Steroidogenic and Lipolytic Activities of 8-Substituted Derivatives of Cyclic 3′,5′-Adenosine Monophosphate*

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ABSTRACT: Thirteen eight-substituted derivatives of cyclic 3′,5′-adenosine monophosphate (cAMP) were examined for their ability to activate the processes of steroidogenesis in the isolated rat adrenal cell and lipolysis in the rat epididymal lipocyte. cAMP, N^6,2′-*O*-dibutyryl-cAMP, and most of the 8-substituted derivatives were capable of stimulating the cells to the same level of activity produced by either epinephrine or adrenocorticotropin in the lipocyte, or adrenocorticotropin in the adrenal cell. Stimulatory potencies of the derivatives were measured from the concentrations required to produce half-maximal activation of steroidogenesis or lipolysis. Twelve of the eight-substituted derivatives were more potent activators than cAMP, and three compounds, the 8-methylthio, 8-bromo, and 8-hydroxy derivatives, exceeded the potency of N^6,2′-*O*-dibutyryl-cAMP in both types of cells. Although the compounds were generally more potent in the adrenal cell than in the lipocyte, their steroidogenic:lipolytic potency ratios varied from 0.66 for 8-mercapto-cAMP to 23 for 8-dimethylamino-cAMP, indicating a degree of cellular specificity in their stimulatory actions. Comparisons between structure–activity relationships in this study and those in others reports suggest that steroidogenic and lipolytic activities of the eight-substituted derivatives result from their function as alternate activators of cAMP-dependent protein kinases of the adrenal cell and lipocyte.

Cyclic 3′,5′-adenosine monophosphate (cAMP[1]) has occupied an increasingly central position as an intracellular mediator of the actions of many hormones (Robison *et al.*, 1968). In a number of instances, the mechanism of action of cAMP has been shown to involve the activation of kinases that catalyze the phosphorylation, with consequent alterations in activity, of specific cellular proteins. Walsh *et al.* (1968) first reported the isolation of a cAMP-dependent protein kinase from rabbit skeletal muscle, and similar kinases were detected subsequently by Kuo and Greengard (1969) in a wide variety of tissues from many animal species; the latter authors proposed that activation of kinases may serve as a common mechanism of all cAMP actions. Recent work from several laboratories (Gill and Garren, 1970; Tao *et al.*, 1970; Kumon *et al.*, 1970; Reimann *et al.*, 1971) has shown that activation of kinases by cAMP involves the binding of the nucleotide to a regulatory subunit of the enzyme, with a consequent dissociation of the enzyme to an activated catalytic subunit and a cAMP–regulatory subunit complex.

The synthesis of biologically active derivatives of cyclic AMP (Posternak *et al.*, 1962; Falbriard *et al.*, 1967) was initiated with a series of analogs bearing substituents at the N^6 and 2′-O positions on the molecule. Although these derivatives were generally less potent than cAMP when assayed for the ability to activate glycogen phosphorylase in cell-free extracts from dog liver, most were considerably more potent than cAMP when examined for the same activity in dog liver slices (Henion *et al.*, 1967). More recently, the active derivatives of cAMP have been extended to include eight-substitution of the purine ring (Michal *et al.*, 1970a; Muneyama *et al.*, 1971; Posternak *et al.*, 1971). Biological activities reported for the eight-substituted cAMP derivatives have included activation of cAMP-dependent protein kinase (Muneyama *et al.*, 1971), activation of glycogen phosphorylase (Michal *et al.*, 1970a) and release of anterior pituitary hormones (Posternak *et al.*, 1971). The eight-substituted derivatives also have been shown to inhibit cyclic nucleotide phosphodiesterase (Muneyama *et al.*, 1971; D. N. Harris and M. Chasin, 1971, unpublished data) the enzyme catalyzing the hydrolysis of cAMP in tissue.

In this paper we report an investigation of the biological activities, at the cellular level, of a series of eight-substituted derivatives of cAMP. The cAMP-mediated cellular processes chosen for study were adrenal secretion of corticosterone and lipolysis in adipose tissue. The respective processes were measured in suspensions of isolated cells prepared by collagenase treatment of rat adrenal glands and rat epididymal fat pads.

Experimental Section

Adrenal Steroidogenesis. Suspensions of rat adrenal cells were prepared by the technique of Kloppenborg *et al.* (1968), as modified in our laboratory (Rivkin and Chasin, 1971). Decapsulated adrenal quarters from male Sprague–Dawley rats were suspended in a buffer of Krebs–Ringer bicarbonate–albumin–glucose (KRBAG) (pH 7.4) prepared according to DeLuca and Cohen (1964) and containing bovine albumin (3 g/100 ml) and glucose (0.2 g/100 ml). Collagenase (5 mg/ml) was added to quarters of 32 adrenals in 10 ml of KRBAG. The tissue was digested for 1 hr at 35°, under 95% O_2–5% CO_2, in a New Brunswick gyrotory bath oscillating at 120 cycles/min. After digestion, the tissue was gently dispersed by repeated passage through a Pasteur pipet. The suspended cells were collected by centrifugation at 4° for 10 min at 480*g*, followed by two cycles of washing and recentrifugation in the original volume of KRBAG. The washed cell pellet was then resuspended in KRBAG (1 adrenal/ml) and filtered

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[1] Abbreviations used are: cAMP, cyclic 3′,5′-adenosine monophosphate; KRBAG, Krebs–Ringer bicarbonate–albumin–glucose buffer; KRPA, Krebs–Ringer phosphate–albumin buffer; dibutyryl-cAMP, N^6,2′-*O*-dibutyryl-cAMP; ACTH, adrenocorticotropic hormone.

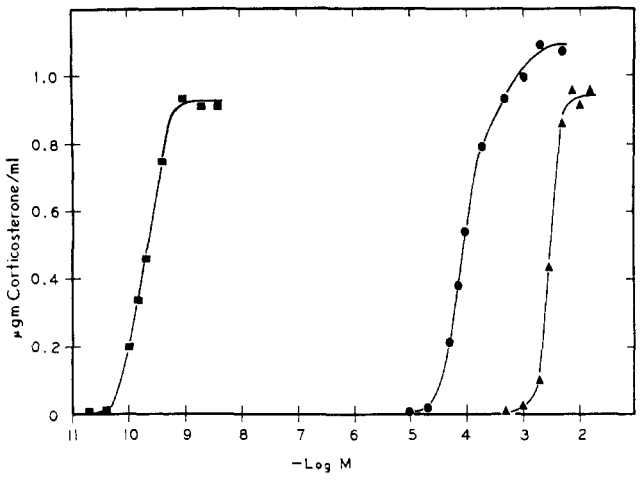

FIGURE 1: Corticosterone secretion by rat adrenal cells in response to ACTH (■), dibutyryl-cAMP (●) and cAMP (▲). All points represent individual incubations within a single experiment.

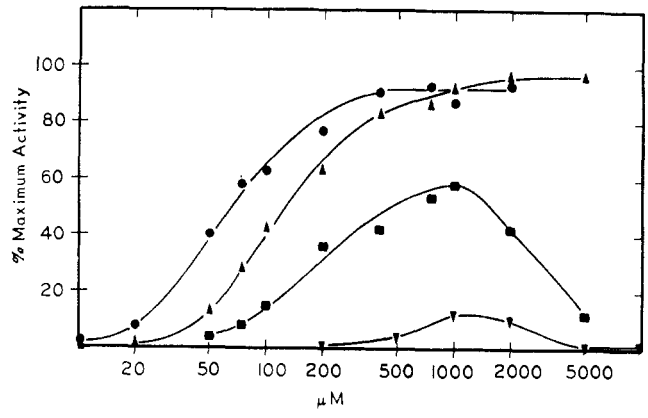

FIGURE 2: Per cent of maximum activation of steroidogenesis in rat adrenal cells in response to 8-methylthio-cAMP (●), 8-dimethylamino-cAMP (▲), 8-methylamino-cAMP (■), and 8-(2-hydroxyethyl)amino-cAMP (▼). Each curve is from an experiment that included multiple concentrations of the derivative and dibutyryl-cAMP. Individual points represent single incubations, and are expressed in per cent of the maximum response to 1000–2000 μM dibutyryl-cAMP.

through a stainless steel sieve with perforations of 0.2 mm, in order to remove any large particles of undigested tissue.

Incubations of cells and determinations of corticosterone were conducted essentially as described previously (Rivkin and Chasin, 1971), with minor modifications. Incubations were conducted at 35° for 2 hr, under 95% O_2–5% CO_2. Each 2.5-ml incubation mixture contained 1 ml of adrenal cell suspension. The net corticosterone secretion for each incubation mixture was determined by subtraction of the fluorescence of a nonstimulated adrenal cell blank. Apparent corticosterone secretion by such blanks did not exceed 0.02 μg/ml, and generally fell in the range of 0.5–3.0% of the maximum steroidogenic capacity of any given cell preparation.

Lipolysis. Epididymal fat pads were obtained from male Sprague–Dawley rats that had been allowed access to food and water prior to sacrifice. After decapitation of the animals, isolated fat cell (lipocyte) suspensions were prepared from distal segments of the excised fat pads according to the method of Rodbell (1964). The buffer employed for the preparation and incubation of lipocytes was a Krebs–Ringer phosphate–albumin buffer (KRPA) (pH 7.4), prepared according to DeLuca and Cohen (1964), but containing one-half the specified concentration of $CaCl_2$, no glucose, and human albumin (2 g/100 ml) substituted for bovine albumin. Collagenase digestions were conducted using a ratio of 5 mg of collagenase per g of minced fat tissue. After three washes, the packed lipocytes were suspended in seven volumes of KRPA. Aliquots (1 ml) of lipocyte suspension, up to 0.1 ml of test substance, and KRPA to a final volume of 2.5 ml were added to plastic scintillation counting vials. The capped vials were incubated at 37° for 1 hr in a New Brunswick Gyrotory bath oscillating at 160 cycles/min. After incubation, the reactions were terminated by the addition of 0.5 ml of 3 M $HClO_4$. The vials were vigorously agitated and then centrifuged for 15 min at 2000 rpm in an International Model 5 centrifuge. The infranatant fractions were decanted into sample cups and assayed for glycerol in a Technicon Auto-Analyzer coupled to an Aminco-Bowman spectrophotofluorometer (Ko and Royer, 1968). This automated fluorimetric procedure measures conversion of oxidized NAD^+ into NADH during the concomitant conversion of glycerol into dihydroxyacetone phosphate, in the presence of glycerol kinase, ATP, and glycerol 3-phosphate dehydrogenase; the method allows quantitation of glycerol in subnanomole quantities.

Materials. The following materials were obtained from the indicated sources: bovine albumin (fraction V), Miles Laboratories, Inc., human albumin (fraction V), E. R. Squibb and Sons, Inc., collagenase (Code CLS), Worthington Biochemical Corp.; glycerol kinase and glycerol 3-phosphate dehydrogenase (EGAQ), Boehringer Mannheim Corp.; *l*-epinephrine bitartrate, Calbiochem; cAMP, Research Plus Laboratories, Inc., sodium dibutyryl-cAMP, Zellstoffefabrik Waldhof (West Germany) and Calbiochem. Experiments with ACTH were performed using a preparation purchased from Sigma Chemical Co. Potency of the Sigma ACTH preparation was estimated at 112 IU/mg by comparison to the Third International Working Standard for Corticotrophin, obtained from the World Health Organization (Bangham *et al.*, 1962). The synthetic eight-substituted cyclic nucleotides were provided by Dr. R. Robins, International Chemical and Nuclear Corp. All cyclic nucleotides were dissolved in water immediately prior to use, and were titrated to neutrality before addition to incubation mixtures.

Results

Stimulation of Adrenal Steroidogenesis. Figure 1 illustrates the steroidogenic response of rat adrenal cell preparations to ACTH, to cAMP, and to a commonly employed active derivative of the latter, N^6,2′-*O*-dibutyryl-cAMP. It is evident from the experiment shown in the figure that the two nucleotides elicited similar maximum levels of corticosterone secretion, although dibutyryl-cAMP was approximately 35 times as potent as cAMP, in terms of the concentration required to produce half-maximal stimulation. Both cyclic nucleotides, moreover, elicited approximately the same maximum response as that produced by stimulation of the cells with ACTH.

Thirteen eight-substituted derivatives of cAMP were examined for their ability to stimulate adrenal steroidogenesis as a function of concentration. To allow comparison of the concentration–response curves obtained from different experiments, dibutyryl-cAMP was included in each experiment

TABLE I: Activation of Steroidogenesis and Lipolysis by Eight-Substituted Derivatives of cAMP.

Compound	Adrenal A_{50} (μM)	Lipocyte A_{50} (μM)
cAMP	3300	8,500
Dibutyryl-cAMP	95	500
8-SCH_3-cAMP	65	180
8-Br-cAMP	85	440
8-N_3-cAMP	85	1,400
8-OH-cAMP	90	260
8-SC_2H_5-cAMP	110	230
8-$N(CH_3)_2$-cAMP	130	3,000
8-SCH_2CH_2OH-cAMP	150	850
8-NH_2-cAMP	150	1,200
8-OCH_3-cAMP	150	1,600
8-$SCH_2C_6H_5$-cAMP	190	1,400
8-SH-cAMP	380	250
8-$NHCH_3$-cAMP	460	3,300
8-$NHCH_2CH_2OH$-cAMP	Inactive[a]	14,000

[a] The greatest activation produced by this derivative was to 12% of maximum activity (see Figure 2).

as a reference compound. Dibutyryl-cAMP consistently produced a maximum steroidogenic response at concentrations of 1000–2000 μM; the maximum activity for the cell preparation used in each experiment was consequently defined as the greatest steroidogenic response to dibutyryl-cAMP in this concentration range. Stimulatory potency of each cAMP derivative was subsequently measured in terms of the "A_{50}" concentration, defined as that interpolated concentration that stimulated a cell preparation to 50% of maximum activity. In a series of 11 experiments, dibutyryl-cAMP gave A_{50} values ranging from 80 to 115 μM (mean ± SE = 95 ± 3 μM).

Representative concentration–response curves for a group of four eight-substituted cyclic AMP derivatives are shown in Figure 2. Two of the compounds, 8-methylthio-cAMP and 8-dimethylamino-cAMP, reached an activity plateau equivalent to that produced by dibutyryl cyclic AMP, and yielded A_{50} concentrations of 65 and 130 μM, respectively. A third compound, 8-methylamino-cAMP, demonstrated an A_{50} of 460 μM, but stimulated the cell preparation to only 58% of maximum activity. The fourth derivative, 8-(2-hydroxyethyl)-amino-cAMP, was essentially inactive, producing only 12% of the maximum response at 1000 μM. The remaining nine eight-substituted derivatives, like 8-methylthio- and 8-dimethylamino-cAMP, produced maximum activation of the cells. The plateau stimulations elicited by these 11 compounds fell within the range of 82–114% of that produced by dibutyryl-cAMP at its optimal concentration. The A_{50} values determined for the eight-substituted derivatives, as well as those of cAMP and dibutyryl-cAMP, are summarized in Table I.

Stimulation of Lipolysis. The lipolytic responses of epididymal lipocyte suspensions to ACTH, epinephrine, cAMP, and dibutyryl-cAMP are shown in Figure 3. Each of these agents elicited a similar maximum lipolytic activity, although the effective concentrations varied for each agent. As in the adrenal cells, the stimulatory potency of cAMP was considerably less than that of dibutyryl-cAMP; half-maximal stimulation by cAMP and dibutyryl-cAMP occurred at concentrations of 8500 and 500 μM, respectively.

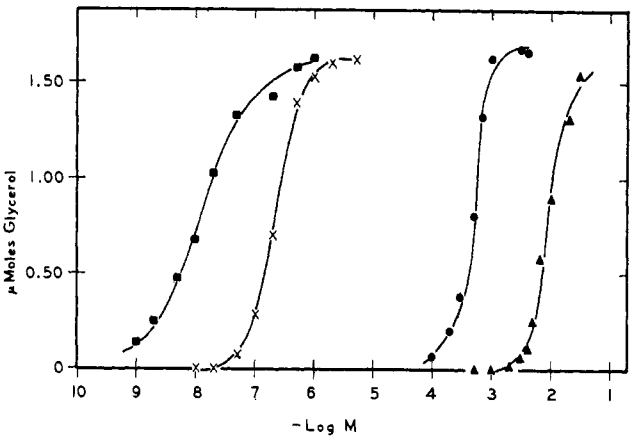

FIGURE 3: Glycerol production by rat epididymal lipocytes in response to ACTH (■), epinephrine (×), dibutyryl-cAMP (●), and cAMP (▲). All points represent individual incubations within a single experiment.

The same group of eight-substituted derivatives of cAMP that was tested for adrenal steroidogenic activity was also subjected to concentration–response studies in the lipocyte preparations. At the highest concentrations employed in the tests, the majority of the derivatives produced plateau values of maximum activity equivalent to those produced by ACTH, epinephrine, cAMP, or dibutyryl-cAMP. For the lipocyte preparation used in each experiment, the maximum lipolytic activity was defined as the average of the maximum activity plateaus of at least three stimulatory compounds. The concentration (A_{50}) producing half-maximal stimulation was then interpolated from a plot of per cent maximum activity *vs.* concentration for each derivative. Figure 4 shows representative concentration–response plots for three eight-substituted derivatives, 8-mercapto-, 8-(2-hydroxyethyl)thio-, and 8-(2-hydroxyethyl)amino-cAMP, yielding respective A_{50} con-

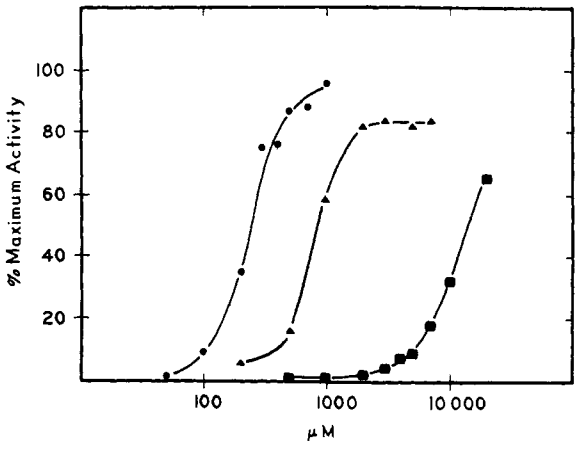

FIGURE 4: Per cent of maximum activation of lipolysis in rat epididymal lipocytes in response to 8-mercapto-cAMP (●), 8-(2-hydroxyethyl)thio-cAMP (▲), and 8-(2-hydroxyethyl)amino-cAMP (■). Each curve is from an experiment that included at least three compounds that produced a plateau of maximum response. Individual points represent single incubations, and are expressed in per cent of the average maximum activity obtained within that particular experiment.

centrations of 250, 850, and 14,000 μM. The lipolytic A_{50} concentrations of the cAMP derivatives are summarized in Table I.

Structure–Activity Correlations. Most of the thirteen eight-substituted derivatives of cAMP compared in Table I were many times more potent than cAMP as stimulators of steroidogenesis and lipolysis, displaying potencies up to 50-fold that of cAMP. In both adrenal cells and lipocytes, only 8-(2-hydroxyethyl)amino-cAMP was less potent than the unsubstituted nucleotide. Several of the eight-substituted derivatives were also more potent than dibutyryl-cAMP in both cell types. One group of compounds, including the 8-hydroxy, 8-methylthio, 8-ethylthio, and 8-bromo derivatives, displayed relatively high potency in both adrenal cells and lipocytes. In general, however, the derivatives were more potent in the adrenal cell than in the lipocyte; two extreme examples were 8-azido- and 8-dimethylamino-cAMP, with respective steroidogenic:lipolytic potency ratios of 16 and 23. One exception, 8-mercapto-cAMP, was more potent in the lipocyte, displaying a steroidogenic:lipolytic potency ratio of 0.66. Derivatives of cAMP containing a sulfur atom at the 8 position generally displayed high steroidogenic and lipolytic potency, in some instances (*e.g.*, methylthio > methoxy > methylamino) considerably greater than that of the analogous nitrogen- and oxygen-containing substituents. Potent stimulatory activity of the derivatives persisted even when the eight-substituent consisted of a bulky aromatic group, as in the case of 8-thiobenzyl-cAMP.

Discussion

The role of cAMP as a hormone mediator in the adrenal cortex and in adipose tissue has been amply documented (Robison *et al.*, 1968). Our observations that cAMP elicits a maximum response equal to that of ACTH or epinephrine in the lipocyte, and to that of ACTH in the isolated adrenal cell, are consistent with this mediating role. In both cell systems, moreover, it is evident that dibutyryl-cAMP and other 3′,5′-cyclic nucleotides are capable of stimulating the cells to the same maximum activity produced by direct hormonal stimulation. The observation that dibutyryl-cAMP and ACTH produce equal maximal rates of corticosterone secretion was also reported recently for an adrenal cell preparation obtained by a trypsin digestion technique (Sayers *et al.*, 1971). Existence of a common maximum response to various cAMP derivatives has allowed direct comparison of their potencies in terms of the concentrations required to produce half-maximal activation.

A previous investigation of the specificity of activation of steroidogenesis by cAMP in isolated adrenal cells showed a decrease of activity when nucleotides other than adenylate were used, as well as an absolute dependence on the presence of the 3′,5′-phosphodiester structure (Rivkin and Chasin, 1971). Perhaps the most striking observation resulting from comparison of the stimulatory potencies of cAMP and the thirteen eight-substituted derivatives was that eight-substitutions nearly always increased the potencies relative to that of cAMP. Similar increases in biological activity were observed (Posternak *et al.*, 1971) with a series of five eight-substituted cAMP derivatives in an assay of the release of growth hormone from isolated rat anterior pituitary tissue. Substitutions at the N^6 or 2′-O positions of cAMP (Henion *et al.*, 1967; Posternak *et al.*, 1969; Cehovic *et al.*, 1970) have also generally led to increased biological activity in intact tissue preparations. Assay of the N^6- or 2′-O-substituted derivatives in cell-free systems, in contrast, has shown activities (Posternak *et al.*, 1962) lower than that of cAMP. Similarly, in comparing the eight-substituted derivatives of cAMP, Muneyama *et al.* (1971) demonstrated that although the compounds were generally active at levels of 0.01 μM as activators of the protein kinase of bovine brain, none was more than 1- to 2-fold as effective as cAMP. One oft-proposed explanation for these discrepancies in relative potency in cell-free *vs.* cellular systems has been the possibility that the derivatives penetrate cell membranes more readily than does cAMP (Robison *et al.*, 1968); a second is that they may be resistant to hydrolysis by cAMP phosphodiesterase, a situation that has been confirmed in the case of the eight-substituted derivatives (Michal *et al.*, 1970b; Muneyama *et al.*, 1971).

Although the eight-substituted derivatives of cAMP are highly potent activators of cAMP-dependent protein kinase (Muneyama *et al.*, 1971), their activity as inhibitors of cyclic nucleotide phosphodiesterase (D. N. Harris and M. Chasin, 1971, unpublished data) raises the question of their mechanism of action at the cellular level. Three lines of evidence suggest that the derivatives exert a primary effect *via* protein kinase activation, rather than through inhibition of phosphodiesterase: (1) twelve of the thirteen eight-substituted derivatives examined in this study demonstrated protein kinase activation, over the concentration range of 1 to 100 μM, similar to that of cAMP (Muneyama *et al.*, 1971) (the single exception, 8-(2-hydroxyethyl)-amino-cAMP, showed less than 20% of the kinase activation shown by cAMP, and was also the only derivative that demonstrated activity less than that of cAMP in the adrenal cells and lipocytes); (2) two compounds that displayed little or no inhibitory activity against cyclic nucleotide phosphodiesterase, 8-hydroxy-cAMP and 8-dimethylamino-cAMP (D. N. Harris and M. Chasin, 1971, unpublished data) were potent stimulators of steroidogenesis and lipolysis; and (3) it has been observed (C. A. Free and M. Chasin, 1971, unpublished data) that inhibitors of cyclic nucleotide phosphodiesterase that potentiate steroidogenic and lipolytic responses of cells to cAMP produce little or no effect when acting alone. These observations were made with theophylline in adrenal cells, and with SQ 20,009, a highly potent new phosphodiesterase inhibitor with the structure 1-ethyl-4-(isopropylidenehydrazino)-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxylic acid ethyl ester·HCl (Chasin, 1971; Free *et al.*, 1971), in adrenal cells and lipocytes. The eight-substituted derivatives of cAMP, in contrast to the above phosphodiesterase inhibitors, produced maximum stimulation of adrenal cells or lipocytes in the absence of cAMP.

Our studies indicate the likelihood that various eight-substituted derivatives of cAMP will be more effective than cAMP in simulating the effects of cAMP-mediated hormones at the cellular level. Comparison of the effects of the compounds in two cell types has also indicated the possibility of finding derivatives that act selectively on specific cells or tissues. The 8-mercapto derivative of cAMP, for example, was more effective in the lipocyte than in the adrenal cell, whereas the 8-dimethylamino derivative demonstrated a steroidogenic:lipolytic potency ratio greater than 20. Although the primary mechanism of action of the eight-substituted derivatives appears to be *via* alternate activation of intracellular protein kinases, it is likely that this activity may be augmented *via* the preservation of intracellular cAMP, in the cases of those derivatives that also function as inhibitors of phosphodiesterase. Such bimodal activity would be of particular significance *in vivo*, or under conditions in which

hormonal stimulation results in the formation of intracellular cAMP.

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