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Cyclic Adenosine 3',5'-Monophosphate in *Escherichia coli*

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The concentration of cyclic adenosine 3',5'-monophosphate (c-AMP) in *Escherichia coli* growing on different sources of carbon was studied. Cultures utilizing a source of carbon that supported growth relatively poorly had consistently higher concentrations of c-AMP than did cultures utilizing sugars that supported rapid growth. This relationship was also observed in strains defective in c-AMP phosphodiesterase and simultaneously resistant to catabolite repression; in such strains the c-AMP concentration was slightly higher for several sources of carbon tested. Cultures continued to synthesize c-AMP and secreted it into the medium, under conditions that brought about an inhibition of the intracellular accumulation of the cyclic nucleotide. Transient repression of the synthesis of β-galactosidase was not associated with an abrupt decrease in the cellular concentration of c-AMP.

Adenosine 3',5'-cyclic monophosphate (c-AMP) is required for the synthesis of a number of inducible enzymes that catalyze catabolic reactions in *Escherichia coli* (14, 15, 16) as well as for the formation of bacterial flagella (3, 21). An additional, unidentified regulatory role for c-AMP has been suggested recently on the basis of a mutant which required exogenous c-AMP for growth even on a rich medium (13). c-AMP, in combination with a "catabolite gene activator protein," facilitates the transcription of genes which code for proteins, the formation of which is controlled by catabolite repression (4, 19).

The mechanism by which the cellular concentration of c-AMP is regulated is not understood at present. Evidently the activities of at least two enzymes, i.e., of the synthetic adenyl cyclase and of the degradative c-AMP phosphodiesterase, affect the cellular concentration of the cyclic nucleotide. It has been suggested (7, 18) that the release of c-AMP into the medium may also play a regulatory role.

Mutants of *E. coli* with either reduced adenyl cyclase (13, 17, 21) or c-AMP phosphodiesterase (8, 9) activities have been isolated. We found earlier that strains defective in c-AMP phosphodiesterase had higher intracellular concentrations of c-AMP than did the parent strain and that they were more resistant to catabolite repression than the parent (8, 9).

In this paper we describe experiments which confirm and extend our preliminary observations. We conclude that, whereas c-AMP phosphodiesterase plays a regulatory role, it cannot be the sole, or even primary, element of control of the cellular concentration of c-AMP.

MATERIALS AND METHODS

Bacterial strains. *E. coli* AB257, CR^+ (sensitive to catabolite repression [Strain AB257 is unusually sensitive to catabolite repression. Thus glucose, at 10 mM, inhibited the synthesis of β-galactosidase in strain AB257 by well over 95%, whereas in other CR^+ strains tested the inhibition ranged from 70 to 90%]), a spontaneous mutant $AB257^{pc-1}$, CR^- (resistant to catabolite repression) (8), and Crookes strain ATCC 8739, also CR^-, were employed. Strains AB257 and $AB257^{pc-1}$ require methionine for growth; Crookes strain is prototrophic. Strains $AB257^{pc-1}$ and Crookes had been shown by us (9) to be defective in their c-AMP phosphodiesterase.

Medium and conditions of growth. Bacteria were grown aerobically at 37 C in a salts medium (10) containing (per liter): 13.6 g of KH_2PO_4, 2.0 g of $(NH_4)_2SO_4$, 0.01 g of $CaCl_2$, 0.5 mg of $FeSO_4 \cdot 7H_2O$, and 0.2 g of $MgSO_4 \cdot 7H_2O$. The pH of the medium was adjusted to 7 with KOH. The source of carbon is indicated for individual experiments; methionine, where required, was employed at a concentration of 0.4 mM. Unless otherwise indicated, cultures were grown for several generations under the conditions in which c-AMP was to be determined. Bacterial growth

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was measured turbidimetrically in a Klett-Summerson colorimeter (red filter), and the protein content was determined by reference to a calibration curve relating bacterial density to protein concentration. In the case of the strains and conditions of culture employed, a Klett reading of 100 corresponded to 5×10^8 bacteria and 200 μg of protein per ml. Presentation of intracellular concentrations of c-AMP in units of molarity is based on an accessible volume of 7.5×10^{-13} ml/bacterium.

Measurement of c-AMP. The protein binding assay of Gilman (5) was employed. In our early experiments we used a c-AMP-binding protein and a protein kinase inhibitor prepared as described by Gilman. We found, however, that a c-AMP-binding protein derived from beef kidney by the relatively simple procedure of Cheung (2) yielded as reliable results as did the "Gilman protein," and we therefore used the preparation obtained from kidney in the absence of the protein kinase inhibitor. The concentration of c-AMP in bacterial extracts was calculated by reference to a standard curve, based on the decrease in the binding of ^{3}H-c-AMP brought about by the addition of known amounts of unlabeled c-AMP. Fresh standard curves were established for every assay. Figure 1 shows two curves representing the competition between known amounts of ^{3}H-labeled c-AMP and non-radioactive c-AMP. (In a few experiments the radioimmunoassay of Steiner et al. (20) was also employed and yielded comparable data.)

For the determination of intracellular concentrations of c-AMP, either 1 or 2 ml of culture was filtered rapidly through membrane filters (Millipore, HAWP 02500; HA 0.45 μm; 25 mm diameter); the filters, without prior washing, were suspended immediately in 2 ml of 0.1 N hot HCl and kept for 10 min at 95 C. (Appropriate reconstruction experiments, in which bacteria of an adenyl cyclase-negative strain were mixed with tritiated c-AMP and filtered immediately, indicated that contaminating, extracellular c-AMP on the filter constituted no more than 2 to 3% of the values obtained for the intracellular concentration of c-AMP.) The heated and acidified bacterial extracts were dried under a stream of air and suspended in 0.4 to 0.5 ml of 50 mM acetate buffer at pH 4.0. Samples (50 μliters) of the extracts, diluted in the same buffer where appropriate, were then assayed in duplicate or triplicate for their concentration of c-AMP. We found in control experiments that addition of c-AMP phosphodiesterase to extracts abolished the inhibition of the binding of ^{3}H-c-AMP by the unlabeled c-AMP in the bacterial extracts; conversely, known amounts of c-AMP added to the extracts were recovered quantitatively. Triplicate assays differed rarely by more than 10%.

For the determination of total (i.e., intracellular and extracellular) c-AMP, unfiltered samples of the culture were boiled for 10 min; for the determination of extracellular c-AMP, a fraction of the filtrate was treated in the same manner. When either total or extracellular c-AMP was measured, the c-AMP employed for establishing the standard curve was dissolved in the salts medium.

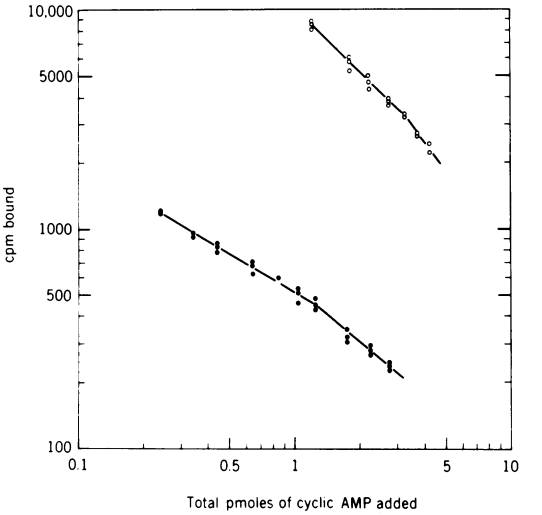

FIG. 1. *Standard curves for assay of c-AMP. The binding protein was prepared by the method of Cheung (2). The buffer was 100 mM sodium acetate, pH 4, containing ethylenediaminetetraacetic acid at a final concentration of 12.5 mM. Prior to filtration, assay mixtures were kept for 60 to 90 min at 0 C in 1.5-ml conical polyethylene tubes (Microsample tubes; Bel-Art Products; Pequannock, N.J.). (○) Total volume 210 μliters; binding protein [in 20 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.5], 16.9 μg; bovine serum albumin, 125 μg; ^{3}H-c-AMP, 1.2 pmol; standard or sample in 50 μliters. (●) Total volume 110 μliters; binding protein (in 20 mM Tris, pH 7.5), 6.7 μg; bovine serum albumin, 50 μg; ^{3}H-c-AMP, 0.24 pmol; standard or sample in 50 μliters.*

The radioactivity of the protein-bound ^{3}H-c-AMP on the filters was determined by scintillation counting after the filter in the counting vial had been dissolved in 1 ml of Cellosolve. Bray solution (1) was employed as scintillant.

Assay of β-galactosidase. β-Galactosidase was assayed as described previously (12). Methyl-thio-β-D-galactoside (TMG) at a concentration of 0.5 mM served as inducer of the enzyme.

Chemicals. c-AMP was purchased from Sigma Chemical Co., and ^{3}H-c-AMP (20.8 or 28 Ci/mmol) was from Schwarz BioResearch.

RESULTS

Cellular concentration of c-AMP during exponential growth. Bacteria using sources of carbon that support rapid growth had lower levels of c-AMP than did bacteria using relatively poor sources of carbon (Table 1). The data also indicate that the two CR$^-$ strains had generally somewhat higher levels of c-AMP than did the wild-type CR$^+$ strain. When grown on glucose, the sugar employed traditionally for

TABLE 1. *Cellular concentration of c-AMP and β-galactosidase activity during exponential growth*

Strain	Source of carbon[a]	Generation time (min)	Intracellular c-AMP (10^{-5} M)	β-galactosidase specific activity[b]
AB257 (CR+)	Glucose + gluconate	57	1.2	10
	Glucose	56	1.2	200
	Glycerol	96	4.3	12,500
	Succinate	170	5.0	14,500
	Acetate	270	5.5	19,800
	L-proline	218	24.0	43,800
AB257^{pc-1} (CR-)	Glucose + gluconate	58	0.8	4,000
	Glucose	72	2.3	11,000
	Glycerol	66	10.2	13,000
	Succinate	157	9.3	13,400
Crookes (CR-)	Glucose + gluconate	48	0.8	3,500
	Glucose	53	2.2	11,000
	Glycerol	59	4.5	13,000
	Succinate	86	6.2	15,400

[a] The respective concentrations of glucose and gluconate were 10 mM each; of glycerol, 20 mM; of succinate, 15 mM; of acetate, 30 mM; and of L-proline, 30 mM.

[b] Specific β-galactosidase activity is given as nanomoles of o-nitrophenol formed per minute per milligram of bacterial protein at 37 C.

the measurement of catabolite repression, the strains resistant to catabolite repression had approximately twice as much c-AMP as did the parent strain. In other experiments, not shown here, the differences between the concentrations of c-AMP found in CR+ and CR- strains were greater than those shown in Table 1; typically, however, the differences were relatively slight.

It will be noted that the mixture of glucose and gluconate inhibited the synthesis of β-galactosidase severely in the CR+ strain and distinctly less severely in the two CR- strains. This difference in sensitivity to catabolite repression could not be related in any obvious manner to the cellular concentration of c-AMP insofar as the two CR- strains had, if anything, even lower concentrations of c-AMP than did the CR+ strain.

Concentration of c-AMP in bacteria and medium upon exhaustion of and readdition of glucose. Makman and Sutherland's experiment (7), as well as our earlier experiments (9), indicated that the cellular concentration of c-AMP rose sharply as the source of carbon disappeared from the medium. In one experiment, strain AB257 was grown on a limiting concentration of glucose (3.5 mM), and the intracellular and extracellular concentrations of c-AMP were monitored at frequent intervals (Fig. 2). It may be seen that, as growth stopped, the intracellular concentration of c-AMP rose sharply (over a 15-min period) from 10^{-5} M to approximately 30 times that value and then fell slowly. The extracellular concentration of c-AMP rose from approximately 3×10^{-8} M c-AMP to 4×10^{-7} M. At the onset of starvation, the bacteria in 1 ml of culture occupied a volume of approximately 2.6×10^{-4} ml. It may be calculated that the *cellular* concentration of c-AMP at its peak was approximately 80 pmol/ml of culture and that it fell to 40 pmol by the end of the experiment. During the same period, the *extracellular* c-AMP rose from 60 pmol/ml to 400 pmol. The release of c-AMP from the bacteria alone cannot account for the increase of the cyclic nucleotide in the culture fluid. It appears that c-AMP continues to be formed and secreted into the medium at a time when intracellular accumulation of the cyclic nucleotide has come to a halt.

In another experiment, the intracellular concentration of c-AMP rose from 10^{-5} M to 2×10^{-4} M over a period of 15 min (Fig. 3). The addition of glucose (3.5 mM) to the starving culture brought about a very rapid fall in the intracellular concentration of c-AMP. The onset of a second period of starvation was accompanied by a second rise in the cellular concentration of cyclic AMP. The extracellular concentration of c-AMP rose to 5×10^{-7} M.

Figure 4 shows that in the CR- strain (Crookes) the onset of starvation was paralleled by an approximately 30-fold increase over a period of 20 min in the cellular concentration of c-AMP. The addition of glucose to the starving

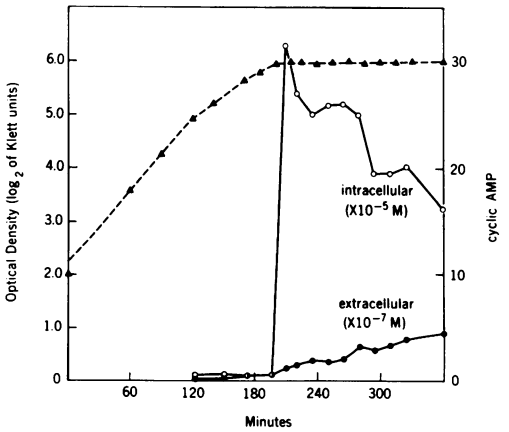

FIG. 2. *The intracellular and extracellular concentrations of c-AMP in a culture of AB257. Growth was limited by 3.5 mM glucose. Procedures are described in Materials and Methods. Symbols: ▲, bacterial growth; O, intracellular c-AMP; ●, extracellular c-AMP.*

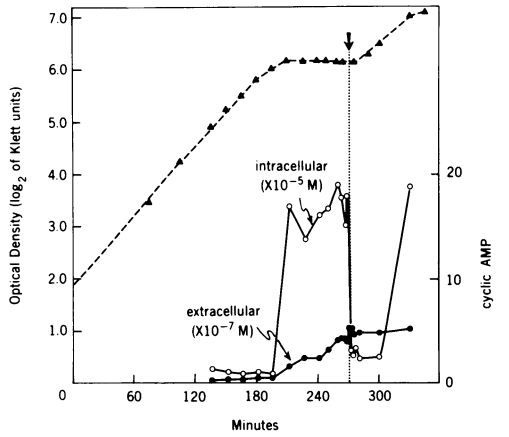

FIG. 3. *The intracellular and extracellular concentrations of c-AMP in a culture of AB257. Growth was limited by 3.5 mM glucose. The arrow denotes the time when the starving culture was replenished with glucose (3.5 mM final). See Fig. 2 for symbols.*

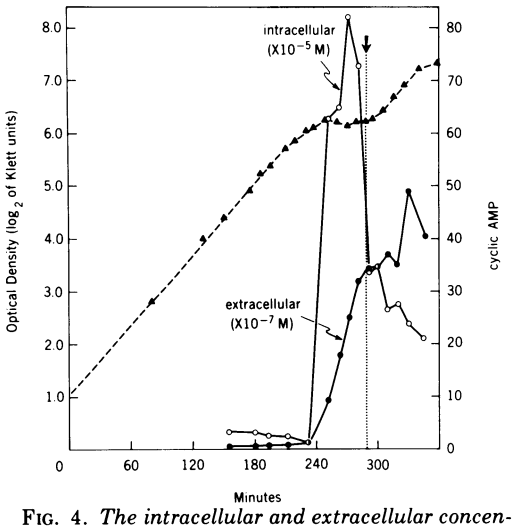

FIG. 4. *The intracellular and extracellular concentrations of c-AMP in a culture of strain Crookes. Growth was limited by 3.5 mM glucose. Symbols as in Fig. 2 and 3; however, note difference in scale.*

culture led to a rapid decrease in cellular c-AMP. When glucose was not added, the c-AMP was released gradually in much the same manner as in the case of strain AB257 (Fig. 2). The concentration of extracellular c-AMP increased about 30-fold, and the increase in extracellular c-AMP occurred at the same time as the accumulation of intracellular c-AMP (Fig. 4). Extracellular c-AMP continued to increase after intracellular accumulation was reversed. As in the case of AB257, the increase in the concentration of c-AMP in the culture fluid from 2,500 pmol/ml at the peak of the intracellular concentration to 5,000 pmol/ml at the end of the experiment is not accounted for by the decrease of *cellular* c-AMP from 200 pmol to 100 pmol/ml during the same period of time.

Cellular concentration of c-AMP during transient repression. The inducibility of β-galactosidase is inhibited severely and for a varying period of time (ranging in our experiments from 20 to 80 min) when glucose is added to a culture growing on glycerol. This phenomenon has been termed "transient repression" (reviewed in reference 6). We felt that a possible explanation for transient repression might be a severe lowering of the cellular concentration of c-AMP brought about by the addition of glucose to a culture growing on glycerol. We tested this hypothesis; the findings are shown in Fig. 5. Glucose (10 mM) and TMG (0.5 mM) were added simultaneously to a culture growing on 20

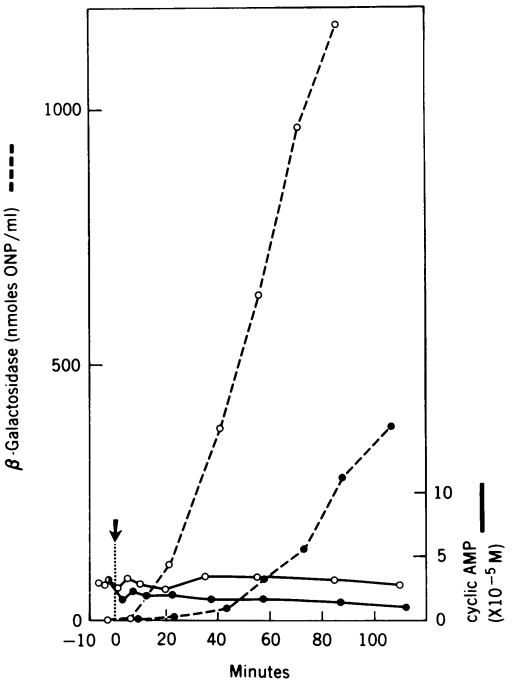

FIG. 5. *Cellular concentration of c-AMP and synthesis of β-galactosidase during transient repression. Strain AB257 was grown on medium containing 20 mM glycerol as source of carbon. At zero time (arrow), TMG (0.5 mM) and glycerol (20 mM) were added to 1 sample, and TMG (0.5 mM) and glucose (10 mM) were added to the second sample of the parent culture. Symbols: O——O, intracellular c-AMP of sample to which glycerol was added; ●——●, intracellular c-AMP of sample to which glucose was added; O----O, β-galactosidase of sample to which glycerol was added; ●----●, β, galactosidase of sample to which glucose was added.*

mM glycerol. A control culture received TMG and a second increment of 20 mM glycerol. The addition of glucose reduced the cellular concentration of c-AMP from 3.3×10^{-5} M to approximately 1×10^{-5} M; this latter concentration is typical of the concentration of c-AMP of cultures of strain AB257 during steady-state growth on glucose (Table 1). The addition of glucose inhibited the synthesis of β-galactosidase completely for approximately 20 min. In other experiments, isopropyl-thio-β-galactoside at 1 mM was employed as inducer of β-galactosidase instead of TMG with quite similar results. It appears that transient repression cannot be explained on the basis of a drastic lowering of the cellular concentration of c-AMP.

DISCUSSION

Pastan and Perlman found that exogenously added c-AMP overcame catabolite repression in *E. coli* (14). This finding suggested the possible existence of an inverse relationship between the cellular concentration of c-AMP and the intensity of catabolite repression. It became of interest, therefore, to determine cellular concentrations of c-AMP under a variety of conditions of growth.

The observations reported in this paper are in general agreement with our earlier findings (8, 9). There is an inverse relationship between the effectiveness of a given compound as source of carbon and the cellular concentration of c-AMP. Thus, for example, in strain AB257, which is wild-type with respect to catabolite repression, the generation time when glucose served as source of carbon was 56 min, and the concentration of c-AMP was 1.2×10^{-5} M. The generation time of the same strain when L-proline served as sole source of carbon was 218 min, and the concentration of c-AMP was 24×10^{-5} M. The relationship between the cellular concentration of c-AMP and the intensity of catabolite repression is illustrated by the same example. The culture grown on glucose had a specific β-galactosidase activity of 200 nmol of *o*-nitrophenol formed per min per mg of protein, whereas that grown on proline had a specific activity of 48,000 (Table 1).

Mutant AB257^{pc-1}, which is resistent to catabolite repression and defective in its c-AMP phosphodiesterase (8), had approximately twice the cellular concentration of c-AMP found in the wild-type parent when it was grown on glucose, glycerol, or succinate (Table 1). This finding is of interest for two reasons. First, it suggests that catabolite repression, rather surprisingly, is regulated by threshold concentrations of c-AMP; e.g., an increase of the cellular concentration of c-AMP from 1.2×10^{-5} M to 2.3×10^{-5} M permitted escape from catabolite repression. Second, it appears that, whereas the concentration of c-AMP was generally higher in mutant AB257^{pc-1} than in wild-type AB257, the fourfold difference found between, for example, cultures grown on succinate and cultures grown on glucose, was observed in both strains (Table 1). It will be recalled that the mutant has only a trace of c-AMP phosphodiesterase activity. Clearly the virtual absence of c-AMP phosphodiesterase permitted the accumulation of generally higher than wild-type levels of c-AMP. However, the fact that even in the mutant levels of c-AMP still differed with the source of carbon on which the culture was grown suggests that the c-AMP phosphodiesterase cannot be the primary site of regulation. One may assume, therefore, that either adenyl cyclase or release of c-AMP into the medium play a major regulatory role.

It is clear that under a number of conditions an inverse relationship obtains between the cellular concentration of c-AMP and the intensity of catabolite repression. It is also evident, however, that this relationship does not hold under all conditions. The complete inhibition of the synthesis of β-galactosidase brought about by growth on a mixture of glucose and gluconate (or glucose-6-phosphate, not shown) is accompanied by a cellular concentration of c-AMP no lower than that found during growth on glucose. Similarly, during transient repression (Fig. 5), the cellular concentration of c-AMP also does not fall below 10^{-5} M, typical of growth on glucose. Conversely, there is considerable synthesis of β-galactosidase in strains AB257^{pc-1} and Crookes during growth on the mixture of glucose and gluconate, despite the low concentration of c-AMP (Table 1). No interpretation is offered at this time for the apparent anomalies. Conceivably the synthesis of another cyclic nucleotide, such as cyclic guanosine monophosphate, or an effect on the formation of the "catabolite gene-activator protein" may provide an explanation.

Several workers (11, 18) failed to detect intracellular c-AMP under a variety of conditions of growth and suggested that c-AMP occurred mainly in the medium. Our findings indicate that, depending upon conditions, the intracellular concentration of c-AMP exceeded the *concentration* of c-AMP in the culture fluid by a factor of 50 to 1,000. The *total amount* of c-AMP was invariably higher in the medium than in the bacteria. This was anticipated in view of the fact that under the conditions of our

experiments the bacteria in 1 ml of culture occupied maximally a volume of 2.6×10^{-4} ml or, approximately 0.025% of the total volume of a given sample. It is for precisely this reason that attempts (18) to determine intracellular c-AMP by the subtraction of the c-AMP found in a cell-free filtrate from the c-AMP found in the unfiltered sample of the culture (i.e., including bacteria and medium) led to the erroneous conclusion that there was very little intracellular c-AMP. It should also be recalled that the c-AMP accumulates in the medium and that, therefore, the concentration of c-AMP in the culture fluid is affected by the length of time a given culture has resided in a given medium.

The finding that, when glucose was added to starving cultures, the cellular concentration of c-AMP dropped rapidly and the concentration of c-AMP in the medium increased concomitantly (Fig. 3, 4) is in agreement with a similar finding, obtained, albeit, under somewhat different circumstances by Makman and Sutherland (7). It is surprising, however, that the amount of c-AMP which appeared in the medium was greatly in excess of the amount of c-AMP found in the bacteria when this attained its maximal concentration. Clearly, c-AMP continued to be synthesized and was released into the medium at a time when intracellular accumulation had ceased. We are now exploring the mechanism of this "change in direction" of the flow of c-AMP.

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