Studies of glucagon resistance in large rat adipocytes: ^{125}I-labeled glucagon binding and lipolytic capacity

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Abstract This study is concerned with potential modifications of large fat cells from adult rats (400–450 g) that make them resistant to stimulation by glucagon. The lipolytic capacity and ^{125}I-labeled glucagon-binding capability of these cells were compared with these properties of small glucagon-sensitive cells from young rats (130–160 g). As determined by maximal stimulation with theophylline, dibutyryl cAMP, or epinephrine, the lipolytic capacity of large cells was not markedly different from small cells, which suggests that an alteration contributing to glucagon insensitivity is not present in the enzymes involved with hormone-mediated lipolysis. Glucagon-binding studies did indicate a difference between the two cell types. Both large cells and particulate fractions from large cells bound less ^{125}I-labeled glucagon than small cells or small-cell particles. That diminished binding is not a consequence of glucagon degradation is indicated by the similar amounts of ^{125}I-labeled glucagon degraded by both cell types. The decrease in ^{125}I-labeled glucagon binding was not as marked as the decrease in lipolytic response to glucagon stimulation. This lack of correlation and the relationship between elevated phosphodiesterase levels and glucagon insensitivity described in the accompanying report suggest that diminished binding explains only in part the marked resistance to glucagon found in large cells.

Supplementary key words isolated fat cells · hormone-stimulated lipolysis · phosphodiesterase · resistance to glucagon · ^{125}I-labeled glucagon binding · glucagon degradation

Manganiello and Vaughan (1) have recently shown that large fat cells from adult rats were less sensitive to glucagon stimulation of lipolysis than small cells from young animals. The underlying mechanism responsible for the insensitivity was not determined, although a reduced responsiveness of adenyl cyclase to glucagon was found in membranes isolated from resistant cells.

Hormone-stimulated lipolysis is the end result of a complex sequence of events initiated by hormone–receptor interaction (2–5). In glucagon-resistant cells, an alteration may exist in the glucagon receptor or in enzymes that catalyze the intracellular events associated with hormone-mediated lipolysis or with degradation of 3′,5′-cAMP.

The present studies demonstrate a decrease in the capability of large cells to specifically bind ^{125}I-labeled glucagon when compared with binding by small, more sensitive cells. This modification coupled with the elevation in phosphodiesterase levels found in large cells, discussed in the following report (6), apparently contributes significantly to the glucagon-resistant state.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, Mass.) were maintained on standard Purina rat chow and water ad lib. Rats weighing 130–160 g were used to obtain "small" fat cells having a mean diameter of 45 μm. "Large" fat cells with a mean diameter of 78 μm were isolated from rats allowed to reach weights of 400–450 g. The cell preparations from large rats contained 28 μmoles of triglyceride/μg of DNA compared with 9 μmoles of triglyceride for small rat cell preparations. This indicates that the mean volume of large cells is roughly three times greater than that of small cells. However, if the volumes are calculated using mean diameters, the large cell volume is five times greater. The difference between the two methods for determining the relative cellular volume probably results from adipocyte heterogeneity, especially as found in preparations of small cells (7).

Tissue preparation and assay methods

Rats were killed by a sharp blow to the head, and the fat cells were isolated from epididymal fat pads after exposure to collagenase according to the method of Rodbell (8). Adipose tissue was routinely treated with 1.0 mg of collagenase/ml for 30 min at 37°C. The use of collagenase concentrations ranging from 0.5 mg/ml to 1.0 mg/ml and incubation times of 30–60 min to disperse the cells did not cause a difference in the response of fat cells to insulin or

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

glucagon stimulation. Fat cells were suspended in Krebs-Ringer bicarbonate buffer, pH 7.4, which contained 1.0% albumin (w/v). Fat cell number in the suspension was estimated by determining the DNA content in a concentrated cell preparation that contained 20–30 μg of DNA. DNA was precipitated by adding 5 ml of a solution of absolute ethanol–20% sodium acetate 9:1 (v/v) to 1 ml of cell suspension. Residual lipids in the precipitate were extracted by washing it twice with 5 ml of hot absolute ethanol and ethyl ether 3:1 (v/v). The precipitate was further washed with 5 ml of 0.5 N perchloric acid, and the DNA content was estimated with diphenylamine as described by Burton (9).

The rate of lipolysis was determined by measuring glycerol release into the incubation medium by isolated adipocytes. 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 adipocyte layer. The amount of glycerol in the incubation medium was determined by the spectrophotometric method of Korn (10).

^{125}I-labeled glucagon (0.88 Ci/μmole) and ^{125}I-labeled insulin (1.1 Ci/μmole) for hormone-binding studies were prepared by iodination with Na^{125}I using chloramine-T and purified on talc as described by Cuatrecasas for insulin (11). The number of iodine atoms per molecule of labeled hormone was approximately 0.5 and 0.63 for glucagon and insulin, respectively. 95% of the radioactivity of both ^{125}I-labeled insulin and ^{125}I-labeled glucagon was precipitated by 5% trichloroacetic acid.

Hormone binding by intact fat cells was determined using an oil flotation technique (12, 13). Fat cells (1.5–3.0 μg of DNA) were routinely incubated with ^{125}I-labeled glucagon in concentrations ranging from 0.5 to 10 $\times$ 10^{-9} M in 0.3 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 1.0 g of bovine albumin per 100 ml. Cells were incubated for 15 min at 24°C in the presence or absence of excess unlabeled glucagon (1 $\times$ 10^{-6} M). The incubation mixture was transferred to plastic microcentrifuge tubes (Beckman, Fullerton, Calif.) containing 75 μl of dinonyl phthalate oil. Fat cells were separated from the aqueous incubation mixture by rapid centrifugation at 10,000 *g* for 45 sec in a Beckman microfuge. Centrifugation packs the fat cells in a layer over the oil, which is interspersed between the cells and the aqueous media. The tubes were cut at the cell–oil interface, and the radioactivity of the portion containing the cells was determined by a Nuclear-Chicago (Des Plaines, Ill.) gamma detector with 55% counting efficiency. No difference was found in the amount of labeled hormone bound when the phthalate oil was layered over the fat cell incubation suspension, which required the cells to travel through the oil during centrifugation. ^{125}I-labeled hormone contamination in the oil was 0.1–0.2% of the total amount added to the incubation mixture.

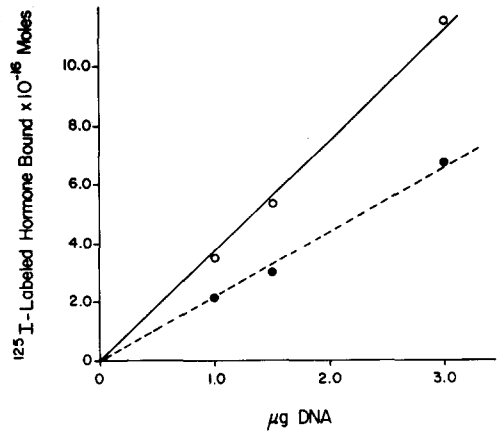

Fig. 1. Specific binding of ^{125}I-labeled insulin (○) and glucagon (●) as a function of fat cell number. Intact fat cells were incubated in the presence of 3.7 $\times$ 10^{-10} M iodinated insulin or 5.0 $\times$ 10^{-9} M iodinated glucagon, and specific binding was determined as indicated in Methods using the oil separation technique.

^{125}I-labeled insulin-binding studies were performed in a manner similar to ^{125}I-labeled glucagon-binding studies. ^{125}I-labeled insulin in a concentration range of 0.1–4.0 $\times$ 10^{-9} M was incubated with fat cells in the presence or absence of 8 $\times$ 10^{-6} M unlabeled insulin for 30 min under conditions described for glucagon-binding experiments.

For the purposes of this study, "specific" binding is defined as the difference between total binding of ^{125}I-labeled hormone (no unlabeled hormone) and binding in the presence of a large excess of unlabeled hormone (11). This component of binding presumably represents the interaction of the hormone receptor with its specific hormone (11). All binding results are reported as specific binding.

The amount of specific binding by fat cells regardless of size was linearly related to the number of cells (1–3 μg of DNA) used in the assay (Fig. 1). Specific binding of ^{125}I-labeled glucagon ranged from 33 to 66% of the total binding in isolated cells and from 50 to 75% of the total binding in particulate fractions from fat cells. In large cells and particles from large cells, nonspecific binding was usually greater than that found in small cells and small-cell particles. "Displacement" in both cell types was observed with concentrations of unlabeled glucagon as low as 1.0 $\times$ 10^{-9} M. When the concentration of labeled glucagon was 5.0 $\times$ 10^{-9} M, maximum "displacement" in both cell types was observed with concentrations of unlabeled hormone as low as 0.1 $\times$ 10^{-6} M. For routine studies the concentration of unlabeled glucagon used was 1.0 $\times$ 10^{-6} M.

The particulate cell fraction used in binding studies was prepared from isolated fat cells. Cells in Krebs-Ringer bicarbonate buffer, pH 7.4, 0.1% albumin, were homogenized for 30 sec by a Brinkmann Polytron PT-10 (Westbury, N.Y.) set at position 3.0. The homogenate was centrifuged at 30,000 *g* for 30 min to obtain maximum collection of glucagon-binding sites. The pellet was washed

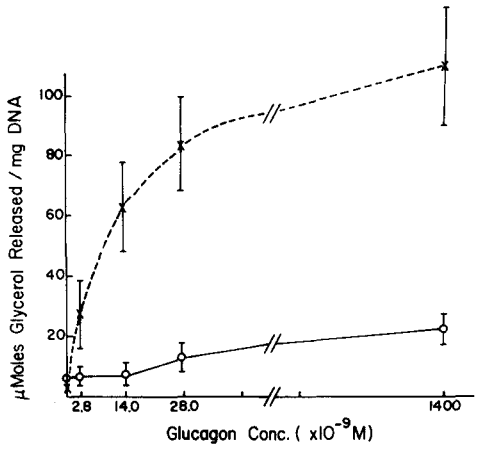

Fig. 2. Effects of various concentrations of glucagon on lipolysis in large (○) and small (×) rat adipocytes. Glycerol release was measured after incubation of isolated fat cells for 1 hr at 37°C under conditions described in Methods. Each point is the mean ± SEM of four experiments.

twice with 50 mM Tris buffer, pH 7.4, and the protein content was determined by the method of Lowry et al. (14) after heating the pellet at 70°C for 30 min in 1 N NaOH.

Recovery of membranes was estimated by labeling intact fat cells with ^{125}I-labeled wheat germ agglutinin. This plant lectin binds tightly to glycoproteins located on the plasma membrane surface of fat cells (15). Fat cells (150 μg of DNA) were incubated with 1.0 μg of ^{125}I-labeled wheat germ agglutinin per ml for 15 min. Cells were washed four times with twice their volume of Krebs-Ringer bicarbonate buffer, after which the radioactivity in a sample of cell suspension was determined. The cells were then homogenized and the particulate fractions were isolated as described above. Approximately 40% of the radioactivity was recovered in the particulate fraction prepared from small cells in comparison with 47% recovery calculated in the particulate fraction of large cells.

Binding of ^{125}I-labeled glucagon to particulate fractions was determined using a Millipore filtration technique (11, 16). Particles (1.0–1.5 mg of protein/ml) were incubated in 0.2 ml of 50 mM Tris buffer, pH 7.4 (1.0% albumin), with ^{125}I-labeled glucagon in a concentration range of 0.1–2.0 × 10^{-9} M. After incubation for 30 min at 24°C, the particles were collected on EGWP Millipore filters (Bedford, Mass.) and rapidly washed; radioactivity of the filter was then determined. Nonspecific binding was determined by the addition of 5 × 10^{-6} M unlabeled glucagon to appropriate incubation mixtures. ^{125}I-labeled insulin binding by particulate fractions was assayed in the presence and absence of 8 × 10^{-6} M unlabeled insulin under similar conditions used for glucagon-binding studies.

Degradation of ^{125}I-labeled glucagon by isolated fat cells was measured as previously reported for liver membranes (16). Fat cells (2–3 μg of DNA) were incubated with ^{125}I-labeled glucagon in the absence of unlabeled hormone under the binding assay conditions described for intact cells. After removal of fat cells by the oil flotation method, the infranate was incubated with isolated liver membranes in the presence and absence of large amounts of unlabeled glucagon. ^{125}I-labeled glucagon binding was determined as described for fat-cell particles using the Millipore filtration technique. The specific binding by liver membranes incubated with infranate was compared with specific binding by a control preparation.

TABLE 1. Comparative effects of lipolytic agents on lipolysis in large and small fat cells

Addition	Small Cells	Large Cells
	nmoles glycerol/μg DNA/hr	
Dibutyryl cyclic AMP		
3 × 10^{-3} M	460 ± 34[a]	601 ± 44[b]
Theophylline		
2 × 10^{-3} M	389 ± 38	486 ± 45[c]
Epinephrine		
0.55 × 10^{-6} M	320 ± 52	119 ± 49[b]
1.6 × 10^{-6} M	415 ± 26	165 ± 37[b]
4.8 × 10^{-6} M		365 ± 11
Glucagon[d]		
1.4 × 10^{-6} M	110 ± 20	22 ± 4[b]

Values are maximal rates of lipolysis induced by dibutyryl cyclic AMP and theophylline.
[a] Means ± SEM of four experiments.
[b] $P < 0.05$ as determined by paired t test.
[c] Not statistically significant.
[d] Taken from Fig. 1.

Materials

Crystalline porcine zinc–insulin and crystalline glucagon were obtained from Eli Lilly (Indianapolis, Ind.). ^{125}I-labeled wheat germ agglutinin was prepared and purified as previously described (15). Dibutyryl cAMP, theophylline, and calf thymus DNA used as DNA standard were obtained from Sigma (St. Louis, Mo.); epinephrine was purchased from Parke, Davis & Co. (Detroit, Mich.). Fatty acid-poor bovine albumin (lot 24) was supplied by Pentex (Kankakee, Ill.); Na^{125}I in 0.1 N NaOH was purchased from Union Carbide (New York); and dinonyl phthalate oil was supplied by Eastman (Rochester, N.Y.).

RESULTS

Results of this study confirm the previously reported marked resistance of large fat cells to stimulation by glucagon (1). As shown in Fig. 2, the lipolytic response elicited by large concentrations of glucagon in large cells was approximately one-fifth of that obtained in small cells. No response was found in large cells at hormone concentrations of 1.4 × 10^{-8} M, although glucagon concentrations of 0.28 × 10^{-8} M elicited measurable lipolysis in the more sensitive cells.

Theophylline, db cAMP, and epinephrine were used to determine the adequacy of the protein kinase and hormone-sensitive lipase in resistant cells. At appropriate concentrations, these lipolytic agents stimulated lipolysis

much more than glucagon in both glucagon-sensitive and insensitive fat cells (Table 1). Large cells produced greater amounts of glycerol than small cells when incubated with relatively large concentrations of db cAMP, while no significant difference in lipolysis was detected when these cells were incubated with a high concentration of theophylline. Epinephrine, which mediates a physiological activation of the kinase–lipase system, produced less lipolysis in both types of cells. At the lowest concentration of catecholamine used, large cells were markedly less sensitive than small cells to epinephrine stimulation. However, the insensitivity was reduced as larger concentrations of epinephrine were employed. Maximum stimulation of large cells required a greater concentration of catecholamine and resulted in a slightly lower lipolytic response than that found in small cells. Although the response of large cells to epinephrine is diminished, it is many times greater than the maximum lipolysis stimulated by glucagon. These results indicate that the alteration(s) responsible for insensitivity to glucagon does not directly involve the ability of large cells to hydrolyze triglycerides or to activate hormone-sensitive lipase.

The association between glucagon and cells was investigated by comparing the specific binding of ^{125}I-labeled glucagon in large and small cells. As shown in Fig. 3, small, sensitive fat cells bound significantly more ^{125}I-labeled glucagon at all concentrations of labeled hormone above 0.5×10^{-9} M than did large, resistant cells. Specific binding was detected in both cell types at glucagon concentrations as low as 0.5×10^{-9} M, and saturation of binding sites was approached at concentrations near 4×10^{-8} M. A close correlation between ^{125}I-labeled glucagon binding and the glucagon dose-response curve (Fig. 2) is evident for small cells. Maximum lipolysis and maximum binding occurred with similar concentrations of iodinated and unlabeled glucagon; furthermore, in a concentration range of $1–5 \times 10^{-9}$ M, both lipolysis and binding were linearly related to the amounts of these agents. A similar correlation cannot be made in large cells because of their marked insensitivity to glucagon.

Since the decrease in ^{125}I-labeled glucagon binding may have resulted from preferential destruction of large cells during the rapid centrifugation required in the binding assay, ^{125}I-labeled insulin-binding studies were carried out. Previous work using a Millipore filtration technique demonstrated an equal number of insulin receptors on large and small cells (17). Similar results were found when ^{125}I-labeled insulin binding was assayed by the oil separation technique (Table 2). This correlation between the two methods, one of which does not employ rapid centrifugation, indicates that there was no significant difference in the amount of cell destruction between the two cell types during the ^{125}I-labeled glucagon-binding assay. As expected, when binding of ^{125}I-labeled glucagon and ^{125}I-labeled insulin was determined in parallel experiments, ^{125}I-labeled glucagon binding by large cells was approximately half that of small cells when expressed on the basis of ^{125}I-labeled insulin binding (Table 2). The ratio values presented in Table 2 represent a relative measurement of glucagon and insulin binding and do not indicate ratios of absolute receptor numbers because saturating concentrations of the iodinated hormones were not used.

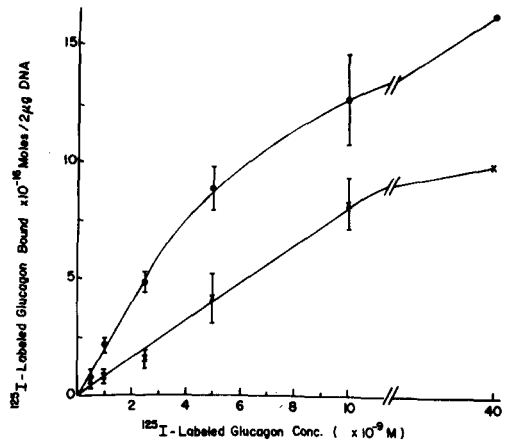

Fig. 3. Specific binding of ^{125}I-labeled glucagon by large (×) and small (●) rat adipocytes. Each point is the mean ± SEM of four experiments. Conditions for determining binding are described in Methods. Glucagon binding was significantly different between large and small cells ($P < 0.05$) at all ^{125}I-labeled glucagon concentrations except 0.5×10^{-9} M as determined by paired t test.

Membranes from liver (16, 18) and pancreatic beta cells (19) have been shown to degrade glucagon rapidly. Since the magnitude of ^{125}I-labeled glucagon binding in this study is a function of ^{125}I-labeled glucagon concentration, an increase in the rate of glucagon degradation by large cells could explain their diminished binding capability. For this reason, the relative amounts of ^{125}I-labeled glucagon inactivated by large and small fat cells was determined (Table 3). Glucagon degradation is shown by the diminished binding of ^{125}I-labeled glucagon in liver membranes after incubation of the labeled hormone with intact fat cells. However, large and small cells inactivated similar amounts of ^{125}I-labeled glucagon. Although it appears that degradation does not contribute to diminished glucagon binding by large cells, degradation may be responsible for the relatively large concentrations of hor-

TABLE 2. ^{125}I-labeled insulin binding and binding ratio of ^{125}I-labeled glucagon to ^{125}I-labeled insulin in isolated fat cells

^{125}I-labeled Insulin Concn $\times 10^{-10}$ M	^{125}I-labeled Insulin Bound $\times 10^{-16}$ moles/2 µg of DNA	
	Small Cells	Large Cells
1.3	2.8 ± 0.5[a]	4.2 ± 0.2
3.7	7.6 ± 1.1	9.7 ± 0.6
7.5	13.5 ± 1.6	16.3 ± 1.3
18.8	28.8 ± 4.1	31.3 ± 1.8
37.7	51.8 ± 9.4	43.4 ± 5.6
^{125}I-labeled glucagon[b]/ ^{125}I-labeled insulin[c]	0.28 ± 0.08	0.14 ± 0.04

[a] Means ± SEM of three experiments.
[b] Concentration of ^{125}I-labeled glucagon in assay was 1×10^{-8} M.
[c] Concentration of ^{125}I-labeled insulin in assay was 1.9×10^{-9} M.

TABLE 3. Degradation of ^{125}I-labeled glucagon by isolated fat cells

^{125}I-labeled Glucagon Concn $\times 10^{-9}$ M	% Reduction in ^{125}I-labeled Glucagon Binding[a]	
	Small Cells	Large Cells
2.5	43	44
5.0	40	43

This experiment was performed in parallel with ^{125}I-labeled glucagon-binding studies which demonstrated a reduction in binding by large cells. For the degradation experiment, fat cells (2.5 μg of DNA) were incubated with the indicated concentration of ^{125}I-labeled glucagon for 15 min at 24°C. Cells were removed from the incubation medium by the oil flotation method. The infranate was incubated with isolated liver membranes as described in Methods. ^{125}I-labeled glucagon inactivation is given as percentage reduction in liver-membrane binding by comparison with a control preparation. The "infranate" used for the control was treated as described above except fat cells were absent.

[a] ^{125}I-labeled glucagon binding by liver membranes.

mone required in vitro to stimulate lipolysis maximally and to saturate glucagon-binding sites.

^{125}I-labeled glucagon-binding studies in particulate fractions isolated from both cell types were used to further establish the diminished glucagon-binding capability of large cells (Table 4). A Millipore filtration method was employed to determine binding by the particulate fractions. "Specific" binding could be measured using this method because of the greater numbers of glucagon-binding sites in the particulate fraction assay. Also, the greater concentration of receptors allowed detection of binding at lower concentrations of ^{125}I-labeled glucagon than was required in the binding assays for intact cells. However, as shown in cells, ^{125}I-labeled glucagon binding by particles from large cells was less than that bound by particles from small cells. The difference is evident regardless of whether binding is based on the number (DNA) of cells from which the particles were isolated or on the protein content of the particulate fraction. As an added control, ^{125}I-labeled insulin binding was used as a basis for ^{125}I-labeled glucagon binding by particles from large and small cells.

TABLE 4. Binding of ^{125}I-labeled glucagon by particulate fractions from large and small fat cells

^{125}I-labeled Glucagon Concn $\times 10^{-10}$ M	^{125}I-labeled Glucagon Bound $\times 10^{-16}$ moles/30 μg of DNA[a]	
	Small Cells	Large Cells
1	5.9	2.8
2	12.2	5.0
4	24.4	8.5
20	94.0	34.0

These data are averages of two experiments in which a total of 8 large and 18 small rats were killed and the particulate fraction of isolated fat cell prepared.

[a] DNA content was determined in an aliquot of isolated fat cells from which the particulate fraction was prepared. Protein content in the binding assay for both large and small particulate fractions was approximately 0.24 mg.

TABLE 5. ^{125}I-labeled insulin binding and binding ratio of ^{125}I-labeled glucagon to ^{125}I-labeled insulin in particulate fractions of fat cells

Cell Particles	^{125}I-labeled Insulin Binding[a] $\times 10^{-16}$ moles/30 μg of DNA	^{125}I-labeled Glucagon[b]/ ^{125}I-labeled Insulin[a]
Small	34.9 ± 3.6[c]	1.14
Large	32.9 ± 7.1	0.56[d]

[a] ^{125}I-labeled insulin concentration in binding assay was 2.5×10^{-10} M.

[b] ^{125}I-labeled glucagon concentration in binding assay was 5.0×10^{-10} M.

[c] Means ± SEM of three experiments.

[d] $P < 0.05$ as determined by paired t test.

Results of this study again indicate diminished ^{125}I-labeled glucagon binding by particles from large cells (Table 5). Furthermore, the similar amounts of ^{125}I-labeled insulin bound by particles from large and small cells suggest that recovery of plasma membranes was similar (Table 5). This is further supported by the results of prelabeling the plasma membrane of intact fat cells with ^{125}I-labeled wheat germ agglutinin. As indicated in the methods section, recovery of labeled membrane was similar in the particulate fractions prepared from large and small cells.

DISCUSSION

Alterations in one or more processes involved in hormone-mediated lipolysis must be responsible for the glucagon-resistant state found in large rat adipocytes. One potential site of such an alteration is the first event associated with glucagon-stimulated lipolysis, i.e., hormone–cell interaction. Other potential sites include the enzymes involved in the intracellular events elicited by this interaction. Adenyl cyclase (2, 3), protein kinase (4, 5), and hormone-sensitive lipase (20, 21) are essential components of the complex process that link the membrane phenomenon (glucagon–receptor association) with the observed physiological response (lipolysis). An alteration in the glucagon receptor or in any of the enzymes could impair the capability of fat cells to respond to glucagon. In this study, an effort was made to systematically investigate each of these sites for alterations in the large fat cells.

The investigation was facilitated by the characteristic type of lipolytic response found with glucagon stimulation. Even in small, sensitive cells, maximal lipolysis elicited by glucagon is much less than that induced by more potent lipolytic agents. By using large concentrations of db cAMP, theophylline, and epinephrine to stimulate lipolysis, it was possible to show that protein kinase and hormone-sensitive lipase levels must not be diminished in large resistant cells; instead, the enzyme levels may be greater than those of small cells. The alterations responsible for the resistant state must therefore reside at a site or process in lipolysis that precedes these two enzymes. However, the increased lipolytic capacity could be respon-

sible for the elevation in basal (unstimulated) lipolysis reported in large fat cells (22, 23).

Glucagon and other lipolytic hormones mediate their biological effects through activation of a common adenyl cyclase (2, 3, 24). No direct measurements of adenyl cyclase activity were made in this study. However, the observation that epinephrine-induced lipolysis is much greater in large cells than that stimulated by glucagon suggests adenyl cyclase levels are adequate to provide a "normal" response to glucagon. The results of studies by Manganiello and Vaughan (1) and by Hartman et al. (22), which report the levels of adenyl cyclase activity in membranes prepared from large and small adipocytes, agree with this suggestion. Adenyl cyclase activities, when stimulated by NaF or catecholamines, were similar in membranes from both cell types. Manganiello and Vaughan (1) further reported a diminished adenyl cyclase response to glucagon in large cell membranes even though the NaF or catecholamine responses were similar to those of small cell membranes. They suggested a modification was present either in the glucagon receptor or in coupling of the receptor to adenyl cyclase.

Not all the findings in studies of adenyl cyclase activity in large and small fat cells concur. Forn et al. (25) demonstrated a decrease in both NaF- and norepinephrine-stimulated adenyl cyclase activities in membranes from large cells of old rats. Recently, Gorman, Tepperman, and Tepperman (26) reported that the feeding of fat to rats for a short period of time is associated with an increase in fat cell size and a decrease in epinephrine and glucagon stimulation of adenyl cyclase activity.

Glucagon–cell association in the glucagon-resistant state was investigated by binding studies in which glucagon labeled with an average of 0.5 atom of ^{125}I per molecule of hormone was used. Goldfine, Roth, and Birnbaumer (19) demonstrated that similarly labeled glucagon (0.5 atom of iodine/1.0 glucagon) had binding characteristics that closely paralleled glucagon activation of adenylate cyclase of particles prepared from pancreatic beta cells. Furthermore, Rodbell et al. (18) have shown that glucagon having an average of one iodine per molecule has the full biological activity of native hormone. Also, under the proper conditions of iodination, other ^{125}I-labeled polypeptide hormones such as insulin (11) and adrenocorticotropic hormone (27) do not lose their ability to effect a biological response.

The close correlation between the binding curve and the lipolytic dose-response curve for glucagon in small adipocytes suggests that ^{125}I-labeled glucagon binding by intact fat cells represents a physiologically significant event. Saturation of glucagon-binding sites was approached at ^{125}I-labeled glucagon concentrations of 4×10^{-8} M, which agrees with the lipolytic dose-response curve and with ^{125}I-labeled glucagon-binding results reported for liver membranes (18).

The amount of glucagon bound by fat cells was based on cell numbers as indicated by DNA content of the isolated cells and on the amount of ^{125}I-labeled insulin bound by these cells. It was possible to use insulin binding in this manner because our previous studies (17) as well as this investigation showed similar numbers of insulin receptors on large and small fat cells. Insulin binding provides an excellent means of comparing the glucagon-binding capability of fat cells. Possible variables such as preferential destruction of large cells in the binding assay, unequal recovery of membranes, or differences in membrane protein content were adequately controlled. Although large, resistant fat cells specifically bind ^{125}I-labeled glucagon, the amount of binding was less in these cells than in small cells regardless of whether binding was based on cell number or on ^{125}I-labeled insulin binding. Studies with cell particles also demonstrated a loss in glucagon-binding ability based on any of three criteria, i.e., DNA content of the starting material, insulin binding, or protein content of the particles. It is important to note that two different methods for determining glucagon binding were used involving different adipose tissue preparations and that both methods showed diminished binding by large fat cells.

Binding of ^{125}I-labeled glucagon by fat cells under the conditions employed is related not only to numbers and affinity of glucagon receptors but also to the concentration of the labeled hormone. Since studies have shown that membranes from other tissues degrade glucagon (16, 18, 19), it was necessary to determine if increased degradation by large cells was responsible for diminished glucagon binding. However, as shown in Results, no difference was found in the amounts of glucagon inactivated by large and small cells. Degradation does prevent an accurate measurement of total binding sites and kinetic constants of binding (18, 19), but, because degradation is similar, a relative comparison of binding capability in large and small cells is possible. That increased degradation is not the cause of insensitivity to glucagon agrees with results of a previous study (1) and with the observation that resistance is not corrected by large amounts of hormone.

Glucagon binding by large cells does not correlate well with their lipolytic response to glucagon. The amount of binding, although less than that found in small cells, is too great to explain the marked reduction in lipolysis stimulated by glucagon. However, the reduction in binding does parallel the 50% reduction in effect of glucagon on adenyl cyclase activity reported by Manganiello and Vaughan (1) for large cell membranes. Similarly, Kahn, Neville, and Roth (28) have demonstrated diminished glucagon-binding capacity in liver cell membranes of obese–hyperglycemic mice that was comparable to the decrease found in glucagon-stimulated adenylate cyclase activity.

The following report (6) shows that large cells have an additional alteration that contributes to their diminished lipolytic response to glucagon stimulation. In these cells, both high- and low-affinity phosphodiesterase activities are approximately twice those of small cells. Inhibition of the "excess" phosphodiesterase activity by small amounts of aminophylline markedly enhanced glucagon-stimulated lipolysis. Under these conditions, the response to glucagon by large cells was slightly more than half the response produced by small cells, which corresponds to their rela-

tive abilities to bind glucagon. These findings strongly suggest that the change in glucagon-binding capability of large rat adipocytes contributes to the glucagon-resistant state but that the additional alteration of elevated phosphodiesterase activity is required for attainment of the marked insensitivity.

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