Epinephrine binding and the selective restoration of adenylate cyclase activity in fat-fed rats

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Abstract Fat feeding results in a progressive loss of epinephrine- and glucagon-stimulated adenylate cyclase activity in adipocyte plasma membrane sacs (ghosts). Basal and NaF-stimulated adenylate cyclase activities in fat-fed animals are not significantly different from those in preparations obtained from chow-fed rats. The high fat diet increases the mean adipocyte diameter rapidly, but increased cell size, at least in the case of epinephrine stimulation, is not responsible for the decreased hormone-stimulated adenylate cyclase activity. Diet shifts to high carbohydrate or high protein regimens result in the restoration of the epinephrine-stimulated, but not the glucagon-stimulated, activity without a significant reduction in mean cell diameter. Both hormone-resistant adipocyte ghosts from fat-fed animals and ghosts obtained from hormone-sensitive adipocytes bind the same amount of [^{3}H]epinephrine per milligram of membrane protein. These data indicate that the fat diet inhibits epinephrine-stimulated adenylate cyclase activity at a point between the hormone receptor and the catalytic unit of adenylate cyclase.

Supplementary key words hormone-stimulated adenylate cyclase · cyclic 3′,5′-AMP · glucagon · hormone receptors

THE MAGNITUDE of the response of adenylate cyclase to epinephrine and glucagon stimulation in rat fat cell plasma membrane sacs (ghosts) is lower in fat-fed animals than in preparations obtained from chow-fed rats. The mechanism responsible for the loss in hormone responsiveness was thought to be associated with a rapid increase in mean cell size which resulted in either a loss of hormone receptors or in alterations in the relationships between the receptors and the adenylate cyclase catalytic unit (1). The present study involves the reversal of the inhibition of epinephrine-stimulated but not glucagon-stimulated adenylate cyclase as a result of fat feeding, without a reduction in mean cell size or change in the ability to bind epinephrine to the plasma membrane of fat cell ghosts.

MATERIALS AND METHODS

Male Holtzman rats weighing initially 110–120 g were used in all experiments. The basal and the epinephrine-, glucagon-, and NaF-stimulated adenylate cyclase activities were measured in animals fed a high fat diet, a high fat diet followed by a 4-day fast, or refed either a high carbohydrate or a high protein, carbohydrate-free diet after an initial 5-day period of fat feeding. Adenylate cyclase was also measured in rats fed one-half their normal daily consumption of the high fat diet for up to 8 days. In all cases of diet shift, or alteration, the rats either maintained or gained in body weight. Except for the high protein regimen, the compositions of the respective diets have been described previously (1). The high protein diet contained on a caloric basis 89% protein and 11% fat, with appropriate vitamin, salt, and fiber supplements.

The measurement of adenylate cyclase activity in adipocyte ghosts has been described in detail previously (1). Briefly, free fat cells and ghosts were prepared according to Rodbell (2) from epididymal fat pads. Adenylate cyclase activity was determined from the rate of formation of cyclic 3′,5′-AMP from [^{32}P]ATP according to Krishna, Weiss, Brodie (3) with modifications as reported by Pohl, Birnbaumer, and Rodbell (4). The recovery (about 35%) of 3′,5′-AMP was calculated by adding 0.15 μCi of ^{3}H-labeled 3′,5′-AMP to each reaction. Reactions were initiated at 30°C by the addition of 40–60 μg of membrane protein. Enzyme activity is reported as nanomoles of ^{32}P-labeled 3′,5′-AMP/10

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min/mg protein determined by the method of Lowry et al. (5). Final hormone concentrations for both epinephrine and glucagon were 10 μg/ml unless otherwise stated. NaF was used at a final concentration of 10 mM.

Lipolysis was measured in fat pads according to Bizzi and Carlson (6) by determining glycerol release into a medium containing 2 ml of Krebs-Ringer bicarbonate buffer with 3% bovine serum albumin and 0.1% glucose, adjusted to pH 7.4. Hormones were added at concentrations of 0.5 μg/ml for epinephrine and 5 μg/ml for glucagon. Glycerol was determined by the method of Lambert and Neish (7) as modified by Korn (8).

Mean adipocyte diameters were determined by using a micrometer eyepiece (American Optical no. 1407a). At least 150 fat cells were measured from each animal, and the mean value for three or more animals is reported.

The binding of DL-[7-^{3}H]epinephrine to adipocyte ghost plasma membranes was determined by a modification of the method of Tomasi et al. (9) reported for liver membranes. DL-[7-^{3}H]Epinephrine was purchased from New England Nuclear Corp., Boston, Mass. L-DOPA and DL-metanephrine were obtained from Calbiochem.

TABLE 1. Adenylate cyclase activity after fat feeding or restriction of the fat diet to one-half the normal daily consumption

Treatment	Additions[a]	Adenylate Cyclase Activity[b]	Cell Size
			μm
None, chow diet	None	0.39 ± 0.04 (5)[c]	
	Epi	1.41 ± 0.07 (5)	55 ± 0.44
	Gluc	0.77 ± 0.06 (5)	
	NaF	2.21 ± 0.38 (5)	
Fat diet ad lib. for	None	0.45 ± 0.03 (6)	
7 days	Epi	0.58 ± 0.04 (6)[d]	81 ± 1.25[d]
	Gluc	0.37 ± 0.05 (6)[d]	
	NaF	2.66 ± 0.41 (6)	
One-half fat diet for	None	0.41 ± 0.06 (5)	
1 day	Epi	1.39 ± 0.04 (5)	58 ± 0.66
	Gluc	0.79 ± 0.07 (5)	
	NaF	2.33 ± 0.44 (5)	
One-half fat diet for	None	0.41 ± 0.05 (5)	
5 days	Epi	1.38 ± 0.09 (5)	64 ± 0.53[d]
	Gluc	0.80 ± 0.06 (5)	
	NaF	2.23 ± 0.40 (5)	
One-half fat diet for	None	0.43 ± 0.06 (5)	
8 days	Epi	1.54 ± 0.09 (5)	66 ± 0.45[d]
	Gluc	0.66 ± 0.05 (5)	
	NaF	2.55 ± 0.09 (5)	

[a] Hormone concentrations were 10 μg/ml for epinephrine and glucagon. NaF concentration was 10 mM in all experiments.

[b] Means ± SEM of enzyme activity reported as nmoles of ^{32}P-labeled 3′,5′-AMP/10 min/mg of protein.

[c] (n), number of experiments. Each experiment represents pooled adipocyte ghosts from three or more rats.

[d] $P < 0.05$ compared with corresponding response in chow-fed animals.

L-Norepinephrine, L-isoproterenol, L-phenylephrine were products of Winthrop Laboratories. L-Epinephrine bitartrate, trypsin, and phospholipases A and C were purchased from Sigma. Phenoxybenzamine was a product of Smith Kline & French, and propranolol was purchased from Ayerst Laboratories. Adipocyte ghosts were incubated for 15 min in 1 ml of 50 mM Tris–HCl, pH 7.4, at 37°C with 1×10^{-7} M [^{3}H]epinephrine. The incubation was stopped by the addition of 2 ml of ice-cold 5.0% trichloroacetic acid, and the membranes were then washed three times with 10 ml of the ice-cold Tris–HCl buffer and collected on HAWP 0.45-μm Millipore filters. The filters were then placed in 15 ml of Bray's scintillation fluid (10) and counted in a Beckman model LS-100 liquid scintillation spectrometer. The binding is calculated from the specific activity of the epinephrine and is reported as pmoles epinephrine bound/15 min/mg membrane protein.

The calculation of epinephrine binding and the final hormone concentration in the incubation system were based on the assumption that only the L isomer of DL-[7-^{3}H]epinephrine was bound to the plasma membrane. Specific hormone binding represents the amount of radiolabeled hormone bound to plasma membranes after the subtraction of the binding that occurred when the 15-min incubation took place in the presence of (1×10^{-4} M) unlabeled L-epinephrine.

RESULTS

Fat feeding and adenylate cyclase hormone responsiveness

As previously reported (1), fat feeding results in a loss of epinephrine and glucagon responsiveness in the adenylate cyclase system of rat adipocyte ghosts when compared with activities of the enzyme from preparations from chow-fed rats. Although the hormonal stimulation of adenylate cyclase is inhibited by fat feeding, the basal and NaF-stimulated activities are not different from those observed in chow-fed animals (Table 1). Fat feeding results in a rapid enlargement of fat cells, and this increase in cellular volume was thought to be the major factor in the loss of hormone sensitivity.

In an attempt to control the rate of cellular enlargement, rats were fed one-half their normal daily consumption of the high fat diet. Rats fed in this manner were able to maintain their normal sensitivity to stimulation of adenylate cyclase by epinephrine and glucagon, but also increased their mean fat cell sizes to some degree (Table 1).

When rats were fed the high fat diet for 5 days and then shifted to a fat-free high carbohydrate diet for 1 day, the responsiveness of adenylate cyclase to epinephrine

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and glucagon was still diminished. After the rats were fed the high carbohydrate diet for 3 days, normal epinephrine but not glucagon sensitivity was restored (Fig. 1). The recovery of the epinephrine sensitivity occurred without a reduction in adipocyte volume, indicating that large cells are not necessarily insensitive to epinephrine stimulation of adenylate cyclase. The refeeding of the high carbohydrate diet did not affect either basal or NaF-stimulated adenylate cyclase activity (Fig. 1).

In two other circumstances, a similar selective restoration of the epinephrine-stimulated but not the glucagon-stimulated adenylate cyclase activity was observed. Ghost preparations from rats that were fed the high fat diet for 5 days and then switched to a high protein, carbohydrate-free diet for 3 days or rats fed the fat diet 5 days and then fasted for 4 days (Table 2) were as responsive to epinephrine as control preparations, but not to glucagon. Refeeding the high protein or high carbohydrate diets or fasting after fat feeding did not significantly alter the basal or NaF-stimulated adenylate cyclase activity. Refeeding with the high protein diet resulted in a slight decrease in mean cell size, and fasting produced a significant reduction in fat cell diameter (Table 2).

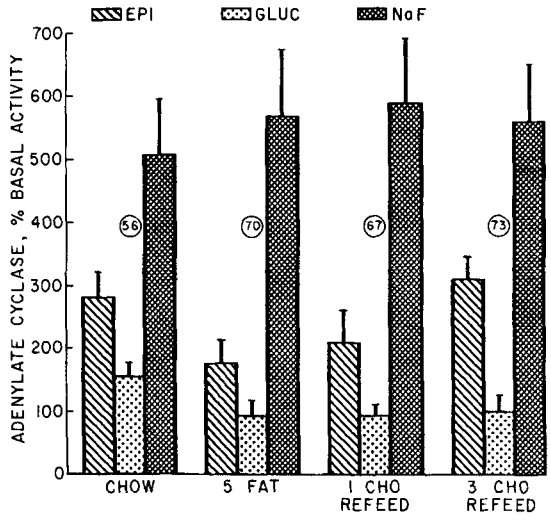

FIG. 1. Hormone-stimulated adenylate cyclase activity in rats fed a high fat diet for 5 days then refed a high carbohydrate, fat-free diet for 1 and 3 days. There is a complete restoration of the epinephrine-stimulated but not the glucagon-stimulated adenylate cyclase activity after 3 days of refeeding the high carbohydrate diet. Circled numerals represent the mean fat cell size in μm of that preparation. Data are reported as percent ± SEM of the basal enzyme activity. Incubation conditions are given in the text (see Methods).

Carbohydrate refeeding and lipolysis

To relate the restoration of the epinephrine sensitivity of adenylate cyclase in fat-fed, carbohydrate-refed rats to whole tissue lipid metabolism, lipolysis was measured in epididymal fat pads from rats fed the high fat diet for 5 days then refed the high carbohydrate diet for 1 and 3 days. As in the case of the adenylate cyclase measurements, the lipolytic response to epinephrine, as indicated by glycerol release, was the same in rats refed 3 days as it was in chow-fed rats, while the response to glucagon was absent (Table 3).

TABLE 2. Adenylate cyclase and high protein refeeding and fasting after fat feeding

Diet Shift after 5 days of Fat Feeding	Additions[a]	Adenylate Cyclase Activity[b]	Cell Size
5 days of fat feeding	None	0.40 ± 0.02 (5)[c]	
	Epi	0.51 ± 0.06 (5)	69 ± 0.49
	Gluc	0.38 ± 0.03 (5)	
	NaF	2.44 ± 0.39 (5)	
High protein diet for 3 days	None	0.36 ± 0.03 (5)	
	Epi	1.29 ± 0.04 (5)[d]	65 ± 0.52
	Gluc	0.35 ± 0.04 (5)	
	NaF	2.10 ± 0.33 (5)	
4-day fast	None	0.39 ± 0.04 (5)	
	Epi	1.44 ± 0.03 (5)	42 ± 0.12[d]
	Gluc	0.36 ± 0.05 (5)	
	NaF	2.20 ± 0.41 (5)	

[a] Hormone concentrations were 10 μg/ml for epinephrine and glucagon. NaF concentration was 10 mM in all experiments.
[b] Means ± SEM of enzyme activity reported as nmoles of ^{32}P-labeled 3′,5′-AMP/10 min/mg of protein.
[c] (n), number of experiments. Each experiment represents pooled adipocyte ghosts from three or more rats.
[d] $P < 0.05$ compared with corresponding response in rats fed the fat diet for 5 days.

TABLE 3. Epididymal fat pad lipolysis

Diet	Additions[a]	μmoles Glycerol/hr/g Tissue[b]	P Value Compared with Corresponding Response in Chow-fed Controls
Chow	None	2.54 ± 0.36	
	Epi	6.43 ± 0.88	
	Gluc	4.66 ± 0.25	
5-day fat	None	2.33 ± 0.44	NS[c]
	Epi	4.33 ± 0.31	<0.05
	Gluc	2.38 ± 0.55	<0.05
5-day fat 1-day carbohydrate refed	None	2.21 ± 0.35	NS
	Epi	4.85 ± 0.22	<0.05
	Gluc	2.01 ± 0.36	<0.05
5-day fat 3-day carbohydrate refed	None	2.42 ± 0.22	NS
	Epi	6.03 ± 0.44	NS
	Gluc	2.34 ± 0.38	<0.05

[a] Hormone concentrations were 0.5 μg/ml epinephrine and 5.0 μg/ml glucagon.
[b] Values are the means ± SEM for six animals.
[c] NS, not significant ($P > 0.05$).

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TABLE 4. Specific binding of [^{3}H]epinephrine to adipocyte ghost plasma membranes

Diet	Additions to Binding System	pmoles Epinephrine Bound/mg Membrane Protein[a]	*P* Value Compared with Corresponding Response in Chow-fed Controls
Chow	None	8.59 ± 0.36 (5)[b]	
Chow	Propranolol, 1×10^{-4} M	3.86 ± 0.24 (3)	<0.05
Chow	Phenoxybenzamine, 1×10^{-4} M	7.42 ± 0.29 (3)	NS[c]
5-day fat	None	8.99 ± 0.35 (5)	NS
5-day fat 3-day carbohydrate refed	None	7.88 ± 0.44 (5)	NS

[a] 60–80 μg of ghost membrane protein incubated at 37°C for 15 min in 50 mM Tris–HCl, pH 7.4, with 0.25 μCi of [^{3}H]epinephrine at a final concentration of 1×10^{-7} M. Data are expressed as pmoles [^{3}H]epinephrine bound/15 min/mg protein.

[b] (n), number of separate experiments pooled for representation.

[c] NS, not significant.

TABLE 5. Inhibition of [^{3}H]epinephrine binding and stimulation of adenylate cyclase by epinephrine analogs

Analog	% Inhibition of [^{3}H]Epinephrine Binding	% Stimulation of Adenylate Cyclase Relative to Epinephrine Stimulation
Epinephrine (unlabeled)	98 ± 6	100
Norepinephrine	96 ± 8	95 ± 9
Isoproterenol	98 ± 8	96 ± 12
DOPA	85 ± 6	0
Metanephrine	8 ± 14	0
Phenylephrine	15 ± 12	0

[^{3}H]Epinephrine binding was conducted in the presence of unlabeled epinephrine and analogs. In addition, each analog was tested as a stimulator of adenylate cyclase with the stimulation by epinephrine representing 100% activation. Epinephrine and all analogs were tested at 1×10^{-4} M for both adenylate cyclase stimulation and inhibition of [^{3}H]epinephrine binding.

[^{3}H]Epinephrine binding to fat cell ghost plasma membrane

We postulated that, since the basal and the total activatable adenylate cyclase activities as measured by NaF stimulation were unchanged in fat-fed animals, the inhibition of hormone-stimulated adenylate cyclase by fat feeding could be associated with either an impaired binding of hormone to a receptor or defective coupling between the receptor and the catalytic unit of adenylate cyclase. To test these alternatives, epinephrine binding studies were conducted on rat fat cell ghosts.

It should be noted that the binding of [^{3}H]epinephrine to membranes isolated from fat cell homogenates is quantitatively the same as the binding to intact adipocyte ghosts. Additionally, extraction and subsequent thin-layer chromatography of radiolabeled hormone from ghost membranes (11) yielded materials that cochromatographed with fresh unreacted [^{3}H]epinephrine, indicating the bound radioactivity to be radiochemically pure epinephrine.[1]

The specificity of binding was tested in several ways: (*a*) inhibition of binding by α or β blocking agents; (*b*) competition for binding sites by structural analogs of epinephrine; and (*c*) competition by polypeptide hormones for binding sites.

The data indicate a specific binding of [^{3}H]epinephrine to adipocyte ghost plasma membranes. Propranolol, a β blocking agent, inhibited by 51% the binding of [^{3}H]epinephrine, but phenoxybenzamine, an α blocking agent, did not affect epinephrine binding (Table 4), indicating [^{3}H]epinephrine is probably binding to a β-type receptor on the plasma membrane.

Structural analogs of epinephrine that are capable of stimulating ghost adenylate cyclase also reduce the binding of [^{3}H]epinephrine to ghost preparations (Table 5). Norepinephrine and isoproterenol stimulate adenylate cyclase and inhibit [^{3}H]epinephrine binding as well as unlabeled epinephrine. Metanephrine and phenylephrine, which do not stimulate adenylate cyclase, fail to reduce [^{3}H]epinephrine binding. The only analog tested that did not stimulate adenylate cyclase but was capable of reducing epinephrine binding to some degree was DOPA (3,4-dihydroxyphenylalanine) (Table 5). In support of our data, Feller and Finger (12) reported that epinephrine, norepinephrine, and isoproterenol are all effective lipolytic agents in rat adipose tissue but phenylephrine and metanephrine are very poor lipolytic agents.

The addition of 10 μg/ml of insulin, secretin, or glucagon did not diminish [^{3}H]epinephrine binding to ghost membranes, indicating that there is no competition for binding sites between these polypeptide hormones and epinephrine for the catecholamine receptor (Table 6).

Preincubation of adipocyte membranes with either proteolytic or lipolytic enzymes altered epinephrine binding. Trypsin digestion proved to be inhibitory, while both phospholipases A and C slightly enhanced [^{3}H]epinephrine binding (Table 7). The addition of soybean trypsin inhibitor protected the membranes from

[1] Gorman, R. R. Unpublished experiments.

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TABLE 6. [^{3}H]Epinephrine binding and competition with polypeptide hormones for catecholamine binding sites

Polypeptide Hormone	pmoles Epinephrine Bound/mg Protein	% Control Value
None	6.25 ± 0.32	
Insulin	6.39 ± 0.57	102
Glucagon	6.11 ± 0.36	98
Secretin	5.96 ± 0.65	95

Membrane samples (60–80 μg protein) were added to an incubation system that contained 1×10^{-7} M epinephrine and 10 μg/ml of either insulin, glucagon, or secretin. The samples were incubated in 1 ml of 0.05 M Tris buffer for 15 min at 37°C. The membranes were then collected on Millipore filters and counted in a liquid scintillation spectrometer. Data of three separate experiments are expressed as mean ± SEM pmoles epinephrine bound/15 min/mg protein.

TABLE 7. [^{3}H]Epinephrine binding after proteolytic and lipolytic enzyme perturbation

Lytic Enzyme	pmoles Epinephrine Bound/mg Protein	% Control Value
None	7.92 ± 0.39	
Trypsin	4.33 ± 0.25	54
Trypsin + trypsin inhibitor	7.66 ± 0.33	97
Phospholipase A	8.49 ± 0.42	107
Phospholipase C	11.04 ± 0.98	139

Membrane samples (400 μg of protein in 1.0 ml of 0.05 M Tris buffer, pH 7.4) were incubated with 0.5 mg/ml trypsin, or 1.0 mg/ml of phospholipase A or C, or 4 mg/ml trypsin inhibitor, at 37°C for 10 min, centrifuged, and washed twice with 0.05 mM Tris buffer. The washed membranes were then resuspended in buffer, and binding of [^{3}H]epinephrine was determined as usual.

trypsin digestion and epinephrine binding was not affected (Table 7).

Dunnick and Marinetti (13) reported that delipidized liver membranes bind epinephrine to the same extent as intact membranes, and our binding data after phospholipase incubation also indicate that lipids are not obligatory for epinephrine binding. It is possible that the increased binding that occurs after phospholipase attack represents an unmasking of receptors similar to the uncovering of insulin receptors with phospholipase C (14), and the loss of binding capacity after treatment with trypsin represents a loss of proteins necessary for normal epinephrine receptor activity.

After the specificity of the binding assay was established, the system was used to determine epinephrine binding in fat-fed, epinephrine-insensitive animals. Our data indicate that membrane preparations from large epinephrine-resistant fat cells of fat-fed rats, and those obtained from "recovered" epinephrine-sensitive, fat-fed, carbohydrate-refed animals, selectively bind the same amount of [^{3}H]epinephrine per milligram of protein as do preparations from smaller cells obtained from chow-fed animals (Table 4). Apparently, the hormone-resistant animals possess a functioning catecholamine receptor but do not respond to the biological signal to their adipose tissue.

DISCUSSION

The inhibition of epinephrine- and glucagon-stimulated adenylate cyclase activity and the reduction in hormone-stimulated lipolysis by fat feeding appears to be associated with increased adipocyte diameter (1, 15, 16). However, the data reported in this paper indicate that, at least in the case of epinephrine stimulation, increased cell size is not the major factor responsible for the reduced hormonal sensitivity. Every instance of diet shift after fat feeding resulted in the complete restoration of the epinephrine, but not glucagon, sensitivity. The recovery of the epinephrine response was accomplished without a significant decrease in mean cell size except after a 4-day fast. The restriction of the fat diet to one-half the normal daily consumption maintained the sensitivity of adenylate cyclase to both epinephrine and glucagon without completely arresting cellular enlargement, indicating that the amount of saturated fat ingested may play a role in the regulation of hormone-stimulated lipolysis in fat cells. High fat diets may inhibit hormone-stimulated adenylate cyclase in several ways. A natural inhibitor of adenylate cyclase related to the inhibitor reported by Ho and Sutherland (17) in hormone-stimulated fat cells could accumulate in fat-fed animals. Palmitate is known to inhibit cyclic AMP accumulation in fat cells stimulated by epinephrine (18); therefore, direct inhibition by the increased level of circulating fatty acids is a possibility. Prostaglandins (19, 20) and insulin (21) also inhibit adenylate cyclase, but circulating insulin concentrations are low during fat feeding (22), and the highly saturated lard diet would not be an excessively rich source of prostaglandin precursors. Conversely, fat feeding may reduce the level of a hormone that is essential for maintenance of hormone sensitivity. Dexamethasone (23), growth hormone (24), and thyroxine (25) all have been reported to induce or maintain hormone sensitivity in fat cells. The possible effect of fat feeding on the balance and interaction of these hormones is not known.

Regardless of the mechanism responsible for the inhibition of hormone-stimulated adenylate cyclase activity in fat-fed animals, membranes from epinephrine-resistant fat cells clearly bind epinephrine as well as do preparations from epinephrine-sensitive cells. Since the binding of epinephrine is unimpaired, as are the basal and NaF-stimulated adenylate cyclase activities, the hypothetical "transducer" (23, 26) between the catechol-

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amine receptor and the catalytic unit of adenylate cyclase appears to be a likely region of the membrane that is affected by the high fat diet.

Persistence of glucagon unresponsiveness in the face of recovery of sensitivity to epinephrine raises the question of the site of glucagon resistance. The obvious possibilities are *1*) failure of glucagon binding or *2*) failure of transmission of the glucagon-receptor message to adenylate cyclase. Pending completion of glucagon binding experiments now in progress, we cannot select between these alternatives. If glucagon binding should turn out to be unimpaired, the interesting possibility of hormone-specific transduction in the membrane is suggested by these results.

It is known that several agents will antagonize or block the response of one hormone without affecting the other (27, 28), and without pharmacological intervention the glucagon response of adenylate cyclase decreases in 350-g rats when compared with 125-g rats, while normal epinephrine sensitivity is maintained (24). It has been implied that as cells enlarge and form new membrane, epinephrine receptors are also synthesized and the content of receptors per unit area of membrane is constant (29). Our specific binding studies with [^{3}H]-epinephrine support this concept. This potential to synthesize or replace receptors may be absent in the case of the glucagon receptor. Therefore, as the fat cell enlarges during fat feeding, the epinephrine but not the glucagon receptors may be maintained, and after a diet shift occurs and the inhibitory effects of the high fat diet are lost, the epinephrine response recovers, but the glucagon response remains depressed.

Interpreting the loss of hormonal responsiveness in the adenylate cyclase system resulting from diet manipulation is difficult. However, the specific loss of epinephrine and glucagon sensitivity after fat feeding and the selective recovery of the epinephrine response upon carbohydrate refeeding afford an opportunity to study hormonal control mechanisms in fat cells without resorting to enzyme probes of membrane constituents or to pharmacological blocking agents.

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