increased transport of substrate by the carrier-mediated process previously established for muscle (Morgan et al, 1960), the effects of phloretin, N-ethyl maleimide and 3-O-methyl glucose on basal and PHL-A-stimulated glucose utilization were examined. Phloretin, like phloridzin, inhibits sugar transport into cells (Wilbrandt, 1950). N-ethyl maleimide apparently reduces the intracellular space available to sugars by combining with plasma membrane sulfhydryl groups (Battaglia and Randle, 1960). A characteristic of the carrier-mediated glucose transport system is competition between structurally similar sugars for entry into cells (Rosenberg and Wilbrandt, 1963), and 3-O-methyl glucose has been shown to be a competitive inhibitor of glucose transport in diaphragm (Battaglia and Randle, 1960) and heart muscle (Morgan and Park, 1958).

TABLE 2

Effects of N-Ethyl maleimide and Phloretin on Phospholipase A -Stimulated Glucose Utilization by Adipose Cells

Additions	Carbon dioxide		Total lipids		Glucose	
	- A	+ A	- A	+ A	- A	+ A
Basal	2.04	3.46	2.46	3.92	1.21	1.69
NEMI, 0.5 μM	2.14	3.02	2.60	3.74	1.04	1.52
5.0 μM	1.66	1.80	1.80	2.38	0.54	1.33
50.0 μM	0.04	0.02	0.06	0.08	0	0.33
Basal	2.69	6.20	3.14	8.06	1.36	4.40
Phloretin						
2.5 μM	1.84	6.36	2.04	7.90	1.36	3.82
25.0 μM	1.24	3.16	1.06	3.32	0.52	1.50
250.0 μM	0.06	0.06	0.06	0.06	0.09	0.24

See text for conditions and Table 1 for units of activity. Where present, PHL-A (+A) was 2.5 μg per ml.

The data of Table 2 indicate that both phloretin and N-ethyl maleimide inhibited both basal and PHL-A-stimulated utilization of glucose by adipose cells. It should be noted, however, that, in the phloretin experiments, the percent effect of PHL-A on each parameter was undiminished by the presence of inhibitor. These results suggest that these inhibitors, within certain concentration ranges, merely attenuated the PHL-A stimulated glucose entry mechanism of adipose cells.

3-O-Methyl glucose (15 and 30 mM) was a competitive inhibitor of both basal (Fig. 2A)and PHL-A stimulated (Fig. 2B) utilization of glucose (1.9 to 11.3 mM). 3-O-Methyl glucose has also been reported to be a competitive inhibitor of insulin-stimulated, as well as basal, glucose utilization in adipose cells, and to be an inhibitor of PHL-C stimulated glucose metabolism in the same tissue (Rodbell, 1966).

From the foregoing and previously reported evidence it seems apparent that a facilitative, carrier-mediated mechanism for glucose

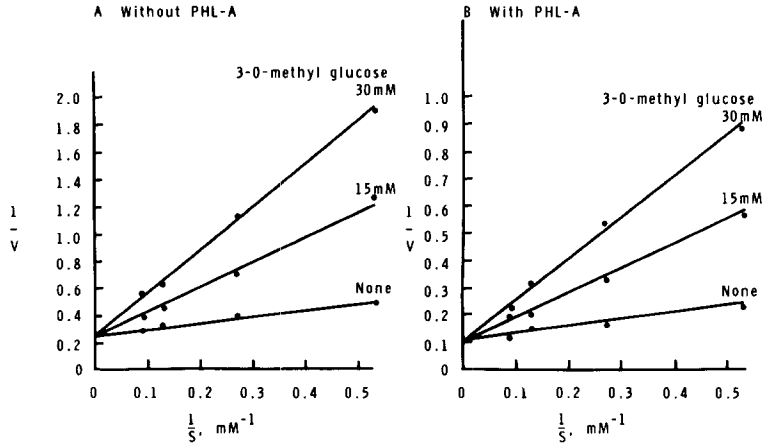

Fig. 2. Lineweaver-Burk plots of rates of basal (2A) and PHL-A-stimulated (2B) utilization of glucose by adipose cells in the presence of 3-O-methyl glucose. PHL-A concentrations in Fig. 2B were 2.5 μg per ml. Velocity (V) is expressed as μmoles U-^{14}C-glucose carbon converted to ^{14}C-total lipids per mg cell protein in 2 h. Similar curves were obtained when formation of $^{14}CO_2$ was the parameter plotted.

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entry is operative in the boundary membrane of adipose cells lacking a basement membrane, and that this process can be stimulated by low concentrations of insulin and phospholipases A and C. Although these phospholipases attack different types of bonds in plasma membrane phospholipids, their effects on the glucose entry mechanism were quite similar

in direction and extent. Furthermore, at relatively high concentrations of both phospholipases, not only was glucose utilization inhibited in unfortified incubation mixtures, but, in addition, the stimulatory effects of insulin, and the inhibitory effects of cortisol[1], were abolished. These observations support previous suggestions (Blecher, 1965; Rodbell, 1966) that insulin and these phospholipases, and perhaps cortisol as well, affect a common parameter that involves the phospholipoprotein of the plasma membrane. This common parameter is likely the configuration of such phospholipoproteins, and the action common to the above factors may be alteration of this configuration so as to stimulate (insulin and low concentrations of phospholipases) or to inhibit (cortisol) the facilitative mechanism for glucose entry. Simple hydrolysis of phospholipids may not be responsible for this alteration of configuration, since preliminary chromatographic experiments have failed to equate phospholipase action on glucose entry with appearance of hydrolytic products. Alteration of configuration of plasma membrane lipoproteins could occur by formation of enzyme-substrate complexes between phospholipases, Ca^{++} and their phospholipid substrates in the membrane; a similar interaction between insulin, Zn^{++} and membrane lipoproteins (perhaps via the quaternary nitrogens of phosphatides) has been proposed (Krahl, 1961).

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