A Novel Assay for Evaluating Glycogenolysis in Rat Adipocytes and the Inability of Insulin To Antagonize Glycogenolysis in This Cell Type[†]

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ABSTRACT: We report here on a novel procedure for measuring glycogenolysis in rat adipocytes. In this procedure, cells are incubated for 30 min at 37 °C with insulin or vanadate, and with [U-^{14}C]glucose to label the glycogen pool with radioactive glucose. The cells are washed and preincubated for an additional 1 h, before being assayed. The extent of glycogenolysis is determined by the decrease in radioactivity in precipitated glycogen, which was quite substantial under experimental conditions facilitating glycogenolysis. From the assay, we determined the following. (a) Glycogenolysis is activated in rat adipocytes in response to lipolytic hormones (i.e. catecholamines and adrenocorticotropic hormone). (b) Other agents and conditions elevating intracellular adenosine 3′,5′-monophosphate levels (i.e. cholera toxin, dibutyryladenosine 3′,5′-monophosphate, and isobutylmethylxanthine) also activate glycogenolysis. (c) Glycogenolysis (as opposed to lipolysis) is activated at concentrations of adrenocorticotropic hormone or isoproterenol 7−11-fold lower and at adenosine 3′,5′-monophosphate concentrations 7-fold lower. (d) Calyculin A, a specific inhibitor of protein phosphatase 1, activates glycogenolysis as well. Calyculin A also activates lipolysis at an equimolar potency. (e) Insulin does not antagonize glycogenolysis in rat adipocytes. In conclusion, the assay allowed us to compare glycogenolysis to lipolysis within the same cell, and to find that the sensitivity to hormones and adenosine 3′,5′-monophosphate was about 1 order of magnitude higher for glycogenolysis than for lipolysis. A more striking finding was the inability of insulin to antagonize glycogenolysis in the rat adipose cell, an effect which occurs readily in liver and muscle cells via protein phosphatase 1-activating machinery. This rules out a role for adipose protein phosphatase 1 activation in the mechanism by which insulin antagonizes lipolysis and supports the contention that the insulin effect in lowering adenosine 3′,5′-monophosphate levels is the central mechanism by which insulin antagonizes lipolysis.

Insulin inhibits lipolysis mediated by physiological concentrations of lipolytic hormones in rat adipocytes [reviewed in Avruch et al. (1972) and Meisner and Carter (1977)]. These lipolytic hormones (i.e. catecholamine, adrenocorticotropic hormone, and glucagon) share the ability to activate adenylate cyclase and increase adenosine 3′,5′-monophosphate (cyclicAMP)[1] levels (Meisner & Carter, 1972; Steinberg et al., 1975). CyclicAMP-dependent protein kinase is then activated followed by phosphorylation and activation of hormone-sensitive triglyceride lipase (HSL; Huttunen et al., 1970). Little is known about the mechanism(s) by which insulin antagonizes lipolysis. Several sites along the lipolytic cascade are potentially sensitive to insulin. Adenylate cyclase may be inhibited (Stock & Prilop, 1974; Trost & Stock, 1977; Fain & Malbon, 1979); low-K_m cyclicAMP phosphodiesterase (cyclicAMP-PDE) may be activated (Manganiello et al., 1991; Shibata et al., 1991; Degerman et al., 1990; Rahn et al., 1994), and protein phosphoserine phosphatases, which dephosphorylate HSL at site 2, may be activated (Olsson & Belfrage, 1987). Many studies either support or disprove an insulin effect at any of the above-mentioned sites. The possibility that the hormone acts in concert at several points along this cascade is certainly feasible. Protein phosphoserine phosphatase 1 (PP1) and protein phosphoserine phosphatase 2A (PP2A) are the principal phosphoserine/phosphothreonine phosphatases in rat adipocytes (Olsson & Belfrage, 1987). PP1 was shown to be activated by insulin in muscle and liver (Dent et al., 1990; Wera & Hemmings, 1995).

Insulin activates glycogen synthase in all three insulin-responsive tissues, and antagonizes glycogenolysis, evoked by physiological concentrations of hormones elevating the cyclicAMP level, in liver and muscle [Dent et al., 1990; reviewed in Wera and Hemmings (1995), Barford (1996), Lawrence (1992), and Cohen (1989)]. The mechanism involved is believed to include insulin-dependent activation of PP1, and dephosphorylation of active (phosphorylated) phosphorylase kinase (Dent et al., 1990; Wera & Hemmings, 1995; Barford, 1996; Lawrence, 1992; Cohen, 1989). Bearing in mind that glycogenolysis relates mechanistically to lipolysis (Belfrage et al., 1987), we have tried to establish a glycogenolytic assay in rat adipocytes. Second, we intended to investigate the antiglycogenolytic effect of insulin.

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[1] Abbreviations: KRB, Krebs-Ringer bicarbonate; BSA, bovine serum albumin; cyclicAMP, adenosine 3′,5′-monophosphate; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; OKA, okadaic acid; IBMX, isobutylmethylxanthine; ACTH, adrenocorticotropic hormone; HSL, hormone-sensitive triglyceride lipase; cyclicAMP−PDE, low-K_m cyclicAMP phosphodiesterase.

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Finally, we wished to elucidate the common denominators and the differences between antiglycogenolysis and antilipolysis, hoping to gain more insight into the latter (more obscure) bioeffect of insulin in the rat adipose cell.

MATERIALS AND METHODS

D-[U-^{14}C]Glucose was purchased from New England Nuclear (Boston, MA), and collagenase type I (134 units/mg) was obtained from Worthington Biochemicals (Freehold, NJ). Okadaic acid, calyculin A, adrenocorticotropic hormone (ACTH), cholera toxin, isoproterenol, and dibutyryl cyclicAMP were purchased from Sigma Chemicals (St. Louis, MO). Porcine insulin was obtained from Eli Lilly Co. All other chemicals used here were of analytical grade. Krebs Ringer bicarbonate (KRB) buffer (pH 7.4) contained 110 mM NaCl, 25 mM $NaHCO_3$, 5 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, and 1.3 mM $MgSO_4$.

Cell Preparation and Bioassays

Rat adipocytes were prepared from fat pads of male Wistar rats (150−200 g) by collagenase digestion as described by Rodbell (1964). Cell preparations showed more than 95% viability by Trypan blue exclusion, at least 3 h after digestion.

Assay of Glycogenolysis. A "short cut" simple procedure for glycogenolysis was performed as follows. Freshly prepared rat adipocytes (∼20% suspension in KRB buffer at pH 7.4 with 0.7% BSA) were preincubated for 30 min at 37 °C with 1.0 mM [U-^{14}C]glucose (3000 cpm/nmol) and with either insulin (10 ng/mL) or sodium metavanadate ($NaVO_3$, 0.1−0.3 mM). Cells were then washed, diluted 10-fold with the same buffer, divided into vials (0.5 mL per vial), and subjected to glycogenolysis for 30 min at 37 °C in the presence and absence of isoproterenol and/or other hormones or agents as specified.

When the antiglycogenolytic effect of insulin was under study or when the glycogenolytic agent was cholera toxin, the preincubated, washed cells were incubated for an additional 1 h at 37 °C in fresh KRB buffer, prior to glycogenolysis (prolonged procedure). Following glycogenolysis, cells were disrupted and glycogen was extracted using 2 M KOH and precipitated with 66% ethanol. The precipitate was washed twice with ice cold ethanol and its radioactive content counted essentially as described by Lawrence et al. (1977) and Hess et al. (1991).

Lipolysis and antilipolysis were performed for 1 h at 37 °C with adipocytes (∼3×10^5 cells/mL) suspended in KRB buffer (pH 7.4 with 0.7% BSA) in the presence and absence of lipolytic hormones and insulin or for 3 h when cholera toxin was the lipolytic agent. Aliquots from the medium were then taken; BSA was precipitated by trichloroacetic acid, and the glycerol content in the medium was determined by a spectroscopic assay [triglyceride C-37 rapid/stat test, Pierce Chemical Co., Rockford, IL, and in Shechter (1982)].

Protein concentration was determined by the method of Bradford (1976). All the assays were performed in duplicate or triplicate. Each figure is a representative experiment performed at least three to five times with identical results.

RESULTS

Establishing a Glycogenolytic Assay in Rat Adipocytes. In establishing the assay in the adipose cell, we had to label the adipose glycogen pool extensively and, if labeling was achieved with insulin, to remove the hormone and terminate its activating effects prior to the glycogenolytic assay. Incubation of rat adipocytes for 30 min at 37 °C with insulin or vanadate, in the presence of [U-^{14}C]glucose, led to widespread labeling of the adipose glycogen pool. Thus, insulin or vanadate increased by 10−20-fold the incorporation of labeled glucose into glycogen (Figure 1). Half-maximal stimulation (ED_{50}) was 3.0 ± 0.3 ng/mL and 20 ± 4 μM for insulin and vanadate, respectively (Figure 1). The ED_{50} for insulin-activated lipogenesis in rat adipocytes is 0.2 ± 0.02 ng/mL [i.e. see Shechter and Ron (1986)]. Thus, there is 15-fold difference in sensitivity to insulin between glycogenesis and lipogenesis, in this cell type.

In Figure 1B, glycogenesis was followed by washing the adipocytes (to remove insulin, vanadate, or glucose) and incubating them for an additional 1 h at 37 °C in fresh KRB buffer (pH 7.4 with 0.7% BSA), containing no insulin, vanadate, or glucose. As shown in Figure 1, no significant reduction in radioactivity of the adipose glycogen pool could be detected. Thus, upon prolonged incubations, spontaneous glycogenolysis is minimal. An alternative option for achieving an "insulin-free" experimental system was the activation of glycogenesis by vanadate (Figure 1A). Vanadate mimics the biological effects of insulin using different pathways in an insulin receptor-independent fashion [Shisheva & Shechter, 1993; Elberg et al., 1994; reviewed by Shechter et al. (1995)].

Activation of Glycogenolysis in Rat Adipocytes, the Effect of Isoproterenol and ACTH, and Comparison to Lipolysis. Figure 2 shows the effect of isoproterenol on glycogenolysis in adipocytes in which the glycogen pool had been previously labeled with [U-^{14}C]glucose and vanadate. Addition of isoproterenol efficiently reduced radiolabeling of adipose glycogen (Figure 2A). The same results were obtained when the glycogen pool was labeled with insulin, rather than with vanadate (not shown). Glycogenolysis was half-maximally activated (ED_{50}) at 30 ± 3 nM, whereas the ED_{50} for lipolysis was 340 ± 40 nM (Figure 2B). Thus, glycogenolysis is half-maximally activated at 11-fold lower concentrations of isoproterenol than lipolysis.

ACTH activated glycogenolysis as well (Figure 2C) and, like isoproterenol, was a more potent activator of glycogenolysis. Half-maximal values (ED_{50}) were 0.28 ± 0.03 and 2.0 ± 0.2 nM for glycogenolysis and lipolysis, respectively (Figure 2C).

Activation of Glycogenolysis by Nonhormonal Agents Elevating CyclicAMP Levels in Rat Adipocytes. Glycogenolysis was also activated by agents acting downstream of cell surface receptor sites, and which have previously been shown to elevate cyclicAMP levels and to activate lipolysis in the rat adipose cell. These include cholera toxin, an activator of adenylate cyclase; IBMX which inhibits cyclicAMP−PDE; and dibutyryl cyclicAMP, a cell permeable derivative of cyclicAMP that activates protein kinase A, downstream to adenylate cyclase (Gilman, 1987; Shechter, 1984; Beebe et al., 1985; Honnora et al., 1985; Gabbay & Lardy, 1986). All three agents activated glycogenolysis (Figure 3). IBMX appears to be a considerably more potent activator of glycogenolysis than of lipolysis [ED_{50} = 5.0 ± 0.5 μM, Figure 3; see Shechter (1984) for comparison].

The observation that glycogenolysis was activated by concentrations of ACTH or isoproterenol 7−11-fold lower than that required for lipolysis (Figure 2) suggested a greater sensitivity of glycogenolysis to cyclicAMP. To analyze this

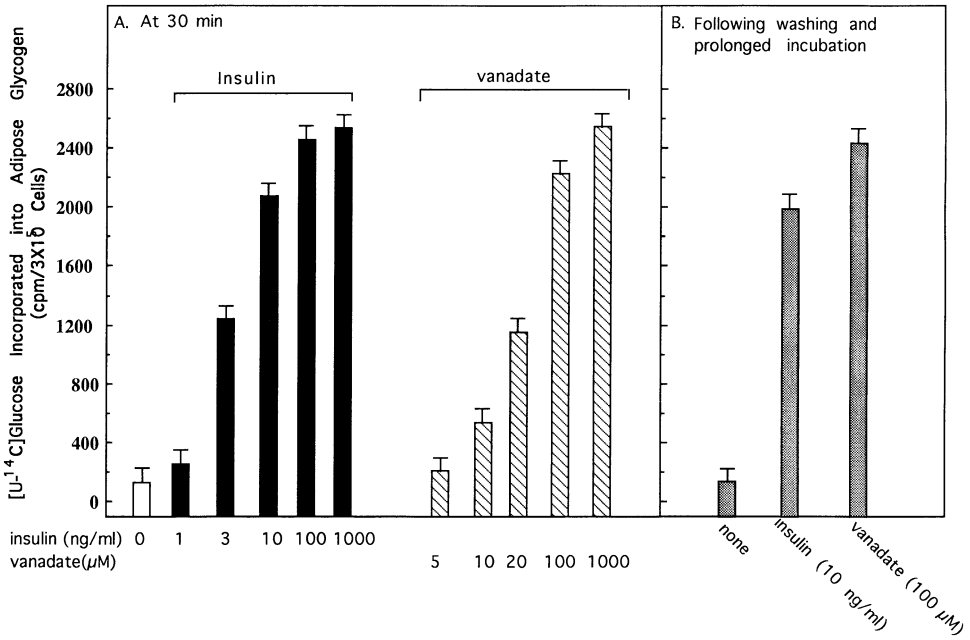

FIGURE 1: Effects of insulin and vanadate on the incorporation of labeled glucose into glycogen in intact rat adipocytes. (A) Freshly prepared rat adipocytes in KRB/0.7% BSA (pH 7.4) buffer were divided into plastic vials (0.5 mL per vial, $\sim 3 \times 10^5$ cells) and incubated for 30 min at 37 °C, under an atmosphere of 95% O_2/5% CO_2, with 1 mM [U-^{14}C]glucose (3000 cpm/nmol) in either the presence or absence of the indicated concentrations of insulin or vanadate. The reaction was terminated by the addition of KOH (final concentration of 1 M). Glycogen was precipitated, extracted, and counted for its radioactive content. (B) Adipocytes (20% v/v suspension) were incubated with 1.0 mM [U-^{14}C]glucose with or without the indicated concentrations of either insulin or vanadate for 30 min at 37 °C. Cells were then washed and incubated for additional 1 h at 37 °C, in KRB buffer containing no glucose, insulin, or vanadate. The radioactive content in glycogen was then determined (prolonged procedure).

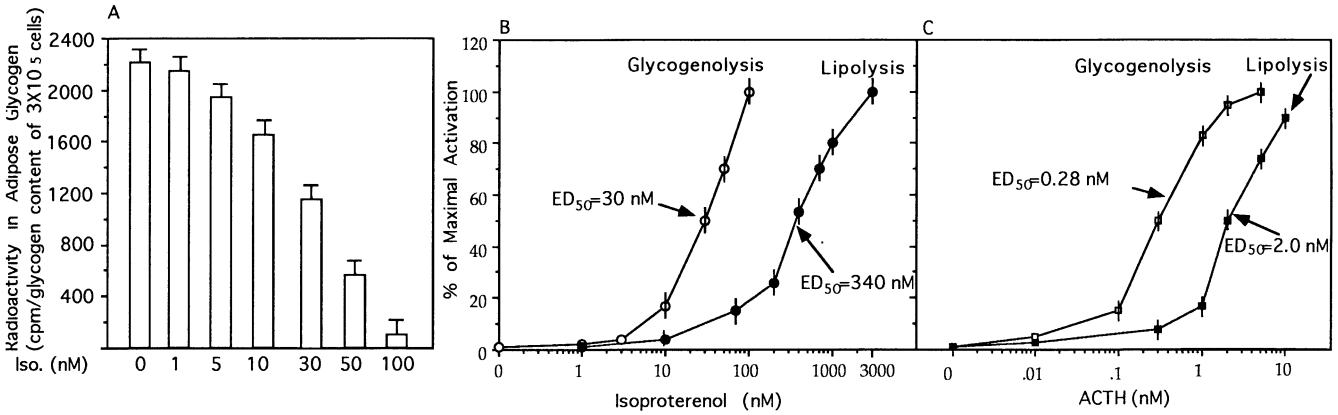

FIGURE 2: Activation of glycogenolysis by isoproterenol and ACTH and a comparison to lipolysis. (A) Adipocytes (20% suspension) were incubated for 30 min at 37 °C with 1.0 mM [U-^{14}C]glucose and 0.1 mM $NaVO_3$. Cells were washed, incubated for additional 1 h at 37 °C in fresh KRB buffer, diluted 10-fold, and distributed into plastic vials (0.5 mL per vial, $\sim 3 \times 10^5$ cells). Glycogenolysis was then performed for 30 min at 37 °C, with (or without) the indicated concentrations of isoproterenol. The amount of radioactivity in glycogen was then determined. (B) Concentration-dependent activation of glycogenolysis and lipolysis by isoproterenol. The results in part A are presented as a percentage of maximal activation of glycogenolysis. Maximal response (100%) is that obtained at 100 nM isoproterenol. Lipolysis was performed for 1 h at 37 °C in the presence and absence of the indicated concentrations of isoproterenol, prior to determining the amount of glycerol released to the medium. (C) Concentration-dependent activation of glycogenolysis and lipolysis by ACTH. Procedures were carried out as specified in parts A and B in the presence and absence of the indicated concentrations of ACTH.

point more directly, both bioeffects were activated at increasing concentrations of dibutyryl cyclicAMP. Glycogenolysis and lipolysis were half-maximally activated at 60 $\pm$ 3 and at 430 $\pm$ 30 μM dibutyryl cyclicAMP, respectively (Figure 4). Thus, glycogenolysis was triggered by concentrations of the cyclic nucleotide 7.2-fold lower than those that were required for lipolysis.

Activation of Glycogenolysis by NoncyclicAMP-Elevating Agents and Effects of Calyculin A and Okadaic Acid. Calyculin A, a potent inhibitor of protein phosphatase 1 (PP1, ID_{50} = 0.2 nM), and okadaic acid, a potent inhibitor of protein phosphatase 2A (PP2A) and a weak inhibitor of PP1 (PP2A, ID_{50} = 0.2 nM; PP1, ID_{50} = 20 nM; Ishihara et al., 1989; Cohen et al., 1990), also activated glycogenolysis in the rat adipose cell (Figure 5). Half-maximal activation was 20 $\pm$ 3 and 340 $\pm$ 30 nM for calyculin A and okadaic acid, respectively. Lipolysis was activated by the same concentrations of the two agents. Thus, with the noncyclicAMP-elevating agents, calyculin A and okadaic acid, activation of lipolysis and glycogenolysis overlap (Figure 5).

Insulin Does Not Antagonize Glycogenolysis in Rat Adipocytes. In the experiments summarized in Figure 6, the glycogen pool of freshly prepared rat adipocytes was labeled (for 30 min at 37 °C) with [U-^{14}C]glucose and with 0.1 mM

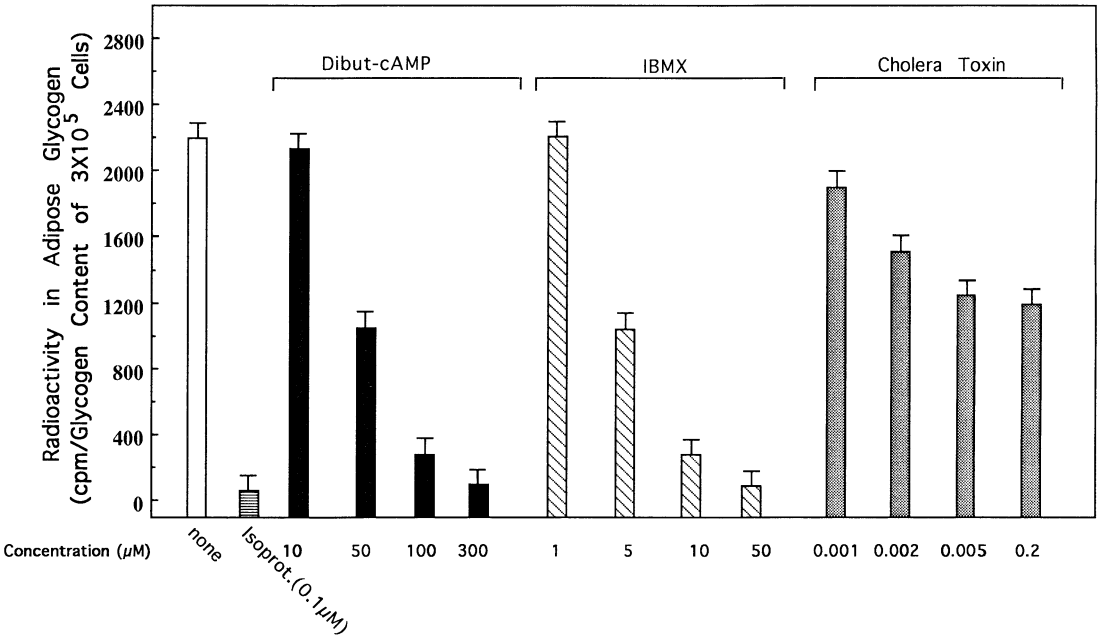

FIGURE 3: Activation of glycogenolysis by nonhormonal agents which elevates cyclicAMP levels. Incorporation of [U-^{14}C]glucose into glycogen was performed by incubating the cells with 0.2 mM [U-^{14}C]glucose and 0.1 mM $NaVO_3$ followed by washing and incubating the cells for an additional 1 h (prolonged procedure described in detail in Figure 1B). The assay of glycogenolysis was then carried out for 45 min at 37 °C with the indicated concentrations of dibutyryl cyclicAMP or IBMX. In the case of cholera toxin, the latter was added to the cells at time 0 (together with or prior to the addition of glucose and vanadate). This is because the effect of cholera toxin occurs following a lag period of ~1 h [i.e. Shechter (1984)].

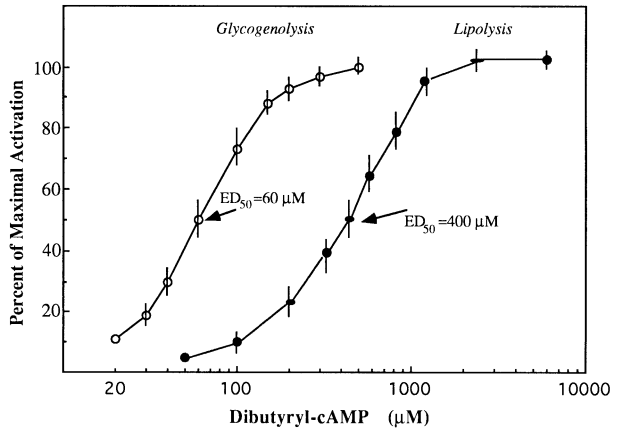

FIGURE 4: Concentration-dependent activation of glycogenolysis and lipolysis by dibutyryl cyclicAMP. Glycogenolysis and lipolysis were performed for 45 min at 37 °C in the absence and presence of the indicated concentrations of dibutyryl cyclicAMP. Glycogenolysis was performed with the prolonged procedure (experimental details in legends of Figures 1B and 2A). Results are expressed as a percentage of maximal activation (100% activation is that obtained with 0.1 and 3 μM isoproterenol for activating glycogenolysis and lipolysis, respectively).

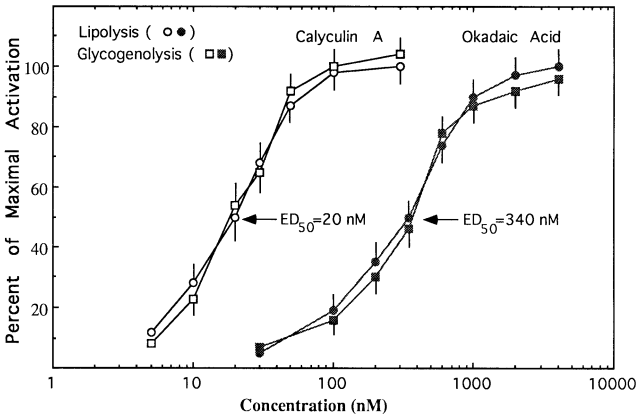

FIGURE 5: Dose-dependent activation of glycogenolysis and lipolysis with calyculin A and okadaic acid. Glycogenolysis (□ and ■) and lipolysis (○ and ●) were carried out for 1 h at 37 °C, in the absence and presence of the indicated concentrations of okadaic acid (■ and ●) and calyculin A (□ and ○).

$NaVO_3$ to obtain an insulin-free experimental system. Following the washing procedure and the 1 h incubation in fresh KRB buffer (experimental procedure), glycogenolysis was assayed with low isoproterenol concentrations in the presence or absence of 17 nM insulin. Insulin did not antagonize glycogenolysis evoked by 30−60 nM isoproterenol. This was valid also when insulin had been used instead of vanadate during the labeling of the adipose glycogen pool with glucose (not shown). On the other hand, insulin readily antagonized lipolysis induced by isoproterenol concentrations, producing 30−90% of the maximal lipolytic response (Figure 6B).

The ability of insulin to act as an antagonist is nullified at elevated levels of cyclicAMP (Shechter, 1984). Since glycogenolysis is much more sensitive than lipolysis to cyclicAMP (Figures 2 and 4), the impaired antiglycogenolytic effect of insulin may originate from a high cyclicAMP level, relative to that required for activating glycogenolysis. Cholera toxin differs from the lipolytic hormones in producing only a modest (almost undetectable) elevation in cyclicAMP levels in rat adipocytes. Therefore, we examined whether insulin is capable of antagonizing cholera toxin-evoked glycogenolysis. As shown in the figure, insulin produced a small (~10%) decrease in cholera toxin-evoked glycogenolysis. In contrast, the inhibitory effect of insulin on cholera toxin-evoked lipolysis was greater than 90% [Figure 6B and Shechter (1984)].

DISCUSSION

The intact adipose cell is a convenient *in vitro* model for studying the rapid (physiologically relevant) bioeffects of

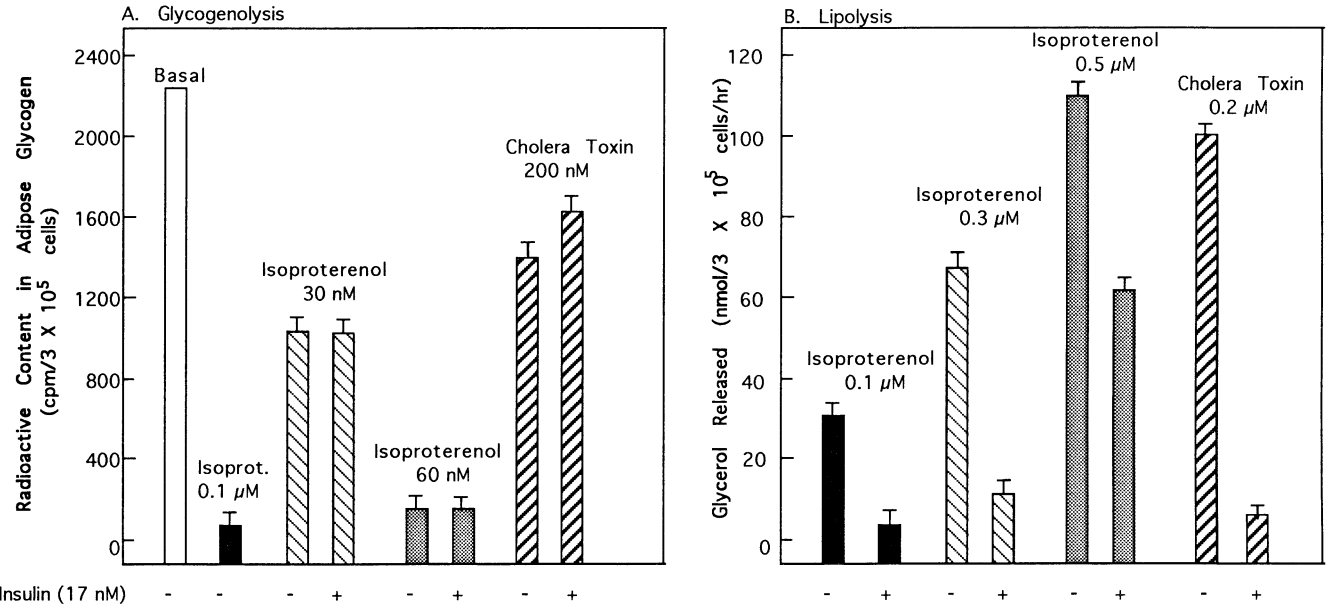

FIGURE 6: Lack of an insulin effect on antagonizing glycogenolysis in rat adipocytes and a comparison to lipolysis. (A) Incorporation of [U-^{14}C]glucose into glycogen was performed by incubating the cells with 1.0 mM [U-^{14}C]glucose and 0.1 mM $NaVO_3$ followed by washing and incubating the cells for an additional 1 h (prolonged procedure described in detail in Figure 1B). The assay of glycogenolysis was then carried out for 45 min at 37 °C, with the indicated concentrations of isoproterenol, in the presence and the absence of 17 nM insulin. In the case of cholera toxin, the latter was added to the cells at time 0 (together or prior to the addition of glucose and vanadate). (B) Lipolysis was carried out for 1 h at 37 °C with the indicated concentrations of isoproterenol and insulin or for 3 h at 37 °C when cholera toxin was the lipolytic agent.

insulin. In this study, we aimed at developing an assay of glycogenolysis in rat adipocytes that is distinctly separated from and independent of insulin's effect in activating glycogenesis. Incubation of adipocytes with [U-^{14}C]glucose in the presence of insulin (ED_{50} = 3 ng/mL) or vanadate (ED_{50} = 20 μM) led to a substantial radioactive labeling of the adipose glycogen pool, which was not reduced upon washing and prolonged incubation of the cells at 37 °C (Figure 1A,B) but was decreased upon adding isoproterenol or ACTH (Figure 2). Glycogenolysis resembled lipolysis in being activated by lipolytic hormones, and by nonhormonal agents which elevate cyclicAMP levels (Figures 2−4). Two fundamental differences however were subsequently found. (a) Glycogenolysis is activated at 7−11-fold lower concentrations of lipolytic hormones and of cyclicAMP, and (b) glycogenolysis is not antagonized by insulin (Figures 2, 4, and 6).

These findings indicated to us that, to promote its classical antilipolytic effect, the adipose cell utilizes a mechanism different from the well-characterized insulin-dependent activation of PP1 in muscle and liver cells. We now had to determine whether the activation of adipose PP1 is capable, in theory, of antagonizing lipolysis. As shown in Figure 5, inhibition of PP1 by calyculin A does activate lipolysis, as it does glycogenolysis, at equimolar potencies for both bioeffects. It is therefore conceivable that activation of adipose PP1 could in principle reduce the rate and extent of lipolysis. The presence of insulin-activatable PP1 in rat adipocytes has been recently demonstrated (Begum, 1995).

How then does insulin antagonize lipolysis? With the ruling out of the PP1 activation mechanism, this study supports a decrease in tissue levels of cyclicAMP as the central mechanism by which insulin antagonizes lipolysis. Insulin activates low-K_m cyclicAMP phosphodiesterase in rat adipocytes (Manganiello et al., 1991; Shibata et al., 1991; Degerman et al., 1990; Rahn et al., 1994), and several lines of evidence support a role for this activation in inhibiting lipolysis. These include the inability of insulin to antagonize lipolysis when cyclicAMP levels are substantially elevated or in the presence of the cyclicAMP−PDE inhibitor, IBMX (Shechter, 1984; Beebe et al., 1985; Honnora et al., 1985; Gabbay & Lardy, 1986), or when lipolysis is evoked by dibutyryl cyclicAMP, a cyclicAMP analog that is not hydrolyzed by cyclicAMP−PDE. Reports, not favoring this notion, included the inability of insulin to decrease total cellular cyclicAMP levels, or the decrease was slight and not sufficient to account for the degree of inhibition of lipolysis (Jarrett et al., 1972; Fain & Rosenberg, 1974; Kono & Barham, 1973; Siddle & Hales, 1974). However, it remains possible that because of the intracellular compartmentalization of cyclicAMP, the total cyclic nucleotide content is not an effective index with respect to the regulation of lipolysis or its inhibition by insulin. Adipocytes definitely have a well-defined insulin-sensitive low-K_m phosphodiesterase whose maximal velocity (V_{max}) is increased if insulin is applied to intact cells before cell homogenization (Manganiello et al., 1991; Shibata et al., 1991; Rahn et al., 1994). The inability of insulin to inhibit glycogenolysis (Figure 6) may simply be due to our finding of glycogenolysis being nearly 1 log unit more sensitive to cyclicAMP than lipolysis (Figures 2 and 4). This implies that the threshold concentration of cyclicAMP required to activate glycogenolysis is well below the K_m value of the insulin-sensitive low-K_m phosphodiesterase, and therefore, the latter is inefficient in reversing glycogenolysis.

Finally, it is worth mentioning that the establishment of this glycogenolytic assay in the rat adipose cell has enabled us for the first time to compare two distinct cyclicAMP-dependent events within the same cell. The finding of glycogenolysis being activated at substantially lower concentrations of lipolytic hormones provides an *in vitro* biochemical−physiological explanation for the preferential

mobilization of glucose, rather than of fatty acids *in vivo* upon slight increase in hormones which activate adenylate cyclase. From a pathophysiological standpoint, one of the parameters characterizing insulin resistance is an increased rate of lipolysis accompanied by a decrease in the ability of insulin to antagonize it (Reynisdottir et al., 1994; Hennes et al., 1996). Now, reinvestigating whether insulin resistance is accompanied by enhanced sensitivity of the adipose cell to lipolytic hormones is of interest. If lipolysis is manifested at lower cyclicAMP concentrations, it explains the known decrease in the ability of insulin to antagonize it (manuscript in preparation).

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