Role of phosphodiesterase in glucagon resistance of large adipocytes

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Abstract The role of phosphodiesterase in glucagon resistance of large adipocytes was investigated. A comparison was made of phosphodiesterase activities of homogenates prepared from isolated small (mean diameter $\simeq$ 45 μm) and large (mean diameter $\simeq$ 78 μm) adipocytes, using various concentrations (5×10^{-4} to 1×10^{-7} M) of 3′,5′-cAMP. Kinetic analyses revealed two distinct catalytic activities (high and low affinities) in both cell types; however, the activities of both high- and low-affinity enzymes were significantly elevated in large adipocytes. Lipolysis was measured in isolated adipocytes in the presence of different concentrations (0.1–0.6 mM) of the phosphodiesterase inhibitor aminophylline. Large adipocytes were less responsive to low levels of methylxanthine, suggesting that greater amounts of phosphodiesterase must be inhibited before lipolysis can be stimulated. To evaluate the influence of phosphodiesterase during glucagon-stimulated lipolysis, small and large adipocytes were incubated with a maximally effective concentration of glucagon (1.5×10^{-6} M) in combination with various concentrations (0.1–0.6 mM) of aminophylline. Although the glucagon effect was potentiated in both cell types, the maximum lipolytic response of large adipocytes (at 0.4 mM aminophylline) was approximately 36% lower than that observed in small adipocytes (at 0.2 mM aminophylline). This reduction correlates closely with the decreased glucagon binding present in large cells; therefore, it appears that the glucagon-resistant state is adequately explained by elevations in phosphodiesterase levels and diminished glucagon–cell association.

Supplementary key words isolated fat cells · hormone-stimulated lipolysis · aminophylline · fat cell size

It has been demonstrated that large adipocytes exhibit a marked resistance to the lipolytic action of glucagon when compared with small cells (1, 2). These studies also provided evidence that this resistance is not related to an alteration of adenyl cyclase, protein kinase, or hormone-sensitive lipase.

Livingston, Cuatrecasas, and Lockwood (2) have demonstrated that reduced glucagon binding by large adipocytes contributes to the insensitivity to glucagon. It is evident, however, that the moderate decrease in binding of glucagon does not correlate well with the marked impairment of the lipolytic response of large fat cells to glucagon stimulation. This finding suggests an additional alteration in cellular metabolism.

Forn et al. (3) have observed an increase in total phosphodiesterase activity in large adipocytes and suggested that this elevated enzymatic activity contributes to the resistance of these cells to lipolytic hormones. No attempt was made, however, to separately measure the two catalytic activities (high and low affinities) of the enzyme known to be present in adipose tissue (4, 5) or to establish the contribution of elevated phosphodiesterase activity to the hormone-resistant state. We have consequently measured the phosphodiesterase activities in small and large adipocytes and have examined the role of this enzyme(s) in the lipolytic hormone resistance of large adipocytes.

MATERIALS AND METHODS

Tissue source and handling

Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, Mass.) were maintained on standard Purina rat chow fed ad lib. Adipocytes were isolated by the method of Rodbell (6) from epididymal fat pads of small (130–160 g) and large (400–450 g) rats.

Determination of phosphodiesterase

Isolated adipocytes (100–200 μg of DNA) were washed free of albumin and suspended in 5 ml of 250 mM sucrose, 1.0 mM Tris, pH 7.4, and 8.5 mM $MgCl_2$. The cells were disrupted with 15 strokes of a Dounce homogenizer and the homogenate was centrifuged at 2000 *g* for 20 min. After removing the fat cake, the precipitate was resuspended in the infranate and assayed for phosphodiesterase activity.

Abbreviations: 3′,5′-cAMP, cyclic 3′,5′-adenosine monophosphate; 5′-AMP, 5′-adenosine monophosphate.

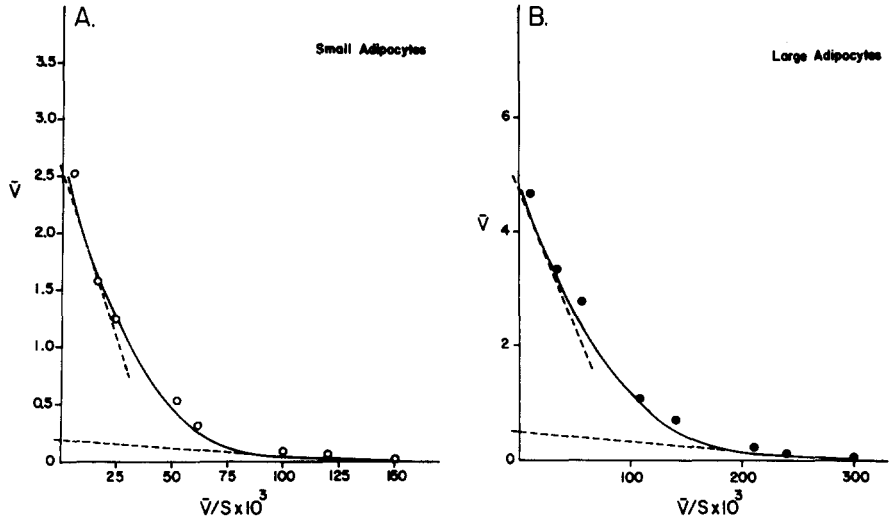

Fig. 1. Eadie-Hofstee plots of phosphodiesterase activities of small and large adipocytes. Phosphodiesterase activity was assayed in homogenates of isolated adipocytes using various concentrations (S) of 3′,5′-cAMP (5×10^{-4} to 1×10^{-7} M). Parallel measurements of enzyme activities were made in both small and large cells on a given day. Each point on the graph represents the mean enzyme activity ($\bar{V}$) of four experiments expressed as nmoles of [^{3}H]adenosine formed/10 min/10 μg of DNA. A best-fit curve was drawn by computer using a nonlinear least squares method for the results of the four experiments. Michaelis-Menten constants (K_m) were determined from the slope of the tangent line. Maximum enzyme activity was obtained from the $\bar{V}$ intercept.

The phosphodiesterase activity in a 50-μl aliquot (1–3 μg of DNA) of the cell preparation was assayed in an incubation mixture of 8.3 mM $MgCl_2$, 83 mM glycylglycine buffer, pH 8.5, 5×10^{-1} to 1×10^{-4} mM 3′,5′-cAMP, 83 mM sucrose, 0.33 mM Tris buffer, pH 7.4, and ^{3}H-labeled 3′,5′-cAMP (0.5×10^{-6} mM) (7). The assay mixture in a total volume of 0.3 ml was incubated at 30°C for 10 min. The reaction was terminated by the addition of 75 μl of a mixture composed of 0.33 N HCl, 3.3 mM 3′,5′-cAMP, and 4.4 mM 5′-AMP. The preparation was heated at 70°C for 4 min followed by neutralization of the mixture with 50 μl of 0.5 N NaOH in 0.5 M Tris buffer (pH 8.0). The generated ^{3}H-labeled 5′-AMP was converted to [^{3}H]adenosine with *Crotalus adamanteus* venom, and the product was chromatographed on a 3.0 × 0.5 cm Sephadex Q-25 column as described by Murad, Manganiello, and Vaughan (7).

Incubation of adipocytes for lipolysis

A 0.5-ml aliquot of isolated adipocytes (2–4 μg of DNA) was incubated in the absence of glucose in 1.5 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, which contained 4 g of fatty acid-poor bovine albumin per 100 ml. The incubations were conducted in plastic scintillation vials at 37°C under 95% O_2–5% CO_2 in the presence or absence of appropriate lipolytic agents. After 1 hr the incubations were terminated by rapidly centrifuging the incubation medium at 200 *g* for 30 sec at room temperature followed by aspiration of the resulting adipocyte layer. The amount of glycerol liberated by fat cells into the incubation medium was determined by the spectrophotometric method of Korn (8).

The DNA content of isolated fat cells was assayed by the diphenylamine method as previously described (2).

Statistical significance of data was determined by the paired *t* test (9).

Materials

Cyclic 3′,5′-AMP, 5′-AMP, calf thymus DNA used as DNA standard, aminophylline, and *Crotalus adamanteus* venom were obtained from Sigma (St. Louis, Mo.). ^{3}H-labeled 3′,5′-cAMP (0.0144 mg/1.0 mCi) was purchased from New England Nuclear (Boston, Mass.); fatty acid-poor bovine albumin (lot 24) was supplied by Pentex (Kankakee, Ill.); and Sephadex Q-25 was obtained from Pharmacia Fine Chemicals (Piscataway, N.J.).

RESULTS

Phosphodiesterase activities of homogenates from isolated small and large adipocytes were assayed at various concentrations (5×10^{-4} to 1×10^{-7} M) of 3′,5′-cAMP. The mean results of four separate experiments are plotted in Fig. 1 and summarized in Table 1. Both small and large fat cells possess two catalytically distinct phosphodiesterase activities (high and low affinities). The observed K_m values for the high- and low-affinity enzymes were similar in both cell types and agree with the K_m values determined by Solomon (10) for rat adipose tissue. At all substrate concentrations the phosphodiesterase activity of large cells was significantly greater ($P < 0.05$) than that found in homogenates of small cells. The maximum veloc-

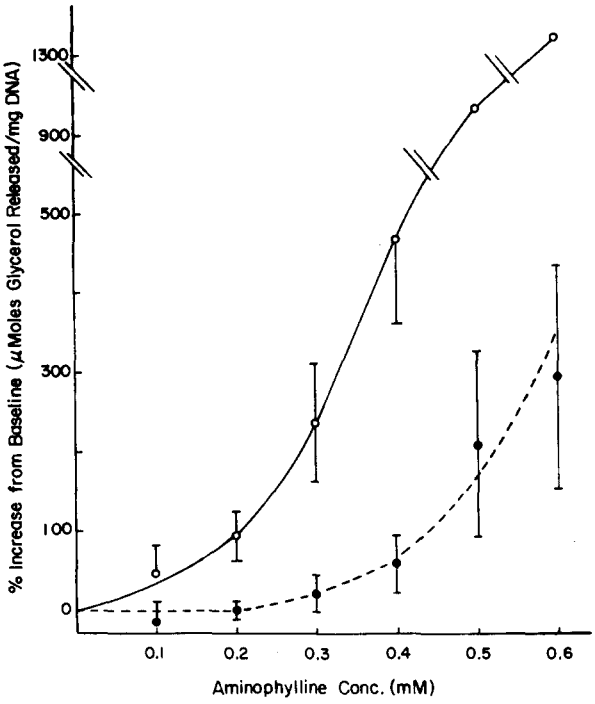

Fig. 2. Aminophylline-stimulated lipolysis of small (○) and large (●) adipocytes. Fat cells (2–4 μg of DNA) were assayed for lipolytic activity in the presence of various concentrations of aminophylline (0.1–0.6 mM), as described in Methods. Lipolysis was determined by the quantity of glycerol released per milligram of DNA during a 1-hr incubation period. Results are expressed as percentage increase above basal lipolysis. Each point is the mean of five experiments ± SEM.

ities of both high- and low-affinity enzymes of large adipocytes were approximately twice those of small cells.

Evidence that increased phosphodiesterase activity plays a role in lipolysis was shown by studies using low concentrations of the phosphodiesterase inhibitor aminophylline (Fig. 2). Aminophylline concentrations of 0.3–0.4 mM and 0.1–0.2 mM were required to stimulate lipolysis in large and small cells, respectively. The diminished lipolytic response of large adipocytes to these concentrations of aminophylline did not reflect a reduced lipolytic capacity, since aminophylline at 2.0 mM stimulated lipolysis to a greater degree than that seen in small cells (2). Furthermore, it appears that elevated phosphodiesterase levels are

TABLE 1. Michaelis-Menten constants and maximum velocities of phosphodiesterase activities in fat cell homogenates

Cell Type	K_m[a]	V_{max}[a,b]
Small adipocytes, high affinity	1.3×10^{-6} M	0.2 ± 0.02
Small adipocytes, low affinity	6.3×10^{-5} M	2.55 ± 0.28
Large adipocytes, high affinity	1.8×10^{-6} M	0.5 ± 0.09
Large adipocytes, low affinity	4.6×10^{-5} M	4.75 ± 0.65

[a]Calculated from Fig. 1.
[b]nmoles/10 min/10 μg of DNA ± SEM.

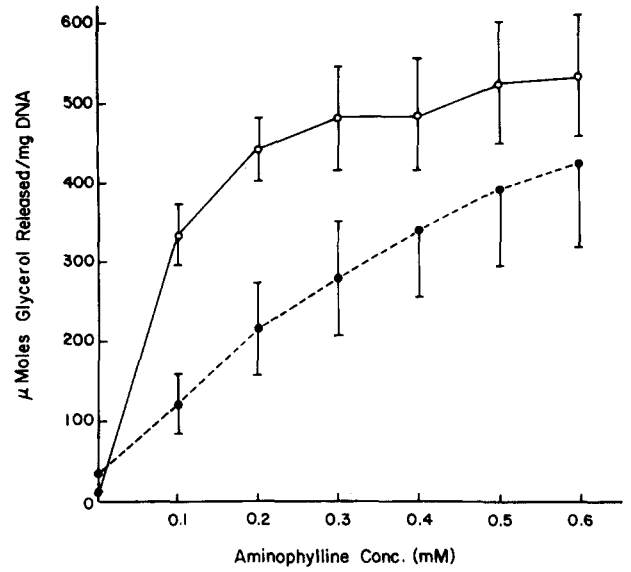

Fig. 3. Potentiation of fat cell lipolysis with combined aminophylline–glucagon stimulation. Glucagon at a concentration of 1.5×10^{-6} M was incubated with small (○) and large (●) fat cells in the presence of various concentrations of aminophylline (0.1–0.6 mM). Each point is the mean of five experiments ± SEM.

responsible for the reduced sensitivity of large cells to low concentrations of aminophylline because activities of the other enzymes involved in hormone-mediated lipolysis are apparently unaltered (1, 2).

The influence of phosphodiesterase during glucagon-mediated lipolysis was evaluated by incubating small and large cells with a maximally effective dose of glucagon (1.5×10^{-6} M) in combination with various concentrations of aminophylline (Fig. 3). The combined lipolytic response in both cell types was greater than the sum of effects produced when cells were incubated with either agent alone (2) (Fig. 2). At each concentration of aminophylline used, the lipolytic response was significantly greater ($P < 0.05$) in small than in large adipocytes, as indicated by the paired t test. In small cells maximal lipolysis was achieved at 0.3 mM aminophylline. Maximal lipolysis was not reached in large cells at any of the aminophylline concentrations used in combination with glucagon.

Because both aminophylline and glucagon contribute to stimulation of lipolysis, it is difficult to determine exactly the influence of glucagon on the lipolytic process during suppression of phosphodiesterase activity. However, by subtracting the amount of lipolysis stimulated by aminophylline alone from that produced by a combination of aminophylline and glucagon (Fig. 4), an approximation of the glucagon effect on lipolysis can be determined between small and large cells. The highest points on the curve (Fig. 4) occur between 0.2 and 0.3 mM aminophylline for small cells and at 0.4 mM aminophylline for large cells. At these concentrations of methylxanthine, the amount of functioning (noninhibited) enzyme should be approxi-

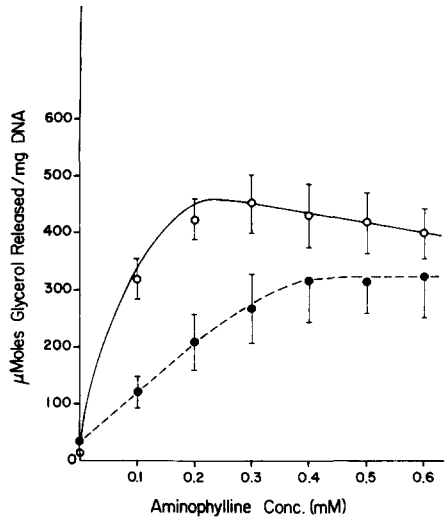

Fig. 4. Approximation of the glucagon effect on lipolysis during phosphodiesterase suppression in small (○) and large (●) adipocytes. The curves were obtained by subtraction of aminophylline-stimulated lipolysis from lipolysis induced by the combination of aminophylline with glucagon (1.5×10^{-6} M). Results were taken from day-matched experiments; each point is the mean of five experiments ± SEM.

mately equal for both cell types. Consequently, the amount of lipolysis at these concentrations of aminophylline provides a relative measurement of the effectiveness of glucagon stimulation. Under these conditions the amount of lipolysis produced by large cells is 64% of that of small adipocytes. The 0.1 and 0.2 mM aminophylline concentrations for small and large adipocytes, respectively, may also be used to determine the relative glucagon effect because phosphodiesterase levels in large adipocytes are approximately twice those in small cells. Using these concentrations, the glucagon effect in large cells is 56% of that observed in small cells.

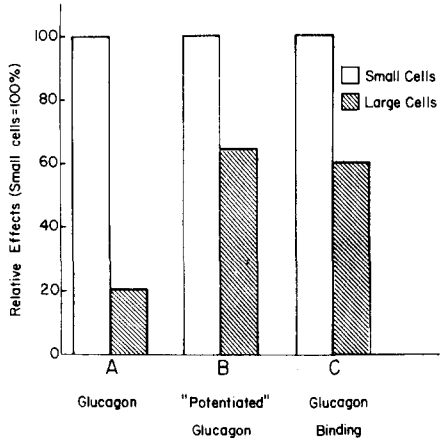

Fig. 5. Comparison of the relative effects (small cells = 100%) of lipolysis and glucagon binding in small and large adipocytes. A comparison of the relative effects of lipolysis between small and large adipocytes incubated for 1 hr in the presence of (*A*) glucagon (1.5×10^{-6} M) and (*B*) "potentiated" glucagon (at 0.2 mM aminophylline for small and 0.4 mM for large fat cells) is shown. The relative effects in the binding of saturating concentrations of glucagon (1×10^{-8} M) to small and large adipocytes is also presented (*C*).

DISCUSSION

Previous studies have indicated that resistance of large fat cells to glucagon stimulation apparently does not result from alterations in adenyl cyclase (1, 2) or in the protein kinase–hormone-sensitive lipase system (2). Manganiello and Vaughan (1) suggested that resistance to glucagon was caused by either a diminished number of glucagon receptors or an uncoupling of the receptors to adenyl cyclase. In support of the former suggestion, Livingston et al. (2) demonstrated a reduction in glucagon binding by large glucagon-resistant adipocytes. However, the reduction was not large enough to explain the marked glucagon insensitivity found in these cells. These observations suggest that an additional modification contributing to the resistant state is present in large adipocytes.

In agreement with previous investigations (4, 5), the present study demonstrates the presence of two catalytically different phosphodiesterase activities in rat epididymal adipose tissue. Homogenates of both small and large fat cells exhibit a high- and low-affinity phosphodiesterase activity. The similar K_m values found for each component of phosphodiesterase activity in small and large cells suggest that degradation of 3′,5′-cAMP is catalyzed by the same species of enzyme(s) in both cell types. However, large cells had greater maximal activities of both high- and low-affinity components than small cells. Forn et al. (3) also reported an elevation in total phosphodiesterase activity in large fat cells from adult rats, although a determination of the separate phosphodiesterase activities was not made. The importance of the high-affinity component in degradation of 3′,5′-cAMP was not determined in our study, but, under physiological conditions, it is possible that this component catalyzes the majority of 3′,5′-cAMP inactivation. The significance of elevated phosphodiesterase activity was examined by using aminophylline to inhibit the degradation of 3′,5′-cAMP by phosphodiesterase (11, 12). As shown in the present study, large fat cells require a greater concentration of aminophylline to stimulate detectable lipolysis than do small cells. Since alterations in other enzymes involved in hormone-mediated lipolysis were not detected in large adipocytes (1, 2), it would appear that the requirement of greater aminophylline concentrations in these cells is a reflection of elevated phosphodiesterase levels. As shown by Livingston et al. (2), stimulation of large adipocytes with maximally effective concentrations of glucagon resulted in only a small increase in lipolysis. However, in the presence of aminophylline in concentrations that would not in themselves stimulate glycerol release, glucagon-mediated lipolysis was greatly enhanced in isolated fat cells. These findings demonstrate the presence of functional glucagon receptors in the large, glucagon-resistant adipocytes.

The lipolytic effectiveness of glucagon (1.5×10^{-6} M) in a phosphodiesterase-suppressed system was determined

by subtracting the glycerol values resulting from the addition of aminophylline alone from the lipolysis data resulting from the combined administration of aminophylline and glucagon. In Fig. 4 the magnitude of the peak or plateau for each curve represents the maximum lipolytic response produced by glucagon (1.5×10^{-6} M) in a phosphodiesterase-suppressed system. The maximum "glucagon" effect in large fat cells required a higher aminophylline concentration when compared with small adipocytes. This observation suggests that the phosphodiesterase levels of large adipocytes inhibit glucagon-mediated lipolysis to a greater extent than that found in small cells and therefore contribute to the glucagon-resistant state. It is important to note that the maximum lipolytic effect of "glucagon" in large cells during phosphodiesterase suppression is only 64% of the peak effect found in small cells. Fig. 5 summarizes this finding and compares it with ^{125}I-labeled glucagon-binding results (2). When the effect of elevated phosphodiesterase activity is diminished or eliminated (Fig. 5, *B*), lipolysis in large cells increases from 20% (*A*) to 64% of that of small cells. The amount of ^{125}I-labeled glucagon binding by large cells (*C*) then closely correlates with their lipolytic response to glucagon. The glucagon-resistant state therefore appears to involve both a decrease in the glucagon binding capability of large cells and an increase in phosphodiesterase activity. These findings provide an explanation for previously reported results (1) that indicate a moderate reduction in glucagon-stimulated adenyl cyclase activity in membranes of large adipocytes even though the lipolytic action of the hormone was markedly reduced below that found in small cells.

The elevation in phosphodiesterase activity is of particular importance for it represents a metabolic alteration that should uniformly affect the response of large adipocytes to all lipolytic hormones, especially when the stimulatory effect is of a low magnitude. This is supported by the findings that large cells require greater concentrations of epinephrine to achieve maximum lipolysis and that, at low concentrations of the hormone, their lipolytic response is much less than that of small cells (2). Also, the lipolytic response of large cells to small concentrations of ACTH is apparently diminished even though the maximum responses to large concentrations of the hormone are similar in both types of cells.[1] That the insensitivity to epinephrine and ACTH can be counteracted by high concentrations of these agents is probably related to the ability of fat cells to produce more 3′,5′-cAMP than that required for maximum lipolysis after a lipolytic stimulation of large magnitude (13). Thus, the marked resistance of large cells to glucagon stimulation would be expected because glucagon is a weak lipolytic agent even in small cells. In addition, large cells are less able than small cells to interact with this hormone, which magnifies the glucagon resistance.

These findings indicate that phosphodiesterase levels may play an important role in regulating fat cell response to lipolytic hormones. However, it is not known what underlies the elevation in phosphodiesterase activity of large cells. Various factors such as cell size and diet have been shown to affect the sensitivity of rat adipocytes to stimulation by insulin (14) and lipolytic hormones (15, 16). In contrast, age does not appear to contribute to insulin (14) or catecholamine (15) insensitivity. To our knowledge, the possible effects of these factors on phosphodiesterase levels have not been determined.

At present it is unclear whether adipose tissue from obese humans is resistant to lipolytic hormone stimulation (17, 18), although recent reports suggest that norepinephrine-stimulated lipolysis is diminished (19–21). It is also unclear if glucagon plays an important physiological role in stimulating lipolysis in adipose tissue even though it is classified as a lipolytic hormone. Foà (22) has suggested that glucagon does promote lipolysis and transfer of free fatty acids to heart and skeletal muscles, especially during starvation. Also, glucagon was shown to produce a significant rise in the plasma levels of free fatty acids in patients that were insulin resistant after acute myocardial infarction (23). This finding suggests that glucagon can influence the rate of lipolysis in vivo. If elevations in phosphodiesterase are present in obese human adipose tissue, the magnitude of resistance to a weak lipolytic agent such as glucagon might be greater than that found with more potent lipolytic agents. Findings in rats indicate that the marked resistance to low concentrations of epinephrine is overcome in large part by maximum hormone stimulation (2). This may explain why some studies in human adipose tissue employing large amounts of potent lipolytic hormones to determine hormone sensitivity have not shown lipolytic hormone resistance.

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