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Rat hepatocyte protein kinase was activated by incubating the cells with various cAMP analogs. Boiled extracts were then prepared and Sephadex G-25 chromatography was carried out. The G-25 procedure separated the analogs from cAMP since the resin had the unexpected property of binding cyclic nucleotides with differing affinities. Separation was necessary because the analogs would otherwise interfere with the sensitive protein kinase activation method developed for assay of cAMP. The cAMP analogs, but not 5'-AMP, lowered basal cAMP by 50–70%. The effect was rapid, analog concentration-dependent, and occurred parallel with phosphorylase activation, suggesting that the cAMP analogs act through cAMP-dependent protein kinase activation. A cAMP analog completely blocked the cAMP elevation produced by relatively low concentrations of glucagon, but did not block the phosphorylase response, indicating that the cAMP analog substitutes for cAMP as the intracellular activator of protein kinase. One implication of the results is that elevation of cAMP and protein kinase activity by hormones has a negative feedback effect on the cellular cAMP level.

There are present in biological systems certain processes which amplify hormonal effects. A few molecules of glucagon, for example, can cause thousands of glucose molecules to be produced in liver. However, in order for such amplification to be effective, each step involved must not be excessive. For example, changes in cellular cAMP levels usually occur within a very narrow concentration range, physiological processes being maximally stimulated by increases in cAMP of only 2–3-fold (1–3). If the cAMP concentration were to be elevated to very high levels relative to the physiological range, reversal of the stimulatory processes would be difficult. In order that such excessive changes do not occur, "leak" systems are also present for dampening the amplification (4). Phosphodiesterases and protein phosphatases are two types of leak systems in the reactions of the cAMP cascade. These enzyme activities are increased because of increases in substrate levels. They serve, on the one hand, to dampen the increase in cAMP, and, on the other hand, to dephosphorylate, or deactivate, the protein phosphorylation reactions. Cells have also evolved mechanisms to "desensitize," "down-regulate," or produce "refractoriness" after chronic exposure to hormones which elevate cAMP (1, 5–8). These in some cases involve uncoupling of the hormone receptor from the adenylate cyclase. In addition to long-term desensitization effects, there may exist feedback mechanisms for acutely dampening the hormonal effect on cAMP levels. A convenient route of such control would be through activation of cAMP-dependent protein kinase, which thus far has been the only receptor for cAMP shown to be present in higher eukaryotes. It would be difficult to study a dampening effect of kinase activation on cAMP levels by the use of hormones. However, such a study can be accomplished by using cAMP analogs to bypass the hormone-adenylate cyclase steps. Analogs of cAMP are known to enter cells and specifically activate cAMP-dependent protein kinase (9). This report presents a simple method for carrying out purification and assay of cAMP after treatment of hepatocytes with cAMP analogs. Using phosphorylase activation as an indicator of protein kinase activation, it is shown that intracellular stimulation of the kinase dampens the hormonal elevation of cAMP in hepatocytes.

METHODS

Preparation and Incubation of Hepatocytes—Hepatocytes from male Sprague-Dawley rats (200–250 g of body weight) were prepared as previously described (10). Cyclic AMP analogs were added to cell suspensions (~50 mg of cells/ml) in 25-ml Erlenmeyer flasks at 37 °C which were continuously gassed with $O_2:CO_2$ (19:1). At various times aliquots were removed for the measurement of phosphorylase or cAMP. All cAMP analog stock solutions were 100-fold more concentrated than the desired final concentration in the hepatocyte suspensions.

Preparation of Extracts and Assay of Phosphorylase—At the end of the hepatocyte incubations, 500-µl aliquots of the cell suspensions were removed, immediately frozen in liquid nitrogen, and stored at −20 °C. The pellets were thawed at room temperature for 10 min. One ml of homogenizing buffer containing 150 mM β-glycerophosphate, 30 mM EDTA, 150 mM NaF, 600 mM sucrose, and 6.5 mg/ml L-cysteine was added, and the suspension was homogenized for 10 s using an Ultraturrex Tissuemizer®. Homogenates were immediately assayed for phosphorylase activity in the absence of 5'-AMP (10).

Preparation of Boiled Extracts for Sephadex G-25 Purification of cAMP—Four ml of cell suspension was centrifuged at 500 × g, the supernatant was removed, and the cell pellet was frozen in liquid nitrogen and stored at −70 °C. Frozen cell pellets were removed from −70 °C and thawed at room temperature for 3–5 min. One ml of ice-cold homogenizing buffer (25 mM NH_4HCO_3, pH 7.8, 1 mM 3-isobutyl-1-methylxanthine, and 20 mM EDTA) was added, and the suspension was placed on ice. The partially thawed pellets were homogenized for 10 s using an Ultraturrex Tissuemizer®. The entire homogenate was placed in a 1.5-ml microfuge tube and placed in a boiling water bath for 5 min. The boiled homogenates were centrifuged for 7 min in a Beckman Microfuge B. The boiled extract was carefully removed and chilled on ice before purification of cAMP by Sephadex G-25 chromatography of 0.5-ml aliquots as described under "Results."

Assay of cAMP—The type I cAMP-dependent protein kinase used for the assay was purified from rabbit skeletal muscle through the DEAE-cellulose step (11). The enzyme was frozen and stored at −70 °C in small aliquots which were thawed immediately before use by hand-warming. Protein kinase assays were performed at different times and temperatures as shown in Fig. 3. To 20 µl of reaction

mixture containing 40 mM Tris-HCl, pH 7.4, 0.1 mM 3-isobutyl-1-methylxanthine, 130 μM heptapeptide (Kemptide), 20 mM magnesium acetate, and 0.2 mM [³²P]ATP (~100 cpm/pmol) was added 60 μl of fractions from the Sephadex G-25 chromatography or cAMP standards diluted in the same 50 mM NH₄HCO₃, pH 7.8. Reactions were initiated with 20 μl of enzyme diluted to 0.33 nM with 10 mM potassium phosphate, pH 6.8, 1 mM EDTA, and 1 mg/ml bovine serum albumin. After incubation, 50-μl aliquots of the mixture were spotted on phosphocellulose papers which were washed in 75 mM phosphoric acid according to Roskoski (12).

In one experiment cAMP was also assayed by radioimmunoassay (13–15).

Purification of cAMP Analogs—The cAMP analogs which were used for cell incubations were first chromatographed on a 0.9 × 57-cm Sephadex G-25 (superfine) column as described in the legend to Fig. 1. The total volume (V_t) of the G-25 was taken as the elution volume of [³H]H₂O. The elution volume of a particular substance was denoted as V_e.

Materials—The heptapeptide substrate, Leu-Arg-Arg-Ala-Ser-Leu-Gly, was from Peninsula Laboratories. Sephadex G-25 (superfine) and gel filtration columns were purchased from Pharmacia. cAMP and cAMP analogs were from Sigma, and [³H]cAMP was from New England Nuclear. 8-pClϕS-cGMP[1] was synthesized by the method of Miller *et al.* (16). [γ-³²P]ATP was prepared by the method of Walseth and Johnson (17). Glucagon was a gift from Eli Lilly.

RESULTS

Sephadex G-25 Chromatography of Cyclic Nucleotides—Since most cyclic nucleotide analogs are known to interfere in biospecific assays or radioimmunoassays of cAMP (13–15, 18), it was necessary to devise a method for separating the analog from cAMP before assay. Various nucleotides are known to bind to Sephadex columns with different affinities (19). Many have elution volumes (V_e) greater than the total volume (V_t) of the columns. The Sephadex G-25 (superfine) elution profiles for several cyclic nucleotides are shown in Fig. 1. Of the four nucleotides chromatographed, only N^6-butyryl-cAMP eluted at less than the total volume as determined by [³H]H₂O elution. It can be seen that all three analogs were clearly separated from cAMP at this temperature (20 °C). It was found subsequently that even better separation could be obtained at 4 °C. For example, cAMP and cGMP were poorly separated at 20 °C, but were almost completely separated at 4 °C. The elution volume of [³H]H₂O (V_t) was not different at the different temperatures, but the V_e/V_t of cAMP changed from 1.35 at 20 °C to 1.62 at 4 °C. The relatively long column (0.9 × 57 cm) used for the experiment of Fig. 1 allowed for high resolution of cyclic nucleotides, but a shorter column (0.9 × 14 cm) at 4 °C was sufficient to separate most of the analogs and allowed for rapid (~2 h) processing of boiled hepatocyte extracts. Chromatography of a boiled hepatocyte extract on the short column at 4 °C is shown in Fig. 2. It can be seen that 8-pClϕS-cAMP separated well from cAMP. It can also be seen that cAMP was separated from almost all of the E_{260} absorbing material of the extract. The assayable cAMP of the extract coeluted with the trace amount of [³H]cAMP added to the extract. The small ³H peak preceding the [³H]cAMP peak could represent [³H]H₂O, since the V_e was identical to that of [³H]H₂O chromatographed in a different run. The method of Fig. 2 was adopted for almost all of the experiments, although the longer column was necessary for separating some analogs which had a V_e nearly the same as that of cAMP.

Assay of cAMP by Protein Kinase Activation—Because of the dilution which resulted from the Sephadex chromatography, it was necessary to develop a sensitive assay for cAMP. The use of protein kinase activation at 30 °C was reported previously (20). It can be seen in Fig. 3 that the kinase was

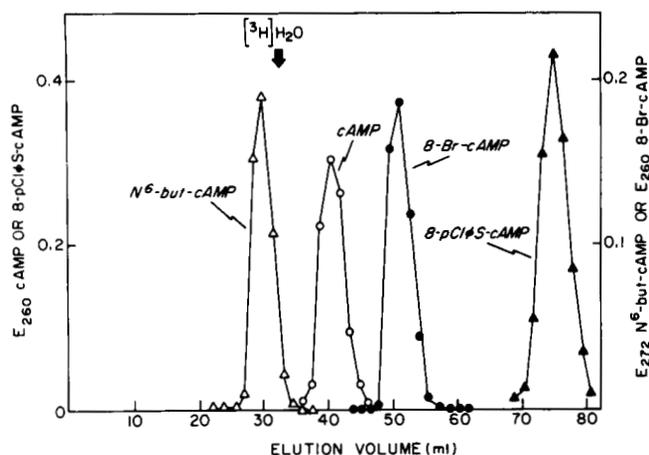FIG. 1. **Chromatography of cAMP analogs on Sephadex G-25.** Samples (0.5 ml of 100 mM N^6-butyryl-cAMP, 0.3 mM 8-pClϕS-cAMP, 20 mM 8-Br-cAMP, or 0.1 ml of 1 mM cAMP) were dissolved in H₂O and ~60 mg of sucrose was added to each and dissolved. They were then applied at 20 °C to a 0.9 × 57-cm Sephadex G-25 (superfine) column which had been equilibrated in 50 mM NH₄HCO₃, pH 7.8. Collection was by gravity flow with 60 cm of hydrostatic pressure. 1.5-ml fractions were collected. N^6-Butyryl-cAMP fractions were diluted 700-fold and 8-Br-cAMP samples were diluted 100-fold before determining absorbance.
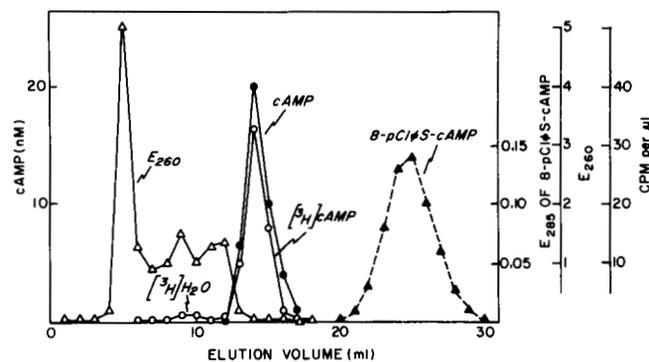

FIG. 2. **Chromatography of a crude hepatocyte extract on Sephadex G-25.** The hepatocyte extract was prepared as described under "Methods." Ten μl of 1.8 × 10⁻⁷ M [³H]cAMP (80,000 cpm) was added at 4 °C to 0.5 ml of extract. Solid sucrose (~60 mg) was then added and dissolved, and the sample was applied at 4 °C to a 0.9 × 14-cm Sephadex G-25 (superfine) column equilibrated in 50 mM NH₄HCO₃, pH 7.8. One-ml fractions were collected by gravity flow (50 cm of hydrostatic pressure). Fifty-μl aliquots were analyzed for radioactivity. Sixty-μl aliquots were used for cAMP determination as described under "Methods," and the E_{260} was measured spectrophotometrically. The elution of 60 μM 8-pClϕS-cAMP was determined in a separate chromatographic run performed identically and the profile is shown for comparison. The presence of the extract did not significantly alter the elution of 8-pClϕS-cAMP (not shown).

much more sensitive to cAMP at 0 °C than at higher temperatures, presumably because the affinity of cAMP binding increased. The K_a obtained at 0 °C (5 nM) was 16 times lower than that at 30 °C. In order to produce sufficient ³²P-heptapeptide at lower temperatures, it was necessary to incubate the reaction for longer times, an overnight incubation at 0 °C being convenient. The type I isozyme of rabbit skeletal muscle was used for the experiments reported here because it was found to be more sensitive to cAMP than was the bovine heart type II isozyme under the conditions used. It can be seen in Fig. 4 that the cAMP level determined using the G-25 fractions of hepatocyte extracts was similar whether performed by protein kinase activation assays or radioimmunoassays.

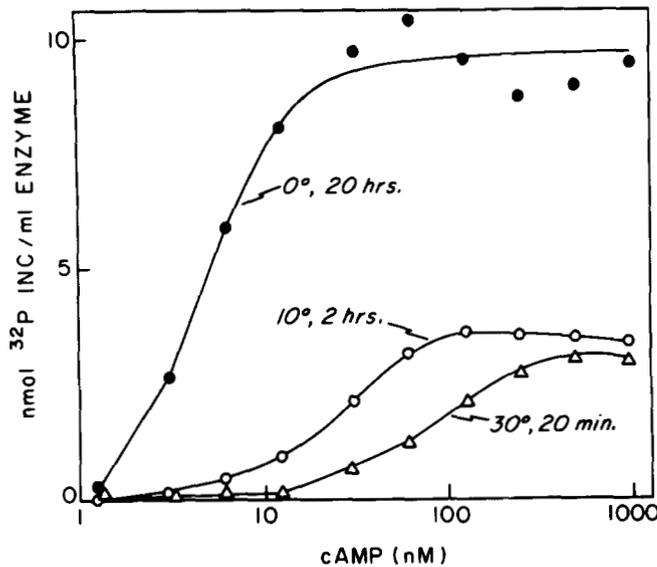[1] The abbreviation used is: 8-pClϕS-, 8-p-chlorophenylthio-.

FIG. 3. **Assay of cAMP by protein kinase activation at different temperatures.** The enzyme used was the type I isozyme of rabbit skeletal muscle. The temperature and times of incubation with various cAMP concentrations are indicated. Heptapeptide was used as substrate. Other conditions are stated under "Methods."
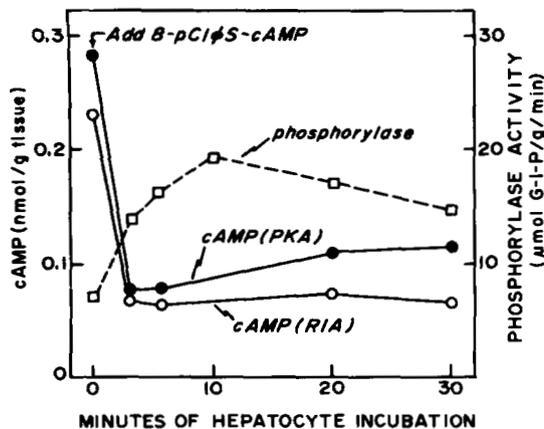

FIG. 4. **Effect of incubation time on 8-*p*ClϕS-cAMP lowering of hepatocyte cAMP levels and activation of phosphorylase.** Hepatocytes were incubated with 1 μM 8-*p*ClϕS-cAMP for the indicated times. Extracts were prepared and processed as described under "Methods." cAMP was assayed by radioimmunoassay (*RIA*) and by protein kinase activation (*PKA*) as indicated.

Effect of cAMP Analogs on Basal Hepatocyte cAMP—In order to ensure the absence of trace contamination of analogs by cAMP, the analogs were first purified on Sephadex G-25 columns before use in cell incubations. If purified 1 μM 8-*p*ClϕS-cAMP was added at zero time to hepatocytes, the cAMP level fell by more than 50% (Fig. 4). The effect occurred in 3 min or less, and the cAMP level was found to be lowered using both types of cAMP assays. Activation of phosphorylase occurred parallel with the lowering of cAMP. This result suggested that the intracellular concentration of 8-*p*ClϕS-cAMP was sufficient to activate cAMP-dependent protein kinase, ultimately causing phosphorylase activation. The effect of 8-*p*ClϕS-cAMP to lower hepatocyte cAMP was analog concentration-dependent. It can be seen in Fig. 5 that concentrations of 0.25 μM or higher of the analog were effective in lowering cAMP. The correlation between the lowering of cAMP and phosphorylase activation was good, except at 0.1 μM analog, where maximal phosphorylase activity was seen
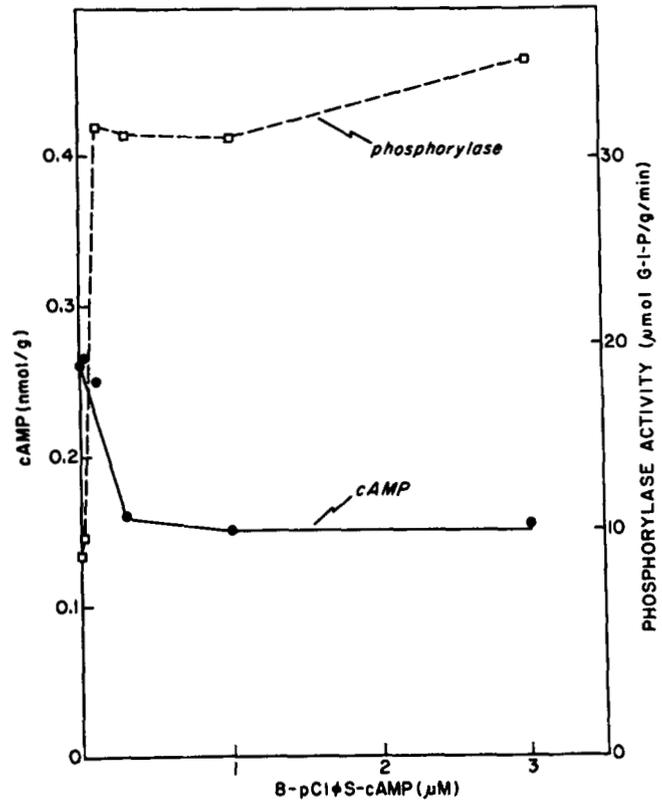

FIG. 5. **Effect of 8-*p*ClϕS-cAMP concentration on cAMP level and phosphorylase activity in hepatocytes.** Hepatocytes were incubated for 5 min with the indicated concentrations of 8-*p*ClϕS-cAMP. Extracts were prepared and processed as described under "Methods." *G-1-P*, glucose 1-phosphate.

with only a slight change in cAMP. It is known, however, that small increases in intracellular kinase activity are sufficient to cause maximal phosphorylase activation in hepatocytes (21). The cAMP-lowering effect was also seen with several cyclic nucleotide analogs other than 8-*p*ClϕS-cAMP (Table I, Experiments 1 and 2) but not with 5'-AMP (Table I, Experiment 3). A cGMP analog, 8-*p*ClϕS-cGMP, also caused lowering of cAMP, but it is known that cGMP analogs stimulate cAMP processes in hepatocytes (22). The lowering of cAMP obtained with cyclic nucleotide analogs was associated in each case with an activation of phosphorylase.

Effect of cAMP Analogs and Glucagon on Hepatocyte cAMP—The cAMP-lowering effect of 8-*p*ClϕS-cAMP was also obtained when hepatocytes were incubated with relatively low glucagon concentrations. It can be seen in Table I (Experiment 4) that 0.1 or 0.2 nM glucagon treatment elevated cAMP appreciably. These effects were completely blocked or nearly completely blocked, respectively, by the addition of 8-*p*ClϕS-cAMP. However, when 100 nM glucagon was used (Table I, Experiment 3), which generated very high cellular cAMP, the analog did not block the effect. A time course for the effect of 0.1 nM glucagon in the absence and presence of 8-*p*ClϕS-cAMP is shown in Fig. 6. It can be seen that the glucagon-induced elevation in cAMP was transient, the level rapidly increasing during the first 5 min and then declining toward the basal value by 10 min. The glucagon-induced elevation in cAMP did not occur when 8-*p*ClϕS-cAMP was present. Glucagon caused phosphorylase activation regardless of whether or not the cAMP analog was present. In the absence of analog, the phosphorylase activity declined slightly after 5 min of incubation.

TABLE I

Effects of cAMP analogs and glucagon on hepatocyte cAMP levels

Hepatocytes were incubated for 5 min. One μM cAMP analogs were added. Except where indicated, values are means of duplicate cell incubations. AHCM-, aminohexylcarbamoylmethyl-; G-1-P, glucose 1-phosphate.

| Experiment	Addition	cAMP	Phosphorylase
		nmol/g tissue	*μmol G-1-P/g/min*
1	0	0.69 ± 0.06 ($n = 5$)	7.7
	8-pClϕS-cAMP	0.37 ± 0.05 ($n = 3$)	21.3
	8-Br-cAMP	0.33 ± 0.08 ($n = 3$)	17.5
	8-pClϕS-cGMP	0.44 ± 0.08 ($n = 3$)	11.5
2	0	0.28	9.9
	8-NH-benzyl-cAMP	0.15	27.5
	8-Benzyl-S-cAMP	0.10	25.3
	N^6-AHCM-cAMP	0.16	23.8
3	0	0.22	
	5'-AMP	0.23	
	100 nM glucagon	5.03	
	100 nM glucagon + 8-pClϕS-cAMP	5.77	
4	0	0.33	
	8-pClϕS-cAMP	0.19	
	0.1 nM glucagon	0.95	
	0.1 nM glucagon + 8-pClϕS-cAMP	0.32	
	0.2 nM glucagon	1.18	
	0.2 nM glucagon + 8-pClϕS-cAMP	0.59	
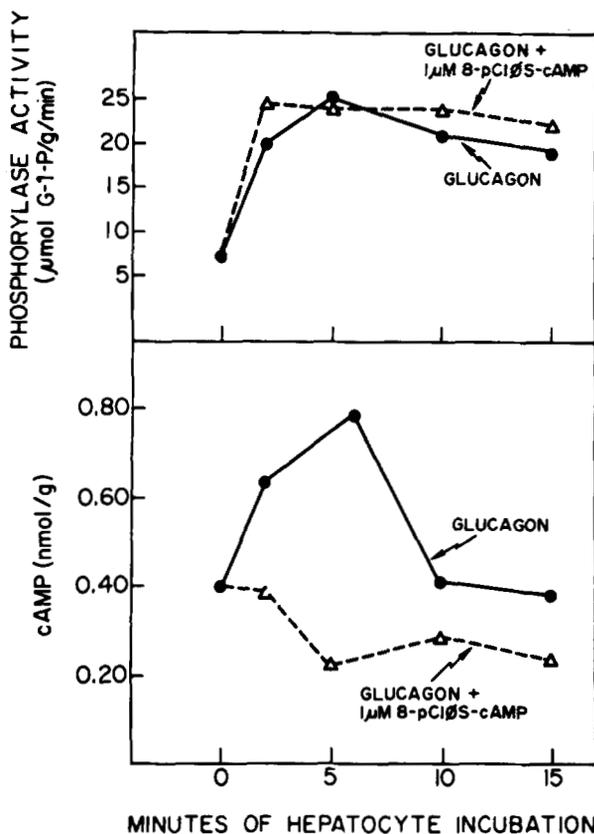
FIG. 6. **Effect of 0.1 nM glucagon in the absence and presence of 1 μM 8-pClϕS-cAMP on hepatocyte cAMP and phosphorylase.** Extracts were prepared and processed as described under "Methods."

DISCUSSION

To our knowledge, the results presented here are the first reported evidence for control of the hepatocyte cAMP level through protein kinase activation. This type of control might be considered as negative feedback and could be beneficial to the cell by preventing excessive cAMP accumulation. It is known that cAMP fluctuates over a narrow concentration range in most cell types. If the effect is found to occur with any hormone which elevates cAMP it would be classified as a heterologous type of desensitization (6). The transient increase in cAMP by glucagon observed here could be taken as one line of evidence for a desensitization produced by cAMP elevation. It has since been found that readdition of glucagon, after the fall in cAMP, produces a smaller rise in cAMP than the original (results not shown).

It will be of interest to determine if the cAMP-lowering effect of cAMP analogs is present in cell types other than hepatocytes. Mulner *et al.* (23) recently reported that microinjection of protein kinase inhibitor into *Xenopus* oocytes causes an increase in cAMP, which is consistent with our findings. Fisher *et al.* (24) observed a rapid (2–3 min) desensitization of β-adrenergic stimulation of heart cells, which is quantitatively related to cellular cAMP. A cAMP-mediated desensitization was also suggested by the finding of Barovsky *et al.* (25) that pretreatment of glioma cells with a cAMP analog produces refractoriness toward forskolin.

The mechanism for the lowering of cAMP by cAMP analogs is not clear, although it seems reasonable to assume that it occurs through protein kinase activation. The results of a recent comprehensive study of cAMP analog effects on lipolysis and glycogenolysis in adipocytes and hepatocytes, respectively, indicated that the analogs are acting by directly stimulating cAMP-dependent protein kinase (9). If protein phosphorylation is involved, then the target protein(s) could be part of either the adenylate cyclase complex or the phosphodiesterase. Presumably β-receptor phosphorylation is not involved since the cAMP analog effects are obtained in the basal state or in the presence of glucagon. Of course, there may exist cAMP regulation of components of adenylate cyclase other than the β-receptor. Several groups have also reported that a hormone-sensitive, low K_m phosphodiesterase is activated in adipose tissue (26, 27) and in liver (28–30) by addition either of hormones which elevate cAMP or of cAMP

analogs. Since the cAMP-dependent protein kinase is the only known mediator of cAMP effects in mammalian tissues, it is reasoned that this enzyme is responsible for these effects. One group has reported that the low K_m phosphodiesterase can be phosphorylated by the protein kinase (30). Paradoxically, insulin also activates the same phosphodiesterase even though this hormone is known to lower cAMP in these tissues (1, 2, 26–30). Other phosphodiesterases cannot be ruled out as targets of the cAMP effect. For example, cAMP analogs could cause an elevation in cellular calcium ion which could activate a calmodulin-sensitive phosphodiesterase (31). There are also known to be present cGMP-binding phosphodiesterases (32). It has been suggested, however, that phosphodiesterases are not the likely target enzymes involved in desensitization processes (7).

Another explanation for the results which should be considered is that the cAMP analogs could compete with endogenous cAMP for the binding sites on the protein kinase. Thus, activation of the kinase by the analogs might occur parallel with an increase in free cAMP. Since free cAMP, but not bound cAMP, is a substrate for phosphodiesterase (33), the cAMP level could be lowered simply because of higher substrate availability. It might be difficult to distinguish between this mechanism and a protein phosphorylation reaction since an increase in free cAMP due to analog competition would be expected to occur in direct parallel with protein kinase activation.

Perhaps the cAMP analog effects are tissue-specific since Jarett and Smith (34) found that adipocyte cAMP levels are actually increased by the addition of $N^6,O^{2'}$-dibutyryl cAMP. It was suggested that the increase in cAMP is brought about by inhibition of phosphodiesterase through competition between the analog and endogenous cAMP. This explanation for cAMP analog effects has been a dominant viewpoint for many years. It would be surprising, however, if the effects of most cAMP analogs on physiological responses are mediated through rises in cellular cAMP caused by competitive inhibition of phosphodiesterase. In general, the analogs have much higher affinities for the protein kinase than for the phosphodiesterase (9). At rate-limiting concentrations of analogs, it would be much more likely that they are acting through protein kinase activation than through phosphodiesterase inhibition. In fact, the results presented here indicate that in hepatocytes the analogs cause a decrease, rather than an increase, in cellular cAMP.

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