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# Short-term Feedback Regulation of cAMP by Accelerated Degradation in Rat Tissues*

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**A recent study showed that cAMP analogs lowered cAMP levels in rat hepatocytes (Corbin, J. D., Beebe, S. J., and Blackmore, P. F. (1985) *J. Biol. Chem.* 260, 8731–8735). The present work demonstrates that cAMP analogs also lowered cAMP in a rapid, concentration-dependent manner in heart and fat cells. In order to determine if the cAMP-dependent protein kinase mediated this effect, techniques were developed to assay the protein kinase activity ratio in hepatocytes treated with cAMP analogs. The activation of protein kinase and phosphorylase in hepatocytes by 8-pClφS-cAMP (where 8-pClφS- indicates 8-parachlorothiophenyl-) was concentration-dependent and occurred in parallel to proportionate decreases in cAMP. More than 20% of the cAMP binding sites on the protein kinase were unoccupied at concentrations of 8-pClφS-cAMP that produced maximal cAMP lowering. Thus, the possibility that 8-pClφS-cAMP lowered cAMP by displacing it from protein kinase binding sites, making it available for hydrolysis, seemed unlikely. In adipocytes, the lowering of cAMP by 8-pClφS-cAMP occurred in parallel with increases in lipolysis and activation of low $K_m$ phosphodiesterase, suggesting that the phosphodiesterase was responsible for the cAMP lowering. Further evidence for this assertion was the finding that in hepatocytes preloaded with low concentrations of 8-pClφS-cAMP, glucagon lowered 8-pClφS-cAMP by about 50%, an amount similar to the cAMP lowering observed with 8-pClφS-cAMP treatment. The results were consistent with a cAMP-dependent protein kinase-catalyzed activation of a phosphodiesterase and suggested that 8-pClφS-cAMP-mediated hydrolysis of cAMP mimicked a physiologically significant response. The observation of this phenomenon in several tissues further suggested that it may be a general mechanism for dampening and terminating the hormonal signal through accelerated degradation of cAMP.**

Chronic exposure of certain tissues to hormones that elevate cAMP produces a state of "refractoriness" to further hormonal stimulation. Dampening mechanisms such as down-regulation of hormone receptors and/or uncoupling of the receptor-adenylate cyclase complex have been described (1–4) and may represent "long-loop" feedback systems. There may also be present "short-loop" feedback systems in tissues, which cause a rapid dampening of cAMP levels. It has been reported that β-adrenergic agonists rapidly increase the activity of low $K_m$ phosphodiesterases in liver (5–6) and fat (7–10), but the relative importance of phosphodiesterase activation in modulating cAMP levels under these circumstances has been difficult to assess since cAMP levels are determined by the composite of adenylate cyclase and phosphodiesterase activities. Cyclic AMP analogs offer the attraction of bypassing adenylate cyclase and activating the cAMP-dependent protein kinase directly (11). Several studies have shown that cAMP analogs activate the low $K_m$ phosphodiesterase (5–6, 10), thus predicting that they would lower cAMP levels in intact cells, although one early experiment showed that the analog $N^6,O^{2'}$-dibutyryl-cAMP elevated cAMP levels in adipocytes (12). More recent work with cAMP analogs having high affinity for protein kinase and relatively low affinity for phosphodiesterase has shown that cAMP analogs lower endogenous cAMP in hepatocytes (13). Preliminary evidence suggests that cAMP-dependent protein kinase mediates this effect, but the mechanism and its physiological significance remain unclear. The objectives of the present study were to examine the mechanism of rapid cAMP lowering in hepatocytes and look for occurrence of this phenomenon in other cell types. Using heart, liver, and fat cells, it is shown that cAMP-dependent protein kinase-mediated lowering of cAMP may be a general mechanism for regulating cAMP levels and may occur through phosphodiesterase activation. It is also suggested that this phenomenon may represent an example of a second messenger regulating its own concentration.

## MATERIALS AND METHODS

*Preparation and Incubation of Hepatocytes*—Hepatocytes were isolated from male Sprague-Dawley rats (200–250 g body weight) by collagenase perfusion as previously described (14). Cyclic AMP analogs were added to continually gassed cell suspensions (50 mg cells/ml) incubated at 37 °C, and aliquots were removed for measurement of phosphorylase, cAMP-dependent protein kinase, and cAMP. 50- and 500-μl aliquots of each cell suspension were removed for subsequent assay of cAMP-dependent protein kinase and phosphorylase, respectively. Cyclic AMP was assayed in cell pellets obtained by centrifuging (500 × *g*) 4 ml of cell suspension and decanting the supernatant. Both aliquots and the cell pellet were immediately frozen in liquid nitrogen and stored at −70 °C.

*Protein Kinase Assay*—The protein kinase activity ratio was determined by a modification of the filter paper assay of Roskoski (15), using a synthetic heptapeptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) as substrate. Preliminary studies were undertaken to assure that the activity ratio could be accurately measured in hepatocyte incubations containing cAMP analogs. The use of larger than normal volumes of homogenizing buffer diluted the cAMP analogs and circumvented the problem of additional activation of the enzyme after homogenization. 4 ml of homogenizing buffer containing 10 mM $KH_2PO_4$, 150 mM KCl, 10 mM EDTA, 0.5 mM 3-isobutyl-1-methylxanthine, and bovine

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serum albumin (BSA,[1] 0.5 mg/ml) were added to the cell pellet, and the suspension was homogenized for 15 s using an Ultra-Turrax Tissuemizer®. Homogenates (25 μl) were immediately assayed for protein kinase activity in the presence and absence of 5 μM cAMP (16). The reaction was terminated after 20 min of incubation at 30 °C. The reaction was linear in the presence and absence of cAMP for at least 30 min.

*Phosphorylase Assay*—Frozen aliquots of the cell suspensions were thawed for 10 min and homogenized for 10 s in 1 ml of buffer containing 150 mM β-glycerophosphate, 30 mM EDTA, 150 mM NaF, 600 mM sucrose, and 6.5 mg/ml cysteine. Homogenates were assayed for phosphorylase activity as previously described (14).

*Purification and Assay of cAMP*—Crude extracts were prepared by homogenizing cell pellets in 1 ml of cold buffer containing 25 mM $NH_4HCO_3$, 1 mM 3-isobutyl-1-methylxanthine, and 20 mM EDTA. The homogenate was boiled for 6 min and centrifuged for 7 min in a Beckman Microfuge B. Tritium-labeled cAMP (~20,000 dpm) was mixed with 0.5 ml of the extract and chilled before Sephadex G-25 (superfine) chromatography (0.9 × 14 cm) at 4 °C. 1-ml fractions were collected, and 50-μl aliquots of the fractions were counted to locate the cAMP peak and calculate recovery in these fractions. Cyclic AMP was assayed in the appropriate fractions (11–15, $V_e/V_t = 1.62$) using Type 1 cAMP-dependent protein kinase purified from rabbit muscle (17). Incubation of the assay was at 4 °C for 16 h, as previously described (13).

Few studies have been reported in which cAMP levels have been measured in tissues treated with cAMP analogs (12–13). It is important to purify cAMP analogs prior to use, since any cAMP contamination of the original preparation will copurify with endogenous cAMP and inflate the estimated concentration. Other contaminating cyclic nucleotides may also copurify and cross-react in the cAMP assay. Preliminary studies in this lab (data not reported) with cardiomyocytes revealed that cyclic nucleotide contamination in commercial analog preparations can be substantial and interfere measurably in cAMP assays. The 8-pClφS-cAMP used for cell incubations was first chromatographed on a 0.9 × 57-cm Sephadex G-25 superfine column as previously described (12). The peak fractions of the elution profile ($V_e/V_t = 2.36$–$2.81$) were combined and standardized on the basis of absorbance at 281 nm.

*Assay of 8-pClφS-cAMP*—In some experiments, hepatocytes were incubated with 0.015 μM 8-pClφS-cAMP in the presence and absence of glucagon ($5 \times 10^{-9}$ M), and 8-pClφS-cAMP was assayed. Incubation of hepatocytes with this concentration of 8-pClφS-cAMP did not detectably activate protein kinase or phosphorylase. Crude extracts were prepared and chromatographed as described for purification of cAMP, except that fractions containing 8-pClφS-cAMP were retained for assay. Sephadex G-25 chromatography at 4 °C separated cAMP ($V_e/V_t = 1.62$) from 8-pClφS-cAMP ($V_e/V_t = 2.78$) completely (13). Since labeled 8-pClφS-cAMP was unavailable, all fractions (20–30) expected to contain 8-pClφS-cAMP ($V_e/V_t = 2.36$–$2.81$) were pooled. The pooled fractions from each incubation were lyophilized and resuspended in 25 mM $NH_4HCO_3$. 60-μl aliquots were assayed using Type 1 cAMP-dependent protein kinase as described for the assay of cAMP. Purified 8-pClφS-cAMP dissolved in the elution buffer was used as the standard. The $K_a$ of Type 1 cAMP-dependent protein kinase for 8-pClφS-cAMP was about half that for cAMP.

*Assay of cAMP Binding Sites*—In some experiments, the protein kinase activity ratio and the number of free and total cAMP binding sites on protein kinase were assayed in the same hepatocyte incubation. Free cAMP binding sites on the regulatory subunit were estimated by taking advantage of the difference between the rates of cAMP binding and dissociation (fast binding, slow dissociation). The procedure was initiated by adding 100 μl of sample to 50 μl of reaction mixture containing 50 mM $KH_2PO_4$ (pH 6.8), 1 mM EDTA, 0.5 mg/ml Type II-A histone mixture (Sigma), 2 M NaCl, 0.1 mM adenosine, and 1.8 μM [$^3$H]cAMP. After 5 min at 0 °C, 1 ml of cold 10 mM $KH_2PO_4$ (pH 6.8) containing 1 mM EDTA was added, and the mixture filtered and washed using Millipore filters (18). The total [$^3$H]cAMP binding sites were measured using the same procedure except that incubation was at 30 °C for 45 min. Exchange of prebound cAMP was complete under these conditions (18). The proportion of free sites was expressed as the ratio of cAMP bound at 0 and 30 °C.

In other experiments, free and total cAMP binding sites were assayed in incubations with Type I cAMP-dependent protein kinase partially purified through the DEAE-cellulose step (17). The activity ratio was varied by the addition of various concentrations (31–1000 nM) of 8-pClφS-cAMP to a total volume of 95 μl reaction mixture containing 6 pmol of holoenzyme, 20 mM Tris-HCl (pH 7.4), 0.05 mM 3-isobutyl-1-methylxanthine, 65 μM heptapeptide (Kemptide), 10 mM $Mg(CH_3COO)_2$, 0.1 mM [$^{32}$P]ATP (~100 cpm/pmol), and 0.39 μM [$^3$H]cAMP in the presence and absence of 2 μM cold cAMP. After incubation at 4 °C, 50-μl aliquots were spotted on phosphocellulose papers for determination of $^{32}$P incorporation. To the 45 μl of reaction mixture remaining, 1 ml of cold 10 mM $KH_2PO_4$ containing 1 mM EDTA was added, and this sample was filtered immediately by Millipore filtration as previously described (18).

*Preparation and Incubation of Adipocytes*—Adipocytes were isolated from epididymal fat pads of 200–250 g male Sprague-Dawley rats by collagenase treatment according to Rodbell (19). The cells were washed and resuspended (10 mg cells/ml suspension) in Krebs-Ringer bicarbonate buffer containing 1 mM $CaCl_2$ and 2% BSA. Duplicate incubations were carried out in closed vials gassed with 95% $O_2$ and 5% $CO_2$ in the presence or absence of various concentrations of 8-pClφS-cAMP. At the end of a 15-min incubation, the cells were washed twice with 0.25 M sucrose in 10 mM TES, and one replicate of each incubation was immediately homogenized with 1 ml of cold buffer containing 25 mM $NH_4HCO_3$, 1 mM 3-isobutyl-methylxanthine, and 20 mM EDTA. The homogenate was boiled for 6 min and centrifuged for 7 min in a Beckman Microfuge B. The aqueous phase was recovered and chromatographed at 4 °C on Sephadex G-25, and cAMP was assayed in the appropriate fractions as described for hepatocytes. The other replicate of each incubation was retained for assay of phosphodiesterase. Glycerol release was determined in similar incubations according to the method described by Beebe *et al.* (20).

*Assay of Low $K_m$ Phosphodiesterase*—Crude microsomal fractions (P-2) were prepared from each adipocyte incubation, and phosphodiesterase activity was measured according to the method described by Kono (21). The assay procedure involved measurement of adenosine formation from tritiated cAMP hydrolysis by phosphodiesterase and snake venom nucleotidase. The reaction was initiated by adding 50 μl of the crude enzyme to 200 μl of reaction mixture containing 50 mM TES (pH 7.5), 0.125 μM unlabeled cAMP, 1 μCi [$^3$H]cAMP (specific activity 31 Ci/mmol), and 5 mM $MgSO_4$. The reaction was stopped after a 5-min incubation at 30 °C by adding 250 μl of an equimolar mixture of cAMP and AMP (1 mM each) and immediately boiling for 3 min. After the tubes cooled, 50 μl of 5 mg/ml snake venom in 0.1 M Tris (pH 8) was added to each tube and incubated for 30 min at 37 °C. This reaction was terminated by a 50-μl addition of 5 mM adenosine in 200 mM EDTA. Tritiated adenosine was separated from [$^3$H]cAMP by QAE-Sephadex and counted. Protein in each original enzyme preparation was determined by the method of Bradford (22), and phosphodiesterase activity was expressed as pmol of cAMP hydrolyzed/min/mg of protein.

*Preparation and Incubation of Cardiomyocytes*—Cardiomyocytes were isolated by collagenase perfusion of rat hearts as described previously (23–24) with slight modification. Hearts were removed quickly from anesthetized rats (250–300 g) and perfused by the Langendorff procedure. The initial perfusion was for 10 min at 60 mm mercury with Krebs-Henseleit bicarbonate buffer (37 °C, pH 7.4) containing 1.25 mM $Ca^{2+}$ and 10 mM glucose (25). The perfusion was continued for 5 additional min (8 ml/min) with no $Ca^{2+}$ in the buffer. The final perfusate was changed to include 12.5 μM $Ca^{2+}$, 0.1% collagenase, and 0.1% BSA, and perfusion continued for 14 min (8 ml/min). The ventricles were excised and incubated for 10 min on a shaker bath at 37 °C in 5 ml of Krebs-Henseleit bicarbonate buffer containing 12.5 μM $Ca^{2+}$, 0.1% collagenase, and 2% BSA. The initial supernatant was discarded and 5 ml of fresh buffer added. Isolated cells were harvested by repeated (three times) 5-min incubations in the collagenase buffer, followed by pouring the suspended cells through nylon mesh after each incubation. Cells were centrifuged at low speed after two washes in Krebs-Henseleit buffer containing 12.5 μM $Ca^{2+}$ and 2% BSA. The cells were subsequently incubated on a shaker bath for 10 min at 37 °C in Krebs-Henseleit buffer containing 12.5 μM $Ca^{2+}$ and 2% BSA. The cells were then allowed to settle twice under gravity; the first time for 10 min at room temperature and the second for 5 min at room temperature in a final Krebs buffer containing 10 mM glucose, 1.8 mM $Ca^{2+}$, and 0.01% BSA. Cells were resuspended in this buffer at a concentration of 180,000 cells/ml and preincubated for 5 min at 37 °C before addition of 8-pClφS-cAMP. Following a 15-min incubation, cells were centrifuged at 500 × *g* for

[1] The abbreviations used are: BSA, bovine serum albumin; 8-pClφS-, 8-parachlorothiophenyl-; $V_e/V_t$, elution volume/total column volume; TES, *N*-tris[hydroxylmethyl]methyl-2-aminoethanesulfonic acid.

5 min at 0 °C. Incubation medium was decanted, and the cell pellet was immediately frozen in liquid nitrogen and stored at −70 °C. Cyclic AMP was assayed in cell extracts after purification as described for hepatocytes. Phosphorylase activity was measured in the cell pellets as previously described (14).

*Methods of Analysis and Response Variable Characterizations*—Response variables from experiments in the present study were characterized using relationship functions appropriate to the shape of the response surface. Responses such as the cAMP-dependent protein kinase activity ratio and phosphorylase activation in relation to concentration of 8-pClϕS-cAMP were adequately characterized by the exponential given as

$$y = \alpha - \beta(2)^{-x/\mu} \quad (1)$$

where $y$ = response variable (protein kinase or phosphorylase activity), $x$ = concentration of 8-pClϕS-cAMP (μM), $\alpha$ = asymptotic value of $y$ at infinite $x$, $\beta = \alpha - y$ at $x = 0$, and $\mu = x$ at $(\alpha/2) + y$ intercept. The cAMP-lowering response in relation to increasing concentrations of 8-pClϕS-cAMP or in relation to time was characterized by

$$y = \delta + \lambda(2)^{-x/\tau} \quad (2)$$

where $y$ = response variable (cAMP concentration), $x$ = concentration of 8-pClϕS-cAMP or time, $\delta$ = concentration of cAMP at infinite $x$, $\lambda = y$ intercept $- \delta$, and $\tau = x$ at (1/2 $y$ intercept) + $\delta$.

Parameter estimates, along with an estimate of their standard errors, were obtained for Equations 1 and 2 using an iterative nonlinear least squares routine in SAS (Statistical Analysis System, Cary, NC). The parameter $\mu$, in the case of cAMP-dependent protein kinase activity ratio or phosphorylase activation, is analogous to the more familiar $K_a$ in the sense that it represents the value of $x$ (8-pClϕS-cAMP concentration) producing a half-maximal response. In the case of the cAMP-lowering response, the parameter $\tau$ also represents the value of $x$ (8-pClϕS-cAMP concentration or time) where the half-maximal response is seen. The parameter estimates for $\mu$ and $\tau$ thus provide a convenient basis for comparing the concentration or time dependence of the various responses within and across tissues.

The results from experiments where 8-pClϕS-cAMP was measured in hepatocytes treated with various cAMP-dependent protein kinase activators were analyzed by a one-way analysis of variance (26). The criterion employed for assessing treatment differences was based on a level of protection against Type I errors of 1% ($\alpha = 0.01$).

## RESULTS

*Protein Kinase Activity Ratio Validation*—Replicate aliquots of hepatocytes were incubated with various concentrations of 8-pClϕS-cAMP, and the cells were homogenized in different volumes (1, 2, 4, 10, and 50 ml) of buffer. Protein kinase activity ratios were assayed with the intent of choosing a homogenization volume which prevented subsequent protein kinase activation by analog carryover when the cells were broken. At higher 8-pClϕS-cAMP concentrations (>0.1 μM), the assayed cAMP-dependent protein kinase activity ratios differed among homogenization volumes (data not shown). This would be expected if extracellular 8-pClϕS-cAMP in the original incubation was not sufficiently diluted in the homogenization volume. A homogenization volume of 4 ml was chosen since the activity ratios using 4, 10, and 50 ml were consistent regardless of 8-pClϕS-cAMP concentration, a result not seen with 1 or 2 ml (data not shown). Further evidence that 4 ml of homogenization buffer provided the conditions for accurate *in situ* activity ratio estimates was obtained by examining the relationship between phosphorylase activation and cAMP-dependent protein kinase activity ratio in hepatocytes incubated with several concentrations of glucagon and 8-pClϕS-cAMP. The original observations and fitted curves are presented in Fig. 1. Assuming that glucagon acts entirely by cAMP elevation, a given increment of protein kinase activation by glucagon or 8-pClϕS-cAMP should produce the same increment of phosphorylase activation. A formal test of the similarity between response curves was provided by comparing maximal phosphorylase activation ($\alpha$) and the cAMP-dependent protein kinase activity ratio at half-maximal phosphorylase activation ($\mu$) from the fitted glucagon ($\alpha = 12.71 \pm 0.30$, $\mu = 0.242 \pm 0.025$) and 8-pClϕS-cAMP ($\alpha = 12.98 \pm 1.01$, $\mu = 0.311 \pm 0.083$) curves. No evidence was seen to suggest that the response curves were different.

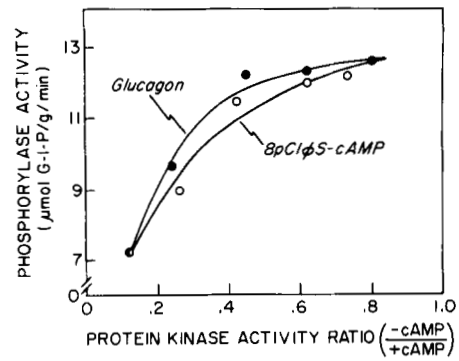


FIG. 1. **The relationship between protein kinase activity ratio and phosphorylase activity in hepatocytes treated with 8-pClϕS-cAMP (○) or glucagon (●).** The original observations are the mean of four replicates within each of four experiments, and the fitted curves were obtained by least squares. Hepatocytes were incubated with various concentrations of 8-pClϕS-cAMP or glucagon for 5 min. Aliquots were retained and assayed for protein kinase activity ratio and phosphorylase activity as described under "Materials and Methods." *G-1-P*, glucose 1-phosphate.

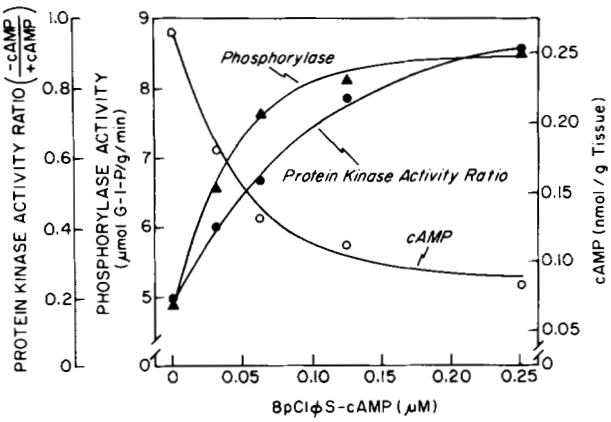


FIG. 2. **Effect of 8-pClϕS-cAMP concentration on protein kinase activity ratio (●), phosphorylase activity (▲), and endogenous cAMP (○) in hepatocytes.** The original observations are the mean of duplicate determinations within each of five experiments, and the fitted curves were obtained by least squares. Hepatocytes were incubated for 5 min with the indicated concentrations of 8-pClϕS-cAMP. Extracts were prepared and processed as described under "Materials and Methods." *G-1-P*, glucose 1-phosphate.

It should be noted that this technique of using large homogenization volumes is ineffective in most cell types, since much greater concentrations of analog (500–2000 times greater) are required to produce cellular responses. Thus, the carryover of analog when the cells are broken artifactually elevates the cAMP-dependent protein kinase activity ratios.

*Effect of cAMP Analogs on Hepatocyte cAMP*—The cAMP analog, 8-pClϕS-cAMP, activated hepatocyte protein kinase, phosphorylase, and lowered cAMP in a concentration-dependent manner. The concentrations required to produce changes in each response were similar among all responses (Fig. 2). The concentrations of 8-pClϕS-cAMP required to produce half-maximal protein kinase activation ($\mu = 0.071 \pm 0.010$ μM), half-maximal phosphorylase activation ($\mu = 0.031 \pm 0.006$ μM), and half-maximal cAMP lowering ($\tau = 0.036 \pm 0.006$ μM) were similar among the measures, as was the concentration of 8-pClϕS-cAMP (0.125 μM), producing nearly

maximal responses of all measures.

*Hepatocyte cAMP Binding Studies*—cAMP analogs, including 8-pCl$\phi$S-cAMP, are known to bind to the R-subunit of cAMP-dependent protein kinase with high affinity (27–28). It was important to rule out the possibility that 8-pCl$\phi$S-cAMP was lowering endogenous cAMP by displacing bound cAMP from cAMP-dependent protein kinase, thus making it available for hydrolysis by phosphodiesterase. It can be seen in Fig. 2 that cAMP-dependent protein kinase activation occurred in parallel to the decline in cAMP, so evidence of free binding sites at concentrations of 8-pCl$\phi$S-cAMP that maximally lowered cAMP would argue against a competitive mechanism for the lowering. Reference to Fig. 3 indicates that as the activity ratio increased from 0.2 to 0.7 (maximal activation = 1), the percentage of free cAMP binding sites decreased proportionately. Concentrations of 8-pCl$\phi$S-cAMP that produced an activity ratio of 0.7 also lowered cAMP maximally. These results suggested that cAMP binding sites were not saturated at this concentration of analog, and in fact free cAMP binding sites were detected. Similar studies in a cell-free system using physiological concentrations of partially purified cAMP-dependent protein kinase indicated the presence of free cAMP binding sites at activity ratios of 0.90 or lower (Fig. 4). Using both intact cells and the partially purified enzyme, free cAMP binding sites declined with increased concentrations of 8-pCl$\phi$S-cAMP, but maximal cAMP lowering occurred at activity ratios where excess cAMP binding sites were still available.

*Assay of 8-pCl$\phi$S-cAMP in Hepatocytes*—Incubation of hepatocytes with 0.015 $\mu$M 8-pCl$\phi$S-cAMP resulted in no detectable activation of cAMP-dependent protein kinase, but the analog could readily be assayed in extracts of the hepatocytes. Addition of glucagon (5 nM), forskolin (20 $\mu$M), or $N^6,O^{2'}$-dibutyryl-cAMP (20 $\mu$M) resulted in lowering of 8-pCl$\phi$S-cAMP in hepatocytes preloaded with the analog (Table I). The $N^6,O^{2'}$-dibutyryl-cAMP lowered 8-pCl$\phi$S-cAMP significantly but was less effective than glucagon or forskolin. Glucagon produced the most substantial lowering of 8-pCl$\phi$S-cAMP (~50%), and the percentage decrease was quite similar to the percentage decrease in endogenous cAMP seen when optimal concentrations of 8-pCl$\phi$S-cAMP (0.25 $\mu$M) were used to activate cAMP-dependent protein kinase. Addition of glucagon or forskolin to hepatocytes at the indicated concentrations resulted in nearly maximal cAMP-dependent protein kinase activation (Table I).

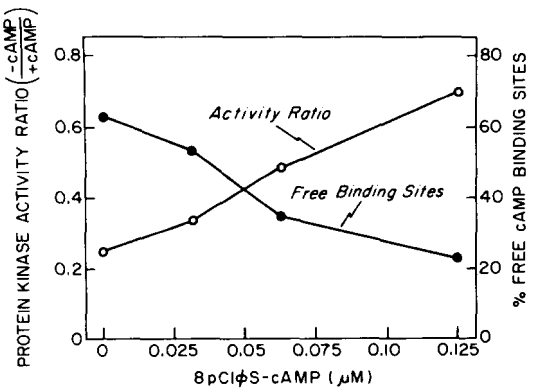


FIG. 3. **The relationship between cAMP binding site occupancy (●) and protein kinase activity ratio (○) in hepatocytes treated with various concentrations of 8-pCl$\phi$S-cAMP.** The original observations are the mean of duplicate determinations within each of three experiments. Hepatocytes were incubated for 5 min with various concentrations of 8-pCl$\phi$S-cAMP. Cells were homogenized, and free and total cAMP binding sites were determined as described under "Materials and Methods."

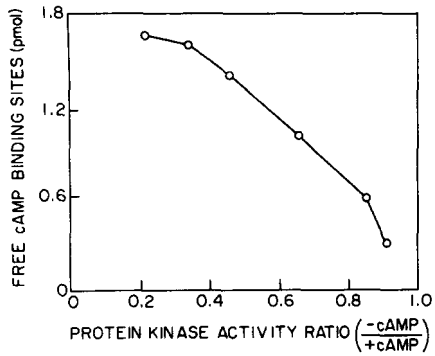


FIG. 4. **The relationship between protein kinase activity ratio and free cAMP binding sites on Type 1 cAMP-dependent protein kinase incubated with various concentrations of 8-pCl$\phi$S-cAMP.** The original observations are the mean of duplicate determinations within each of three experiments. The enzyme was partially purified from rabbit skeletal muscle through the DEAE-cellulose step (33), and 2.8 pmol of the holoenzyme was used for each binding determination.

TABLE I

*Assay of 8-pCl$\phi$S-cAMP in hepatocytes preloaded with the analog and treated with various agents to activate cAMP-dependent protein kinase*

| Treatment | 8-pCl$\phi$S-cAMP[a] | Protein kinase activity ratio |
|---|---|---|
| | *pmol/g tissue* | |
| Control | 36.47 | 0.24 |
| 5 nM glucagon | 18.36 | 0.81 |
| 20 $\mu$M forskolin | 21.40 | 0.80 |
| 20 $\mu$M dibutyryl-cAMP | 30.20 | —[b] |
| S.E.[c] | 0.63 | 0.04 |

[a] Hepatocytes were preincubated with 0.015 $\mu$M 8-pCl$\phi$S-cAMP for 5 min and treated in the indicated manner for 5 additional min. Aliquots were removed for determination of the cAMP-dependent protein kinase activity ratio, and the concentration of 8-pCl$\phi$S-cAMP in the cell pellet was determined. The means and their corresponding standard errors were estimated from four experiments.

[b] Accurate estimates of the cAMP-dependent protein kinase activity ratio were not obtained in this incubation.

[c] Standard error of the mean from analysis of variance.

It was presumed that 20 $\mu$M $N^6,O^{2'}$-dibutyryl-cAMP also activated protein kinase substantially. Accurate estimates of cAMP-dependent protein kinase activity could not be obtained with 20 $\mu$M $N^6,O^{2'}$-dibutyryl-cAMP due to analog carryover, but previous studies have shown that this concentration of the analog activated phosphorylase.[2]

*Effect of cAMP Analogs on Adipocytes*—Glycerol release, low $K_m$ phosphodiesterase activation, and cAMP lowering occurred in response to increasing concentrations of 8-pCl$\phi$S-cAMP in adipocytes (Fig. 5). The concentration of analog required to produce half-maximal glycerol release (171 ± 14 $\mu$M) and phosphodiesterase activation (140 ± 11 $\mu$M) were similar, but were somewhat higher than the concentration (106 ± 9 $\mu$M) required for half-maximal cAMP lowering. Higher concentrations of 8-pCl$\phi$S-cAMP were required to elicit all responses in fat cells than in hepatocytes. This finding was consistent with previous studies using cAMP analogs in different tissues (11) and may be due to differences in cell permeability. Nevertheless, the percentage decrease in endogenous cAMP produced by optimal 8-pCl$\phi$S-cAMP concentrations (~60%) was similar to the cAMP decrease seen

[2] P. F. Blackmore, unpublished observations.

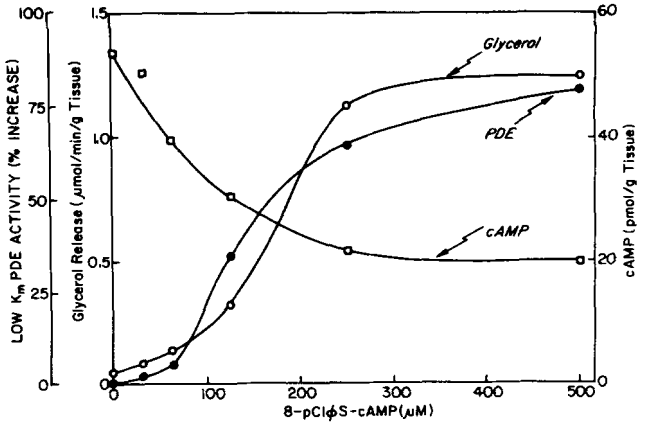


FIG. 5. **Effect of 8-pClφS-cAMP concentration on glycerol release (○), low $K_m$ phosphodiesterase (*PDE*) activation (●), and endogenous cAMP (□) in adipocytes.** The original observations are the mean of duplicate determinations within each of five experiments. Adipocytes were incubated for 15 min with the indicated concentrations of 8-pClφS-cAMP. Extracts were prepared and processed as described under "Materials and Methods." Estimates of the concentration of analog required to produce half-maximal glycerol release and phosphodiesterase activation were obtained from fitted logistic ogives as described previously (51).

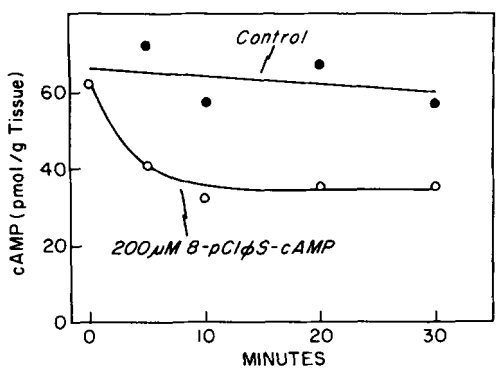


FIG. 6. **Time course of cAMP in control adipocytes (●) and adipocytes treated with 200 μM 8-pClφS-cAMP (○).** The original observations are the mean of duplicate determinations within each of four experiments, and the fitted lines were obtained by least squares. Extracts were prepared and processed as described under "Materials and Methods."

in hepatocytes (~50%). Incubation of adipocytes with 200 μM 8-pClφS-cAMP produced a rapid 49% decline ($\tau = 2.23 \pm 0.67$ min, half-time for maximal lowering) in cAMP, whereas basal cAMP in control cells decreased slightly during the 30-min incubation (Fig. 6). A previous report demonstrated rapid cAMP lowering in hepatocytes (13) and reanalysis of those data produced an estimate for $\tau$ (half-time for maximal cAMP lowering) of $0.55 \pm 0.16$ min. Inasmuch as the process appeared to be somewhat slower in fat cells than hepatocytes, the hypothesis that the half-times were different in the two tissues was rejected.

*Effect of cAMP Analogs on Cardiomyocytes*—The concentration of 8-pClφS-cAMP required to activate phosphorylase in cardiomyocytes (Fig. 7) was somewhat less than the concentration required to lower cAMP in adipocytes, but was still substantially higher than in hepatocytes. Incubation of cardiomyocytes with 70 μM 8-pClφS-cAMP achieved approximately 80% maximal activation of phosphorylase; a concentration of $37.2 \pm 4.2$ μM (μ) produced half-maximal activation. Under the conditions used, it was not possible to determine accurately the cAMP-dependent protein kinase activity ra-

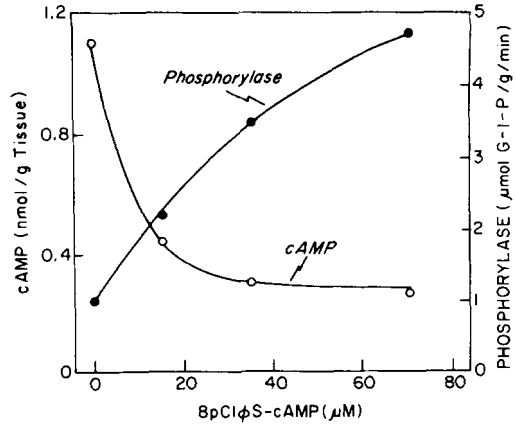


FIG. 7. **The relationship between phosphorylase activity (●) and endogenous cAMP (○) in cardiomyocytes treated with various concentrations of 8-pClφS-cAMP.** The original observations are the mean of four replicates within each of four experiments, and the fitted curves were obtained by least squares. The cardiomyocytes were incubated for 15 min with various concentrations of 8-pClφS-cAMP. Extracts were prepared and processed as described under "Materials and Methods." *G-1-P*, glucose 1-phosphate.

tios, since carryover of the analog when the cells were broken produced additional enzyme activation. Basal cAMP was lowered in the same 8-pClφS-cAMP concentration range that activated phosphorylase, but the concentration producing half-maximal lowering of cAMP ($\tau = 13.99 \pm 5.46$ μM) was less. Even so, reference to Fig. 7 illustrates the good correlation between phosphorylase activation and cAMP lowering. The percentage decline (~60%) in basal cAMP in cardiomyocytes treated with 70 μM 8-pClφS-cAMP was similar to the cAMP decline seen in adipocytes (~60%) and hepatocytes (~50%) and the 8-pClφS-cAMP decline (~50%) seen in glucagon-treated hepatocytes.

## DISCUSSION

Previous studies have shown that interactions among various hormones modify cellular cAMP concentrations in some cell types. Some hormones are thought to exert their effects by decreasing cAMP levels. The ability of insulin to decrease cellular cAMP levels in glucagon-stimulated hepatocytes (29–33) and epinephrine-stimulated adipocytes (34–35) is well documented. Buxton and Brunton (36) have described in cardiomyocytes a decreased β-agonist-induced cAMP accumulation by an $\alpha_1$-agonist-mediated activation of phosphodiesterase. Dumont *et al.* (37) have shown that norepinephrine ($\alpha_2$) decreases thyroid-stimulating hormone-stimulated adenylate cyclase activity in thyroid slices, and $\alpha_2$ agents are known to cause decreases in cAMP in several other tissues by acting through the $N_i$ (inhibitory guanyl nucleotide-binding regulatory protein of adenylate cyclase) pathway (38). It has also been reported that increased cytosolic calcium activates a calmodulin-sensitive phosphodiesterase in some tissues (39). These heterologous types of cAMP control may be important in modifying cellular responses to hormones. This laboratory has recently described a homologous type of cAMP control in which cAMP-dependent protein kinase activation lowers hepatocyte cAMP. Additional evidence that cAMP may act to regulate its own concentration can be found in reports which have shown that hormones which elevate cAMP activate a hormone-sensitive phosphodiesterase in several cell types (5–8, 10, 40), and it has been reported that a low $K_m$ phosphodiesterase can be phosphorylated by protein kinase (41). Other examples of homologous regulation of second messenger con-

centrations can be found in reports which show that elevation of cytoplasmic $Ca^{2+}$ results in activation of mechanisms which quickly lower cytoplasmic $Ca^{2+}$ (42), and elevation of cytoplasmic diacylglycerol has also been reported to cause a subsequent decline in cytoplasmic levels of this second messenger (43).

The present results support the suggestion by this laboratory that cAMP-dependent protein kinase mediates the lowering of cAMP. Likely targets for cAMP-dependent protein kinase would be the adenylate cyclase complex or low $K_m$ phosphodiesterases, the former decreasing cellular cAMP by slowing synthesis and the latter by enhancing cAMP degradation. The present observation that glucagon enhances 8-pCl$\phi$S-cAMP breakdown in hepatocytes suggests that a cellular phosphodiesterase(s) is being activated by protein kinase. The finding that 8-pCl$\phi$S-cAMP activated a low $K_m$ phosphodiesterase in the same concentration range that produced cAMP lowering in adipocytes further suggests that cAMP is being lowered by phosphodiesterase activation. This does not rule out the possibility that cAMP analogs may have an additional effect to lower cAMP by modifying adenylate cyclase activity, but it should be pointed out that glucagon-mediated lowering of 8-pCl$\phi$S-cAMP was nearly the same, on a percentage basis, as analog-mediated cAMP lowering. It will be of interest to confirm that phosphodiesterases are responsible for cAMP lowering under these circumstances and identify which form of phosphodiesterase is the target enzyme. The finding that the lowering of cAMP in adipocytes is well-correlated with activation of the low $K_m$ form of phosphodiesterase is strongly suggestive that this enzyme is the one acted on by protein kinase. Another possibility considered is that cytoplasmic 8-pCl$\phi$S-cAMP displaces cAMP from its binding sites on cAMP-dependent protein kinase, thus stimulating phosphodiesterase activity by increasing substrate availability. The results obtained in binding studies with intact hepatocytes and with partially purified holoenzyme argue against such a mechanism. Free binding sites are detected in both systems with 8-pCl$\phi$S-cAMP concentrations and cAMP-dependent protein kinase activity ratios which produced maximal cAMP lowering. These findings suggest that additional 8-pCl$\phi$S-cAMP would have to fill remaining binding sites before significant cAMP displacement would occur. Although in the basal state the proportion of total endogenous cAMP bound to cAMP-dependent protein kinase compared to unbound cAMP is large, previous studies have shown that addition of low concentrations of cAMP analogs to the holoenzyme actually stimulates cAMP binding (44). The results presented here argue in favor of a cAMP-dependent protein kinase-mediated mechanism rather than an artifactual lowering through competition and displacement from binding sites.

This laboratory has recently suggested that lowering of hepatocyte cAMP by protein kinase activation may be a form of negative feedback. The earlier findings and the results presented here provide evidence that cAMP-dependent protein kinase regulates cellular cAMP levels and may represent a short-loop feedback system for cAMP. Although it seems plausible that this feedback system serves to dampen cAMP levels, other functions are possible. Recent studies have shown that $\beta$-agonists increase the fractional turnover rate for cAMP (45), and it has been suggested that cAMP turnover may be a central component in stimulus-response coupling (46). Obviously, the coupled stimulation of adenylate cyclase and phosphodiesterase activities would increase the turnover rate of cAMP. Long-loop feedback systems involving mechanisms which produce desensitization and/or down-regulation of hormone receptors have also been described (1–4). The action of these systems results in diminished cAMP accumulation after chronic exposure to hormones, and refractoriness to further hormonal stimulation. It has been assumed generally that the long-loop feedback systems act through the receptor-adenylate cyclase, although a role for phosphodiesterase has not been ruled out. Recent work has demonstrated a 2-fold induction of the low $K_m$ phosphodiesterase in cultured cells constantly exposed to isoproterenol or cAMP analogs (47). Perhaps part of the refractoriness of these cells to hormonal stimulation can be explained by cAMP-dependent protein kinase-mediated activation of the additional phosphodiesterase that is present. It seems likely that the interaction of these various mechanisms is important in determining the hormone sensitivity of particular cells at any given time.

Previous studies have demonstrated the existence of low $K_m$ phosphodiesterases in cardiac tissue (48–50), but to our knowledge it has not been shown that these forms of the enzyme are activated by either insulin or hormones which elevate cAMP. Comparable forms of the enzyme from liver (5–6) and adipose tissue (7–8, 10) are hormone-sensitive. The observation that cAMP analogs lower endogenous cAMP in cardiomyocytes suggests the presence of a low $K_m$ hormone-sensitive phosphodiesterase in this tissue, and this technique may provide a convenient method for detecting the cAMP-activated enzyme in other tissues.

All tissues examined (hepatocytes, cardiomyocytes, and adipocytes) to date have shown an 8-pCl$\phi$S-cAMP-induced lowering of cAMP, which is analog concentration-dependent. The analog-induced cAMP lowering in the examined cell types is a consistent response in the sense that the proportion of cAMP decline is similar among all cell types. These findings suggest that the 8-pCl$\phi$S-cAMP induced lowering of cAMP mimicks a physiologically significant response and may be mediated by a common mechanism. The occurrence of this phenomenon in several tissues further implies that it may be a general mechanism for dampening and terminating hormonal signals.

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