crease in enzyme activity occurred (*9*). Apparently this 5-minute time period is required for the cyclic nucleotide to reach an intracellular concentration that will maintain *N*-acetyltransferase activity. This finding of complete antagonism of the effects of *l*-propranolol by dibutyryl cyclic AMP suggests to us that all the effects of *l*-propranolol on *N*-acetyltransferase activity are through an adrenergic receptor and are mediated by cyclic AMP. In addition, if we inhibit the *l*-propranolol-induced decrease in cyclic AMP by treating cells with either theophylline (10 m*M*) or isobutylmethylxanthine (0.5 m*M*), compounds which inhibit cyclic nucleotide phosphodiesterase activity (*10*), *N*-acetyltransferase activity also does not decrease.

It would appear from these observations that hours after the start of adrenergic stimulation, the low concentration of cyclic AMP that persists serves to maintain *N*-acetyltransferase activity at increased levels; the "turnoff" of enzyme activity could primarily be a result of a decrease in cyclic AMP below a critical intracellular concentration necessary for maintenance of the enzyme in an active state. This decrease might in part be due to a decrease in synthesis; alternatively, the decrease in cyclic AMP might reflect its release from a bound form and subsequent metabolism.

Previous experiments have indicated that a cessation of protein synthesis does not produce a rapid decrease in *N*-acetyltransferase activity (*2*, *3*). Hence, it seems improbable that "turnoff" is a result of a halt in the synthesis of a specific protein; the rapid rate of disappearance of enzyme activity is more consistent with an inactivation mechanism that might involve rapid reversal of the adrenergic-cyclic AMP-induced changes in membrane potential required for stimulation of *N*-acetyltransferase (*6*). It is interesting that induced *N*-acetyltransferase activity is highly unstable in broken cell preparations, and that it is stabilized by acetyl coenzyme A (*11*), and also that depolarizing agents can, albeit slowly, turn off *N*-acetyltransferase activity (*3*). In view of this, it seems possible that cyclic AMP, perhaps acting in part by way of its effects on membrane physiology (*6*), might facilitate the interaction between acetyl coenzyme A and enzyme molecules to maintain the enzyme in the active and stable form; alternatively, cyclic AMP might accomplish this by stimulating the phosphorylation of *N*-acetyltransferase molecules by protein kinase, a generally occurring mode of enzyme regulation.

The pinealocyte preparation in these experiments can be used to study the mechanism through which cyclic AMP acts to mediate rapid transmitter-regulated changes in enzyme function. Further information about this mechanism might be helpful in understanding the nature of rapid changes in neurochemical transduction, a fundamental issue in neurobiology.

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Regulatory Role of Guanosine 3′,5′-Monophosphate in Adrenocorticotropin Hormone-Induced Steroidogenesis

Abstract. *The relation between steroidogenesis induced by adrenocorticotropic hormone and the concentrations of adenosine 3′,5′-monophosphate (cyclic AMP) and guanosine 3′,5′-monophosphate (cyclic GMP) was studied at different time intervals in isolated adrenal cells. Submaximal and supramaximal steroidogenic concentrations of the hormone did not cause detectable changes in cyclic AMP during the first 30 minutes, whereas there was an increase in the concentration of cyclic GMP that was accompanied by phosphorylation and steroidogenesis. It is therefore suggested that cyclic GMP, rather than cyclic AMP, is the physiological mediator of adrenocorticotropic hormone–induced adrenal steroidogenesis.*

Isolated adrenal cells consisting mainly of fasciculata cells (*1*) respond to microunit amounts of adrenocorticotropin hormone (ACTH) (*1*) in the synthesis of corticosterone, have undetectable phosphodiesterase activity (*2*), and can convert the exogenously added precursors, (20*S*)-20-hydroxycholesterol, pregnenolone, progesterone, and deoxycorticosterone to corticosterone (*3*, *4*). This system thus permits direct investigation of ACTH action in an isolated fasciculata cell.

Previous studies (*4*) with this system provided evidence that was not compatible with the hypothesis (*5*) that adenosine 3′,5′-monophosphate (cyclic AMP) is the sole obligatory mediator of ACTH-induced steroidogenesis. Indirect evidence bringing into question the mediatory role of cyclic AMP in adrenal steroidogenesis has also been presented by other laboratories (*6*). In more direct studies (*7*), we showed that the physiological concentration of ACTH, less than 10 μU, stimulated phosphorylation and corticosterone synthesis but did not cause the corresponding increment in cyclic AMP synthesis. Under identical conditions, however, guanosine 3′,5′-monophosphate (cyclic GMP) concentrations were raised with the corresponding increase in the protein kinase activity and corticosterone synthesis (*7*).

To demonstrate unequivocally the correlation of cyclic GMP levels with the ACTH-induced steroidogenesis, we adopted a simple, sensitive, and specific assay for this nucleotide (*8*). We used crude *Escherichia coli* fractions instead of the highly purified *E. coli* polypeptide chain elongation factor Tu (EF-Tu) used by Arai *et al.* (*9*). This method, which is based on the highly specific binding property of the EF-Tu with guanosine diphosphate (GDP) includes the con-

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version of cyclic GMP to 5′-GMP by phosphodiesterase (E.C. 3.1.4.1) and the transfer of ^{32}P from [γ-^{32}P]adenosine triphosphate to GMP to yield [β-^{32}P]-GDP by GMP kinase. The radioactivity of [β-^{32}P]GDP bound to the crude *E. coli* fraction is determined by filtration through a Millipore disk. This method detects as little as 50 fmole of cyclic GMP in the incubation medium and does not cross react with cyclic AMP, inosine 3′,5′-monophosphate (cyclic IMP), cytidine 3′,5′-monophosphate, and uridine 3′,5′-monophosphate. In contrast to our findings. Shibuya *et al.* (*8*) found that cyclic IMP interfered in the assay based on the binding of GDP with purified EF-Tu fraction. Here we report the time course of the regulation of cyclic GMP under physiological and supraphysiological hormonal conditions and compare these values with those obtained for cyclic AMP under identical conditions. From our data we conclude that cyclic AMP is not the physiological mediator of ACTH action for the process of steroidogenesis.

Figure 1 shows that as little as 5 μU of ACTH (25 pg) induces the process of steroidogenesis in a linear fashion with an initial lag period of approximately 5 minutes. In contrast, cyclic AMP levels do not change in any time interval. However, when one examines the time course of cyclic GMP formation and phosphorylation in response to this concentration of the hormone, a perfect correlation between the onset of the synthesis of the cyclic nucleotide, protein kinase activity, and steroidogenesis is observed. The formation of cyclic GMP and the activation of protein kinase activity precede the onset of steroidogenesis. The addition of 100 μU of ACTH (500 pg), which stimulates maximum corticosterone synthesis (*1*), does not change the basal cyclic AMP concentration during the first 30 minutes, but the activation of steroidogenesis occurs along with the stimulation of cyclic GMP formation and phosphorylation (Fig. 2).

Figure 3 depicts the correlation between the synthesis of corticosterone and the endogenous concentrations of cyclic AMP and cyclic GMP formed in response to the varying concentrations of ACTH at 60-minute time intervals. The results again show that in the intact isolated adrenal cell low concentrations of ACTH, 2.5 to 10 μU (< 50 pg), do not increase the concentration of cyclic AMP but do stimulate steroidogenesis. In contrast, these concentrations of the hormone stimulate the peak synthesis of cyclic GMP with a concomitant increase in the protein kinase activity and corticosterone synthesis. It is only at supraphysiological concentrations of ACTH that a significant increase in cyclic AMP is observed. These results are in agreement with the previous (*7*) values for cyclic GMP except that the present more sensitive assay of cyclic GMP did not show the complete decline of cyclic GMP to the basal value with the higher concentration of the hormone.

These data indicate that cyclic AMP is not the physiological mediator of ACTH-induced steroidogenesis. We suggest that cyclic GMP fulfills the criterion of the second messenger and this cyclic nucleotide acts by way of the activation of cyclic GMP–dependent protein kinase.

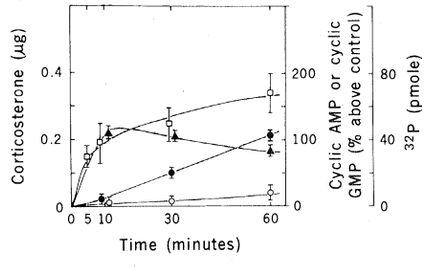
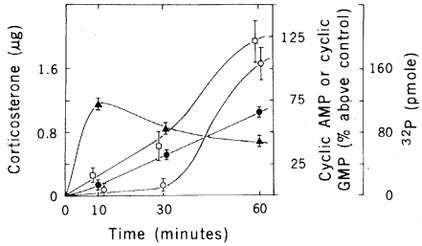


Fig. 1. Time response curve for the production of cyclic AMP (○), cyclic GMP (□), corticosterone (●), and phosphorylation (▲) in isolated adrenal cells in response to 5 μU of ACTH. For the incubation systems, 2×10^6 isolated adrenal cells were suspended in 0.8 ml; reagents were dissolved in 0.2 ml of Krebs-Ringer bicarbonate buffer, *p*H 7.4, containing 4 percent albumin and 0.2 percent glucose, or protein kinase buffer. The total volume of first incubation mixture was 1 ml and of the second incubation mixture, 0.5 ml. Experiments were conducted in sextuplicates: two of the samples were used for the determination of corticosterone, two for the measurement of cyclic AMP (*7*) and cyclic GMP, and two for the assay of phosphorylation (*7*). 5′-GMP and other noncyclic nucleotides were removed by alumina column (*10*). That the separation of cyclic GMP from 5′-GMP was complete was assured by applying to the alumina column a sample of ^3H-labeled cyclic GMP and ^{14}C-labeled 5′-GMP (each with 10,000 disintegrations per minute) and counting a portion of the eluate to check that there was no ^{14}C radioactivity in this fraction. The eluate was then absorbed on a QAE-Sephadex A-25 column in the formate form. A first elution with 7 ml of 0.5*N* formic acid yielded cyclic AMP and a second elution with 7 ml of 4*N* formic acid gave cyclic GMP. The recovery of cyclic AMP and cyclic GMP was 60 to 80 percent. The samples were lyophilized, dissolved in 700 μl of water, and the appropriate portion was used for the measurement of cyclic AMP and cyclic GMP. Results are expressed as the mean values (± standard deviation) of six separate determinations from three different experiments. Basal values have been subtracted from the experimental results.



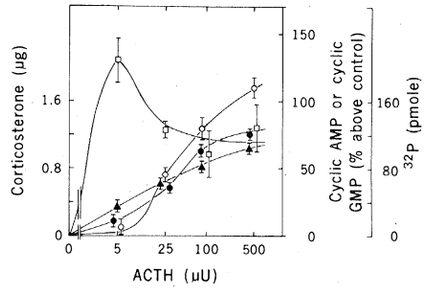


Fig. 2 (left). Time response curves for the production of cyclic AMP (○), cyclic GMP (□), corticosterone (●), and phosphorylation (▲) in isolated adrenal cells in response to 100 μU of ACTH. The conditions of the experiment are identical to those in Fig. 1. Fig. 3 (right). Concentration response curve for the production of cyclic AMP (○), cyclic GMP (□), corticosterone (●), and phosphorylation (▲) in isolated adrenal cells incubated for 60 minutes in the presence of 0 to 500 μU of ACTH. The conditions of the experiment are identical to those in Fig. 1.

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