Glycogen Synthesis Stimulation by Adenylate Cyclase Inhibition in Rat Epididymal Adipocytes†

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ABSTRACT: The effects of adenylate cyclase inhibition on the transport of glucose and fructose and their incorporation into glycogen were investigated in order to assess the extent to which lowered cAMP levels can take part in the various components of glycogen synthesis regulation in isolated rat epididymal adipocytes. The dose–response characteristics of (*R*)-*N*-(2-phenylisopropyl)adenosine (PIA), a potent and specific adenylate cyclase inhibitor, on glycogen synthesis were compared with those effectively inhibiting lipolysis, a measure of functional cAMP levels. PIA had no effect on basal glucose or fructose transport but stimulated glucose and fructose incorporation into glycogen. Their respective incorporation was 10 and 69% of that achieved in the presence of insulin. These effects of PIA were shown to be in part the result of increased glycogen synthase I activity. PIA was 20% as effective as insulin in this action. Thus, were insulin to lower cAMP levels and/or inhibit cAMP-dependent protein kinase, this action would be irrelevant to glucose transport but would contribute to the stimulation of glycogen metabolism. However, an additional mechanism(s) involving neither increased glucose transport nor lowered cAMP levels is required to account for the full action of insulin. Fat cells in the absence of medium glucose and in the presence of 10^{-7} M PIA and adenosine deaminase constitute a system functionally depleted of cAMP where this mechanism can be studied in isolation.

Glycogen synthesis is regulated primarily by the activity state of glycogen synthase. Apparently in all tissues this enzyme is subject to allosteric activation by D-glucose 6-phosphate (Glc-6-P)[1] as well as to regulation by phosphorylation–dephosphorylation. Glc-6-P enters the regulation both as an allosteric activator of the enzyme and apparently by affecting the rate of dephosphorylation of the enzyme by phosphoprotein phosphatase, a phenomenon termed mechanism II by Lawrence & Larner (1978). Thus glycogen synthesis in adipose tissue and muscle is under physiologic conditions linked via Glc-6-P to the regulation of glucose transport. However, the glycogen synthase activity state can also be altered in the absence of medium glucose, i.e., by insulin (Jungas, 1966). In rabbit skeletal muscle glycogen synthase seven phosphorylation sites are currently recognized. Three sites (sites 1a, 2, and 1b) may be phosphorylated by cAMP-dependent protein kinase, one site (site 2) may be phosphorylated by either of four kinases, i.e., cAMP-dependent protein kinase, phosphorylase kinase, glycogen synthase kinase 4, and a Ca^{2+}–calmodulin dependent glycogen synthase kinase, another three sites (sites 3a, 3b, and 3c) are specifically phosphorylated by glycogen synthase kinase 3, and one final site (site 5) is phosphorylated by glycogen synthase kinase 5 (Cohen, 1982). Multiple protein kinases capable of phosphorylating glycogen synthase are present also in adipose tissue, but cAMP-dependent protein kinase reportedly dominates (Schlender & Reimann, 1977).

In the muscle enzyme, each of the phosphorylation sites except sites 1b and 5 apparently makes its own contribution to the activity state of the enzyme (Cohen, 1982). Insulin action on skeletal muscle in vivo results in dephosphorylation of glycogen synthase mainly at sites 3a–c, and to some extent at sites 2, 1a, and 1b. It is not known yet to what extent this action involves mechanism II mentioned above and may thus be the result of activation of glucose transport. Moreover, insulin-induced hypoglycemia in vivo may have elevated concentrations of glucagon and catecholamines, agents that may modulate the action of insulin. Clarification of these issues will require dissection of insulin action into glucose transport dependent and independent components and further dissection of the transport-independent action into components involving cAMP and cAMP-dependent protein kinase and components not involving that system.

Glycogen synthesis regulation in adipocytes has been described (e.g., Jungas, 1966; Lawrence & Larner, 1978; Kaslow et al., 1979) although glycogen synthase from this tissue has not yet been characterized as extensively as the enzyme from skeletal muscle. Because insulin may lower cAMP levels in rat adipocytes (Kono & Barham, 1973; Desai et al., 1973; Butcher et al., 1968; Burns et al., 1979; Wong & Loten, 1981) and cAMP-dependent protein kinase appears to be an important contributor to total glycogen synthase kinase activity (Schlender & Reimann, 1977), it was important to clarify the extent to which a lowering of cAMP levels alone would result in insulin-like effects on glycogen synthesis and to dissect the effects of lowered cAMP levels into glucose transport dependent and independent components. The present work ex-

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[1] Abbreviations: PIA, (*R*)-*N*-(2-phenylisopropyl)adenosine; ACTH-(1–24), corticotropin-(1–24)-tetracosapeptide; KRB and KRP, Krebs–Ringer bicarbonate and, respectively, phosphate buffers as specified under Methods; BSA V, bovine serum albumin, Cohn fraction V; Bt_2cAMP, N^6,$O^{2'}$-dibutyryladenosine cyclic 3′,5′-phosphate sodium salt; Glc-6-P, D-glucose 6-phosphate; EC_{50}, 50% effective concentration; zinterol, (*RS*)-*N*-[5-[2-[(1,1-dimethyl-2-phenylethyl)amino]-1-hydroxyethyl]-2-hydroxyphenyl]methanesulfonamide hydrochloride; Ro 20-1724/1, *d*-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone.

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amines the effects of adenylate cyclase inhibition in isolated rat epididymal fat cells on glucose and fructose transport, on glucose and fructose incorporation into glycogen, and on glycogen synthase I activity, i.e., the ratio of activity in the absence and presence of 7.2 mM Glc-6-P. It relates the effects on glycogen metabolism to rates of lipolysis, because lipolysis most closely reflects functional cAMP levels. The effects of adenylate cyclase inhibition are quantitatively compared with the effects of insulin.

Note that it is not the intention of this paper to show under what circumstances and to what extent insulin actually lowers cAMP levels. Rather the studies with adenylate cyclase inhibition are used to set an upper bound on the extent to which a potential cAMP lowering action and/or an inhibitory action on cAMP-dependent protein kinase of insulin could account for the observed effects. Finally, conditions of fat cells are described in which insulin action can be studied in the absence of functional cAMP levels.

Experimental Procedures

Materials

Sprague-Dawley rats (120–150 g) were purchased from Tyler Laboratories, Bellevue, WA, fed standard laboratory chow and water ad libitum, and kept for at least 24 h in a light-controlled room (12-h cycle; 600–1800 h) prior to use. Animal weights on the day of experimentation ranged from 120 to 200 g. BSA V[1] was purchased from Reheis Chemical Co. (lot S11709), and solutions (20% w/v) were dialyzed against three changes of distilled water and three changes of KRB.[1] Crude collagenase (*Clostridium histolyticum*) type I was purchased from Worthington (lot 4197 CLS 48P003). D-[U-^{14}C]Glucose (specific activity 2.2 mCi/mmol), D-[U-^{14}C]fructose (specific activity 359.1 mCi/mmol), 3-*O*-[^{3}H]-methyl-D-glucose (specific activity 80 Ci/mmol), uridine (5′)diphospho(1)-α-D-[U-^{14}C]glucose (specific activity 300 mCi/mmol), 2-deoxy-D-[^{14}C]glucose (282 mCi/mmol), and [^{14}C]methoxyinulin (15.07 mCi/g) were products of New England Nuclear Corp. Dinonyl phthalate was purchased from ICN, 3-*O*-methyl-D-glucose was from Aldrich Chemical Co., and D-fructose, phloretin, 2-deoxy-D-glucose, Bt_2cAMP,[1] Glc-6-P, and calf intestinal mucosa adenosine deaminase type I [specific activity 195–250 units/mg of protein in 3.2 M $(NH_4)_2SO_4$] were purchased from Sigma Chemical Co. Adenosine deaminase was added to incubations without further purification. Control experiments showed that $(NH_4)_2SO_4$ in concentrations similar to those added along with adenosine deaminase had discernible but negligible effects on glycerol release and glucose-C1-oxidation. Bovine insulin was purified according to May et al. (1978). (*R*)-*N*-(2-Phenylisopropyl)adenosine (Th 162, mp 140–142 or 183–183.5 °C depending on crystallization conditions), henceforth referred to simply as PIA, was bought from Boehringer Mannheim Biochemical, Indianapolis, IN, under the name of N^6-(L-2-phenylisopropyl)adenosine. PIA solutions were prepared freshly each day either as a 0.1 M stock solution in dimethyl sulfoxide or as a 0.01 M solution in warm KRP.[1] Dimethyl sulfoxide in the resulting low final concentrations used was shown, with the exception of 3-*O*-methylglucose transport studies, not to interfere with metabolic assays, in agreement with Wieser et al. (1977). We did notice that PIA stock solutions in dimethyl sulfoxide stored at −20 °C lost activity with time. Assay of PIA effects on 3-*O*-methyl-D-glucose uptake required purely aqueous PIA solutions. Synthetic ACTH-(1–24)[1] was obtained from Organon Pharmaceuticals. Zinterol[1] was a gift from Mead Johnson Co., Evansville, IN, and *d*-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724/1) (Batch 3P-3053) was a generous gift from Hoffmann-La Roche Inc., Nutley, NJ. All other reagents were analytical reagent grade from standard laboratory suppliers.

Methods

Buffers. KRB buffer, pH 7.4, was prepared as usual except that the $CaCl_2$ concentration was 1.3 mM. KRP buffer, pH 7.4, was prepared according to Lawrence et al. (1977).

Isolation of Fat Cells. Epididymal fat pads from 6–12 rats, depending on the size of the experiment, were pooled, and adipocytes were prepared according to the method of Rodbell (1964).

3-O-Methyl-D-glucose Uptake. Incubation and rapid filtration procedures were those described previously (Muchmore et al., 1981), but with the inclusion of adenosine deaminase (280 milliunits/mL) in the incubation medium (Fain & Wieser, 1975). Uptake was measured over a 4-s period.

2-Deoxyglucose Uptake. The method described by Muchmore et al. (1981) was used. Uptake was measured over a 2-min period at 25 °C.

D-*Fructose Uptake.* The rapid filtration methodology and buffers were the same as in the case of 3-*O*-methyl-D-glucose uptake measurements. Cells [(8–10) × 10^5] were incubated for 30 min in a total volume of 450 μL containing adenosine deaminase (280 milliunits/mL) and additions as indicated. The addition of D-[U-^{14}C]fructose (50 μL of 100 mM solution, specific activity 90 μCi/mmol) was used to start uptake. Ten milliliters of ice-cold stopping solution as in 3-*O*-methylglucose uptake experiments was used to quench the reaction. Fructose uptake was measured at 0, 2, 4, 10, and 20 s.

Glycerol Release. Isolated fat cells [(0.5–1.5) × 10^5 cells/2 mL] were incubated for 2 h at 37 °C in polyethylene scintillation vials containing KRB, 2% BSA V, and adenosine deaminase (280 milliunits/mL) with a 95% air/5% CO_2 atmosphere and various drug additions as indicated. After incubation, the samples were transferred into polystyrene tubes (12 × 75 mm) on ice and centrifuged for 2 min, following which the cells were removed by aspiration. The infranatant was assayed for glycerol according to Chernick (1969). Each experimental parameter was performed in triplicate and assayed in duplicate. Glycerol standard curves were examined in the presence of 10^{-7} M PIA. No interference of the drugs with the coupled enzymatic assay could be observed.

Glycogen Synthesis. D-[U-^{14}C]Glucose (10.5 μCi/mL incubation) incorporation into glycogen was measured according to Lawrence & Larner (1978) with minor modifications described earlier (Muchmore et al., 1981). D-[U-^{14}C]Fructose incorporation (4.8 μCi/mL incubation) was measured in an identical fashion. However, the final incubation concentration was 10 mM fructose rather then the 1 mM used with glucose. All incubation vials contained adenosine deaminase (280 milliunits/mL).

Glycogen Synthase I Activity. Isolated adipocytes were incubated in the presence of various additions at 37 °C for either 10 or 30 min and glycogen synthase I activity (−Glc-6-P/+Glc-6-P) was assessed as described by Lawrence et al. (1977). UDP-D-[U-^{14}C]glucose incorporation assay conditions were identical with those of Lawrence et al. (1977) with the exception of a 20-min incubation time, addition of adenosine deaminase (280 milliunits/mL), and a substrate specific activity of 3.8 μCi/mL. The glycogen precipitation conditions were those specified by Muchmore et al. (1981). In recent experiments Whatman ET 31 paper was replaced by silica-coated chromatography paper (Whatman SG-81), which resulted in improved reproducibility. Use of the latter paper in

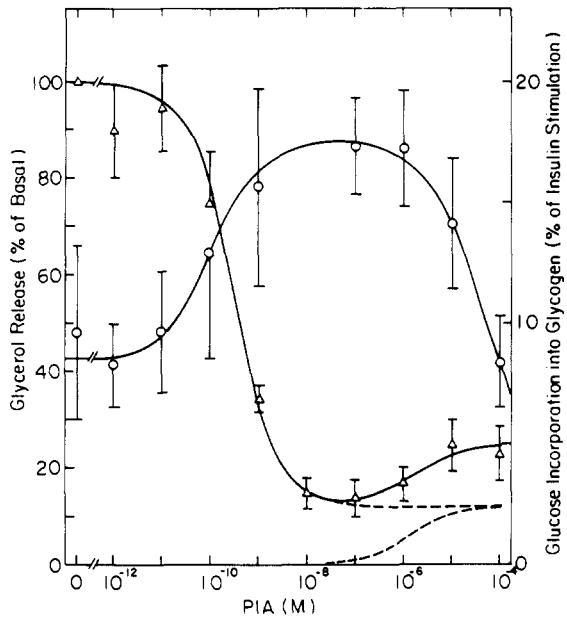

FIGURE 1: PIA effects on lipolysis and on glucose incorporation into glycogen. Lipolysis measured as glycerol release (△) into the incubation medium is expressed as percent of a basal rate of 31 ● 8 nmol h^{-1} (10^5 cells)$^{-1}$. Cell density on the average was 1.34×10^5 cells/mL. Glucose incorporation data (○) are expressed as percent of incorporation achieved by 10^{-10} M insulin [2.2 ± 0.3 nmol h^{-1} (10^5 cells)$^{-1}$]. Cell density on the average was 1.10×10^6 cells/mL. The glucose concentration was 1 mM. Adenosine deaminase was present in all experiments at a concentration of 280 milliunits/mL. Data are $\bar{x}$ ± SEM from three experiments, in which both parameters were measured on the same cell batch. Glycerol release was measured in triplicate and glucose incorporation into glycogen in quintuplicate incubations within each experiment. (Solid curves) Weighted nonlinear least-squares fits to the data for a biphasic concentration–effect relationship as described under Methods. (Broken curves) Decomposition of solid curve for lipolysis into the tow component concentraton–effect curves.

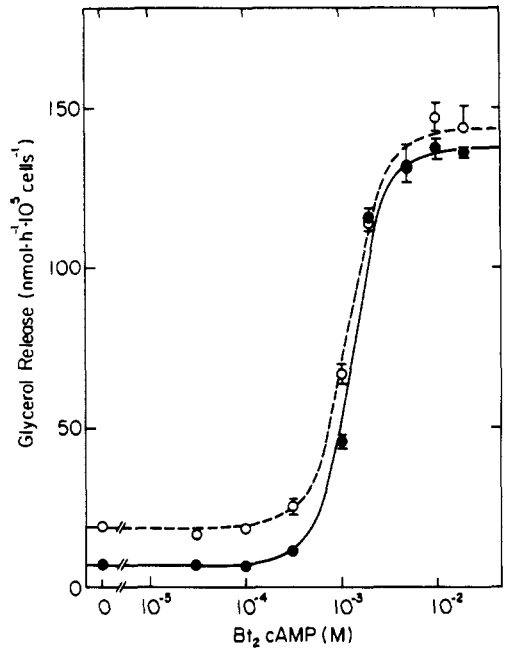

FIGURE 2: Comparison of the effect of Bt_2cAMP on lipolysis in the presence and absence of PIA. Effects in the absence (○) and presence (●) of 10^{-7} M PIA. The adenosine deaminase concentraton was 280 milliunits/mL. Data are $\bar{x}$ ± SEM of triplicate incubations. Data lacking an error bar had standard errors smaller than the symbol size. Curves are weighted nonlinear least-squares fits to the equation given under Methods with $h = 2.25$ in the absence and $h = 2.41$ in the presence of PIA.

the assay of glucose incorporation into glycogen by intact cells conferred no advantage over the method described above.

Calculations. Simple concentration–effect curves were analyzed by weighted nonlinear regression to the Hill equation $y = (y_{max} - y_{min})/[(EC_{50}/x)^h + 1] + y_{min}$, using the Levenberg–Marquart modification of the Gauss–Newton iterative procedure (Magar, 1972). Statistical weights were $(SEM)^{-2}$. h is the Hill coefficient, x is the concentration of the agent, y_{min}, y_{max}, and y are the minimal, maximal, and intermediate effects observed, respectively, and EC_{50} is the 50% effective concentration. Biphasic concentration–effect curves were analyzed by fitting the data to two terms of the kind used in the above equation, with h set to 1 for both terms. Data are given as $\bar{x}$ ± SEM from N separate experiments. When a typical experiment is shown, the error in the data is the SEM of n determinations within a single experiment. Fitted parameters are given as $\bar{x}$ ± SEM or $\bar{x}^{\times}_{\div}$ SEM, where appropriate.

Results

(Phenylisopropyl)adenosine Effects on Lipolysis. PIA, a fat cell adenylate cyclase inhibitor (Londos et al., 1980), was used to inhibit cAMP production in isolated rat epididymal fat cells. The incubation medium contained adenosine deaminase (280 milliunits/mL) for the reduction of extracellular adenosine accumulation. Under these circumstances PIA is well documented to lower cAMP levels (Fain, 1973; Stock & Prilop, 1974; Prilop, 1975; Wieser & Fain, 1975; Fain & Malbon, 1979). Glycerol release was inhibited by PIA in the low concentration range, while at higher PIA concentrations some of the inhibition was lost (Figures 1 and 3). Analysis of the observed curve by the method of least squares indicated that it was the sum of two component concentration–effect curves (Figure 1). The first component with an EC_{50} of $(3.3^{\times}_{\div}1.3) \times 10^{-10}$ M ($N = 3$) described inhibition of lipolysis from a basal rate of 31 ± 8 nmol h^{-1} (10^5 cells)$^{-1}$ ($N = 3$) to a maximally inhibited rate of 4.2 ± 0.4 nmol h^{-1} (10^5 cells)$^{-1}$ ($N = 3$) (Figure 1). The second component described weak stimulation of lipolysis with an EC_{50} of $(1.2^{\times}_{\div}1.3) \times 10^{-6}$ M ($N = 3$) from 0 to 3.7 ± 1.8 nmol h^{-1} (10^5 cells)$^{-1}$. At a PIA concentration of 10^{-7} M the contribution of the derived second phase lipolytic effect of PIA to the observed rate of glycerol release was negligible, while the antilipolytic effect of PIA was essentially fully expressed. Inhibition of lipolysis required progressively higher PIA concentrations with increasing concentrations of a lipolytic hormone (data not shown), in agreement with the competitive nature of the inhibition (Westermann & Stock, 1970).

The effect on lipolysis by phosphodiesterase inhibition in the presence of PIA (10^{-7} M) was measured to assess whether 10^{-7} M PIA had completely inhibited cAMP generation. The specific phosphodiesterase inhibitor Ro 20-1724/1 (Sheppard et al., 1972) was used. When adenylate cyclase was inhibited maximally by PIA (10^{-7} M), inhibition of phosphodiesterase by Ro 20-1724/1 resulted in only minimal stimulation of glycerol release, i.e., in a typical experiment from a maximally inhibited rate of 0.4171 ± 0.0003 to 3.254 ± 0.004 nmol h^{-1} (10^5 cells)$^{-1}$, whereas in the absence of PIA Ro 20-1724/1 stimulated glycerol release from a basal rate of 38 ± 6 to 82 ± 14 nmol h^{-1} (10^5 cells)$^{-1}$.

In the presence of PIA (10^{-7} M) exogenously added cAMP (data not shown) and Bt_2cAMP (Figure 2) could still stimulate lipolysis. These data show that PIA did not interfere with the action of cAMP on lipolysis in steps subsequent to cAMP production, and it may be inferred that, in the presence of 10^{-7} M PIA, fat cells can be considered depleted of functional

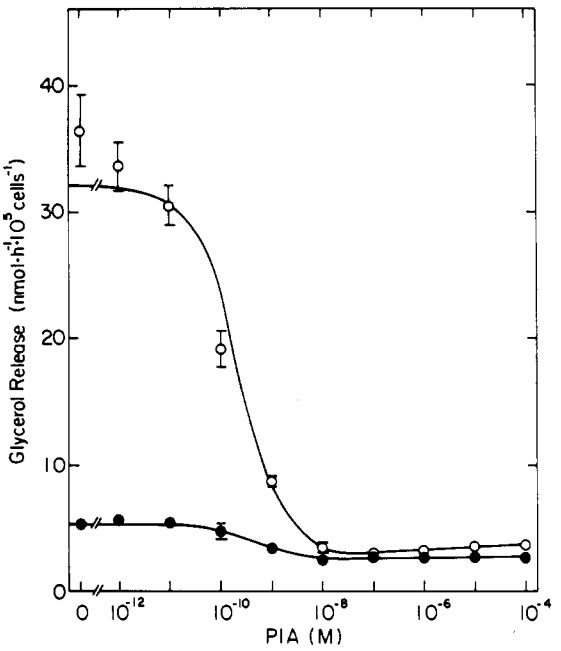

FIGURE 3: PIA effects on lipolysis in the absence and presence of insulin. No insulin (○); 10^{-10} M insulin (●). Adenosine deaminase was present at 280 milliunits/mL. Data are $\bar{x} \pm$ SEM from triplicate incubations. Points without error bars had standard errors smaller than symbol size. Curves are weighted nonlinear least-squares fits to the data for biphasic concentration–effect relationships as described under Methods.

Table I: 3-*O*-Methyl-D-glucose Uptake in Isolated Adipocytes in the Presence of Adenosine Deaminase and Various Adenylate Cyclase Modulators[a]

drug	3-*O*-methyl-D-glucose uptake [pmol s^{-1} (10^5 cells)$^{-1}$]
control	0.53 ± 0.07
ACTH-(1-24) (10^{-8} M)	0.67 ± 0.06
zinterol (10^{-7} M)	0.59 ± 0.02
Bt_2cAMP (2×10^{-3} M)	0.59 ± 0.02
PIA (10^{-7} M)	0.61 ± 0.02
insulin (10^{-9} M)	1.04 ± 0.06[b]

[a] Cells (4.26×10^6/mL) were preincubated for 20 min in the presence of adenosine deaminase (280 milliunits/mL) before 4-s uptake measurements at a 3-*O*-methylglucose concentration of 1 mM. Values are $\bar{x}$ ± SEM of quintuplicate observations. [b] A significant difference to control values at $p < 0.05$.

cAMP. When lipolysis was inhibited by 10^{-10} M insulin, a concentration corresponding approximately to the nadir of the biphasic antilipolytic dose–response curve for insulin (e.g., Kono & Barham, 1973; data not shown), PIA could additionally inhibit lipolysis. However, the lowest rates achieved by the combination of agents was not significantly lower than the effect of maximally inhibitory concentrations of PIA (10^{-7} M) alone (Figure 3).

cAMP Effects on Glucose Transport. Adipocyte cAMP production was elevated by incubation with either ACTH-(1–24), zinterol, a β-adrenergic agonist (Little & de Haën, 1980), or Bt_2cAMP, or it was depressed by the adenylate cyclase inhibitor PIA. Cells were preincubated with these agents for between 15 and 45 min before measurement of initial rates of 3-*O*-methylglucose uptake (0–4 s). Initial rates in pretreated cells were compared to rates in cells without pretreatment and in cells which were exposed to a maximally stimulatory concentration of insulin (5×10^{-9} M). Table I shows that altering adenylate cyclase activity, and thus increasing or decreasing intracellular levels of cAMP, by various

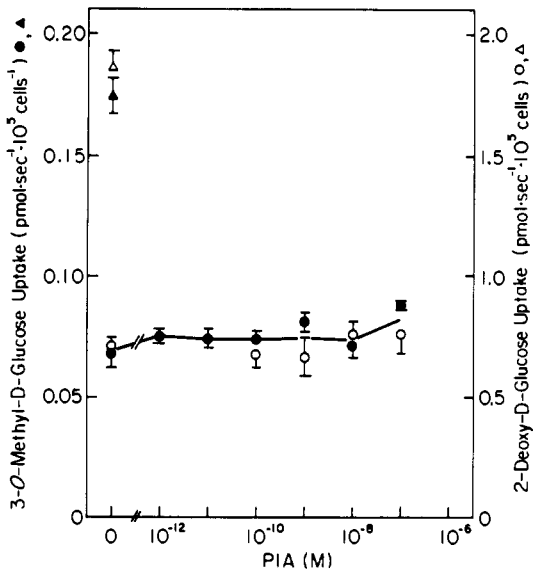

FIGURE 4: Concentration–effect relationship of PIA and 3-*O*-methyl-D-glucose or 2-deoxyglucose uptake. 3-*O*-Methyl-D-glucose uptake (●, ▲): Isolated fat cells (5.1×10^6 cells/mL) were preincubated at 37 °C with PIA for at least 30 min and assayed as described under Methods. The 3-*O*-methyl-D-glucose concentration was 0.2 mM. Data are $\bar{x} \pm$ SEM from quadruplicate incubations. Insulin (1×10^{-7} M) (▲) in the absence of PIA stimulated uptake 2.57-fold over control values. 2-Deoxyglucose uptake (○, △): Isolated fat cells (8.9×10^5 cells/mL) were preincubated at 24 °C with PIA for at least 30 min prior to measuring uptake as described under Methods. The 2-deoxyglucose concentration was 0.275 mM. Data are expressed as $\bar{x} \pm$ SEM of four incubations measured in duplicate. Insulin (10^{-8}M) (△) in the absence of PIA stimulated uptake 2.65-fold as compared to control values. Adenosine deaminase (280 milliunits/mL) was present in both experiments.

drugs had no significant effect on the initial uptake rates of 3-*O*-methylglucose when compared to control. Insulin (5×10^{-9} M), however, stimulated 3-*O*-methylglucose uptake approximately 2-fold as compared to control. For a more complete assessment of the effects of PIA on the glucose transport system, PIA concentration–effect relationships on 3-*O*-methylglucose and 2-deoxyglucose uptake were obtained (Figure 4). No significant stimulation of transport in either of the two assays was observed at concentrations that are known to lower cAMP. In contrast, insulin stimulated uptake in both experimental systems approximately 2.5-fold. Thus, altered adenylate cyclase activity, and by inference lowered or elevated intracellular levels of cAMP, appeared to have no effect on basal carrier-facilitated glucose transport as measured by 3-*O*-methylglucose uptake over 4 s or as measured by the 2-deoxyglucose method over 2 min.

Effects of PIA and Insulin on Glycogen Synthesis. For comparison of the effects of an inhibitor of adenylate cyclase with the effects of insulin, concentration–effect curves for PIA and insulin action on glucose incorporation into glycogen and on rates of lipolysis were determined in adipocytes from a single cell pool. Figures 1 and 5 demonstrate that PIA stimulated glucose incorporation into glycogen with an EC_{50} of ($1.5 \overset{\times}{\div} 10$) $\times 10^{-10}$ M ($N = 3$), the same EC_{50} that characterized inhibition of lipolysis (Figures 1 and 3). At high PIA concentrations glycogen synthesis was inhibited with an EC_{50} of ($3 \overset{\times}{\div} 3$) $\times 10^{-5}$ M ($N = 3$). Although not identical, the latter EC_{50} is very similar to that characterizing the second phase lipolytic component of PIA effects (Figure 1). However, maximal stimulation of glycogen synthesis by PIA, i.e., from a basal rate of 0.191 ± 0.001 to 0.394 ± 0.002 nmol h^{-1} (10^5 cells)$^{-1}$ ($N = 3$), was only about 10% of the maximal stimulation from 0.107 ± 0.004 to 2.04 ± 0.01 nmol h^{-1} (10^5 cells)$^{-1}$

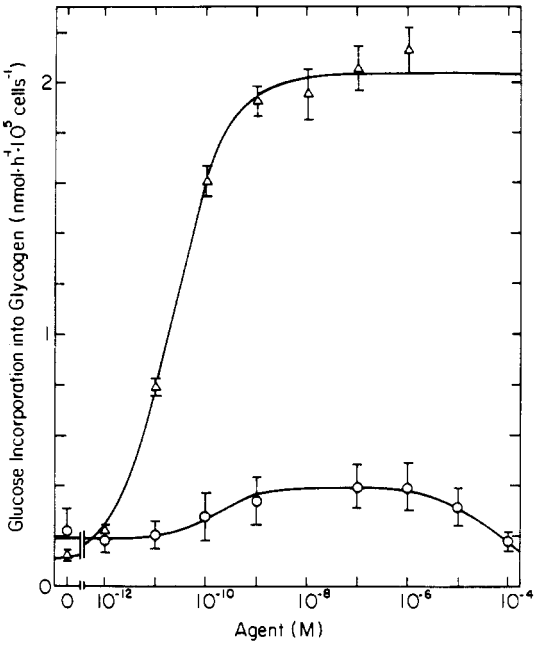

FIGURE 5: Comparison of the concentration–effect relationship of insulin and PIA on glucose incorporation into glycogen. Incubations with insulin (△) were performed in quintuplicate. The cell density was 1.77×10^6 cells/mL. The curve for insulin effects is a weighted nonlinear least-squares fit for a simple dose–response relationship with $h = 1$ as described under Methods. Data and fitted curve representing stimulation by PIA (○) are identical with those shown in Figure 1 and are expressed as absolute values for comparison purposes. In all instances the glucose concentration was 1.0 mM. Adenosine deaminase was present at 280 milliunits/mL. For other experimental details see legend to Figure 1.

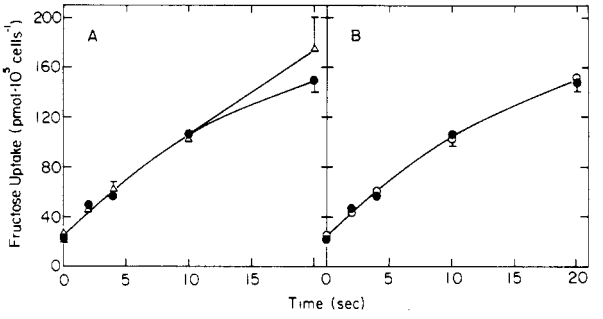

FIGURE 6: Effects of insulin and PIA on initial fructose uptake rates. Isolated fat cells (5×10^6 cells/mL) were preincubated alone, with insulin or with PIA at 37 ° C for at least 30 min before assay of uptake of D-[U-^{14}C]fructose (10 mM) as described under Methods. In this same cell batch insulin stimulated 3-*O*-methylglucose 1.96-fold. Data are $x \pm$ SD of duplicate determinations. Where the error bar is missing, it was smaller than the symbol size. (A) Effect of insulin (10^{-7} M) (△) as compared to control (●). (B) Effect of PIA (10^{-7} M) (○) as compared to control (●).

($N = 1$) achievable with insulin (Figure 5).

It is well-known that insulin stimulates carrier-facilitated D-glucose diffusion into adipocytes and could therefore account for the large response observed in Figure 5 via mechanisms involving accumulation of glucose 6-phosphate. PIA and insulin were compared with respect to D-fructose incorporation into glycogen in order to dissect glucose transport independent effects of insulin on glycogen synthesis from those mediated by insulin effects on D-glucose transport. Figure 6 demonstrates that neither insulin (10^{-7} M) nor PIA (10^{-7} M) stimulated initial fructose uptake into adipocytes, in the same cells in which insulin did stimulate initial 3-*O*-methylglucose uptake approximately 2-fold. Figure 7 shows that both insulin and PIA have the ability to stimulate fructose incorporation into

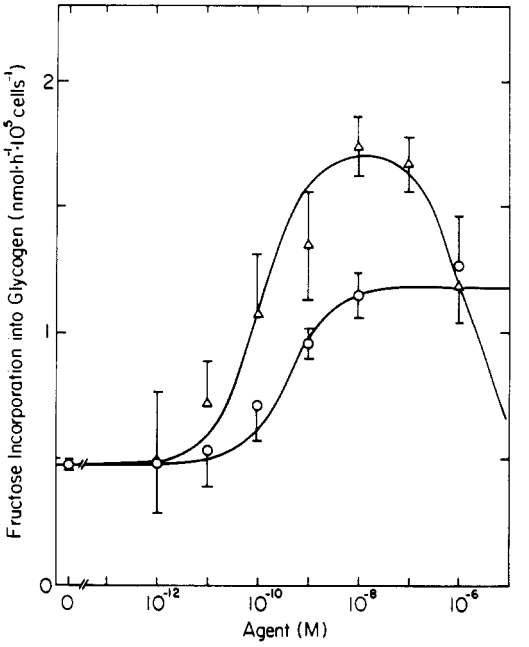

FIGURE 7: Concentration–effect relationship of PIA and insulin on fructose incorporation into glycogen. Stimulation of fructose incorporation into glycogen by insulin (△) and by PIA (○). Average cell density was 1.63×10^6 cells/mL. The fructose concentration was 10 mM. Data are $\bar{x} \pm$ SEM from quintuplicate incubations from one experiment, in which the effects of both agents were determined on the same cell batch. The value in the absence of drugs is the $\bar{x}$ ± SEM of 10 different experiments. Curves are weighted nonlinear least-squares fits to the data with $h = 1$ as described under Methods.

glycogen, demonstrating that both agent may affect glycogen synthesis independently of transport stimulation. While the highest PIA concentration used in these experiments was not high enough to know whether the PIA effect was reversed in the high concentration range, insulin did show a biphasic concentration–effect relationship. PIA stimulated fructose incorporation into glycogen with an EC_{50} of 4.2×10^{-10} M from a basal rate of 0.478 nmol h^{-1} $(10^5$ cells$)^{-1}$ to a maximally stimulated rate of 1.18 nmol h^{-1} $(10^5$ cells$)^{-1}$, whereas maximal stimulation by insulin, which occurred at 10^{-8} M hormone, was 1.72 nmol h^{-1} $(10^5$ cells$)^{-1}$. The EC_{50} for insulin stimulation was 1.13×10^{-10} M.

When the question of additivity of the effects of insulin and PIA on glycogen synthesis was addressed, it was found that PIA over a concentration range of 7 orders of magnitude failed to alter the effects of 10^{-9} M insulin on glucose incorporation into glycogen both in the absence and in the presence of 10^{-4} M phloretin, a potent inhibitor of glucose transport (data not shown).

Comparison of PIA-Stimulated with Insulin-Stimulated Glycogen Synthase I Activity. The glycogen synthase I activity ratio (–Glc-6-P/+Glc-6-P) was assessed in isolated cells which were exposed for various lengths of time (10–30 min) to either insulin or PIA. The concentration–effect relationship of the two agents is shown in Figure 8. In the absence of medium glucose during preincubation and drug exposure, the glycogen synthase I activity ratio was 0.105 ± 0.008 ($N = 12$). Insulin was able to elevate this ratio with an EC_{50} of $(7.8 \overset{\times}{\div} 2.1) \times 10^{-11}$ M to a maximum of 0.193 ± 0.004 ($N = 3$), corresponding to an 84% increase over basal. PIA also elevated the basal ratio, with an EC_{50} of $(2.1 \overset{\times}{\div} 3.8) \times 10^{-10}$ M ($N = 6$), but only to a level of 0.123 ± 0.002 at 10^{-7} M, corresponding to a 17.1% increase over basal. Note that the best fit to the averaged data shown in Figure 8 yielded somewhat different EC_{50} values as compared to the values given in the

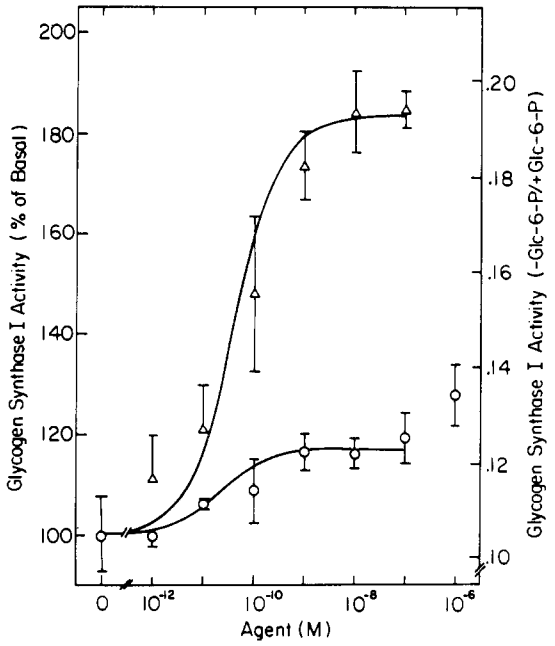

FIGURE 8: Comparison of insulin and PIA stimulation of glycogen synthase I activity. The effects of insulin (Δ) and PIA (O) in the presence of adenosine deaminase at 280 milliunits/mL but in the absence of glucose were determined on the same cell batch for each experiment. Data are $\bar{x} \pm$ SEM from 3–12 experiments performed in duplicate and are expressed on the left-hand ordinate as percent of basal rate of glycogen synthase I activity from control experiments in the absence of drug. The right-hand ordinate expresses absolute activity ratios (–Glc-6-P/+Glc-6-P). Curves are weighted nonlinear least-squares fits to the data with $h = 1$ as described under Methods.

text. The latter values, although not significantly different from those shown in the graph, were obtained from the mean of best fit values of the individual experiments. This was done to obtain a realistic estimate of variance of EC_{50} values. Thus PIA, in the concentration range in which it has antilipolytic activity, displayed on the average only one-fifth the efficacy of insulin in stimulating glycogen synthase I activity. It should be noted that PIA at concentrations higher than 10^{-7} M tended to stimulate glycogen synthase I activity further, just as it commences to have a stimulatory effect on 3-*O*-methylglucose transport (Figure 4) and on lipolysis (Figure 1). Because the mechanisms involved are obscure and outside the scope of the present communication, it was not further investigated.

Discussion

In order to delineate the role of cAMP in the regulation of glycogen synthesis in isolated rat epididymal fat cells, we investigated the effects of the adenylate cyclase inhibitor, PIA. PIA was chosen as adenylate cyclase inhibitor because its action at extraordinarily low concentrations helped assure specificity. We wanted to find conditions under which cAMP levels were lowered enough to be functionally irrelevant. The best controlled studies have shown that at submaximally stimulated rates of lipolysis, these rates reflected cAMP levels well (Robison et al., 1971; Kono & Barham, 1973; Siddle & Hales, 1974; Hjemdahl & Fredholm, 1976; Burns et al., 1979; Schimmel et al., 1980; Wong & Loten, 1981; Fan & Ho, 1981; Sengupta et al., 1981). The relationship between the two is not linear; rather, rates of lipolysis are related to cellular cAMP levels in a saturable and positively cooperative fashion. Zero lipolysis seems to coincide with zero cAMP concentration (Kono & Barham, 1973). Moreover, cAMP-dependent protein kinase is known to be involved in the regulation of hormone-sensitive triglyceride lipase (Steinberg et al., 1975). Rates of lipolysis can therefore be used to gauge functional rather than absolute cAMP levels.

Our data establish an EC_{50} of 3.3×10^{-10} M for the inhibition of basal lipolysis by PIA, in agreement with data by Londos et al. (1980). Evidence that PIA (10^{-7} M) effectively inhibited the generation of cAMP was obtained by showing that in the presence of PIA (10^{-7} M) stimulation of lipolysis by the specific phosphodiesterase inhibitor Ro 20-1724/1 was negligible. At high concentrations PIA was found to be weakly lipolytic. This may explain the biphasic dose response on glycerol and free fatty acid levels previously observed in vivo (Westermann et al., 1969). Theoretical decomposition of the biphasic dose–response curve into two components demonstrates that at 10^{-7} M PIA, where inhibition of lipolysis via adenylate cyclase inhibition is almost maximal, there is as yet no stimulation of lipolysis by PIA. We therefore concluded that in the presence of 10^{-7} M PIA cellular cAMP levels are so low as to prevent significant action of cAMP-dependent protein kinase. The cells thus may be considered depleted of functional cAMP, a situation approximating an adenylate cyclase deficient cell.

Next, the potential role of cAMP in glucose transport activation was addressed. This issue has, to date, attracted surprisingly little attention and only recently have attempts been made to assess the effects of cAMP on 3-*O*-methylglucose transport. Thus, while Grinstein & Erlij (1976) failed to find an effect of exogenously provided Bt_2cAMP on 3-*O*-methylglucose efflux from rat skeletal muscle, in barnacle muscle Baker & Carruthers (1980) demonstrated an inhibitory effect of intracellularly provided cAMP on 3-*O*-methylglucose uptake. We showed that neither elevation of cAMP levels with lipolytic hormones or Bt_2cAMP, nor lowering cAMP levels with PIA had any appreciable effect on basal 3-*O*-methylglucose or 2-deoxyglucose uptake rates in rat adipocytes. Thus cAMP or cAMP-dependent protein kinase has no primary role in glucose transport regulation of fat cells. A conclusion to the contrary (Taylor et al., 1976; Taylor & Halperin, 1979) was based on a complex indirect assay for glucose transport, interpretation of which has remained open to question.

Turning to glycogen synthesis, PIA was shown to stimulate incorporaton of both glucose and fructose into glycogen 2-fold, with EC_{50}'s identical with that for the effect of PIA on lipolysis. Since PIA had no effect on the glucose transport system or on fructose transport, these effects can involve neither allosteric activation of glycogen synthase by glucose 6-phosphate at a constant phosphorylation state of the enzyme nor enhancement of dephosphorylation by that metabolite via mechanism II of Lawrence & Larner (1978). Rather it seems that PIA effects reflect a change in the phosphorylation state of the enzyme brought about by alteration in the activity of protein kinases and/or phosphoprotein phosphatases. cAMP-dependent protein kinase is capable of altering the activity of glycogen synthase (Cohen, 1982). Indeed PIA was found to increase glycogen synthase I activity even in the absence of medium glucose.

Because insulin is well-known to activate low K_m cAMP phosphodiesterase in rat adipocytes (Loten & Sneyd, 1970; Manganiello & Vaughan, 1973; Zinman & Hollenberg, 1974; Sakai et al., 1974; Kono et al., 1975), it would be expected that insulin lowers cellular cAMP levels. Also, if insulin were to inhibit adenylate cyclase (Illiano & Cuatrecasas, 1972; Renner et al., 1974; Torres et al., 1978; Londos et al., 1978), the same effect of insulin on cAMP levels could be anticipated. Indeed a lowering of cAMP levels has frequently (Butcher et al., 1968; Kono & Barham, 1973; Desai et al., 1973; Burns

et al., 1979; Wong & Loten, 1981), but not always (Koo et al., 1973; Siddle & Hales, 1974), been observed. Failure to observe such lowering may be in part due to the inherent difficulties in measuring cAMP levels below basal values and in part due to failure of controlling adenosine accumulation in the fat cell incubation medium, which by itself lowers cAMP levels. The importance of cAMP lowering for insulin action has been disputed (Fain, 1980), but the evidence on which the criticism was based can in turn be severly criticized. Thus the available evidence favors the idea that lowering of cAMP levels may be an important determinant in some actions of insulin. It has also been suggested that insulin induces the generation of an inhibitor of cAMP-dependent protein kinase (Larner et al., 1979) and that insulin lowers the phosphorylation state and thus the activity of protein phosphatase inhibitor 1 (Foulkes et al., 1980), a protein that requires phosphorylation by cAMP-dependent protein kinase for activity. To learn the maximum extent to which a cAMP-lowering effect of insulin and/or an inhibitory effect on cAMP-dependent protein kinase could explain its metabolic effects, we chose to compare the effects of insulin with the effects of PIA on a variety of processes.

Given the lack of a role for cAMP in glucose transport regulation (discussed above), stimulation of transport by insulin obviously cannot be related to its effects on the cAMP system. These results, obtained with direct transport experiments, added substance to the widely held belief based on glucose oxidation data (e.g., Fain & Rosenberg, 1972) that cAMP was not involved in transport activation by insulin. In contrast, the observation that PIA inhibited lipolysis to such a degree that insulin was unable to inhibit it any further is at least consistent with a crucial role of cAMP in the control of lipolysis by insulin. However, this leaves open the possibility that could lipolysis be stimulated by routes not involving cAMP [reviewed in Fain (1980)], insulin action may also involve a different mechanism.

Adipocytes possess distinct transport systems for fructose and glucose (Froesch, 1965). Our short-term (0–20 s) fructose uptake experiments provide the most direct evidence yet that insulin does not stimulate the fructose-specific transport system, a conclusion previously deduced from more indirect experiments involving 45-min fructose uptake measurements (Froesch, 1965; Schoenle et al., 1979). Schoenle et al. (1979) suggested that the glucose-specific transport system may contribute to some degree to total fructose transport. This should render fructose transport slightly insulin sensitive. Our data did not support this interpretation and neither did data from 4-min fructose uptake experiments (Dr. James M. May, personal communication). The results of Schoenle et al. (1979) obtained by measuring fructose uptake over a 45-min period in the presence and absence of 3-*O*-methylglucose as a competitive inhibitor of the glucose-specific transport system thus may have to be interpreted as an insulin effect on fructose metabolism beyond the transport site but sensitive to 3-*O*-methylglucose.

Turning to the comparison of PIA effects with those of insulin on glucose incorporation into glycogen, we found that the effect of PIA on glycogen synthesis was much smaller in magnitude, namely, about 10% that of insulin. Since, in contrast to insulin, PIA does not activate glucose transport, we attribute the difference to a large degree to the contribution of insulin's effect on the glucose transport system and the Glc-6-P-mediated mechanisms discussed in the introduction. However, insulin's action on glycogen synthesis is likely to include components independent of glucose transport. If lowered cAMP levels were to explain all of the glucose transport independent effects of insulin, one might expect PIA and insulin to be equally efficacious in stimulating fructose incorporation into glycogen. PIA stimulated fructose incorporation into glycogen with the same EC_{50} as incorporation of glucose into glycogen or inhibition of lipolysis. PIA was about 57% as efficacious as insulin, suggesting that lowered cAMP levels could only account for part of the glucose transport independent action of insulin.

The insulin effect on glucose incorporation into glycogen is a simple saturable process as are the effects of insulin on glucose transport (Lawrence et al., 1977) and glucose transport dominated phenomena such as glucose-C1-oxidation (Gliemann, 1965) and glucose incorporation into triglycerides (Anderson et al., 1977). In contrast, we found insulin effects on fructose incorporation into glycogen to be biphasic in a manner reminiscent of the biphasic effect of insulin on lipolysis and cAMP levels (e.g., Kono & Barham, 1973), adenylate cyclase activity (Renner et al., 1974), and phosphodiesterase activity (Kono et al., 1975). PIA stimulated glycogen synthase I activity to the extent of 20% of the activity in the presence of insulin. This percentage was somewhat lower than that expected based on the data on fructose incorporation into glycogen but is consistent with a lowering of cAMP levels by insulin significantly contributing to the regulation of glycogen synthesis.

Studying rat adipocytes in the absence of medium glucose, Lawrence & Larner (1979) found stimulation of glycogen synthase I and inhibition of phosphorylase activity by insulin in the absence of measurable changes in cAMP levels. They concluded that it was unlikely that the effects of insulin on these enzymes resulted from an action of the hormone to decrease the concentration of cAMP. However, in their studies accumulation of adenosine in the incubation medium was not controlled. Conceivably, adenosine, a potent adenylate cyclase inhibitor, masked the contribution of altered cAMP levels to the action of insulin in their experiments. Still, we agree with Lawrence & Larner (1979) in concluding that a major component of insulin action ($\sim$80%) on glycogen synthase I activity in the absence of medium glucose is unrelated to cAMP levels. But we are able to go one step further. The major component of insulin action is also not explicable by any other cAMP-dependent mechanism such as inhibition of cAMP-dependent protein kinase (e.g., Larner et al., 1979) or alteration in levels of phosphorylation of phosphoprotein phosphatase inhibitor 1 (Foulkes et al., 1980), a process again involving cAMP-dependent protein kinase.

In summary, lowering of cAMP levels and/or interference with cAMP-dependent protein kinase was found to have the potential of explaining all of insulin's inhibitory action on basal lipolysis in rat epididymal adipocytes. In contrast, these cAMP-related mechanisms could not explain the effects of insulin on glucose transport at all. They do, however, have the potential for contributing significantly to the regulation by insulin of glycogen synthase I activity. Our study did not intend to answer the question of whether insulin actually used these mechanisms, rather it was aimed at putting a limit on the extent to which they conceivably could contribute to the total effects of insulin. Finally, development of a fat cell system depleted of functional cAMP provides a controlled system in which the components of insulin action independent of the above mechanisms can be studied in isolation.

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Registry No. cAMP, 60-92-4; dibutyryl-cAMP, 362-74-3; insulin, 9004-10-8; 3-*O*-methyl-D-glucose, 146-72-5; 2-deoxy-D-glucose, 154-17-6; D-glucose, 50-99-7; D-fructose, 57-48-7; glycogen synthase, 9014-56-6; glycogen, 9005-79-2.

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