STEROIDOGENESIS IN ISOLATED ADRENAL CELLS OF RAT

II. Effect of Caffeine on ACTH and Cyclic Nucleotide-Induced Steroidogenesis and Its Relation to Cyclic Nucleotide Phosphodiesterase (PDE)

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Summary

Corticosterone production in isolated adrenal cells (IAC) of rat has been measured in response to ACTH or ribonucleoside 3',5'-cyclic phosphate of adenosine (c-AMP), guanosine (c-GMP), inosine (c-IMP) and N^6-2'-O dibutyryl adenosine monophosphate (dc-AMP) in the presence and absence of caffeine. Caffeine inhibited ACTH-induced steroidogenesis in a manner independent of its effect on PDE. Study of PDE in whole adrenal homogenate showed hydrolysis of c-AMP, c-GMP and c-IMP but not of dc-AMP and other cyclic nucleotides. No PDE activity was demonstrable in IAC. High sensitivity of IAC to minute quantities of ACTH and various cyclic nucleotides may be due in part to lack of PDE activity in these preparations.

Introduction

We have recently reported on homogenous preparation of fasiculta cells of the rat adrenal cortex which is sensitive to physiologic concentrations of ACTH and thus is a suitable tool for bioassay of ACTH (1). It was demonstrated that in these preparations corticosterone synthesis is stimulated in response to minute quantities of synthetic corticotropin and cyclic 3',5' nucleotides of adenosine (c-AMP), guanosine (c-GMP), inosine (c-IMP) and dibutyryl 3',5'-AMP (dc-AMP) but not cytosine (c-CMP), thymidine (c-TMP) and uridine (c-UMP) (2). Unlike the quartered adrenal gland which shows 3-5 fold response to milli unit quantities of ACTH, corticosterone production in isolated adrenal cells (IAC) is stimulated at least 100 fold above the base line value with only 20-50 μU of ACTH/400,000 cells (one adrenal). The recent observations in our laboratories (2,3), as well as the results of others (4-10) on the biologic activities of

various cyclic nucleotides on hormonally sensitive tissues, have raised the possibility of involvement of more than a single cyclic nucleotide (i.e., c-AMP) as the mediator of hormone action.

It is known that the effective intracellular concentration of any cyclic nucleotide is partially controlled by the activity of phosphodiesterase (PDE) (11), and it has been assumed that the effects of methylxanthine are exerted through PDE activity. In an effort to elucidate the mechanism of steroidogenesis by various cyclic nucleotides, the effect of caffeine on steroidogenesis as well as the metabolism of exogenous cyclic nucleotides was investigated in IAC. Whereas whole adrenal tissue PDE could hydrolyze purine containing cyclic nucleotides, IAC were found to contain no PDE activity. Caffeine which is expected to stimulate corticosterone production through increased concentration of intracellular c-AMP not only did not stimulate, but it inhibited steroidogenesis in IAC. The relationship of this finding to the apparent lack of PDE in IAC is discussed.

Materials and Methods

The method of adrenal cell preparation was a modification (2) of the trypsin digestion method (12). In general, for each IAC preparation, adrenals from 16 rats were used, and cells from each adrenal (approximately 400,000 cells) were resuspended in 0.8 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 4% albumin and 0.2% glucose (KRB-AG). Production of corticosterone (B) from the adrenal cells was measured by fluorometric method (13). Results are reported as µg B produced/ml of incubation/cells from one adrenal in two hours. PDE was assayed by the modified (11) method of Butcher et al (14) or by the use of labelled precursor (^{3}H c-AMP) by the method of Thompson and Appleman (15). Cyclic nucleotides were obtained from Sigma Chemical Company or Boehringer-Mannhein. ACTH with activity of 1.5 IU/ampule was purchased from U. S. Pharmacopeia. All other chemicals were reagent grade and were obtained commercially.

Results

As can be seen from Table I, ACTH, c-AMP, dc-AMP, c-IMP and c-GMP all

TABLE I

EFFECT OF CAFFEINE ON CORTICOSTEROIDOGENESIS IN THE ISOLATED ADRENAL CELLS

Experimental Condition	µg Corticosterone / ml/2 hrs
Baseline*	0.047
Caffeine (1 mM)	0.001
(5 mM)	0.005
(10 mM)	0.001
ACTH (5 µU/ml)	0.878
+Caffeine (1 mM)	0.918
+Caffeine (5 mM)	0.656
+Caffeine (10 mM)	0.509
dc-AMP (0.10 mM)	1.016
+Caffeine (1 mM)	1.200
+Caffeine (5 mM)	0.943
+Caffeine (10 mM)	0.698
c-IMP (3 mM)	0.841
+Caffeine (10 mM)	0.794
c-GMP (8 mM)	0.513
+Caffeine (10 mM)	0.384

*Baseline incubation mixture consists of 0.8 ml of adrenal cell suspensions (from one adrenal) in KRB-AG, pH 7.4, and 0.2 ml of vehicle (NaCl in 2% albumin, pH 3.5). Total volume: 1 ml. Incubation was carried out at 37°C in a metabolic shaker under 5% CO_2 and 95% O_2 for two hours.

stimulate corticosterone production at the concentration employed. The concentrations used here are the half-maximal dose obtained from sigmoid curves (2). We have previously reported that other cyclic nucleotides had no stimulatory effect on these preparations (2). This table also demonstrates that the inhibitor of PDE, caffeine, which was expected to stimulate steroidogenesis, was not only an ineffective stimulator, but it exhibited an

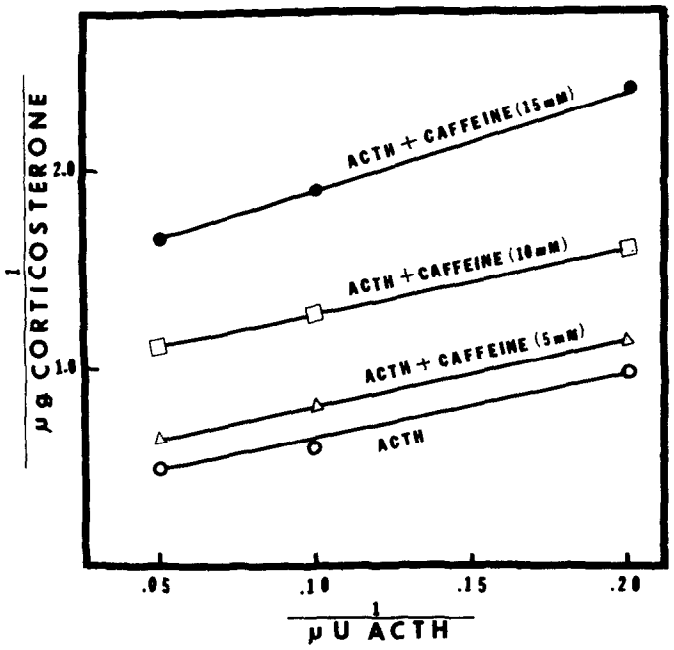

Figure 1. Effect of different concentrations of caffeine on ACTH-induced steroidogenesis in IAC. Condition of experiment is similar to Table I.

inhibitory effect on ACTH and cyclic nucleotide-induced steroidogenesis. Because of the significant inhibitory effect of caffeine, a more detailed study of this compound in combination with ACTH was undertaken. Figure 1 shows a double reciprocal plot on ACTH concentration against corticosterone production with three concentrations of caffeine. As can be seen, a straight line is obtained for effect of ACTH alone on IAC. Furthermore, the inhibitory effect of caffeine with ACTH in Figure 1 appears not to be competitive in nature, contrary to what would be expected if PDE inhibition were the only mechanism by which caffeine acts. Because of this effect of caffeine, studies were made of PDE activity of adrenal cells versus adrenal quarters. As can be seen from Table II, although whole adrenal homogenate has a rich source of PDE which can hydrolyze c-AMP, c-GMP, c-IMP, and to a lesser extent c-CMP, (but not dc-AMP, c-TMP, c-CMP and c-UMP), the homogenate of IAC is almost devoid of PDE activity. Results on this table show also that caffeine is capable of inhibiting and imidazole of stimulating whole adrenal PDE similar

TABLE II

HYDROLYSIS OF CYCLIC NUCLEOTIDES BY ADRENAL PHOSPHODIESTERASE*

Substrate	Addition (conc.)	Adrenal Homog. % Hydrolysis	Adrenal Cells % Hydrolysis†
c-AMP		100**	4.5
c-AMP	Caffeine (0.2 mM)	96	0
c-AMP	Caffeine (1 mM)	96	0
c-AMP	Caffeine (5 mM)	81	0
c-AMP	Caffeine (10 mM)	49	0
c-AMP	Imidazole (5 mM)	184	9.6
c-IMP		92	0
c-GMP		71	0
c-CMP		17	0
c-UMP		8	0
c-TMP		4	0
dc-AMP		4	0

*Incubation Mixture: Tris Hcl 40 mM (pH 7.5); Mg SO_4, 1.8 mM; enzyme, 1 mg protein; substrate, 2 mM. Total Volume: 1 ml. Incubation at 30°C in air for one hour.

**100% hydrolysis for c-AMP is taken as conversion of 370 nanomoles of the substrate/mg protein/60 minutes.

†Phosphodiesterase activity is measured by the use of tritiated c-AMP (15).

to PDE from other tissues (11). Since it has been suggested that, aside from its inhibitory effect on PDE, caffeine in isolated fat cell may produce other effects (3), it is possible that it could also cause inhibition of protein synthesis in adrenal cells similar to theophylline in adrenal quarters (16). Experiments were performed in which adrenal cells were first preincubated with caffeine, washed free of caffeine, and incubated with ACTH and dc-AMP.

No significant change in steroidogenic properties of ACTH and dc-AMP was noted following preincubation with caffeine (data not shown), suggesting that the effect of caffeine is dependent on the continued presence of ACTH and dc-AMP.

Discussion

Studies presented here on PDE activity of adrenal homogenate represent hitherto unknown effects of c-GMP and c-IMP as substrates of adrenal PDE. It is interesting to note that PDE in adrenal homogenate is capable of hydrolyzing c-AMP, c-GMP, and c-IMP, which are capable of stimulating steroidogenesis in IAC. Lack of PDE activity in IAC in comparison to quartered glands may in part explain *1)* the greater sensitivity of IAC to ACTH and cyclic nucleotides, since the latter compounds cannot be as readily metabolized by PDE of IAC, and *2)* the lack of a stimulatory effect of caffeine on steroidogenic properties of ACTH and dc-AMP. Since the action of the latter two compounds on adrenal steroidogenesis is mediated through activation of protein kinase and formation of a new protein for the stimulation of the rate limiting step in steroid synthesis (17), then the effect of caffeine on steroidogenesis could be independent of its effect on PDE but may possibly be explained by the inhibitory effect of caffeine on protein synthesis. The kinetic studies on Figure 1 are not at variance with such a possibility.

It would appear also that because of a negligible level of PDE activity in IAC, this preparation would be a useful tool for studying the kinetics of production of cyclic nucleotides catalyzed by adrenal cyclase(s) in the absence of methylxanthines without significant destruction of the end product by PDE.

The results of the study of pharmacological agents as a means of explaining hormonal action through cyclic nucleotide mediation must be interpreted with caution, since the possibility of the existence of multiple functions for these agents in the cell aside from their effect on PDE is suggested by the present work as well as by previous investigations (3,16).

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