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## Two Classes of cAMP Analogs Which Are Selective for the Two Different cAMP-binding Sites of Type II Protein Kinase Demonstrate Synergism When Added Together to Intact Adipocytes\*

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Twenty-five cyclic nucleotide analogs were tested individually to act as lipolytic agents and to activate adipocyte protein kinase. The lipolytic potency of individual analogs correlated better with their  $K_a$  for protein kinase and their lipophilicity rather than with either parameter alone. Some of the most potent lipolytic analogs had high  $I_{50}$  values for the particulate low  $K_m$  cAMP phosphodiesterase suggesting that their effect was not due to raising endogenous cAMP levels through inhibition of phosphodiesterase. The most potent lipolytic analogs contained a thio moiety at the C-8 or C-6 position. These analogs exhibited concave upward dose-response curves. At high concentrations, some analogs were as effective as optimal concentrations of epinephrine in stimulating glycerol release.

The regulatory subunit of protein kinase has two different intrachain cAMP-binding sites and cAMP analogs modified at the C-8 position (C-8 analogs) are generally selective for Site 1 and analogs modified at the C-6 position (C-6 analogs) are generally selective for Site 2 (Rannels, S. R., and Corbin, J. D. (1980) *J. Biol. Chem.* 255, 7085-7088). Thus, C-8 and C-6 analogs were tested in combination to stimulate lipolysis in intact adipocytes and to activate protein kinase *in vitro*. Each process was stimulated synergistically by a combination of a C-6 and C-8 analog. Two C-8 analogs or two C-6 analogs added together did not cause synergism of either process. For both lipolysis and protein kinase activation, C-8 thio analogs acted more synergistically than C-8 amino analogs when incubated in combination with C-6 analogs, a characteristic of type II protein kinase. It is concluded that the observed synergism of lipolysis is due to binding of cAMP analogs to both intrachain sites and that it is the type II protein kinase isozyme which is responsible for the lipolytic response.

The cAMP-dependent protein kinase mediates most, if not all, of the effects of cAMP in mammalian tissues (1, 2). Of present interest is the regulation of lipolysis in adipocytes via the hormone-sensitive lipase. The only established mechanism of regulation of the lipase is cAMP-dependent phosphorylation-activation (3-5) and  $Mg^{2+}$ -requiring dephosphoryla-

tion-inactivation (6). The cAMP-dependent protein kinase is usually present in tissues as a mixture of type I and type II isozymes (7, 8). By several criteria, the rat adipose tissue protein kinase appears to be primarily a type II isozyme but it may not be identical with the type II kinase from rat cardiac tissue (9) and it has not been determined which isozyme is ultimately responsible for the lipolytic response.

It has been established that both type I and type II isozymes are composed of two monomeric catalytic subunits and an inhibitory, dimeric regulatory subunit containing two cAMP-binding sites per monomeric chain (10). These intrachain cAMP-binding sites can be differentiated based on their cAMP dissociation rates (11, 12) and cAMP analog specificity (12). Site 1 is characterized by a slower dissociation rate and has a relative selectivity for cAMP analogs modified at the C-8 position on the adenine ring (C-8 analogs) while Site 2 has a faster dissociation rate and is more selective for analogs modified at the C-6 position (C-6 analogs). [ $^3H$ ]cIMP (Site 2 selective) and 8-azido- [ $^{32}P$ ]cAMP (Site 1 selective) binding experiments established that binding of cyclic nucleotides at either site stimulates binding at the other site for both type I and type II protein kinase isozymes (13, 14). Further, it was demonstrated for both isozymes that a synergism of protein kinase activation occurs using a combination of Site 1 and Site 2 selective analogs (15). For Site 1-selective analogs, a nitrogen atom attached at C-8 is optimally synergistic with C-6 analogs when using type I while a sulfur atom attachment is most effective using type II (15).

In the present studies, we extend these *in vitro* findings to demonstrate a synergism of the cAMP-mediated lipolytic response in isolated intact adipocytes. In addition, we expand previous cyclic nucleotide structure-activity studies to correlate analog effects on protein kinase activation and lipolysis, and explore the factors responsible for the lipolytic efficacy of the analogs. By adding analog combinations to intact tissues, novel approaches can be made to delineate cellular cyclic nucleotide effector control mechanisms. First, a new test is available to establish whether or not the protein kinase is the mediator of a particular response. Second, the particular isozyme or isozyme mixture responsible for the effect in tissues can potentially be identified. Third, because cyclic nucleotide binding to Sites 1 and 2 is cooperative, an estimation can be made of the magnitude of the sensitivity magnification at the protein kinase step in the intact tissue. Last, since analog combinations are synergistic, this allows for the use of lower total analog concentration to obtain the same cellular response as would be obtained using a single analog.

### EXPERIMENTAL PROCEDURES

*Preparation of Adipocytes and Cell Incubations*—Adipocytes were isolated by collagenase treatment according to the method of Rodbell

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(16), from epididymal fat pads of 130–200-g Sprague-Dawley rats. Epididymal fat was incubated in KRBA<sup>1</sup> containing 1 mM CaCl<sub>2</sub>, 3.5% bovine serum albumin (fraction V). The cells were washed and suspended in the same buffer such that the fat dry weight was 35–55 mg/ml cell suspension. Incubations were carried out in closed polypropylene vials, gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub> in the presence or absence of various concentrations of cyclic nucleotide analogs or other lipolytic agents. When IBMX was present, the concentration was 200 μM. Incubations were routinely done in a shaking water bath (64 cycles/min) at 20 °C. The synergism of lipolysis was quantitated using a synergism quotient which is defined as the ratio of the observed level of glycerol release when C-6 and C-8 analogs are incubated in combination to the expected level of glycerol release determined from the sum of the effects of each individual analog.

**Glycerol Determination**—At various times, aliquots of incubation mixture were made 3% perchloric acid and then 13% (v/v) carbon tetrachloride. The samples were mixed and spun in a desk-top clinical centrifuge for 5–10 min. After KOH neutralization, aliquots of the supernatants were removed and assayed for glycerol according to the method of Wieland (17). Lipolysis is expressed as micromoles of glycerol/g of fat. The fat dry weight was determined by spotting aliquots of incubation mixtures on nitrocellulose papers, drying, and weighing.

**Determination of Partition Coefficients**—The partition coefficients of various cyclic nucleotide analogs were determined using 1-butanol as the organic phase and 0.05 M potassium phosphate (pH 7.4) as the aqueous phase. The two phases were first mixed and allowed to separate overnight, and the resulting phases were used to determine the partition coefficients. 3 ml of 1-butanol were added to 1 ml of cyclic nucleotide analog (50 μM) diluted in 0.05 M potassium phosphate and vigorously mixed for 30 s on a Vortex-Genie (Fisher Scientific) at a setting of 4. The samples were spun in a desk-top clinical centrifuge to speed the phase separation. The absorbance of the analog in potassium phosphate was determined at 260 nm before and after 1-butanol extraction. The difference in absorbance was taken as that fraction of analog which partitioned into the 1-butanol. The partition coefficient was determined by the ratio of the concentration of analog in the 1-butanol to the concentration in potassium phosphate after organic extraction. The analogs partitioned less effectively into *tert*-amyl alcohol but followed the same rank order as with 1-butanol.

**Protein Kinase Assay**—The activity of the cAMP-dependent protein kinase was determined using a modification of the filter paper assay procedure described by Roskoski (18), using the synthetic heptapeptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) as substrate. The reaction mixture contained 50 mM Tris (pH 7.5), 2% glycerol, 1 mM EGTA, 43 mM 2-mercaptoethanol, 65 μM synthetic peptide, 200 μM [ $\gamma$ -<sup>32</sup>P]ATP (100–200 cpm/pmol), 10 mM MgCl<sub>2</sub> in the presence or absence of 5 μM cAMP. When IBMX was present, the concentration was 200 μM. The reaction was terminated after 5 min of incubation at 30 °C. The reaction was linear in the presence or absence of cAMP for at least 10 min. The cAMP-dependent protein kinase activity ratio is defined as the ratio of activity measured in the absence and presence of 5 μM cAMP.

**K<sub>a</sub> Determination for Cyclic Nucleotides**—The K<sub>a</sub> for cAMP and cyclic nucleotide analogs was determined in the standard protein kinase assay in the presence of 200 μM IBMX. The data were fitted to the Hill equation after subtraction of basal activity ratios which were between 0.02 and 0.13. The cyclic nucleotide concentrations were varied between 3 and 10,000 nM. Protein kinase was present at 0.5–1.0 nM regulatory subunit monomer for the DEAE-cellulose concentrated enzyme or 0.05–0.1 nM after separation by sucrose density gradient centrifugation. The K<sub>a</sub> for cAMP was unaffected by pH values between 6.8 and 8.0 or the presence or absence of sucrose (2.5%), glycerol (2%), or IBMX (200 μM).

**Preparation of Adipocyte Particulate Phosphodiesterase**—The low K<sub>m</sub> phosphodiesterase was prepared by modification of the method of Loten *et al.* (19). Adipocytes from four rat epididymal fat pads were prepared as described above. Cells were washed two times with KRBA and three additional times with 10 mM Tris-HCl, 1 mM EDTA (pH 7.5) containing 250 mM sucrose (TSE buffer). Cells were resuspended in 5 ml of TSE buffer and homogenized with 10 strokes of the A pestle using a Dounce homogenizer. The homogenate was centrifuged for 30 min at 20,000 rpm and the supernatant was discarded. The

pellet was washed by gentle resuspension in 5 ml of TSE buffer followed by centrifugation. The resulting pellet was resuspended in 5–10 ml of 10 mM Tris-HCl, 1 mM EDTA (pH 7.5) using 10 strokes of the pestle and centrifuged at 20,000 rpm, and the supernatant fraction was assayed for low K<sub>m</sub> phosphodiesterase activity.

Cyclic nucleotide phosphodiesterase activity was measured as described by Loten *et al.* (19). In all experiments, enzyme was added in a concentration which resulted in the hydrolysis of approximately 25% of the substrate. [<sup>3</sup>H]cAMP was always present at a concentration of 0.1 μM and cyclic nucleotide analogs were added to the reaction in a wide range of concentrations. Their ability to compete for [<sup>3</sup>H]cAMP hydrolysis is expressed as the concentration of analog which inhibited cAMP hydrolysis by 50% (I<sub>50</sub>).

**Isolation of cAMP-dependent Protein Kinase**—The cAMP-dependent protein kinase was partially purified from adipocytes using DEAE-cellulose according to the method of Corbin *et al.* (9). Adipocytes were prepared as described above. After two washes with KRBA, adipocytes were washed twice with 10 mM potassium phosphate, 1 mM EDTA, and 250 mM sucrose (pH 6.8) (buffer A) and suspended in the same buffer except with 10 mM EDTA (buffer B). The adipocytes were hand-homogenized with 20 strokes of the B pestle using a Dounce homogenizer. The homogenate was centrifuged for 30 min at 20,000 × *g*. One main peak of protein kinase activity eluted from DEAE-cellulose at 170 mM NaCl as previously shown for adipose tissue (8).

**Materials**—Bovine serum albumin was purchased from Reheis Chemical Co. Collagenase was obtained from Worthington Diagnostic Systems, Inc. Glycerol kinase, glycerol-3-phosphate dehydrogenase and N<sup>6</sup>-phenylisopropyladenosine were products of Boehringer Mannheim. 2'-5'-Dideoxyadenosine was obtained from P-L Biochemicals, Inc. 8-Thioisopropyl-cAMP and 8-thiobenzyl-cAMP were received from ICN. 8-Aminobutylamino-cAMP was synthesized as previously described (14). The following analogs were generously provided by Drs. Jon Miller and Robert Suva of the Biomedical Research Laboratory, Life Sciences Division, SRI International, Menlo Park, CA: N<sup>6</sup>-carbamoylpropyl-cAMP, 6-thiomethyl-cNMP, N<sup>6</sup>-hydroxy-cAMP, 8-thio-pNO<sub>2</sub>benzyl-cAMP, 8-hydroxy-cAMP, 8-aminoethanol-cAMP, 2-trifluoromethyl-cAMP, and 2-aminodimethyl-cAMP. All other cyclic nucleotide analogs were purchased from Sigma.

## RESULTS

**Effect of Cyclic Nucleotide Analog Concentration on Glycerol Release**—A variety of cyclic nucleotide analogs at various concentration were tested for their lipolytic effects on adipocytes. Adipocyte incubations with analogs or hormones were carried out at 20 °C. When experiments were done at 37 °C, similar dose-response or time-response characteristics were seen except the time-response curves were shifted to the left. Fig. 1 shows the dose-response curves of 16 representative analogs. The studies in Fig. 1 and Table I were carried out by measuring glycerol release after 60 min of incubation with each individual analog at several concentrations. Duplicate glycerol determinations were done on each of two or more adipocyte preparations. Generally, the various analogs fit into three major categories based on their dose-response characteristics. The effects of N<sup>6</sup>-benzoyl- and 8-bromo-cAMP were relatively linear with concentration (Fig. 1C). Analogues which were modified with alkyl chains at the C-6 positions (N<sup>6</sup>-Bt and N<sup>6</sup>-AHCM, Fig. 1, D and B, respectively), a hydroxyl at the C-6 or C-8 position (N<sup>6</sup>-hydroxy- and 8-hydroxy-cAMP, Fig. 1B) or amine-containing substituents at the C-8 position (8-aminomethyl- and 8-aminobenzyl-cAMP, Fig. 1A) had hyperbolic dose-response curves. Concave-upward dose-response curves were characteristic for analogs containing a thio derivative (8-thiomethyl-cAMP, 8-thiobenzyl-cAMP, Fig. 1A and 6-thiomethyl-cNMP, Fig. 1B).

Table I shows values for glycerol release of 23 cyclic nucleotide analogs tested as lipolytic agents. The analogs are listed according to decreasing efficacy for a modification of a given position on the adenine ring. The values for glycerol release (nanomoles/g/μM analog/h) were determined from linear regressions of dose-response curves from experiments

<sup>1</sup> The abbreviations used are: KRBA, Krebs-Ringer bicarbonate buffer; IBMX, 3-isobutyl-1-methylxanthine; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

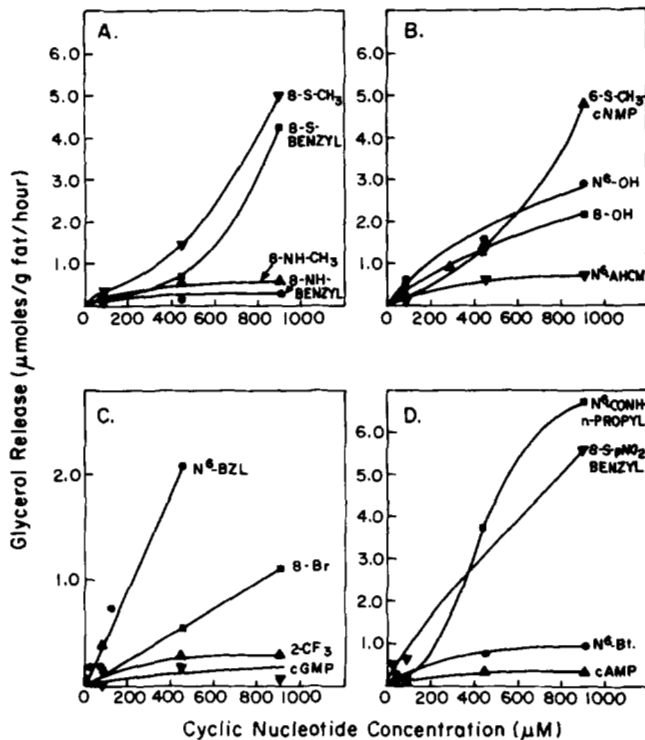


FIG. 1. The effect of cyclic nucleotide analog concentration on lipolysis. Lipolysis was determined by measuring glycerol release after 1 h of incubation at 20 °C. The plotted values have been corrected for glycerol released in the absence of added cyclic nucleotide analogs. *N*<sup>6</sup>-Bt, *N*<sup>6</sup>-butyryl; *N*<sup>6</sup>-AHCM, *N*<sup>6</sup>-aminohexylcarbamoylpropyl.

like those shown in Fig. 1. Since most of the analogs had nonlinear dose-response characteristics, the order of Table I represents an average concentration effect. It follows that the order was mainly determined by the highest concentration of analog tested (910 μM). *N*<sup>6</sup>-Carbamoylpropyl- and *N*<sup>6</sup>-ben-

zoyl-cAMP were the most effective lipolytic agents tested. Analogs modified with a thio moiety at the C-6 or C-8 position were also very potent. On the other hand, analogs containing amino derivatives at the C-8 position were relatively poor at stimulating lipolysis. cAMP, cGMP, 8-bromo-cGMP, 2-trifluoromethyl-cAMP, 2-aminodimethyl-cAMP, and cAMP itself were poor lipolytic agents. When analogs were ordered based on their effects at lower concentrations (90 μM), a similar order of efficacy was obtained except *N*<sup>6</sup>-hydroxy- and 8-hydroxy-cAMP were the most potent and 8-thiomethyl-, 8-thioisopropyl-, and 8-thiobenzyl-cAMP had a somewhat lower rank. However, C-8 analogs with thio modifications were always better lipolytic agents than analogs containing amino substituents.

Several factors were considered to be responsible for the efficacy of the analogs tested in these studies. An analog should be an effective lipolytic agent if it 1) has a good lipophilic character so that it can pass through the plasma membrane and enter the cell, 2) is an effective activator of cAMP-dependent protein kinase which subsequently phosphorylates and activates the hormone-sensitive lipase, and 3) is not hydrolyzed by the phosphodiesterases present in the cell. Therefore, we attempted to measure parameters of the analogs which would be predictive of their ability to stimulate glycerol release. As a measure of lipophilicity, the partition coefficients of several analogs were determined. Several analogs were also tested for their ability to activate the type II cAMP-dependent protein kinase. Since few of these analogs were available with radioactive labels, it was difficult to directly study the hydrolysis of these derivatives. Alternatively, we studied the ability of some analogs to inhibit [<sup>3</sup>H]cAMP hydrolysis by the low *K<sub>m</sub>* phosphodiesterase isolated from the adipocyte high speed pellet. Table II shows the values for glycerol release taken from Table I with the partition coefficient, *K<sub>a</sub>* for protein kinase activation and the *I*<sub>50</sub> for the low *K<sub>m</sub>* cAMP-phosphodiesterase.

Analog with hydrophobic substituents such as alkyl and

TABLE I

## Effect of cyclic nucleotides on glycerol release

Cyclic nucleotide analogs are listed in order from the most to the least potent for a given modification on the purine ring. The average analog concentration effect was determined from linear regression analysis of dose-response curves like those indicated in Fig. 2. Glycerol release was determined as described under "Experimental Procedures" and is expressed as nanomoles of glycerol/g of fat, dry weight/μM of analog/h.

Cyclic nucleotide analog			Glycerol release
Abbreviated name	Substituent		nmol/g/μM/h
C-6 analog	cAMP		0.23
	<i>N</i> <sup>6</sup> -Carbamoylpropyl	<i>N</i> <sup>6</sup> -CONH-C <sub>3</sub> H <sub>7</sub>	7.86
	<i>N</i> <sup>6</sup> -Benzoyl	<i>N</i> <sup>6</sup> -CO <sub>2</sub> -C <sub>6</sub> H <sub>5</sub>	7.54
	6-Thiomethyl nebularine	6-S-CH <sub>3</sub> -cNMP	4.07
	<i>N</i> <sup>6</sup> -Hydroxy	<i>N</i> <sup>6</sup> -OH	2.66
	<i>N</i> <sup>6</sup> -Butyryl	<i>N</i> <sup>6</sup> -CO <sub>2</sub> -C <sub>3</sub> H <sub>7</sub>	0.93
	<i>N</i> <sup>6</sup> -Aminohexylcarbamoylmethyl	<i>N</i> <sup>6</sup> -(CH <sub>2</sub> ) <sub>6</sub> -NH-CONH-CH <sub>3</sub>	0.83
	cIMP		0.01
C-8 analog	8-Thio-pNO <sub>2</sub> benzyl	8-S-pNO <sub>2</sub> -CH <sub>2</sub> -C <sub>6</sub> H <sub>5</sub>	5.71
	8-Thiomethyl	8-S-CH <sub>3</sub>	5.68
	8-Thioisopropyl	8-S-isoC <sub>3</sub> H <sub>7</sub>	4.73
	8-Thiobenzyl	8-S-CH <sub>2</sub> -C <sub>6</sub> H <sub>5</sub>	4.39
	8-Hydroxy	8-OH	2.04
	8-Amino	8-NH <sub>2</sub>	1.63
	8-Bromo	8-Br	1.07
	8-Aminohexylamino	8-NH-(CH <sub>2</sub> ) <sub>6</sub> -NH <sub>2</sub>	1.05
	8-Aminomethyl	8-NH-CH <sub>3</sub>	0.43
	8-Aminobenzyl	8-NH-CH <sub>2</sub> -C <sub>6</sub> H <sub>5</sub>	0.25
C-2 analog	8-Aminoethanol	8-NH-CH <sub>2</sub> -H <sub>2</sub> -OH	0.16
	2-Trifluoromethyl	2-CF <sub>3</sub>	0.0
cGMP analog	2-Aminodimethyl	2-N-(CH <sub>3</sub> ) <sub>2</sub>	0.0
	8-Bromo	8-Br-cGMP	0.0
	cGMP		0.11

TABLE II  
Various parameters of cAMP analogs

Values for glycerol release were taken from Table I. The partition coefficient,  $K_a$  for protein kinase activation and the  $I_{50}$  for the low  $K_m$  phosphodiesterase were determined as described under "Experimental Procedures."

	Glycerol release	Partition coefficient	Protein kinase activation ( $K_a$ )	Phosphodiesterase $I_{50}$
	nmol/g/ $\mu$ M analog/h		$\mu$ M	$\mu$ M
Cyclic AMP analog				
$N^6$ -Carbamoylpropyl	7.9	4.9	1.9	492
$N^6$ -Benzoyl	7.5	3.0	2.1	90
8-Thiomethyl	5.7	1.6	0.3	46
8-Thioisopropyl	4.7	6.6	0.5	60
8-Thiobenzyl	4.4	19.4	0.6	48
8-Amino	1.6	0.4	0.2	40
8-Bromo	1.1	1.6	0.2	14
$N^6$ -Butyryl	0.9	1.3	3.0	597
8-Aminomethyl	0.4	0.9	0.6	33
8-Aminobenzyl	0.3	10.9	4.0	202
cAMP	0.2	0.6	0.2	0.06
2-Trifluoromethyl	0.0	4.6	1.6	0.005
cIMP	0.0	0.2	6.9	0.60

aryl groups had high partition coefficients while analogs with more hydrophilic substituents such as 8-amino derivatives, cIMP, and cAMP had low partition coefficients. There was no clear correlation between the partition coefficient of the analog and its ability to stimulate glycerol release. Likewise, there was no clear correlation between the  $K_a$  of the analog for protein kinase activation and its ability to serve as an effective lipolytic agent. In addition, there appeared to be no correlation between the lipolytic effectiveness of an analog and its ability to inhibit the low  $K_m$  phosphodiesterase as indicated by  $I_{50}$  values. The  $I_{50}$  values in Table II indicate that good lipolytic analogs required high concentrations to inhibit [ $^3$ H]cAMP hydrolysis. When the partition coefficient, the  $K_a$  for protein kinase activation, and the  $I_{50}$  for the phosphodiesterase were all considered, the lipolytic effectiveness of the analogs was explained (Table II).

**Comparison of Effects of Epinephrine and Cyclic AMP Analogs on Lipolysis**—Fig. 2 shows the effect of time on epinephrine and cAMP analog-stimulated glycerol release. Because linear regression was used to determine the rates of glycerol release when possible, all of the lines do not necessarily pass through 0. Fig. 2 shows that 10  $\mu$ M epinephrine in the presence of 200  $\mu$ M IBMX resulted in a linear rate of 127 nmol of glycerol/g of fat/min for at least 90 min. When IBMX was deleted from the incubation media, the rate of lipolysis was decreased by approximately 15–20%. The presence of high concentrations of  $N^6$ -benzoyl-cAMP (455  $\mu$ M) and 8-bromo-cAMP (1364  $\mu$ M) did not alter the rate of epinephrine-induced lipolysis. When 455  $\mu$ M  $N^6$ -benzoyl- and 1364  $\mu$ M 8-bromo-cAMP were incubated with adipocytes in the absence of hormone, a concave-upward time course was seen. The rate of lipolysis was 75 nmol of glycerol/g of fat/min in the first 30 min of incubation in this experiment but 130 nmol of glycerol/g of fat/min in last 30 min. When the concentrations of  $N^6$ -benzoyl- and 8-bromo-cAMP were reduced (136 and 455  $\mu$ M, respectively) and the incubation time was extended, a more pronounced concave-upward time course was evident.

Millimolar concentrations of  $N^6$ -benzoyl-, 8-thiomethyl-, 8-thiobenzyl-, 8-thio-pNO<sub>2</sub>benzyl-, and  $N^6$ -carbamoylpropyl-cAMP stimulated lipolysis at rates approximately equal to epinephrine (not shown). Concave-upward time courses were generally seen with all analogs which were effective lipolytic agents. Since the concave-upward time-effect relationship was

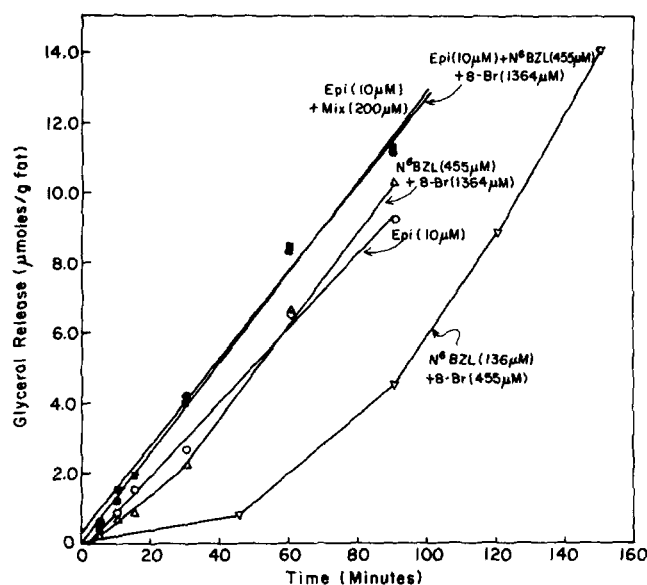


FIG. 2. The effect of epinephrine and cAMP analogs on lipolysis. Adipocytes were incubated in the presence of 10  $\mu$ M epinephrine alone ( $\circ$ ), 10  $\mu$ M epinephrine, and 200  $\mu$ M IBMX ( $\blacksquare$ ), 10  $\mu$ M epinephrine with 455  $\mu$ M  $N^6$ -benzoyl- and 1364  $\mu$ M 8-bromo-cAMP ( $\bullet$ ), 455  $\mu$ M  $N^6$ -benzoyl- and 1364  $\mu$ M 8-bromo-cAMP ( $\Delta$ ), and 136  $\mu$ M  $N^6$ -benzoyl- and 455  $\mu$ M 8-bromo-cAMP ( $\nabla$ ). Glycerol release was determined as a measure of lipolysis at the indicated times.

still seen when the adipocytes were preincubated for 60 min before the addition of the analogs, it appears that the concave-upward time courses were not due to the generation of cyclic nucleotide-independent factor(s) during the experiment (data not shown). The shape of these concave-upward time courses was unaffected by adenosine (100  $\mu$ M), phenylisopropyladenosine (0.1 and 10  $\mu$ M), 2',5'-dideoxyadenosine (50  $\mu$ M), or IBMX (200  $\mu$ M), suggesting that the adenosine receptors are not sites of action of analogs or other factors generated during the incubation which account for the concave-upward time courses (20, 21). Since IBMX antagonizes the inhibitory adenosine receptor of the adipocyte (22) as well as inhibits the phosphodiesterase, these data also suggest that under

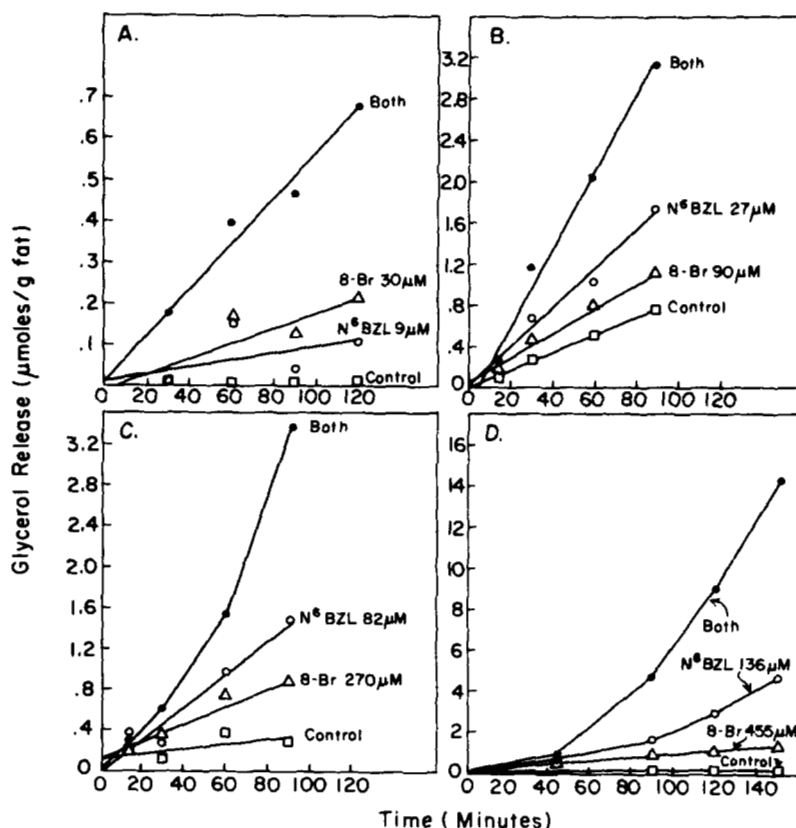
these conditions these processes play only a minor role in analog-stimulated lipolysis.

**Effect of Analog Combinations on Glycerol Release**—Previous studies from this laboratory have demonstrated that binding of a cAMP analog selective for either intrachain site on the regulatory subunit of protein kinase stimulated binding of a cAMP analog selective for the other site (13, 14). A continuation of these studies demonstrated that two such site-selective analogs could also be used in combination to synergistically activate protein kinase (15). The main approach of the present studies was to determine if these *in vitro* findings could be extended to isolated, intact cell preparations to demonstrate a synergism of a metabolic response believed to be mediated by protein kinase. Consequently, isolated adipocytes were incubated in the presence of site-selective cAMP analogs alone and in combination and glycerol release was determined as a measure of lipolysis. The first series of experiments of this type were carried out with the Site 1-selective analog 8-bromo-cAMP and the Site 2-selective analog *N*<sup>6</sup>-benzoyl-cAMP. The first approach was to carry out time courses with increasing concentrations of a fixed ratio of these analogs (Fig. 3). The basal, unstimulated rate of glycerol release varied in this series of experiments from essentially 0 (Fig. 3A) to 9 nmol/g of fat/min (Fig. 3B). This control level of lipolysis was always subtracted from the experimental values to obtain a net value for glycerol release. To quantitate the resulting effects on glycerol release, a synergism quotient was used. The synergism quotient was defined as the net effect of the analog combination on lipolysis divided by the sum of the net individual analog effects on lipolysis. A quotient greater than 1 indicated a synergistic effect while a quotient less than 1 indicated that an inhibitory effect on lipolysis occurred. For experiments using *N*<sup>6</sup>-benzoyl-cAMP and 8-bromo-cAMP, the synergism quotients were generally not higher than 2.5 when the absolute net values at

any given time point were considered. At intermediate concentrations of this analog pair, synergism quotients varied from less than or equal to 1.0 after short periods of incubation (15–30 min) to values of around 2.0 after longer incubation periods (Fig. 3B). At higher concentrations of analog and particularly after longer periods of incubation, synergism quotients were higher (Fig. 3, C and D). When time-effect relationships were linear, synergism quotients were calculated using linear regression analysis. The synergism quotients were 1.35 and 2.0 in Fig. 3, A and B, respectively. However, when time-effect relationships were not linear (Fig. 3, C and D), calculation of the synergism quotient based on absolute net glycerol values at any given point of determination may give conservative estimations of the magnitude of synergism. Since under these conditions the rate of lipolysis was changing, it may be appropriate to consider the relative rates of glycerol release between time points. In Fig. 3C, the rate of lipolysis with each analog alone was linear while the rate of lipolysis when both analogs were present was concave upward. The synergism quotients calculated after 60 and 90 min of incubation were 1.2 and 1.8, respectively. If, however, the relative rates between 30 and 60 min or 60 and 120 min were considered, the synergism quotients were 1.8 and 3.3, respectively. However, the apparent disparity between the values calculated by these two approaches was reduced as the rate of lipolysis due to a single analog varied with time as occurred with higher concentrations of *N*<sup>6</sup>-benzoyl-cAMP (Fig. 3D).

Another approach to investigating the effects of cAMP analog combinations on lipolysis was to keep one analog at a constant concentration while the concentration of the other analog was varied. Fig. 4 shows one experiment of this type in which the Site 2-selective analog *N*<sup>6</sup>-benzoyl-cAMP and the Site 1-selective analog 8-thioisopropyl-cAMP were incubated alone and in combination. In Fig. 4A, glycerol release was measured after adipocytes were incubated for 2 h with 10

**FIG. 3. The effect of increasing concentrations of a fixed ratio of *N*<sup>6</sup>-benzoyl- and 8-bromo-cAMP on synergism of lipolysis.** *N*<sup>6</sup>-Benzoyl- (○) and 8-bromo-cAMP (△) were incubated alone and in combination (●) at a fixed ratio of about 3. Each part represents a different adipocyte preparation. The values at each time point represent glycerol determinations on duplicate incubations. Lipolysis was measured by glycerol release.



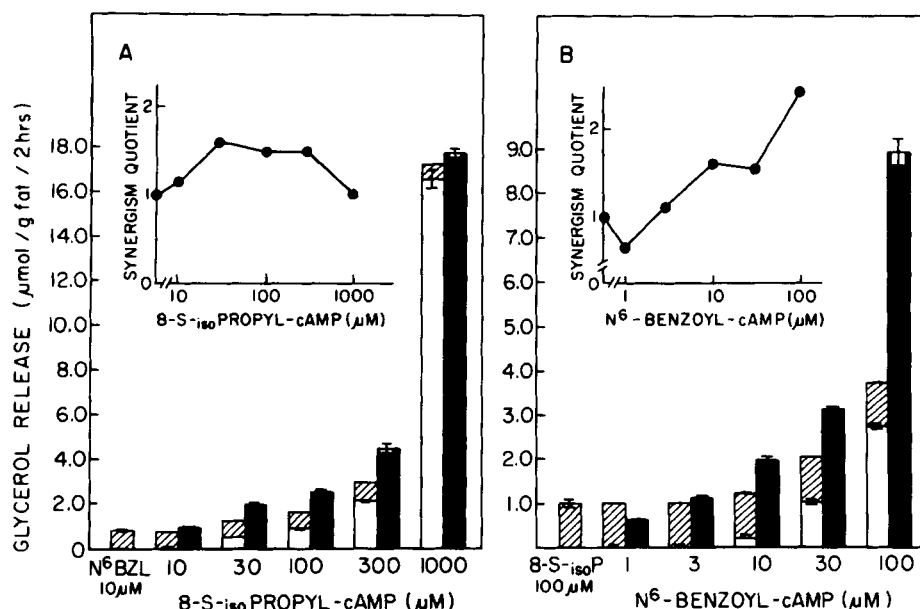


FIG. 4. Synergistic effect of  $N^6$ -benzoyl- and 8-thioisopropyl-cAMP on lipolysis.  $N^6$ -Benzoyl-cAMP ( $10 \mu\text{M}$ ) and 8-thioisopropyl-cAMP ( $100 \mu\text{M}$ ) were incubated alone and in the presence of various concentrations of the other analog and glycerol release was determined after 2 h of incubation. The striped portion of the bars represent glycerol values for  $10 \mu\text{M}$   $N^6$ -benzoyl-cAMP (A) and  $100 \mu\text{M}$  8-thioisopropyl-cAMP (B) when incubated alone. The open portion of the bars represent glycerol values for increasing concentrations of 8-thioisopropyl-cAMP (A) and  $N^6$ -benzoyl-cAMP (B) when incubated alone. The height of the bars on the left of each pair represents the sum of the individual analog effects or the expected glycerol value if analogs were incubated together. The total height of the solid bar indicates the observed glycerol value when analogs were incubated in combination at the indicated concentrations. The differences between the heights of the paired bars reflects the magnitude of synergism of lipolysis. The data represent means and standard errors of triplicate determinations in a representative experiment. The insets show the synergism quotient at each concentration of analog combination. The synergism quotient is defined under "Experimental Procedures."  $N^6$ BZL,  $N^6$ -benzoyl-cAMP; 8-S-isoP, 8-thioisopropyl-cAMP.

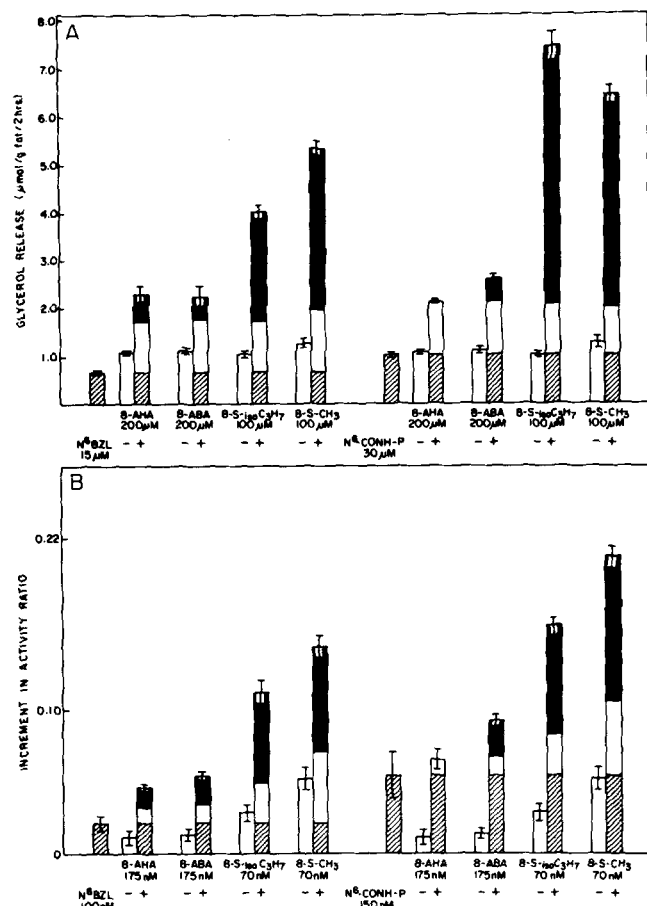
$\mu\text{M}$   $N^6$ -benzoyl-cAMP and various concentrations of 8-thioisopropyl-cAMP and Fig. 5B shows the reverse approach in which  $100 \mu\text{M}$  8-thioisopropyl-cAMP was combined with various concentrations of  $N^6$ -benzoyl-cAMP. The bar on the left of each pair indicates the sum of the analog effects on lipolysis while the bar on the right of each pair indicates the observed lipolytic effects of the analog when combined in the incubation media. The insets show the synergism quotients which were based on the ratios of the heights of these bars.  $10 \mu\text{M}$   $N^6$ -benzoyl-cAMP and  $100 \mu\text{M}$  8-thioisopropyl-cAMP were approximately equipotent as lipolytic agents (striped bars, Fig. 4, A and B, respectively). When  $N^6$ -benzoyl-cAMP ( $10$ – $100 \mu\text{M}$ ) and 8-thioisopropyl-cAMP ( $30$ – $300 \mu\text{M}$ ) were incubated alone, linear dose-response data were seen (open bars, Fig. 4). At higher concentrations ( $1000 \mu\text{M}$ ), 8-thioisopropyl-cAMP, unlike  $N^6$ -benzoyl, demonstrated concave-upward dose-response characteristics typical of the 8-thio-cAMP analogs (Fig. 1).

When  $10 \mu\text{M}$   $N^6$ -benzoyl-cAMP was incubated with concentrations of 8-thioisopropyl-cAMP which alone were in a region of linear dose-response effects ( $30$ ,  $100$ , and  $300 \mu\text{M}$ ), a relatively constant degree of synergism was seen (Fig. 4A, inset). The apparent maximum synergism quotient of about 1.5 was not increased when a lower ( $10 \mu\text{M}$ ) or higher ( $1000 \mu\text{M}$ ) concentration of 8-thioisopropyl-cAMP was incubated in combination with  $N^6$ -benzoyl-cAMP. It is not surprising that synergism was not seen at the highest concentration of 8-thioisopropyl-cAMP tested since this analog concentration alone elicited a maximal lipolytic response. The magnitude of synergism seen with this experimental approach was not dependent on the concentration of 8-thioisopropyl-cAMP. However, when 8-thioisopropyl-cAMP was kept constant and

the concentration of  $N^6$ -benzoyl-cAMP was varied, the synergism quotient was directly proportional to the concentration of  $N^6$ -benzoyl-cAMP (Fig. 4B, inset). In these experiments, the synergism quotient varied between 1.1 and 2.4 as the concentration of  $N^6$ -benzoyl-cAMP was increased from 3 to  $100 \mu\text{M}$ . As the concentration of  $N^6$ -benzoyl-cAMP was increased to yield a maximal lipolytic effect, the synergism quotient again approached 1.0 (data not shown).  $N^6$ -Benzoyl-cAMP also had a dose-dependent synergistic effect on epinephrine-stimulated ( $3$ ,  $25$ , and  $200 \text{ nM}$ ) and IBMX ( $200 \mu\text{M}$ )-stimulated lipolysis (data not shown). At very low concentrations of  $N^6$ -benzoyl-cAMP ( $1 \mu\text{M}$ ), an inhibition of 8-thioisopropyl-cAMP-stimulated lipolysis was seen (synergism quotient = 0.65). When detailed time courses (such as those in Fig. 3) were carried out, synergism quotients of less than 1 were sometimes seen. When this inhibition of glycerol release was present, it was seen when at least one analog was present at low concentrations and/or at early time points when the concentration of the analog at its active site(s) would be expected to be low.  $N^6$ -Benzoyl-cAMP, the most thoroughly studied analog, frequently showed this characteristic.

Experiments similar to those in Fig. 4 carried out with  $N^6$ -benzoyl-cAMP and the Site 1-selective analog 8-bromo-cAMP produced similar results. A relatively constant synergism quotient of about 2.0 was seen when incubations were carried out when the concentration of 8-Br-cAMP was varied in the presence of a constant concentration of  $N^6$ -benzoyl-cAMP between 15 and  $90 \mu\text{M}$  8-bromo-cAMP. An increasing magnitude of synergism was obtained when the concentration of  $N^6$ -benzoyl-cAMP was increased from 10 to  $140 \mu\text{M}$  in the presence of  $30 \mu\text{M}$  8-bromo-cAMP.

Synergism of lipolysis with cAMP analog combinations was



**FIG. 5. Comparison of synergism of lipolysis and synergism of adipocyte type II protein kinase activation using C-6 and C-8 cAMP analogs.** A, synergism of lipolysis was determined by incubating adipocytes with C-6 analogs and C-8 analogs alone and in combination and measuring glycerol released after 2 h of incubation. The striped bars show the effects of *N*<sup>6</sup>-benzoyl-cAMP (left) and *N*<sup>6</sup>-carbamoylpropyl-cAMP (right) when incubated alone. The unshaded bars on the left of each pair of bars show the effects of the indicated C-8 analog when incubated alone. The total height of the bar on the right of each pair indicates the effects on glycerol release when the indicated C-8 analogs were incubated with adipocytes in the presence of *N*<sup>6</sup>-benzoyl-cAMP (left) or *N*<sup>6</sup>-carbamoylpropyl-cAMP (right). The shaded portion of the bar indicates the extent of synergism. The data represent the means and standard errors of four to eight determinations on each of two different adipocyte preparations. B, synergism of protein kinase activation was determined by incubating adipocyte type II protein kinase with C-6 analogs and C-8 analogs alone and in combination in the standard protein kinase assay. The activity ratios represent the increases above basal due to the indicated concentrations of the various analogs. The basal activity ratio determined in the absence and the presence of 5 µM cAMP was 0.09. The protein kinase was isolated by DEAE-cellulose chromatography. The data indicate the means and standard errors of three or four determinations. *N*<sup>6</sup>BZL, *N*<sup>6</sup>-benzoyl-cAMP; *N*<sup>6</sup>CONH-P, *N*<sup>6</sup>-carbamoylpropyl-cAMP; 8-AHA, 8-aminohexylamino-cAMP; 8-ABA, 8-aminobutylamino-cAMP; 8-S-iso C<sub>3</sub>H<sub>7</sub>, 8-thioisopropyl-cAMP; 8-S-CH<sub>3</sub>, 8-thiomethyl-cAMP.

seen when the sum of the lipolytic effects of the individual analogs was between 10 and 50% of the optimally stimulated level of glycerol release as determined by 10 µM epinephrine in the presence of 200 µM IBMX. Synergism of lipolysis was only seen when a Site 1-selective analog was incubated with a Site 2-selective analog but not when two Site 1-selective or two Site 2-selective analogs were combined (Table III). When two C-6 analogs or two C-8 analogs were combined such that

**TABLE III**  
Lack of synergism of glycerol release with pairs of C-6 or pairs of C-8 analogs

Analog combination	Combination divided by sum of individual effects (synergism quotient)
<i>N</i> <sup>6</sup> -Carbamoylpropyl (91 µM) + <i>N</i> <sup>6</sup> -benzoyl (45 µM)	0.86
<i>N</i> <sup>6</sup> -Carbamoylpropyl (91 µM) + <i>N</i> <sup>6</sup> -butyryl (91 µM)	0.86
<i>N</i> <sup>6</sup> -Benzoyl (45 µM) + <i>N</i> <sup>6</sup> -butyryl (91 µM)	0.94
8-Thiobenzyl (91 µM) + 8-aminomethyl (91 µM)	0.98
8-Thiobenzyl (91 µM) + 8-methyl (91 µM)	1.02
8-Aminomethyl (91 µM) + 8-methyl (91 µM)	0.72
8-Thiobenzyl (91 µM) + 8-bromo (91 µM)	1.04

alone they stimulated lipolysis 10–20% each, their synergism quotients were less than or equal to 1.

This laboratory had previously shown that bovine heart type II and rat heart type I protein kinase exhibited a different C-8 analog specificity for stimulation of binding and synergism of activation when incubated with a C-6 analog (14, 15). Specifically, when used in combination with C-6 analogs, those analogs with a sulfur atom attached to C-8 acted more synergistically using type II protein kinase and type I exhibited greater synergism using analogs with a nitrogen atom attached at C-8. Consequently, the use of 8-thio- and 8-amino-cAMP analogs in combination with C-6 analogs could potentially differentiate between a type II and a type I protein kinase-mediated metabolic response. When adipocyte protein kinase was isolated on DEAE-cellulose, it eluted at a salt concentration consistent with a type II protein kinase. It was therefore of interest to see if the site-specific analog pattern of synergism of lipolysis could be correlated with synergism of adipocyte type II protein kinase. Fig. 5A shows the lipolytic effects of individual analogs and the sum of their effects such that they can be compared with the effects of various analog combinations. In Fig. 5A, the total height of the bar on the right of each pair indicates the amount of glycerol released when the adipocytes are incubated with the analog combinations. The shaded portion of the bars indicates the extent of synergism. The observed levels of glycerol release varied among the combinations depending on whether the C-6 analog was incubated with a C-8-thio analog or a C-8-amino analog. Incubation of *N*<sup>6</sup>-carbamoylpropyl-cAMP or *N*<sup>6</sup>-benzoyl-cAMP with either 8-thio-cAMP analog produced the greatest degree of glycerol release over that expected from the sum of the individual analogs alone (synergism quotients = 2.5–3.6). Both of the C-6 analogs acted far more synergistically when incubated in combination with 8-thio-cAMP analogs than when incubated with 8-amino derivatives. Only a limited degree of synergism was expressed when either C-6 analog was incubated in combination with the 8-aminohexylamino- or 8-aminobutylamino-cAMP (average synergism quotient = 1.2). Similar results were achieved with these C-6 analogs in combination with 8-aminobenzyl-cAMP (not shown).

These data for lipolytic effects are in excellent agreement with studies using the same analogs on activation of isolated adipocyte type II protein kinase (Fig. 5B). The basal protein kinase activity ratio, determined in the absence of added cyclic nucleotides, varied between 0.08 and 0.13. In each experiment, the basal activity ratio was subtracted to give the change in activity ratio due to the analogs being tested. The order of effectiveness of the analog pairs for synergism of protein kinase activation follows the same order of effectiveness for glycerol release. Both *N*<sup>6</sup>-benzoyl-cAMP and *N*<sup>6</sup>-carbamoyl-



propyl-cAMP acted most synergistically with 8-thioisopropyl- and 8-thiomethyl-cAMP. The C-6 analogs were much less effective in combination with 8-aminoethylamino- and 8-aminobutylamino-cAMP. Although the increases in activity ratio due to the individual analogs alone were small, the magnitude of change due to the 8-thio derivatives were greater than that due to the 8-amino derivatives. However, other studies on activation of adipocyte protein kinase indicated that increasing the effect on activity ratio by increasing the concentration of 8-amino derivatives or decreasing the effect by decreasing the concentrations of 8-thio derivatives had no bearing on their order of effectiveness for synergism when incubated in combination with C-6 analogs. These studies also indicated that synergism of adipocyte protein kinase could be readily demonstrated as long as the sum of the individual analog effects on the activity ratio did not exceed about 50% of the maximal activity ratio change. This is in agreement with previous studies on the synergism of activation of rat heart type I and bovine heart type II protein kinase (15).

It had previously been shown that 8-thio-cAMP was considerably better in combination with C-6 analogs for binding to and activation of type II than for type I protein kinase (14, 15). However, in results not shown, 8-thio-cAMP was relatively poor when tested for lipolysis with these C-6 analogs although 8-thio-cAMP acted more synergistically with *N*<sup>6</sup>-carbamoylpropyl- than with *N*<sup>6</sup>-benzoyl-cAMP. The inability of 8-thio-cAMP to show strong synergism in combination with C-6 analogs in the isolated adipocyte system or in the adipocyte protein kinase activation studies is not totally understood but may suggest differences in the type II kinases from heart and adipose tissue. Differences in these enzymes have already been suggested (9).

Other studies comparing synergism of glycerol release and synergism of protein kinase activation were also carried out using *N*<sup>6</sup>-butyryl-, *N*<sup>6</sup>-carbamoylpropyl-, and *N*<sup>6</sup>-benzoyl-cAMP in combination with the Site 1-selective analog 8-aminomethyl-cAMP and the Site 2-selective analog 8-thio-benzyl-cAMP. The combination of 8-aminomethyl-cAMP with each of the C-6 analogs did not produce synergism of lipolysis. However, 8-thio-benzyl-cAMP produced synergism quotients between 3.2 and 3.6 when incubated in combination with C-6 analogs. These data were consistent with the experiments carried out measuring protein kinase activity ratios. In each case, the synergism produced with the C-6 analog in combination with 8-thio-benzyl-cAMP was 1.5–1.7 times greater than when C-6 analogs were paired with 8-aminomethyl-cAMP. Again, the overall correlation of lipolysis and protein kinase activation in these experiments is unmistakable.

#### DISCUSSION

The activation of protein kinase by cAMP is a critical step in the process of hormone-stimulated cascade amplification. Koshland *et al.* (23) have defined two forms of signal amplification. Magnitude amplification is characterized by the production of a large number of output molecules (*e.g.* free fatty acids released from adipose tissue) generated by a small number of stimulus molecules (*e.g.* hormone). Sensitivity amplification, on the other hand, is characterized by a greater percentage change in response than expected from the percentage change in stimulus. Studies presented here support other work (12–15) and provide proof that protein kinase activation is an important step in sensitivity amplification. Briefly, it has been established that the positive cooperativity in cyclic nucleotide binding is reflected by cooperativity in

protein kinase activation (15). In the present studies, the synergistic activation of protein kinase is well correlated with a synergistic lipolytic response when isolated protein kinase or adipocytes are incubated in the presence of a combination of a Site 1-selective and a Site 2-selective cAMP analog.

Data presented here not only demonstrate that the protein kinase is the mediator of the cyclic nucleotide analog synergistic effect on lipolysis, but also suggest that it is the type II isozyme which is responsible for the lipolytic effect. For both protein kinase activation and lipolysis, the synergism was greater when a C-6 analog was incubated in combination with a C-8-thio analog than when incubated with a C-8-amino analog, a type II protein kinase response (15). Furthermore, the pattern of selective synergism of lipolysis was closely correlated with the same pattern of synergism of type II protein kinase activation.

The efficacy of analogs as protein kinase agonists in intact cells or in intact organisms is dependent upon the concentration of analog at its site of action. The lipolytic efficacy of all the analogs tested appears to be explained by consideration of at least two of three basic analog properties: high lipophilicity, low  $K_a$  for protein kinase activation, and stability to low  $K_m$  phosphodiesterase hydrolysis. For example, some of the most effective lipolytic agents generally have relatively high  $K_a$  values for protein kinase activation, but have favorable partition coefficients and are relatively resistant to the phosphodiesterase. On the other hand, relatively ineffective lipolytic agents have lower  $K_a$  values but tend to have unfavorable partition coefficients and are less resistant to the phosphodiesterase. Analogs which have either excessively high or low partition coefficients are not so effective lipolytic agents as might be expected from their  $K_a$  values. This could be due to their inability to cross the cell membrane in the case of a low partition coefficient or the inability to partition out of the membrane or the lipid droplet in the case of a high partition coefficient. Although these conclusions seem sound, we have not presently ruled out that some analogs may be metabolized by a mechanism other than the phosphodiesterase(s) to a more effective or a less effective lipolytic agent.

The strong correlation between synergism of lipolysis and synergism of protein kinase activation, in addition to the synergism of binding (14, 15) and type II protein kinase activation previously demonstrated (15), leaves little doubt that protein kinase, and not any other mechanism, is responsible for the synergistic effect of analogs on lipolysis. However, a discussion of other possible mechanisms is presented. The presently available data argue against phosphodiesterase. The analog specificity of the high  $K_m$  phosphodiesterase has not been studied but the effects of cAMP analogs on the low  $K_m$  phosphodiesterase, which is believed to play a major role in the hydrolysis of intracellular cAMP (19, 24), have been investigated. It is difficult to explain the synergism based on analog effects on this enzyme. Although the synergism of lipolysis and protein kinase activation occur over a wide range of concentrations, this synergism is readily seen at low analog concentrations, almost certainly below concentrations required for phosphodiesterase inhibition. In addition, since the best lipolytic agents, *N*<sup>6</sup>-carbamoylpropyl- and *N*<sup>6</sup>-benzoyl-cAMP, have  $I_{50}$  values 492 and 45 times higher than their  $K_a$  values, it seems highly unlikely that the phosphodiesterase would significantly contribute to the analog-stimulated lipolysis. Some of the data presented here also argue against a role for the adenosine receptor in the synergism of lipolysis. Fain and Malbon (25) have demonstrated that adipose tissue is normally under tonic adenosine inhibition, causing adenylate cyclase and lipolysis to be less responsive to hormone stimu-

lation. The experiments here were carried out at relatively high adipocyte concentrations where adenosine inhibition of adenylate cyclase was essentially complete. This conclusion was supported since analog-stimulated lipolysis was not inhibited by  $N^6$ -phenylisopropyladenosine; therefore, under these conditions, endogenous cAMP formation was minimal and the likelihood of endogenously generated cAMP due to an adenosine receptor mechanism was remote. It is possible, however, that if the adenylate cyclase inhibition were released by some adenosine antagonist which could be generated or introduced as a contaminant of the analog preparation the endogenous cAMP levels could increase. This does not appear to be the mechanism of the synergism of lipolysis since IBMX, an adenosine receptor antagonist (26), does not prevent the lipolytic synergism due to  $N^6$ -benzoyl- and 8-bromo-cAMP (data not shown).

There are several possible explanations for the concave-upward dose-response effects of analogs on lipolysis. One possibility is that low concentrations of the analog activate one protein kinase isozyme and higher concentrations activate the other type. This seems unlikely in view of the fact that type I is very low, or absent, in the adipocyte. It has not been totally ruled out that high concentrations of these analogs inhibit either the phosphodiesterase or the adenosine receptor and endogenous cAMP contributes to the concave-upward dose-response curves.

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**Two classes of cAMP analogs which are selective for the two different cAMP-binding sites of type II protein kinase demonstrate synergism when added together to intact adipocytes.**

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