

Isolated Adrenal Cells: Corticosterone Production in Response to Cyclic AMP (adenosine-3',5'-monophosphate)¹ (35325)

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Haynes *et al.* (1) have demonstrated that ACTH and cyclic AMP stimulate sections of rat adrenals to secrete corticosterone. Isolated rat adrenal cells, like adrenal sections, respond to ACTH with increased production of corticosterone (2). In this report, we describe the responses of suspensions of isolated adrenal cells to the addition of cyclic AMP and to its derivative, dibutyryl cyclic AMP.

Materials and Methods. Suspensions of cells of the rat adrenal cortex were prepared by the method of Swallow and Sayers (2) with modifications (Sayers, Swallow, and Giordano, unpublished data). Briefly, the modified method is as follows. Adrenal quarters are placed in Krebs-Ringer buffer (KRB) which contains glucose (0.2 g/100 ml) and trypsin (0.25 g/100 ml); the mixture is agitated with a siliconized glass paddle driven at 500 rpm at 37°. Freed cells in suspension are periodically withdrawn, pooled (total volume about 100 ml) and collected as a pellet by centrifugation. The pellet is suspended in 34 ml of KRB which contains glucose (0.2 g/100 ml), bovine serum albumin (BSA) (0.5 g/100 ml), calcium (2.55 mM, that of KRB or 7.65 mM, a concentration three fold that of KRB) and lima bean trypsin inhibitor (0.1 g/100 ml). Cells number about 500,000/ml of suspension.

Test substances, dissolved in 0.1 ml of vehicle, were added to 0.9-ml aliquots of the cell suspension and the mixture was incubated for 2 hr at 37° in a Dubnoff incubator (66 oscillations/min), atmosphere 95% O₂, 5% CO₂.

Fluorescence in methylene chloride extracts of the incubates was determined by a modification of the method of Silber *et al.* (3). At least 90% of the fluorescent material in the incubates is corticosterone as demonstrated by thin-layer chromatography.

ACTH was USP Standard in ampoules with a potency of 1.5 *Intravenous* IU/ampoule (4). Dibutyryl cyclic AMP, cyclic AMP, and 5'-AMP were obtained from Schwarz BioResearch, Inc.; and adenosine, ATP, and theophylline were from Mann Research Laboratories. The vehicle consisted of 0.9% sodium chloride, 0.5% BSA brought to pH 3.5 with the addition of 0.1 *N* HCl; vehicle only, in a volume of 0.1 ml, was added to duplicate samples of cell suspension and incubated 2 hr to serve as blanks. Response is expressed as *net* production of corticosterone: corticosterone in sample to which test substance had been added minus corticosterone in the vehicle blanks.

Results. *The response of isolated cells to dibutyryl cyclic AMP and to ACTH.* Dibutyryl cyclic AMP was added to aliquots of a suspension of isolated adrenal cells in quantities ranging from 1×10^{-9} to 1×10^{-5} mole/1.0 ml of suspension. The relation between rate of secretion of corticosterone [(B) produced (nmoles/2 hr of incubation)] and log of the moles of dibutyryl cyclic AMP added is represented by a sigmoid curve (Fig. 1). Also plotted in Fig. 1, are the experimental data for the response of the cells to ACTH. Data from 2 other experiments for dibutyryl cyclic AMP are presented in Table I. All three experiments for dibutyryl cyclic AMP are presented in graphic form in Fig. 2; because the values of maximum rate of B production (B_{max}) vary from one experiment to the next, response for each experi-

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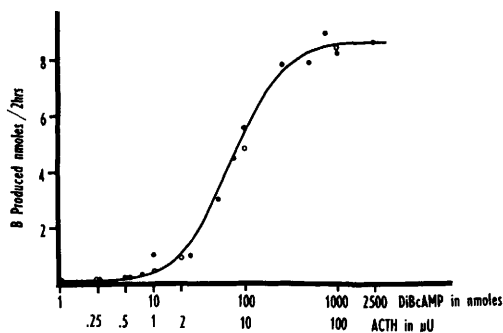


FIG. 1. Net corticosterone production (B) (nmoles/2 hr) plotted against dibutyryl cyclic AMP (moles) or ACTH (μ U) added to isolated cell suspensions: incubation medium contained calcium in a concentration of 7.65 mM; B produced in vehicle blank equaled 0.39 nmoles/2 hr; points are values for single aliquots of suspension; Expt. 12·239.

ment has been expressed as B/B_{\max} . That amount of dibutyryl cyclic AMP which when added to 1-ml aliquots of cell suspension induces half maximum steroid production is between 6 and 8×10^{-8} moles; this amount of dibutyryl cyclic AMP appears to be independent of the value of B_{\max} .

Specificity of the response. Dibutyryl cyclic AMP was clearly the most potent of the nucleotides tested (Table II). An amount equal to 10^{-8} moles added to 1.0 ml of suspension (concentration equal to 10^{-5} M) induced a significant increase in steroidogenesis by the isolated adrenal cells. One hundred times this amount (10^{-6} moles) of 5'-AMP,

TABLE I. Net Corticosterone (B) Production by Isolated Adrenal Cells in Response to Dibutyryl Cyclic AMP.^a

DiBcAMP added (moles)	Net B produced (nmoles/2 hr)	
	Expt. 9·259	Expt. 12·119
10^{-8}	0.66, 0.51	1.41, 1.53
5×10^{-8}	—	4.47, 4.35
10^{-7}	10.68, 10.83	7.20, 7.59
10^{-6}	15.93, — ^b	—
10^{-5}	15.57, 16.14	10.95, 10.65

^a Calcium concentration of incubation medium, 7.65 mM. Vehicle blanks: for Expt. 9·259, 0.51 and 0.48 nmoles/2 hr; for Expt. 12·119, 0.51 and 0.51 nmoles/2 hr.

^b Sample lost.

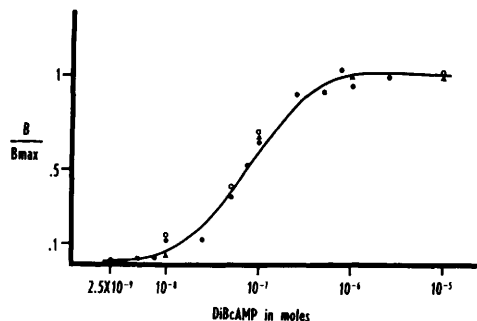


FIG. 2. B/B_{\max} plotted against dibutyryl cyclic AMP added to cell suspensions for three separate experiments (12·239, 9·259, and 12·119).

adenosine, or ATP induced a very slight increase in B production, but very small increases also occurred at 10^{-7} and 10^{-8} moles and most likely represent some general effect on cellular metabolism. Cyclic AMP induced small and barely significant increases at 10^{-8} and 10^{-7} moles; 10^{-6} moles exhibited activity corresponding approximately to that of 2×10^{-8} moles of its dibutyryl derivative. *Theophylline* added to the cell suspensions to make a concentration of 10^{-3} M enhanced the activity of cyclic AMP but not that of ACTH (Table III).

Discussion. The observations of this report confirm those of Haynes *et al.* (1) to the effect that cyclic AMP stimulates steroidogenesis by the cells of the adrenal cortex. This together with the fact that ACTH increases the production of cyclic AMP by adrenal quarters (5) offers strong evidence in support of the thesis that cyclic AMP is an intermediary in the sequence of events initiated by ACTH and ending, in the case of the rat adrenal, with corticosterone secretion.

As expected, dibutyryl cyclic AMP was much more potent in enhancing steroidogenesis than the parent compound, cyclic AMP. The butyryl derivative may be degraded at a slower rate and/or may penetrate the plasma cell membrane more rapidly (6). In this connection, theophylline enhanced the steroidogenic action of cyclic AMP presumably by inhibition of phosphodiesterase (7). In contrast, the methyl xanthine had no significant influence on the activity of ACTH. Halkerton *et al.* (8) found that theophylline failed to enhance ACTH-induced steroidogenesis in

TABLE II. Net Corticosterone (B) Production by Isolated Adrenal Cells in Response to Dibutyryl Cyclic AMP (Expt. 7·109); Cyclic AMP, 5'-AMP, Adenosine, and ATP (Expt. 7·19).^a

Dibutyryl cAMP		Cyclic AMP	5'-AMP	Adenosine	ATP	
Added (moles)	B produced (nmoles/2 hr)	Added (moles)	B produced (nmoles/2 hr)			
10 ⁻⁸	0.54, 0.54	10 ⁻⁸	0.09, 0.09	0.06, 0.06	0.09, 0.06	0.06, 0.03
2 × 10 ⁻⁸	1.44, 1.41	10 ⁻⁷	0.12, 0.09	0.15, 0.15	0.12, 0.12	0.12, 0.15
4 × 10 ⁻⁸	4.77, 4.83	10 ⁻⁶	1.29, 1.23	0.12, 0.12	0.06, 0.09	0.09, 0.12

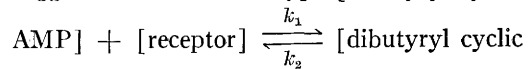
^a Calcium concentration of incubation medium 2.55 mM. Vehicle blanks: for Expt. 7·109, 0.63 and 0.57 nmoles/2 hr; for Expt. 7·19, 0.30 and 0.27 nmoles/2 hr.

rat adrenal bisects. They also found that theophylline inhibited incorporation of ¹⁴C-labeled glycine into protein in the incubated adrenal sections and suggested that the methyl xanthine "... has two antagonistic components, (i) potentiation of the steroidogenic action by protection of 3',5'-AMP, and (ii) reduction of the steroidogenic action by inhibition of protein synthesis."

Kowal and Fiedler (9) have reported that monolayer cultures of adrenal cells derived from murine adrenal tumors exhibit increased steroid production in response to the addition of a variety of nucleotides and to adenosine. Adenosine, 5'-AMP, ADP, ATP, and cyclic AMP were demonstrated to have about the same steroidogenic potency; cyclic AMP was more potent than dibutyryl cyclic

AMP. These observations are in contrast to the findings of Haynes *et al.* (1) for adrenal fragments and the observations reported here for isolated rat adrenal cells. Furthermore, Stollar *et al.* (10) found 5'-AMP to be much less active than cyclic AMP in stimulating steroidogenesis in monolayer cultures of cells derived from a mouse tumor. Perhaps the action of the various nucleotides described by Kowal and Fiedler (9) is not a direct one on steroidogenesis but an indirect one via enhancement of synthesis of cyclic AMP. Sattin and Rall (11) have described increased rate of synthesis of cyclic AMP following addition of various nucleotides to brain tissue.

The sigmoid curve relating corticosterone production and log of the quantity of dibutyryl cyclic AMP added (Figs. 1 and 2) suggests kinetics of the type [dibutyryl cyclic



AMP · receptor] $\xrightarrow{k_3}$ corticosterone. The relation between steroid production and ACTH added is also described by such kinetics (12). In the case of the nucleotide, the rate of transfer across the plasma membrane must be of significance in relating response of the cells to quantity of the nucleotide in the medium. Furthermore, since the site and the mode of action of cyclic AMP to promote steroidogenesis in the cells of the adrenal cortex are still subjects of active investigation we defer a statement as to the significance of the sigmoid relationship illustrated in Figs. 1 and 2.

Summary. Cyclic AMP and dibutyryl cyclic AMP stimulate corticosterone production when added to suspensions of isolated rat

TABLE III. Net Corticosterone (B) Production (nmoles/2 hr) by Isolated Adrenal Cells in Response to ACTH and to Cyclic AMP in the Absence and in the Presence of Theophylline (10⁻³ M). Expt. 7·149.^a

ACTH added (μU)	Without theophylline	With theophylline
1.25	0.12, 0.09	0.12, 0.18
5	0.84, 0.87	1.32, 0.84
20	6.39, 6.51	4.10, 6.75
Cyclic AMP added (moles)		
10 ⁻⁷	0.0, 0.06	0.0, 0.0
10 ⁻⁶	1.95, 2.01	6.93, 6.93

^a Calcium concentration of incubation medium, 2.55 mM. Vehicle blanks: without theophylline, 0.90 and 0.87; with theophylline, 0.81 and 0.78 nmoles/2 hr.

adrenal cells. Theophylline enhances the action of cyclic AMP but not that of ACTH. Adenosine, 5'-AMP, and ATP exhibit no or very slight steroidogenic activity in the isolated cell system.

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