1985

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Original Publication Citation

Beebe, S.J., Redmon, J.B., Blackmore, P.F., & Corbin, J.D. (1985). Discriminative insulin antagonism of stimulatory effects of various camp analogs on adipocyte lipolysis and hepatocyte glycogenolysis. *Journal of Biological Chemistry, 260*(29), 15781-15788.

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 260, No. 29, Issue of December 15, pp. 15781-15788, 1985
Printed in U.S.A.

Discriminative Insulin Antagonism of Stimulatory Effects of Various cAMP Analogs on Adipocyte Lipolysis and Hepatocyte Glycogenolysis*

(Received for publication, June 5, 1985)

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Although insulin effectively blocked hormone-stimulated glycerol output in adipocytes or phosphorylase activation in hepatocytes, the inhibitory effect of insulin on cAMP analog-stimulated cells depended on the cAMP analog used. Of the 20 analogs tested in adipocytes and 13 tested in hepatocytes, the effects of about half of them were effectively blocked by insulin, whereas the effects of many of them were not inhibited at all. In order to approach the explanation for this discriminative insulin action, the inhibitory effects of insulin on the responses to the analogs in the intact cells were correlated with the *in vitro* cAMP analog specificity for the hepatocyte cAMP-dependent protein kinase isozymes and the low K_m, hormone-sensitive phosphodiesterases from both cell types. No correlation was found between insulin resistance of analog-stimulated hepatocyte phosphorylase and the concentration of analog required *in vitro* for half-maximal activation of either type I or type II cAMP-dependent protein kinase from hepatocytes. However, a good correlation was found between insulin resistance of cAMP analog-stimulated responses and the analog I_{50} values for the phosphodiesterase from both cell types. Using a new method capable of measuring hydrolysis at low analog concentrations, several of those analogs which had relatively low, but not high, phosphodiesterase I_{50} values were shown to be directly hydrolyzed by the low K_m adipocyte phosphodiesterase. The insulin inhibition of cell responses when stimulated by hydrolyzable analogs, but not by poorly hydrolyzable analogs, is best explained by insulin stimulation of the low K_m phosphodiesterases from both cell types.

Lipolysis in adipocytes and glycogenolysis in hepatocytes are increased by catabolic hormones which elevate cAMP and activate cAMP-dependent protein kinase. In the presence of the anabolic hormone insulin, these effects are partially or completely blocked, but the mechanism is not certain. It has been demonstrated in a number of laboratories that insulin decreases intracellular levels of cAMP in fat (1, 2) and in liver (3–7) tissues, particularly when they are elevated. Some evidence suggests that this is due to an inhibition of adenylate cyclase (8, 9), but this is not universally accepted (7, 10–12). Alternatively, it has been proposed that insulin stimulates a low K_m cAMP phosphodiesterase to account for the decrease in cAMP (13–17), although the physiological significance of this effect is questioned (18). Some investigators have been unable to detect a lowering of cAMP under certain conditions of insulin action (19–24). Other possible mechanisms include increases in phosphoprotein phosphatase(s) (25) and protein kinase inhibition (26, 27) by this hormone. The latter effect would result, in part, from an inhibition of protein kinase activity secondary to a decrease in cAMP due to phosphodiesterase activation. However, it has been suggested that insulin reduces the sensitivity of protein kinase to cAMP and thereby inhibits the enzyme without lowering cAMP (28). To account for hepatic insulin action, a recent study concluded that inhibition of cAMP-dependent protein kinase occurs independently of cAMP changes and phosphodiesterase activation (23). Several groups (25, 29–31) have partially purified low molecular weight peptide(s) which could mediate some or all of the effects of insulin. In order to account for the many and diverse actions of insulin, it is not unreasonable to consider that after insulin binds to the receptor, there are multiple pathways of action.

A study was recently conducted using cyclic nucleotide analogs to stimulate lipolysis in isolated rat adipocytes (32). The pattern of analog effects indicated that they were acting through cAMP-dependent protein kinase activation. This provided the groundwork for the present study which demonstrates that, although insulin blocks hormonal effects, it does not block the effects of all cAMP analogs used to stimulate adipocyte glycerol output and hepatocyte phosphorylase. In order to examine the reason for the discriminative insulin action, both cell types were thoroughly characterized for cAMP analog potency and specificity in the presence and absence of insulin, and the specificities of the same cAMP analogs were determined for the isolated low K_m phosphodiesterases and cAMP-dependent protein kinases. These experiments present a unique approach to the use of cAMP analogs to study the mechanism of insulin action on cyclic nucleotide-mediated processes. A preliminary report of these findings has previously appeared (33).

EXPERIMENTAL PROCEDURES

Adipocyte Experiments—Adipocytes were isolated by collagenase treatment of rat epididymal fat pads according to the method of Rodbell (34). All aspects of adipocyte experiments were as previously described (32), with some exceptions. Incubations were for 30 min at 37 °C. Cells were preincubated for 8–12 min in the presence or absence of 1 nM insulin before the addition of hormone or cyclic nucleotide analog. There was no effect of insulin on basal lipolytic rates. A maximal lipolytic response (10–12 μmol of glycerol/g of fat, dry weight/30 min) was determined using 10 μM epinephrine in the presence of 200 μM isobutylmethylxanthine. Glycerol release was determined after 30 min of incubation as a measure of lipolysis according to the method of Wieland (35), as previously described (32).

* This work was supported in part by the Research Grant AM 15988 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Hepatocyte Experiments—Hepatocytes were prepared by collagenase perfusion of rat livers, as described by Blackmore and Exton (36). Briefly, hepatocytes were isolated from the livers of 180–220-g rats. The livers were perfused *in situ* at 37 °C with Ca^{2+}-free Krebs-Henseleit bicarbonate buffer, pH 7.4, containing 11 mM glucose, 5 mM sodium pyruvate, 5 mM sodium L-glutamate, and washed human erythrocytes (10% hematocrit). After an initial noncirculating flow through the liver with the above buffer (50 ml), a 0.4 mg/ml collagenase solution (Worthington, type I) was recirculated through the liver for 30 min. The cells were shaken away from the digested liver, poured through nylon mesh, and centrifuged at 50 × *g* for 2 min. Cells were resuspended in the above buffer containing 1.5% gelatin and preincubated for 10 min in the presence or absence of 6 nM insulin before the addition of a series of concentrations of glucagon or cyclic nucleotide analog. After a 5-min incubation, a 500-μl aliquot of the cell suspension was removed, frozen in liquid nitrogen, and stored at −70 °C until assayed. Pellets were partially thawed, homogenized, and assayed for phosphorylase by measuring the incorporation of [^{14}C]Glu-1-P into glycogen in the absence of 5′-AMP, as previously described (36). In the absence of glucagon, there was little or no effect of insulin on the basal activity of phosphorylase. A maximal phosphorylase activity (a 3–4-fold activation) was elicited by 10 nM glucagon (28–32 μmol of Glu-1-P/g, wet weight/min).

Preparation and Assay of Adipocyte and Hepatocyte Particulate Phosphodiesterase—The low K_m phosphodiesterase from adipocytes and hepatocytes were prepared and assayed according to the modified method (31) of Loten *et al.* (16). These enzymes have been shown to be sensitive to insulin stimulation when the phosphodiesterases are partially purified from hormone-treated intact cells (13, 14, 16). Consistent with other reports (13, 14), the concentration of cAMP in this study required for half-maximal activity of the enzymes from both cell types was between 0.4–0.8 μM. I_{50} values were determined, as previously described (32), using a concentration of 0.1 μM [^{3}H]cAMP as substrate in the standard phosphodiesterase assay. Cyclic nucleotide analogs were added to the reaction mixture in a wide range of concentrations. The I_{50} value was taken as a measure of analog competition for [^{3}H]cAMP hydrolysis and was expressed as the concentration of analog which inhibit [^{3}H]cAMP hydrolysis by 50%.

Determination of Cyclic Nucleotide Analog Hydrolysis—cAMP analogs were incubated with adipocyte phosphodiesterase at concentrations equivalent to their I_{50} value. After various times of incubation (0–45 min), aliquots of the reaction mixture were boiled for 5 min and appropriately diluted. The concentration of cAMP analog, at zero time and at various times following incubation with the phosphodiesterase, was determined using a standard curve for each analog in a protein kinase assay. The adipocyte or bovine heart type II protein kinase was used as enzyme and synthetic heptapeptide as substrate, as previously described (32, 37). Duplication incubations of cAMP analog and phosphodiesterase were carried out on at least three different phosphodiesterase preparations, and the cAMP analog concentration was determined from at least two different dilutions of boiled incubation mixture.

Apparent K_a Determination for Cyclic Nucleotides—The type I and type II protein kinases from hepatocytes were completely separated by DEAE-cellulose column chromatography, as previously described (38). The adipocyte type II protein kinase was purified in a similar manner, as described in Ref. 37. The apparent K_a values for cAMP and cAMP analogs were determined in a standard protein kinase assay, using synthetic heptapeptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) as substrate, as described in Ref. 32. The apparent K_a is defined as the concentration of cAMP analog required for half-maximal activation of the enzyme. Experiments for the hepatocyte enzymes were carried out with a total protein kinase activity of 1200–1800 pmol of ^{32}P incorporated per min/ml of enzyme and a basal activity ratio (activity in the absence divided by activity in the presence of 5 μM cAMP) of 0.10–0.15 for type I and 0.02–0.08 for type II. Apparent K_a values for the adipocyte protein kinases were determined as described in Ref. 37. The apparent K_a value is expressed as K_a' which is defined as the ratio of the apparent K_a for cAMP divided by the apparent K_a for the cAMP analog.

Materials—[^{14}C]Glu-1-P was obtained from ICN. 8-Thioparachlorophenyl-cAMP was purchased from Boehringer Mannheim. N^6-Diethyl-, N^6-carbamoylpropyl-, 6-thiomethyl-, 8-thioparanitrobenzyl-, and 2-trifluoromethyl-cAMP were generously provided by Drs. Jon Miller and Robert Suva of Biochemical Research Laboratory, Life Sciences Division, SRI International, Menlo Park, CA. R0-7-2956 was a kind gift from Dr. Peter F. Sorter from Hoffmann-LaRoche. Glucagon and insulin were generous gifts from Lilly. Other materials were as described in Ref. 32.

RESULTS

The Effect of cAMP Analogs on Physiological Responses in Adipocytes and Hepatocytes—From the results of recently conducted studies using cAMP analogs to stimulate lipolysis in isolated rat adipocytes (32) and glycogenolysis in hepatocytes (39), it was concluded that analogs act by crossing the cell membrane to directly activate the cAMP-dependent protein kinase. In the present study, a large number of cAMP analogs were tested in both hepatocytes and adipocytes.

The most interesting feature concerning analog efficacy was the striking difference in sensitivity between adipocytes and hepatocytes (Table I). Adipocyte lipolysis was sensitive to cAMP analogs in the millimolar concentration range, whereas hepatocyte phosphorylase was activated in the micromolar concentration range. For example, the EC_{50} (concentration required for half-maximal activation) for 8-thioethyl-cAMP in adipocytes was about 1 mM, whereas the same extent of phosphorylase activation in hepatocytes required only 0.3 μM. Similarly, half-maximal activation of adipocyte lipolysis required approximately a 3,000-fold higher concentration of 8-bromo-cAMP and a 10,000-fold higher concentration of 8-thioparachlorophenyl-cAMP than was required for the same extent of hepatocyte phosphorylase activation. Generally, hepatocytes were 100–10,000 times more sensitive than adipocytes to cAMP analog stimulation when comparing these two physiological processes.

Another aspect of analog potency concerned a comparison of relative potencies within each cell type. The analogs followed a similar, but not identical, rank order of potency in adipocytes and hepatocytes. For analogs modified at the 8-carbon of the adenine ring, 8-thio derivatives were among the most potent agents and were considerably more potent than 8-amino derivatives in both cell types (Table I). For example, the EC_{50} for 8-thiomethyl-cAMP was 8- and 12-fold lower than that of 8-aminomethyl-cAMP in hepatocytes and adipocytes, respectively. In both cell types, 8-thioethyl- and N^6-benzoyl-cAMP were potent agents, N^6-butyryl- and 8-bromo-cAMP were of intermediate potency while 8-amino derivatives were least potent. However, some analogs varied in relative potency between the cell types. 8-Thioparachlorophenyl- and 8-thioparanitrobenzyl-cAMP were the most potent in hepatocytes and of intermediate potency in adipocytes. Conversely, 8-thioisopropyl-cAMP and N^6-carbamoylpropyl-cAMP were

TABLE I

Potency of cAMP analogs in isolated cells

Cyclic nucleotide analog	EC_{50} adipocyte lipolysis	EC_{50} hepatocyte phosphorylase
	μM	μM
N^6-Carbamoylpropyl-cAMP	500	3.0
8-Thioisopropyl-cAMP	500	2.0
N^6-Benzoyl-cAMP	700	0.5
8-Thioethyl-cAMP	900	0.3
8-Thioparachlorophenyl-cAMP	1,000	0.1
8-Thiomethyl-cAMP	1,000	0.5
8-Thioparanitrobenzyl-cAMP	1,200	0.1
N^6-Butyryl-cAMP	1,900	4.8
8-Bromo-cAMP	3,900	1.3
8-Amino-cAMP	6,100	
8-Aminomethyl-cAMP	7,900	6.0
8-Aminohexylamine-cAMP	8,600	60.0
8-Aminobenzyl-cAMP	>15,000	
cIMP	>15,000	30.0

most potent in adipocytes, but of intermediate potency in hepatocytes.

Correlation of Analog I_{50} Values for Phosphodiesterase with Actual Analog Hydrolysis—The phosphodiesterase I_{50} value of cAMP analogs was assumed to represent competitive inhibition of [^{3}H]cAMP hydrolysis. However, it was possible that some analogs were either acting as noncompetitive inhibitors or binding to the hydrolytic site without being hydrolyzed. It was therefore important to determine if the analogs were in fact being hydrolyzed. Since few of the analogs commonly in use are radioactively labeled, an alternative method was used to determine direct analog hydrolysis. The concentration of several analogs was measured before and after incubation with the low K_m phosphodiesterase isolated from rat fat pads. Analog concentration was determined using a standard curve for protein kinase activation with each analog tested. As a control, hydrolysis of unlabeled cAMP in this procedure was compared with hydrolysis of [^{3}H]cAMP in the standard phosphodiesterase assay. The rates of hydrolysis using the two procedures were in good agreement. Fig. 1 shows a typical time course of hydrolysis using two cAMP analogs tested by this technique. 8-Bromo-cAMP and 8-thioparachlorophenyl-cAMP were incubated with the adipose tissue phosphodiesterase at 17 and 22 μM, respectively. These are near the respective analog I_{50} concentrations (Table II). Under these conditions, about 40% of 8-bromo-cAMP and about 25% of 8-thioparachlorophenyl-cAMP were hydrolyzed in 30 min. Several other cAMP analogs were also tested at concentrations at or near their I_{50} values after a 30-min incubation with the phosphodiesterase (data not shown). 8-Amino-, 8-aminomethyl-, and 8-thiomethyl-cAMP were also significantly hydrolyzed (20–40%) under these conditions. However, hydrolysis of N^6-benzoyl- and N^6-monobutyryl-cAMP was not readily detected.

Under the conditions used in these studies, the rates of hydrolysis of these analogs could not be compared to one another or to cAMP since they were tested at different concentrations. These experiments nevertheless demonstrated that some analogs do serve as substrates for the phosphodiesterase, albeit at higher concentrations than are required for cAMP hydrolysis. For all analogs tested, hydrolyzable analogs (Footnote b in Table II) have relatively low I_{50} values, and nonhydrolyzable analogs (Footnote c in Table II) have relatively high I_{50} values. The analog I_{50} values for the phosphodiesterases from both cell types were in good agreement (Table II and Fig. 4). Only the I_{50} values for N^6-benzoyl- and N^6-carbamoylpropyl-cAMP differed slightly for the phosphodiesterases from the two cell types.

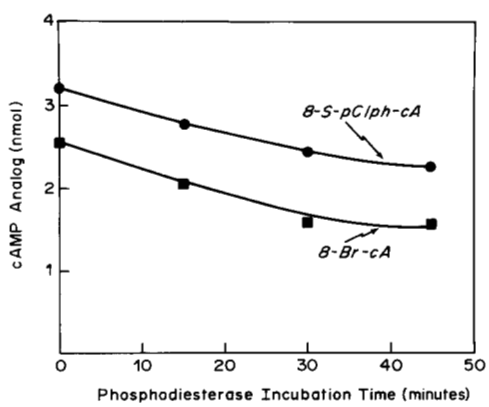

FIG. 1. **Time course for the direct hydrolysis of cAMP analogs by the low K_m phosphodiesterase from rat adipose tissue.** Analog hydrolysis was determined as described under "Experimental Procedures." Each *point* represents the mean of duplicate incubations in a typical experiment. *8-S-pClph-cA*, 8-thioparachlorophenyl-cAMP; *8-Br-cA*, 8-bromo-cAMP.

The Effect of Insulin on Hormone- and cAMP Analog-stimulated Responses in Adipocytes and Hepatocytes—Before the effects of insulin on analog-stimulated cell responses were investigated, control experiments were carried out with several concentrations of epinephrine in adipocytes or glucagon in hepatocytes in the presence and absence of a series of insulin concentrations (data not shown). Insulin (0.1–10 nM) inhibited epinephrine-stimulated lipolysis 90–100% when the lipolytic effect was less than 50% of a maximal response. When lipolysis was stimulated 70–80% of the maximum (1 μM epinephrine), insulin (0.1–1 nM) inhibited glycerol release by only 70–80%. At this higher level of stimulation, insulin at 10 nM was less effective as a lipolytic antagonist (50–60% inhibition). This biphasic insulin inhibition of epinephrine-stimulated lipolysis is in agreement with previously published data (2). Since a maximal insulin inhibition at all levels of epinephrine stimulation was observed by 1 nM insulin, this concentration was chosen as a potential antagonist to analog-stimulated lipolysis.

Similar experiments were carried out in the presence or absence of insulin with glucagon-stimulated hepatocytes. When phosphorylase activity was stimulated to about 70% of the maximum (0.1–1.0 nM glucagon), insulin (0.01–10 nM) caused a concentration-dependent decrease in phosphorylase activity. A maximal inhibition of 70–80% was seen with approximately 6 nM insulin. This concentration of insulin was used in experiments where the effects of insulin on analog-stimulated hepatocytes were investigated.

It was previously shown that insulin blocks exogenous cAMP-stimulated lipolysis or glucose production in adipocytes (2) and hepatocytes (23), respectively. In this study, insulin was tested as a potential antagonist using a large number of cAMP analogs as agonists. Hormone and cAMP analog concentration curves were generated in the presence and absence of insulin for hepatocyte glycogenolysis (Fig. 2) and adipocyte lipolysis (Fig. 3). Five representative analog concentration curves for each cell type are shown. In both cell types, responses stimulated by some analogs, but not others, were sensitive to insulin inhibition. Responses to analogs such as 8-thioethyl-, 8-amino-, and 8-bromo-cAMP in adipocytes and 8-thioethyl-, 8-thioisopropyl-cAMP, and cIMP in hepatocytes were sensitive to inhibition by insulin. In both cell types, responses to N^6-benzoyl- and N^6-monobutyryl-cAMP were resistant to insulin inhibition. The data in Table II for insulin inhibition was compiled from concentration curves like those in Figs. 2 and 3 using 20 cAMP analogs in adipocytes and 13 analogs in hepatocytes. The per cent insulin inhibition was determined from the region of the analog concentration curves where insulin causes the greatest inhibition. In adipocytes, insulin was most effective at inhibiting lipolysis when the analog-stimulated glycerol release was 30–60% of the maximal response. In hepatocytes, insulin was most effective when phosphorylase was stimulated 20–40% of the maximum. It can be seen that, in general, analog-stimulated responses, which were sensitive or resistant to insulin inhibition in adipocytes, were the same in hepatocytes. There were two primary exceptions to this generalization. The response to 8-thioisopropyl-cAMP was blocked by insulin in adipocytes, but not in hepatocytes. The effect of N^6-carbamoylpropyl-cAMP was not inhibited by insulin in adipocytes, but was inhibited in hepatocytes.

It should be pointed out that the effect of insulin on analog-stimulated responses should not be due to differential analog penetration of the cell membranes since all insulin effects are

TABLE II

Comparison of insulin effects with cAMP analog I_{50} values for phosphodiesterases

Cyclic nucleotides	Adipocyte		Hepatocyte	
	Insulin inhibition (lipolysis)	PDE[a] I_{50}	Insulin inhibition (phosphorylase *a*)	PDE[a] I_{50}
	%	μM	%	μM
cAMP[b]	70	0.7	60	1
8-Thioethyl-cAMP	100	79	50	51
8-Aminomethyl-cAMP[b]	100	33	50	83
N^6-Diethyl-cAMP	90	84		
8-Bromo-cAMP[b]	90	14	70	45
8-Thioparachlorophenyl-cAMP[b]	80	27	30	36
6-Thiomethyl-cAMP	80			
8-Thiomethyl-cAMP	75	46	30	80
8-Amino	70	40		
8-Thioisopropyl-cAMP	60	280	0	370
8-Thiobenzyl-cAMP	35	48		
8-Thioparanitrobenzyl-cAMP	15	25	20	28
N^6-Aminohexylcarbamoylmethyl-cAMP	15	>1,000		
N^6,$O^{2'}$-Dibutyryl	0	111		
N^6-Carbamoylpropyl-cAMP	0	492	50	140
N^6-Benzoyl-cAMP[c]	0	90	0	355
N^6-Butyryl-cAMP[c]	0	597	0	980
8-Hydroxy-cAMP	0			
8-Aminohexylamino-cAMP	0	>1,000	0	>1,000
8-Aminobenzyl-cAMP	0	202		
cIMP		0.9	60	
Other agonists				
Epinephrine	90			
Glucagon			70	
R0-7-2956	90	47		
Isobutylmethylxanthine	90	3		

[a] PDE, phosphodiesterase.
[b] Cyclic nucleotides hydrolyzed by the adipocyte phosphodiesterase when tested at their I_{50} concentration.
[c] Cyclic nucleotides *not* hydrolyzed by the adipocyte phosphodiesterase when tested at their I_{50} concentration.

compared at equipotent concentrations of analogs. Therefore, one would not expect a correlation between the effects of insulin on analog-stimulated responses and the butanol/water partition coefficient of the analog (32), and none was apparent (data not shown).

There are several points of interest to the differential insulin inhibition of analog-stimulated responses in these cell types (Table II and Fig. 4). The responses generated by analogs which have modifications at C-8 of the adenine ring were generally inhibited by insulin if the derivative was not bulky and/or hydrophobic. For instance, lipolytic responses to 8-amino- and 8-aminomethyl-cAMP were inhibited 70–90%, but responses to analogs with bulky substituents, such as 8-aminohexylamino- and 8-aminobenzyl-cAMP, were not inhibited significantly by insulin. Likewise, lipolytic responses generated by 8-thioethyl- and 8-thiomethyl-cAMP were blocked by insulin (75–90%). However, lipolytic responses stimulated by 8-thioisopropyl-, 8-thiobenzyl-, and 8-thioparanitrobenzyl-cAMP were inhibited by insulin by 60, 35, and 15%, respectively. These same generalizations were true when phosphorylase activation was analyzed. At least two exceptions to the above generalization were recognized. The lipolytic response of 8-thioparachlorophenyl-cAMP, containing a relatively bulky group, was inhibited by insulin while 8-hydroxy-cAMP, containing a relatively non-bulky group, was not. Although fewer N^6 analogs were studied in the intact cells, responses to these analogs were generally not inhibited by insulin. The exceptions to this are the adipocyte response to N^6-dimethyl- and 6-thiomethyl-cAMP and the hepatocyte response to N^6-carbamoylpropyl-cAMP.

Further studies were designed to find an explanation for the discriminative insulin inhibition of responses to cAMP analogs. It was reasoned that if insulin action on lipolysis and glycogenolysis was due to stimulation of phosphodiesterase activity, insulin would only inhibit those responses generated by analogs which were hydrolyzed by phosphodiesterase. Therefore, the effects of insulin on analog-stimulated responses were correlated with the analog I_{50} value for the low K_m phosphodiesterases from adipocytes and hepatocytes (Fig. 4). The *top* and *bottom panels* of Fig. 4 represent hepatocyte and adipocyte experiments, respectively. The height of each bar represents the analog I_{50} value for the phosphodiesterase isolated from the each cell type. The analog I_{50} is defined as the concentration of analog required to cause a half-maximal inhibition of cAMP hydrolysis by the respective phosphodiesterases using 0.1 μM [^{3}H]cAMP as substrate. Analogs were arbitrarily divided into two general categories. Insulin-sensitive analog responses (Fig. 4, *striped bars*) were inhibited by insulin by ≥60% in adipocytes and ≥30% in hepatocytes. Insulin-resistant analog responses were inhibited little, if any, by insulin. For example, insulin inhibited analog-stimulated responses in hepatocytes and adipocytes to 8-bromo- and 8-thioparachlorophenyl-cAMP, both of which had relative low phosphodiesterase I_{50} values (Fig. 4 and Table II) and were susceptible to phosphodiesterase hydrolysis (Fig. 1). Insulin did not inhibit analog-stimulated responses in either cell type to N^6-benzoyl- and N^6-monobutyryl-cAMP, both of which had relatively high I_{50} values (Fig. 4 and Table II), and were not apparently hydrolyzable. 8-Thiobenzyl-, 8-thioparanitrobenzyl-, and N^6-carbamoylmethyl-cAMP were not included since these analog responses were only weakly inhibited by insulin (Table II). There was clearly a strong correlation between the I_{50} value for an analog and its responsiveness to insulin inhibition in both cell types. Although the correlation

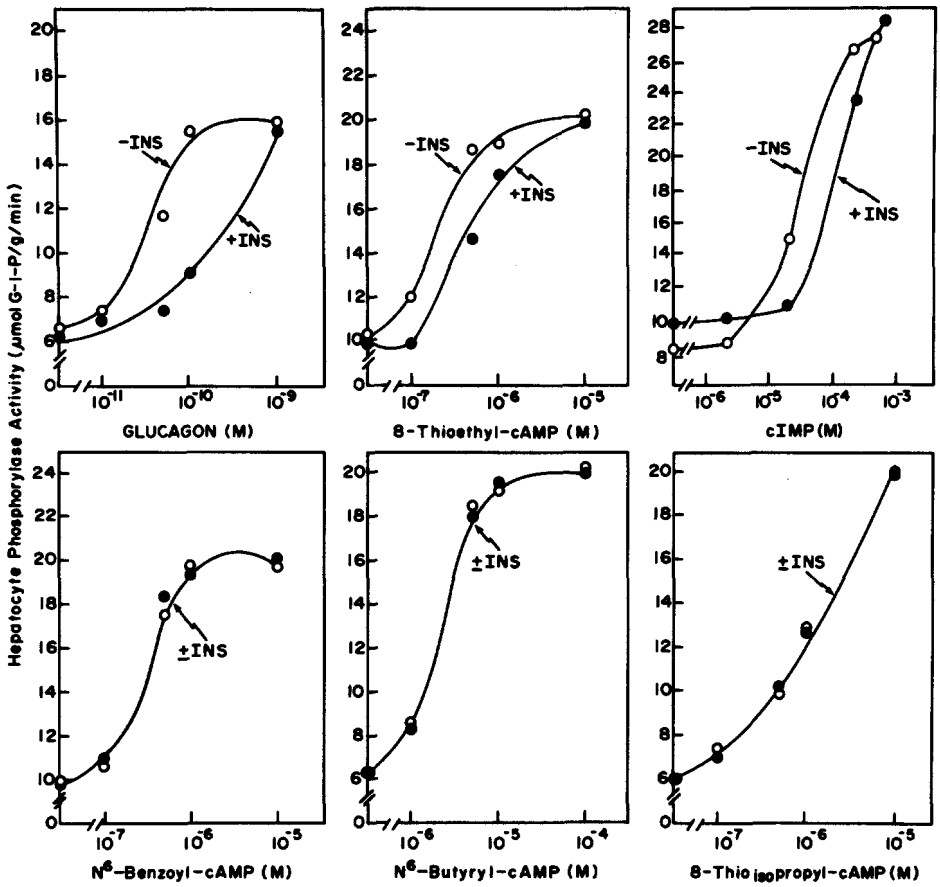

FIG. 2. **The effects of insulin on hormone- and cAMP analog-stimulated phosphorylase activation in isolated hepatocytes.** Hepatocytes were prepared as described under "Experimental Procedures." Cells were preincubated in the absence (○) or presence (●) of 6 nM insulin for 10 min. Hormore or cAMP analogs were added at various concentrations, and cells were incubated for an additional 5 min. Aliquots of 500 μl were removed and immediately frozen in liquid nitrogen and stored at −70 °C until assayed. Pellets were partially thawed, homogenized, and phosphorylase activity was determined in the absence of 5′-AMP by the incorporation of glucose 1-phosphate into glycogen as described under "Experimental Procedures" and as previously described (36). Dose-response curves in the presence or absence of insulin were determined for each cAMP analog on three or more different hepatocyte preparations. Typical experiments are represented. *INS*, insulin; *G-1-P*, glucose 1-phosphate.

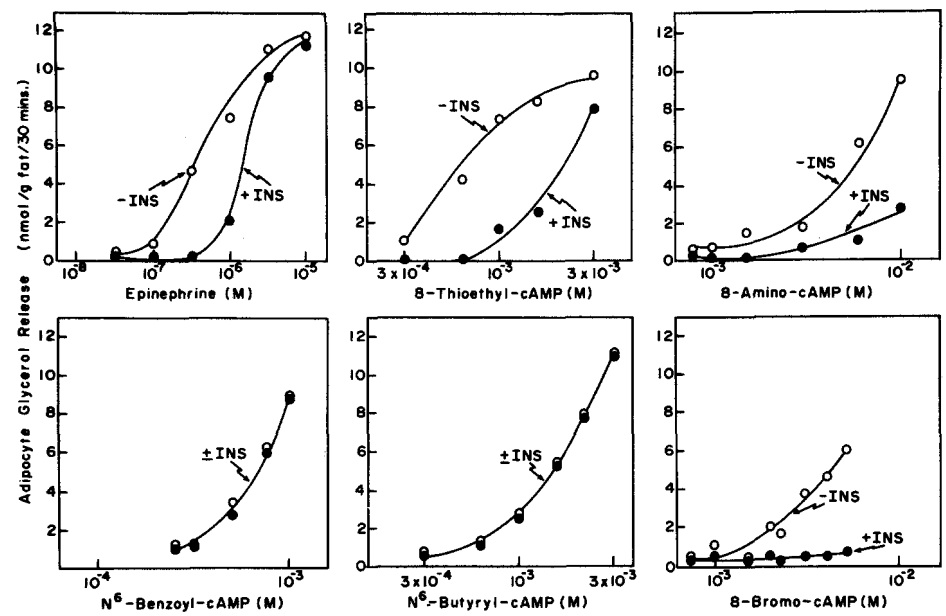

FIG. 3. **The effects of insulin on hormone- and cAMP analog-stimulated lipolysis in isolated adipocytes.** Adipocytes were isolated and incubated in the absence (○) and presence (●) of insulin (1 nM) prior to the addition of hormone or cAMP analog and assayed for glycerol release as a measure of lipolysis as described under "Experimental Procedures." Dose-response curves in the presence and absence of insulin were determined for each cAMP analog on three or more different adipocyte preparations. Typical experiments are represented. *INS*, insulin.

was unmistakable, it was not perfect. 8-Thioisopropyl-cAMP had a similar I_{50} value for the phosphodiesterase from both cell types, but its response was inhibited by insulin in adipocytes and not in hepatocytes. In hepatocytes, N^6-carbamoylpropyl-cAMP had a relatively low I_{50} value and its response was inhibited by insulin, while in adipocytes this analog had a higher I_{50} value and its response was not inhibited by insulin.

Although in both cell types there was a strong correlation between the effects of insulin on cAMP analog-stimulated responses in both cell types and the phosphodiesterase I_{50} values, this did not completely rule out the possibility that insulin acted on the protein kinase. This appeared to be a possibility in adipocytes because there was a vague correlation between the sensitivity of cAMP analog effects to insulin antagonism and the apparent affinity of analogs on adipocyte type II protein kinase (37). Further experiments were therefore carried out to correlate the hepatocyte responsiveness to insulin and the cAMP analog specificity for hepatocyte type I and type II protein kinases. For this evaluation, the apparent K_a values for cAMP analog activation of protein kinase iso-

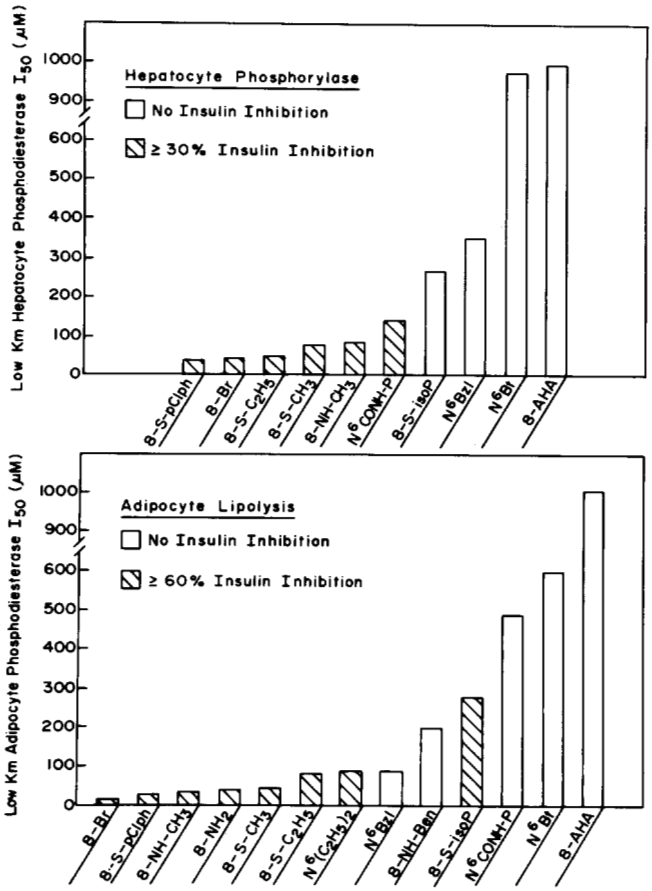

FIG. 4. **Correlation of hepatocyte and adipocyte low K_m phosphodiesterase I_{50} values with the effect of insulin on analog-stimulated cell responses.** The effect of insulin on hepatocyte phosphorylase activation (*top panel*) and adipocyte lipolysis (*bottom panel*) was determined for each cAMP analog as illustrated in Figs. 2 and 3. Cell responses to cAMP analogs which were not inhibited by insulin are shown by *open bars*, and responses which were inhibited by insulin (≥30% in hepatocytes and ≥60% in adipocytes) are shown by *striped bars*. The extent of insulin inhibition was determined at agonist concentrations eliciting 30–50% of a maximal response. The height of each bar indicates the I_{50} value for each cAMP analog using the low K_m phosphodiesterase from each cell type as described under "Experimental Procedures." *8-S-pClph*, 8-thioparachlorophenyl-cAMP; *8-Br*, 8-bromo-cAMP; *8-S-C_2H_5*, 8-thioethyl-cAMP; *8-S-CH_3*, 8-thiomethyl-cAMP; *8-NH-CH_3*, 8-aminomethyl-cAMP; *8-NH_2*, 8-amino-cAMP; *N^6CONH-P*, N^6-carbamoylpropyl-cAMP; *$N^6(C_2H_5)_2$*, N^6-diethylamino-cAMP; *8-S-isoP*, 8-thioisopropyl-cAMP; *N^6Bzl*, N^6-benzoyl-cAMP; *8-NH-Ben*, 8-aminobenzyl-cAMP; *N^6-Bt*, N^6-monobutyryl-cAMP; *8-AHA*, 8-aminohexylamino-cAMP.

zymes (concentration of cyclic nucleotide required for half-maximal activation) are expressed as a relative value or K'_a [K_a (cAMP)/K_a (cyclic nucleotide)]. Consequently, values greater than or less than one indicate that the test compound is superior or inferior, respectively, to cAMP. Hepatocytes were chosen for these studies because they were much more sensitive than adipocytes to cAMP analog stimulation, and therefore more cAMP analogs which were poor activators of protein kinases could be tested. This was particularly true of cIMP which was a poor lipolytic agent, for reasons previously described (32). This analog was an effective glycogenolytic agent, albeit at high concentrations (Fig. 2). Furthermore, hepatocytes were chosen because they contain both type I and type II protein kinases. It is readily apparent in Fig. 5 that no correlation existed between the K'_a of either type I or type II protein kinases and the effect of insulin on hepatocyte phosphorylase. For example, insulin blocked the responses to cIMP (low K'_a for both isozymes), 8-bromo-cAMP (intermediate K'_a for both isozymes), and 8-thioparachlorophenyl-cAMP (high K'_a for both isozymes). Conversely, responses to analogs which had either low or intermediate K'_a values were not blocked by insulin.

DISCUSSION

Cyclic nucleotide analogs act on intact cells by crossing the cell membrane and activating the cAMP-dependent protein kinases. The predominant, if not the only, effect of all tested cAMP analogs is directly on the cAMP-dependent protein kinase (32). This is supported by studies using cAMP analogs which selectively bind to one or the other of two intrasubunit cyclic nucleotide-binding sites on the regulatory subunit (40). Since these sites interact with positive cooperativity (40), addition of a combination of two analogs, one selective for one site and the second for the other site, causes a synergistic activation of the protein kinase (32, 40). In all respects, the behavior of site-selective cAMP analog combinations on the cooperativity of binding and activation of purified protein kinase is equivalent to the behavior of the same analog combinations on protein kinase-mediated metabolic responses in intact cells (32, 39).

Since the low K_m phosphodiesterase from these two cells have been shown to be stimulated by insulin, this enzyme is a logical candidate for the observed discriminative insulin action. For the vast majority of the analogs tested in both cell types, insulin blocks the responses to analogs with relatively low I_{50} values for phosphodiesterase but does not block the cell responses to analogs with relatively high I_{50} values. The simplest interpretation of these results is that insulin blocks the cell responses only if the analogs are relatively hydrolyzable. To test the assumption that a low I_{50} value is correlated with analog hydrolyzability, a method has been developed using the cAMP-dependent protein kinase to directly measure disappearance of the analog following hydrolysis. The disadvantage of this method is that it measures substrate disappearance rather than product formation. However, this method has at least two advantages over previous methods which measured analog hydrolysis by phosphate release or paper chromatography (41). First, very low levels of cAMP analog can be used (≥5 nM) compared to much higher concentrations (~1 mM) required by the previous methods. Second, because of the sensitivity of protein kinase, very small changes in analog concentration can be detected. Five analogs which have been tested for direct phosphodiesterase hydrolysis have relatively low I_{50} values and are shown to be hydrolyzed. The responses to these same analogs are inhibited by insulin. Analogs such as N^6-benzoyl- and N^6-monobutyryl-cAMP have higher I_{50} values and are not hydrolyzed. The responses to these analogs are not inhibited by insulin. Insulin inhibition of responses to only those analogs with low I_{50} values (hydrolyzable analogs) suggests that the activation of the phosphodiesterase is an important mechanism of insulin action. The differences in insulin inhibition do not appear to be due to analog effects on protein kinase since no correlation is found between the K'_a for the hepatocyte protein kinase isozymes and the effects of insulin on hepatocyte phosphorylase.

Although low I_{50} values are well correlated with analog hydrolysis for the analogs tested, this relationship will not necessarily hold for all analogs with low I_{50} values. For instance, if an analog readily binds to the hydrolytic site of the phosphodiesterase but is not hydrolyzed, a low I_{50} value could

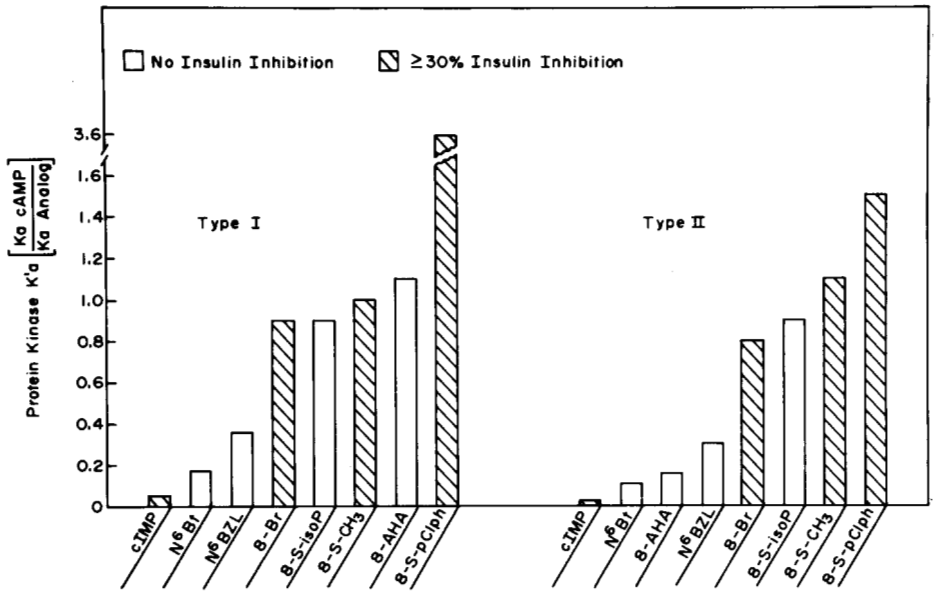

FIG. 5. **Correlation of hepatocyte type I and type II protein kinase K'_a values with the effect of insulin on analog-stimulated phosphorylase activation.** The effect of insulin on hepatocyte phosphorylase activation was determined as described in Fig. 2. The height of each bar indicates the K'_a value for each cAMP analog using partially purified hepatocyte type I and type II protein kinase as described under "Experimental Procedures." The K'_a is defined as the apparent K_a of cAMP (type I, 130 nM; type II, 90 nM) divided by the apparent K_a for cAMP analog. Abbreviations are indicated in Fig. 4.

be obtained. Analogs such as 8-thioparanitrobenzyl- and 8-thiobenzyl-cAMP, whose responses in adipocytes are minimally inhibited by insulin, may fit this classification. I_{50} values have been determined for N^6-benozyl- and N^6-monobutyryl-cAMP, yet hydrolysis is not detected when tested at or near their I_{50} values. This suggests that they inhibit [^{3}H]cAMP hydrolysis by a noncompetitive mechanism, or that they bind to the hydrolytic site, block [^{3}H]cAMP hydrolysis, but are not hydrolyzed themselves. Although direct hydrolysis studies were conducted using only the adipocyte phosphodiesterase, a comparison of phosphodiesterase I_{50} values from both cell types suggests that the enzymes are similar, but probably not identical.

Consideration was given to several possible artifacts which could be responsible for these results. The discriminative action of insulin does not appear to be due to an elevation of endogenous cAMP resulting from inhibition of phosphodiesterase by some analogs but not others. A recent study had demonstrated that cAMP analogs actually decrease endogenous cAMP levels (42). It could possibly be argued that cAMP analogs cause Ca^{2+} mobilization and alter hepatocyte phosphorylase activity by stimulating phosphorylase kinase. It has been shown that high concentrations of glucagon (10^{-8} M), which fully activate protein kinase and phosphorylase, raise intracellular Ca^{2+}, but possibly via a protein kinase mechanism (43). These conditions are in contrast to the conditions used in the present studies where optimal insulin inhibition is observed when phosphorylase is activated by only 20–40% of the maximal response. Nonspecific alterations of intracellular cAMP analogs by mechanisms other than phosphodiesterase hydrolysis have been studied for only some analogs (44). However, that such a mechanism could be responsible for these results seems unlikely due to the diversity of cAMP analogs within the two groups whose responses are blocked, or not blocked, by insulin.

Recently, studies of the effects of insulin on hepatocyte glucose production stimulated by 8-bromo-cAMP (23) and adipocyte lipolysis stimulated by 8-thiomethyl- and 8-thioisopropyl-cAMP have been reported (24). From these experiments and others, and in contrast to the conclusions of the present paper, the authors concluded that insulin does *not* act through phosphodiesterase activation. The results in the present paper, using these same three cAMP analogs in the same cell types, are in agreement, *i.e.* insulin blocks these cAMP analog-stimulated responses. However, the interpretations of these results are completely different. Part of this is due to the use of a large number of cAMP analogs in both cell types in the present paper, and indeed some analog responses are not inhibited by insulin. The prevailing view in the literature that many cAMP analogs are poorly hydrolyzed is correct. Compared to cAMP, most analogs are relatively poor substrates for the phosphodiesterase. However, in the present study, several cAMP analogs with low I_{50} values are shown to be hydrolyzed using a sensitive, direct method.

Several studies have shown that cell responses to isobutylmethylxanthine and other phosphodiesterase inhibitors are inhibited by insulin (2, 23, 24, and this study). It has been argued that retention of an inhibitory effect of insulin in the presence of phosphodiesterase inhibition suggests that insulin does not act through phosphodiesterase (23, 24). While this seems logical, the differential action of insulin in cAMP analog responses suggests that this conclusion requires reconsideration. Although isobutylmethylxanthine has a low K_i for phosphodiesterase inhibition (16), the inhibition is likely not complete, suggesting that only a small amount of phosphodiesterase activity is required for the insulin effect. This is consistent with the observation that some analogs require concentrations 20–100 times greater than cAMP for hydrolysis, yet insulin still blocks the response to these analogs. It is possible that insulin as well as agents which elevate endogenous cAMP stimulate a common (16, 45) or a different (46) phosphodiesterase. A recent study has suggested that the elevation of cAMP and the activation of the protein kinase result in a feedback inhibition to "dampen" the rise in cAMP (42). This system may act in concert with a possible insulin stimulation of phosphodiesterase. Although this has not been proved, it is consistent with results that show endogenous cAMP must be elevated before insulin can lower the cyclic nucleotide.

In the course of these studies, a number of observations have been made on the cAMP analog potency in intact cells. All analogs used in the present study can fully activate protein kinase *in vitro*. Cyclic nucleotide analogs can be good *in vivo* protein kinase agonists if they reach effective concentrations in the cell. The principles of analog efficacy appear to be based on fundamental pharmacological principles of drug

action which have been shown to be applicable to analog action on adipocyte lipolysis (32). Therefore, analog efficacy depends not only on the concentration of the analog required to activate the protein kinase (apparent K_a, *e.g.* agonist potency), but also on the partitioning characteristics of the analog (partition coefficient, *e.g.* an indication of lipid/water solubility) and the susceptibility of the analog to hydrolysis by phosphodiesterases (I_{50} and analog hydrolysis, *e.g.* route of elimination). All of these characteristics have usually not been dealt with in previous reports of cAMP analog effects in intact tissues. Comparison of the efficacy of cAMP analogs in adipocytes and hepatocytes in this paper also suggests that the porosity of the cell membrane and the lipid content of the cell may also be important.

The hepatocyte response is 100–1000 times more sensitive to cAMP analog stimulation than the adipocyte response. Since there are only minor differences in analog specificity of the protein kinases (this study, 37) and low K_m phosphodiesterase between these cell types, other factors are probably responsible for these differences. Perhaps a different level of phosphodiesterase activity, rather than a difference in specificity of this enzyme, could account for the differences. A second possibility may be a relative "leakiness" of hepatocyte membranes. Another factor could be the high lipid to cytoplasm ratio of adipocytes compared to hepatocytes. Analogs with high partition coefficients may not readily reach effective concentrations in the cytoplasm because they become trapped in the lipid of the adipocytes. Mooney *et al.* (47) have suggested that compartmentalization of enzymes involved in the lipolytic response may provide a second permeability barrier to the analog. Although the reasons for these potency differences are not known, one or more of these factors are probably involved. In any case, other cell types will presumably respond differently, and it may be possible to use certain cAMP analogs in the whole organism to selectively stimulate some cell types and not others.

Acknowledgments—Special thanks to Dr. Sharron Francis and Dr. Ronald Uhing for helpful discussions during these studies and the preparation of this manuscript. We thank Alfreda Beasley, Leslie Landiss, and Laura Waynick for excellent technical assistance and Penny Stelling for typing the manuscript.

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