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# REGULATION OF ADRENOCORTICAL STEROIDOGENESIS BY CYCLIC 3'-5'-GUANOSINE MONOPHOSPHATE IN ISOLATED RAT ADRENAL CELLS.*

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SUMMARY

Cyclic 3',5' guanosine monophosphate (cGMP) increased in isolated rat adrenocortical cells in a dose related manner with adrenocorticotropic hormone (ACTH). These increases were closely coupled to increases in steroidogenesis (r=0.95 to 1.00) as measured by corticosterone and summed corticosterone, progesterone, and 11-desoxycorticosterone. The data are compatible with the hypothesis that cGMP is the second messenger for ACTH.

## INTRODUCTION

In 1958 Haynes, *et al.* demonstrated that cAMP levels increase in rat adrenal slices during ACTH stimulated steroidogenesis (1). The functional implications of this observation seemed clear cut, however, in 1972 Beall and Sayers noted that although cAMP did increase during ACTH stimulated steroidogenesis in isolated adrenal cells, steroid production was as much as 70% of maximal before cAMP levels began to rise (2). In addition, Moyle, *et al.* elegantly demonstrated that derivatization of the single tryptophan residue of ACTH produced an analog of ACTH (NPS-ACTH) that was capable of stimulating maximal steroid production in isolated rat adrenal cells but

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ABBREVIATIONS USED: cAMP, cyclic 3'-5'-adenosine monophosphate; cGMP, cyclic 3'-5'-guanosine monophosphate; NPS-ACTH, *o*-nitrophenylsulfenyladrenocorticotrophin; ACTH, adrenocorticotrophin; progesterone, 4-pregnen-3, 20-dione; 11-desoxycorticosterone, 4-pregnen-21-ol-3, 20-dione; corticosterone, 4-pregnen-11β,21-diol-3,20-dione.

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was unable to cause a significant increase in cAMP (3). Finally, Kitabchi, *et al.* presented evidence that cGMP increases during ACTH stimulated steroidogenesis at low doses of ACTH where cAMP accumulation was nil (4). Because of these observations, the role of cAMP as the intermediate in the steroidogenic response is compromised. It is the purpose of this report to present evidence that cGMP rather than cAMP is a more viable candidate for the second messenger during ACTH stimulated steroidogenesis in isolated rat adrenal cells.

## MATERIALS AND METHODS

Materials: Porcine ACTH (91.5 i.u. per mg) was purchased from Sigma Chemical Company. Trypsin and lima bean trypsin inhibitor were purchased from Worthington Biochemical Corporation. Cyclic AMP and cGMP were purchased from Calbiochem as the free acid. Cyclic [8-$^3$H]AMP and cyclic [8-$^3$H]GMP were purchased from New England Nuclear. [1,2-$^3$H]corticosterone, [1,2,6,7-$^3$H]progesterone, [1,2-$^3$H]11-deoxycorticosterone were also purchased from New England Nuclear. Unlabeled corticosterone, progesterone, and 11-deoxycorticosterone were purchased from Steraloids, Inc. All glassware was silylated as described by Fenimore, *et al.* (5).

Preparation of Isolated Adrenal Cells: Cell suspensions were prepared as described by Sayers, *et al.* (6) with some modification. Adrenal glands were placed in clean, silylated Erlenmeyer flask equipped with a micro stirrer (1.5 mm x 8 mm) after dicing with small scissors. The amount of trypsin solution was approximately 1.0 ml per adrenal. Each 20 minutes tissue fragments were allowed to settle and supernatant withdrawn and placed in conical glass centrifuge tubes, an equal volume of trypsin solution was then added back to the fragments. Supernatants were centrifuge at 100xg for 15 minutes at 5°C. The supernatant was removed and cells were resuspended in buffer containing lima bean trypsin inhibitor (2 mg/ml). After five cycles of settling, readdition, centrifugation, and resuspension in lima bean trypsin inhibitor solutions, the conical tubes containing the dispersed cells were centrifuged at 100xg for 20 minutes at 5°C and the cells collected by discarding the supernatant. These cells were then resuspended in Krebs-Ringer bicarbonate buffer without lima bean trypsin inhibitor. Reproducible yields of 4-6 x $10^5$ cells/adrenal were obtained. These cells were judged viable by Trypan-blue exclusion as well as the inability to utilize exogenously added NADPH.

Incubation of Adrenal Cells: Adrenal cells were batch incubated with constant agitation and oxygenation (95% $O_2$, 5% $CO_2$) at 37°C and cell concentration of 1-1.5 x $10^5$ per ml. During a control period before addition of ACTH and at appropriate times after ACTH addition 0.5 ml aliquots of incubate were withdrawn and frozen at -70°C in a dry ice-acetone bath. These were saved

for later analysis of cyclic nucleotides and steroids in the whole incubate.

Measurement of Steroids: Corticosterone was measured by radioimmunoassay as described by Farmer, *et al.* (7). The sum of progesterone plus 11-deoxycorticosterone was measured by a co-specific antibody prepared to the 21-hemisuccinyl conjugate of 11-deoxycorticosterone. Other than these two steroids, the assay discriminated at least 4 orders from other competing steroids.

Measurement of Cyclic Nucleotides: Cyclic AMP was measured by the method of Farmer, *et al.* (8). Cyclic GMP was measured using the sensitive technique of Brooker, *et al.* (9).

## RESULTS

At no dose of ACTH during the short interval incubations (10 minutes & 20 minutes) was cAMP increased over control levels. At later times at the highest dose of ACTH used, cAMP levels showed a slight increase that was not correlated to either steroid production or rate of steroid production. However, throughout the incubation levels of cGMP were found to correlate well with steroid production as shown in Table I. The figures illustrate the observed linear nature of steroid production and cGMP synthesis. In addition, at the earliest time intervals rather than later intervals, (10 minutes & 20 minutes) this correlation can be seen to lie between .99 and 1.0. Generally, at later time intervals the correlations are lower probably because of some residual phosphodiesterase activity as well as degradation of added ACTH (6)(10).

Secondly, measurement of corticosterone by itself does not constitute a measure of total steroid production. At all times during the incubation progesterone plus 11-deoxycorticosterone is a significant proportion of the total steroids produced.

## DISCUSSION

These data clearly show a high degree of coupling between cGMP synthesis and steroidogenesis. At the majority of doses and times, cAMP remained at control level while cGMP levels correlate

TABLE I: Coefficient of correlation for cGMP and steroids depicted in Figure 1.

| 10 min. | | r | p< |
|---|---|---|---|
| cGMP | vs Total (P+B+DOC) | .994 | .050 |
| | Corticosterone (B) | .999 | .005 |
| 20 min. | | r | p< |
| cGMP | vs Total (P+B+DOC) | .995 | .050 |
| | Corticosterone (B) | 1.0 | .025 |
| 40 min. | | r | p< |
| cGMP | vs Total (P+B+DOC) | .950 | .005 |
| | Corticosterone (B) | .980 | .025 |
| 60 min. | | r | p< |
| cGMP | vs Total (P+B+DOC) | .966 | .025 |
| | Corticosterone (B) | .980 | .025 |

quite closely to steroid production. At low doses of ACTH (50μU) that more closely resemble the physiological situation, cGMP levels decline from a high at the 10 minute period of 160 pM above control levels to a point 80 pM below control levels at 60 minutes. Corticosterone synthetic rates estimated over 10 minute periods rise precipitously between 10 and 20 minutes. In the 40-60 minute interval there was no net steroid synthesis at the lower doses. This was paralled by a decline in cGMP to below control levels. Therefore, coupling of steroidogenesis and cGMP is extant in the final phase of the incubation when steroidogenesis has ceased at the lowest dose and is diminished at higher doses of ACTH.

Although minimal to unobservable phosphodiesterase activity is demonstrable with cAMP as substrate in this cell preparation (6), cGMP levels do follow a pattern indicative of the presence of a phosphodiesterase. The fact that levels of cGMP at low doses of ACTH decline below control levels also suggests the

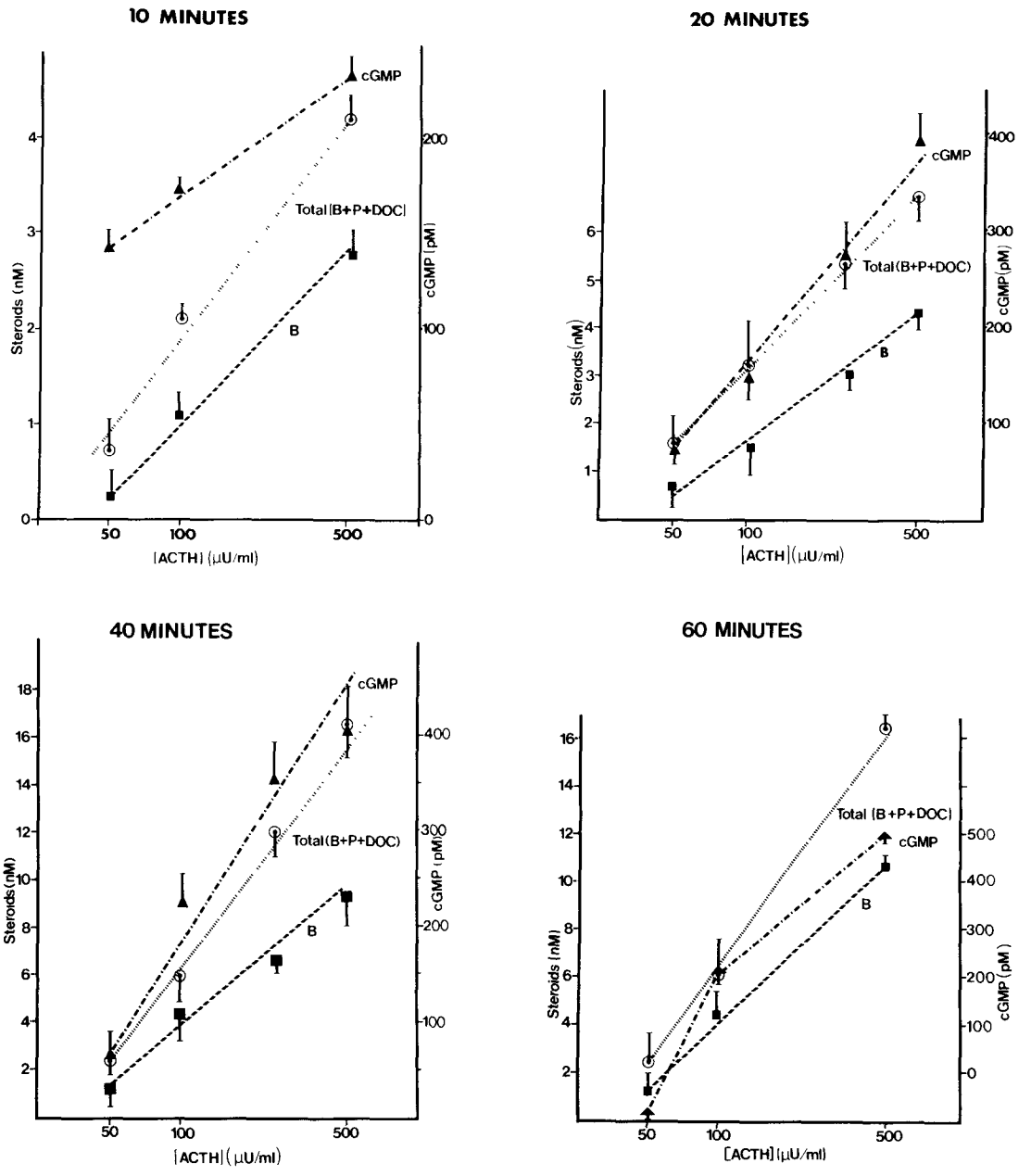


FIGURE 1. Effect of increasing concentrations of ACTH on cGMP, corticosterone = B and progesterone + 11-desoxycorticosterone = Total (B+P+DOC). Error expressed as standard error of the mean. Data are expressed as changes from 'zero ACTH added' controls at each time period. Basal cGMP was 200 pM. Molar concentrations are based on the aliquot withdrawn for analysis (see methods).

potential activation of such an enzyme system. It has been demonstrated that $Ca^{++}$ fluxes in adrenal quarters increase during ACTH stimulation (11). Several other authors has noted the presence of a $Ca^{++}$ dependent activator of phosphodiesterase (12,13, 14) in various tissues. These two observations may explain in part the activation of a cGMP phosphodiesterase.

Corticosterone precursors constitute a significant portion of the steroids produced. At low doses of ACTH progesterone plus 11-desoxycorticosterone exceeded corticosterone by a factor of two. One can hypothesize that at earlier times (<10 minutes) and lower doses of ACTH, the precursors are more important indices of steroidogenesis than corticosterone in this system. The increase above basal levels was much more marked for progesterone plus 11-desoxycorticosterone than was corticosterone at all points. The underlying reason for the increased precursor levels when compared to adrenal venous blood is undefined. It may simply be the result of greater exposure to extracellular fluid *in vitro* as compared to *in vivo* or it may be the result subtle changes in the adrenal membrane not reflected by either NADPH addition or trypan blue exclusion.

Clearly these data point to a more plausible relationship between cGMP synthesis and steroidogenesis than cAMP and steroidogenesis. Therefore it is reasonable to hypothesize that cGMP rather than cAMP mediates ACTH stimulated steroidogenesis.

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