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Steroidogenesis and Cyclic Adenosine 3′,5′-Monophosphate Accumulation in Rat Adrenal Cells

DIVERGENT EFFECTS OF ADRENOCORTICOTROPIN AND ITS *o*-NITROPHENYL SULFENYL DERIVATIVE*

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SUMMARY

Both adrenocorticotropin (ACTH) and its *o*-nitrophenyl sulfenyl derivative (NPS-ACTH) in which the single tryptophan residue of the hormone is modified, were able to stimulate corticosterone synthesis to the same maximal rate in isolated rat adrenal cells. The concentration of NPS-ACTH required for half-maximal steroidogenesis was approximately 70 times that of ACTH. Although both ACTH and NPS-ACTH stimulated cyclic AMP accumulation, the effect of NPS-ACTH was marginal; the maximal stimulation of cyclic adenosine 3′,5′-monophosphate (cyclic AMP) accumulation in response to ACTH was 30 to 100-fold greater than that due to NPS-ACTH. Apparently ACTH increased cyclic AMP accumulation well beyond that required for the stimulation of maximal steroidogenesis. NPS-ACTH appeared to inhibit in a competitive manner the effect of ACTH on cyclic AMP production but not steroid synthesis. The continued presence of ACTH or NPS-ACTH was necessary for the continued stimulation of steroidogenesis indicating that the factor (or factors) mediating the steroidogenic response must be present throughout the time of stimulation. The relationship between steroid synthesis and cyclic AMP accumulation was different for ACTH and NPS-ACTH. Much less cyclic AMP was produced when NPS-ACTH stimulated steroid synthesis to 75% of the maximal level than when ACTH enhanced steroidogenesis half-maximally. Even though cyclic AMP was found to leave the cells during the time of incubation, the same lack of correlation between cyclic AMP generation and rate of steroid synthesis mentioned above was found whether intracellular or total cyclic AMP was measured.

These results suggest that there may be two receptors for ACTH in the adrenal cell population which may be in the same cell or in different cell types. NPS-ACTH stimulates one of these receptors but inhibits the other. Furthermore, these results imply that either very small amounts of cyclic AMP are required for the stimulation of steroidogenesis or factor (or factors) besides cyclic AMP may be involved in mediating this function of ACTH.

Since the classic report of Haynes and Berthet (1) in 1957 implicating cyclic AMP[1] in adrenal steroidogenesis, there has been a burgeoning of the literature with articles relegating the action of ACTH on the adrenal gland to generation of cyclic AMP. Once generated, cyclic AMP stimulates all the processes that lead to the manifestation of the physiological responses attributed to the action of ACTH. Thus, cyclic AMP has been implicated in virtually all aspects of adrenal function ranging from steroidogenesis (2, 3) to ascorbic acid depletion (4) and adrenal growth (5). Most of these studies were designed to show that ACTH stimulated cyclic AMP production and that cyclic AMP could duplicate the effects of ACTH. Relatively few attempts have been made to study the quantitative aspects of the relationship of cyclic AMP generation to the end response that was observed.

We have now investigated the relationship between cyclic AMP accumulation and corticosterone synthesis in isolated rat adrenal cells in response to ACTH and its *o*-nitrophenyl sulfenyl derivative, NPS-ACTH, prepared by chemical modification of the single tryptophan residue in ACTH (6). NPS-ACTH has been shown to be a specific inhibitor of ACTH-induced stimulation of lipolysis in isolated rat fat cells (6) as well as adenylate cyclase in rat fat cell ghosts (7). The measurement of very small amounts of cyclic AMP has been facilitated by the availability of a simple and sensitive method described by Gilman (8).

EXPERIMENTAL PROCEDURE

Materials—Highly purified ACTH (173 i.u. per mg) (9) and NPS-ACTH (6) were prepared according to the published procedures. Collagenase (CLS grade) and lima bean trypsin inhibitor were purchased from Worthington. Cyclic [8-^{3}H]AMP

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[1] The abbreviations used are: cyclic AMP, cyclic adenosine 3′,5′-monophosphate; ACTH, adrenocorticotropin; NPS-ACTH, *o*-nitrophenyl sulfenyl adrenocorticotropin.

(20.8 Ci per mmole) was obtained from Schwarz-Mann. Merck aluminum oxide (neutral, activity I, for column chromatography) was purchased from Brinkmann Instruments, Inc., Westbury, N. Y.

Preparation of Adrenal Cells—Cell suspensions were prepared by modification of previously published procedures (10, 11). Adrenal glands from adult male Sprague-Dawley rats (300 to 350 g) were decapsulated, minced, and placed in tubes containing 5 ml of cold Krebs-Ringer bicarbonate buffer, pH 7.1, 20 mg of collagenase, and 5 mg of glucose. Usually, 20 adrenals were added per tube (50 ml of polypropylene centrifuge tube, Falcon Ind., Ventura, Calif.). When all the adrenals had been obtained, the flasks were gassed with 95% O_2-5% CO_2, capped, and laid sideways in a Dubnoff metabolic incubator. After gentle shaking at 37° for 30 min collagenase digestion was terminated and 5 ml of 0.9% NaCl solution (23°) were added to facilitate removal of the adrenal pieces from a "mucus-like" substance entrapping them. Adrenal fragments were removed from the "mucus" by agitating the mass with a glass rod and allowing the pieces to fall to the bottom of the tube. The collagenase and the mucus material were removed with a Pasteur pipette and discarded. Two milliliters of Krebs-Ringer bicarbonate buffer containing 1 mg per ml of glucose were added to the adrenal fragments which were then repeatedly drawn in and out of a 10-inch segment of Tygon tubing (⅛ inch inside diameter) attached to the needle mount of a 3-ml syringe. The tubing was sufficiently long to prevent aspirating the adrenals into the syringe. When the contents of the Tygon tubing became cloudy due to the release of cells from the adrenal fragments, the nondispersed fragments were allowed to settle and the cells were transferred through two layers of cheesecloth into polyethylene centrifuge tubes. Two milliliters of buffer were added to the remaining fragments and the process of drawing the cells in and out of the tubing was repeated until all the fragments had been dispersed. Near the end of the dispersing process, the Tygon tubing was pressed lightly against the bottom of the tube to aid in breaking up the last fragments. The suspension of cells was centrifuged at 100 × *g* for 10 min at 4° to separate intact and broken cells. This procedure yielded optimally 6 to 7 × 10^5 cells per adrenal; however, lower yields of cells were obtained with some batches of collagenase.

Incubation of Adrenal Cells—The adrenal cells were resuspended in Krebs-Ringer bicarbonate buffer, pH 7.1 (3 ml of buffer per each pair of adrenals), containing 2 mg per ml of bovine serum albumin, 1 mg per ml of glucose, and 0.1 mg per ml of lima bean trypsin inhibitor. One-milliliter aliquots of the cell suspension were incubated at 37° in 12-ml capped polyethylene round bottom tubes gassed with 95% O_2-5% CO_2 for the times indicated in the figures and tables. Hormones were added in 0.05 ml of 0.001 N HCl.

Measurement of Corticosterone—Corticosterone was determined according to the procedure of Peterson (12). At the end of the incubation, the cells and the medium were transferred to a 12-ml glass-stoppered centrifuge tube containing 1 ml of 20% ethanol. Five milliliters of dichloromethane (Mallinckrodt, analytical grade) were added immediately and the mixture shaken on a Vortex mixer until an emulsion formed. The phases were separated by placing the samples in the freezer (−20°) for a few hours. Two milliliters of the dichloromethane layer were removed, added to 2 ml of a mixture of absolute ethanol-concentrated H_2SO_4 (75:175 v/v), and mixed rapidly for 15 s. Fluorescence of the sulfuric acid layer was measured on a Turner fluorometer after 1 hour.

Measurement of Cyclic AMP—Cyclic AMP was measured by the method described by Gilman (8) with minor modifications. The cyclic AMP-binding protein and the inhibitor of the protein kinase were obtained from rabbit hind leg muscles by the procedure of Walsh *et al.* (13) followed by chromatography on DEAE-cellulose according to the procedure of Gilman (8). This preparation of the binding protein had a K_m of 2 nM for cyclic AMP in the absence of the protein kinase inhibitor and a K_m of less than 1 nM in the presence of the inhibitor. The pH optimum was similar to that described for the beef protein. For measuring the cyclic AMP accumulated in the adrenal cells and medium, incubations were terminated by transferring the contents of the incubation tubes into 1 ml of 1 mM theophylline contained in glass centrifuge tubes in a boiling water bath. The samples were then frozen, thawed, and passed through a 3-cm column of neutral alumina contained in a Pasteur pipette. The use of neutral alumina for the separation of cyclic AMP from polyanionic nucleotides such as ATP, ADP, AMP, and P_i has been described previously (14). Residual fluid on the column was forced through the alumina with a stream of air. The eluate was lyophilized to concentrate the cyclic AMP. Recovery of cyclic AMP added to the incubation medium was 80%. The remainder of the procedure was the same as that of Gilman (8). Specifically, sample aliquots were incubated in a total volume of 0.1 ml of 0.05 M sodium acetate buffer, pH 4.0, containing 1 pmole of cyclic [^{3}H]AMP and sufficient kinase and inhibitor to bind 0.25 pmole of cyclic AMP.

Intracellular and Medium Cyclic AMP Determination—Intracellular levels of cyclic AMP were determined after separating the cells from the incubation medium by passing the cell suspensions through Millipore filters (pore size 0.45 μm). The filters were immediately transferred to tubes containing 2 ml of 1 mM theophylline in a boiling water bath and kept at this temperature for 15 min. The medium was added to 1 ml of 1 mM theophylline and kept at 100° for 15 min. The cyclic AMP in the medium was measured after passing through an alumina column as described above for the total cell suspension. Cyclic AMP in the cell fraction was determined after lyophilizing the theophylline extract and reconstituting in 0.1 M acetate buffer, pH 4.0, as required.

RESULTS

Stimulation of Steroidogenesis—Both ACTH and NPS-ACTH are able to stimulate corticosterone synthesis in isolated rat adrenal cells as shown in Fig. 1. NPS-ACTH appears to be approximately one one-hundredth as potent as ACTH. Both peptides stimulate steroidogenesis to the same extent.

Stimulation of Cyclic AMP Accumulation—Since ACTH and NPS-ACTH stimulated steroidogenesis and because the steroidogenic response is considered to be mediated by cyclic AMP, it was expected that NPS-ACTH would stimulate cyclic AMP formation. As shown in Fig. 2, both ACTH and NPS-ACTH stimulated cyclic AMP production, but to vastly different magnitudes. The total amount of cyclic AMP produced in response to NPS-ACTH was only one-thirtieth of that produced by ACTH. Since both peptides enhanced steroidogenesis to the same extent, the amount of cyclic AMP required for steroidogenesis must be only a small fraction of the total amount of cyclic AMP generated in response to the higher concentrations of ACTH. The large difference in the maximal amount of cyclic AMP accumulated in response to ACTH and NPS-ACTH could not be explained on the basis of different kinetics since both peptides elevated cyclic AMP levels throughout the time of measurement (Fig. 3). The

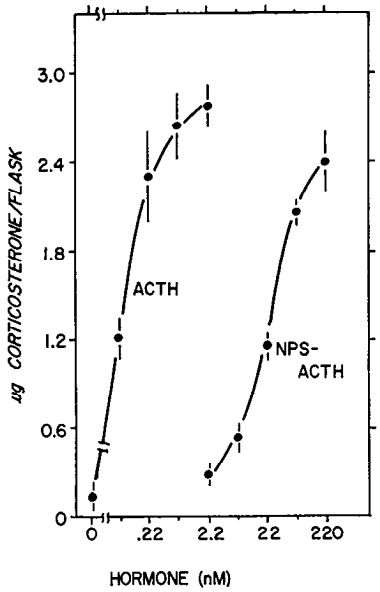


FIG. 1. Stimulation of corticosterone synthesis by ACTH and NPS-ACTH. Isolated adrenal cells (380,000 cells per flask) were incubated in the presence of varying concentrations of the peptides for 60 min. The amount of corticosterone synthesized in each flask was measured as described under "Experimental Procedure." *Vertical bars* extend to the limits of the S.E.M. for duplicate analyses of each of duplicate incubations at each point.

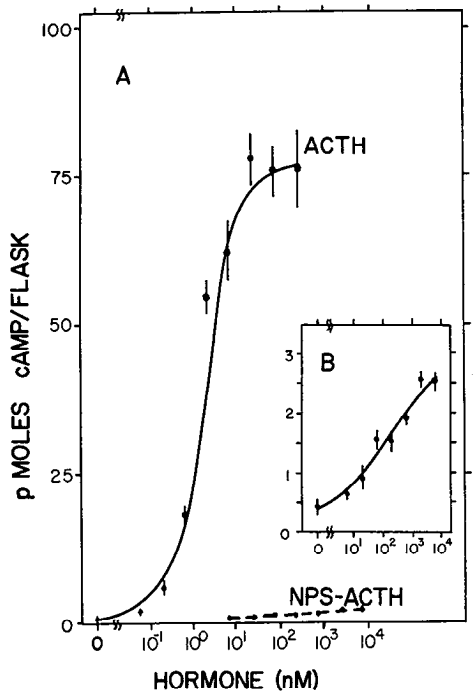


FIG. 2. Stimulation of cyclic AMP accumulation by ACTH and NPS-ACTH. Adrenal cells (300,000 cells per flask) were incubated for 20 min in the presence of the hormones. The amount of cyclic AMP accumulated is shown as a function of ACTH and NPS-ACTH concentration in *A*. *B* shows the production of cyclic AMP as a function of NPS-ACTH concentration on an expanded scale. *Vertical bars* extend to the limits of the S.E.M. for duplicate analyses of each of triplicate incubations at each point.

rate of corticosterone synthesis induced by both ACTH and NPS-ACTH appeared to be linear for at least an hour and was proportional to the concentration of hormone throughout the range examined. The results obtained with the varying concentrations of ACTH and NPS-ACTH are shown in Fig. 4. When both corticosterone synthesis and cyclic AMP accumulation due to ACTH stimulation were measured in the same experiment (Fig. 5), a large difference was observed in the concentrations of ACTH required for maximal steroidogenesis compared to maximal cyclic AMP generation. This further supported the conclusion that only a small fraction of the cyclic AMP produced by ACTH stimulation was required for steroidogenesis.

The concentrations of ACTH and NPS-ACTH required for half-maximal stimulation of steroidogenesis measured in several experiments are listed together with the concentrations of ACTH needed for half-maximal stimulation of cyclic AMP synthesis in Table I. Although the responsiveness of different batches of cells varied, the concentration of NPS-ACTH producing half-maximal stimulation of steroidogenesis was 80 times that of ACTH. Half-maximal stimulation of cyclic AMP synthesis was produced by a concentration of ACTH 16 times that required for half-maximal stimulation of steroidogenesis.

Inhibition of ACTH-induced Cyclic AMP Synthesis by NPS-ACTH—Previous studies (7) had shown that NPS-ACTH inhibited the stimulation of adenylate cyclase in rat fat cell ghosts by ACTH. Since NPS-ACTH produced only a small fraction of the cyclic AMP seen after ACTH stimulation in the adrenal cells, it was felt that NPS-ACTH might inhibit the ACTH-induced increase in adrenal cyclic AMP. The data described by Fig. 6 indicate that NPS-ACTH is a potent inhibitor of ACTH-induced cyclic AMP accumulation. The fact that ACTH was able to overcome the inhibitory action of NPS-ACTH suggests that the latter acts as a competitive inhibitor of the hormone. Further evidence for the competitive nature of the inhibition due to NPS-ACTH was obtained by keeping the ratio of ACTH to inhibitor constant. As seen in Fig. 7, the maximum generation of cyclic AMP was dependent only on the ratio of NPS-ACTH:ACTH and not on the total amount of NPS-ACTH present. This behavior is characteristic of competitive inhibition. In the case of noncompetitive inhibition, the same maximum for cyclic AMP generation would be found for the two ratios of inhibitor to hormone. These inhibition studies showed that the amount of NPS-ACTH required to produce a 50% inhibition of the effect of ACTH was always less than 20 times the concentration of ACTH (four out of four experiments).

In view of the variability in the responsiveness of different batches of cells the inhibition of ACTH-induced cyclic AMP synthesis by NPS-ACTH was studied with the same batch of cells used for measuring steroidogenesis dose-response relationships. As seen from the results shown in Table II, NPS-ACTH produced greater than 50% inhibition of ACTH-induced cyclic AMP synthesis at a concentration of NPS-ACTH less than 10 times that of ACTH and 85% inhibition at a concentration less than 20 times that of ACTH. In the same experiment the concentration of NPS-ACTH required for half-maximal stimulation of steroidogenesis was found to be 57 times that of ACTH.

When NPS-ACTH was added to cells which had been stimulated by ACTH for 5 min (Fig. 8), cyclic AMP levels were rapidly reduced. This marked decrease in cyclic AMP levels seen after the addition of NPS-ACTH was partially prevented when 1 mM theophylline was present in the incubation medium (data not shown). Conversely, when ACTH was added to cells previously exposed to NPS-ACTH for 10 min, there was no significant lag in the synthesis of cyclic AMP compared to the stimulation produced by the addition of ACTH and NPS-ACTH simultaneously, although the concentration of NPS-ACTH employed was sufficient to produce a 50% inhibition of cyclic AMP

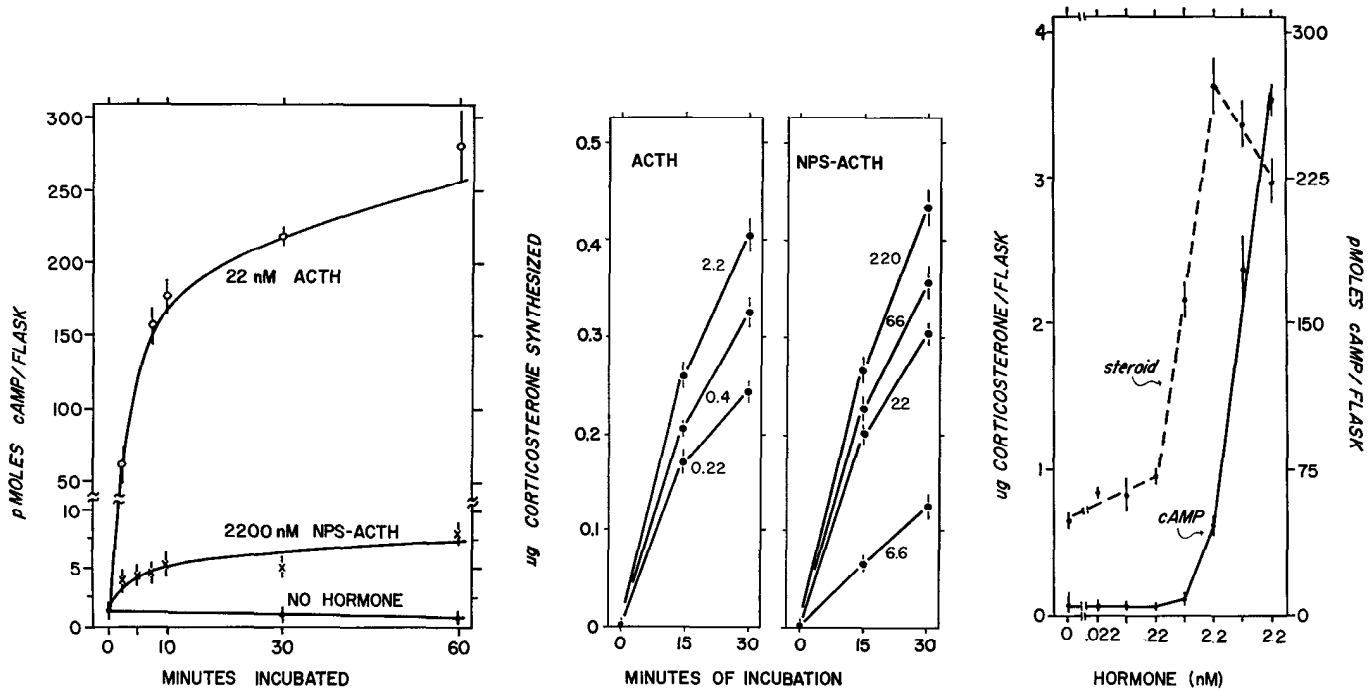


FIG. 3 (*left*). Accumulation of cyclic AMP in adrenal cells in response to ACTH and NPS-ACTH as a function of time. Adrenal cells (410,000 per flask) were preincubated for 15 min in the absence of hormones. At 0 min, ACTH or NPS-ACTH was added in a volume of 0.05 ml to give a final concentration of 22 nM ACTH, 2,200 nM NPS-ACTH, or no hormone. *Vertical bars* extend to the limits of the S.E.M. for duplicate determinations of each of triplicate incubations per point.

FIG. 4 (*center*). Stimulation of steroidogenesis by ACTH and NPS-ACTH as a function of time. Adrenal cells (225,000 cells per flask) were incubated in triplicate with ACTH or NPS-ACTH at the concentrations indicated. At varying periods the total corticosterone per flask was measured by procedures described in the text. *Vertical bars* extend to the limits of the S.E.M. for single measurements of each of the three flasks. The cells used to obtain these data were the same as in Experiment 1 of Table IV.

FIG. 5 (*right*). Corticosterone and cyclic AMP accumulation as a function of ACTH concentration. Adrenal cells (400,000 cells per flask) were incubated in the presence of varying concentrations of ACTH in sextuplicate. At the end of 1 hour corticosterone was measured using three flasks per point and cyclic AMP was determined in each of the remaining three flasks.

TABLE I

Concentrations of ACTH and NPS-ACTH required for stimulation of steroidogenesis and cyclic AMP synthesis

The values shown refer to the nanomolar concentration of ACTH or NPS-ACTH required to produce half-maximal stimulation of corticosterone synthesis or cyclic AMP formation.

Experiment	Steroidogenesis[a]			Cyclic AMP synthesis[b] stimulated by ACTH
	ACTH	NPS-ACTH	NPS-ACTH: ACTH	
1	0.093	14.9	160	
2	0.120	10.6	88	
3	0.280	25.5	91	
4	0.700	25.8	37	
5	0.407			5.50
6	0.660			8.81
7	0.484			6.38
8	0.210	12.0	57	7.00
9	0.530	25.5	48	3.52
Average	0.387	19.1	80	6.22

[a] Both ACTH and NPS-ACTH stimulated steroidogenesis to the same maximum.

[b] Stimulation of cyclic AMP synthesis by NPS-ACTH was too small (always less than 3% of that produced by ACTH) for accurate evaluation of the half-maximal concentration.

synthesis (Fig. 9). In other experiments in which higher concentrations of ACTH and NPS-ACTH were tested it was found that the initial rate of cyclic AMP synthesis was the same irrespective of whether NPS-ACTH was added before or simultaneously with ACTH. The results shown in Figs. 8 and 9 imply that the interaction of both ACTH and NPS-ACTH with the adrenal cells is rapid and reversible.

Relationship between Cyclic AMP Formation and Corticosterone Synthesis—In view of the observation that very little of the cyclic AMP produced by ACTH was required for stimulation of steroidogenesis, experiments were designed to ascertain whether a truly proportional relationship existed between the amounts of cyclic AMP generated and the quantities of corticosterone synthesized in response to low concentrations of ACTH and NPS-ACTH. Twice the usual amount of adrenal cells were added per incubation flask in order to obtain more accurate measurements of the small cyclic AMP levels expected. The results of measurements of both cyclic AMP and corticosterone in representative experiments are shown in Table III. A general correlation between the amount of cyclic AMP formed and the amount of corticosterone synthesized was apparent; however, the quantitative relationships between the effects of ACTH and NPS-ACTH were surprising. Cells producing less than half-maximal quantities of corticosterone in response to ACTH generated more cyclic AMP than those producing half or three-

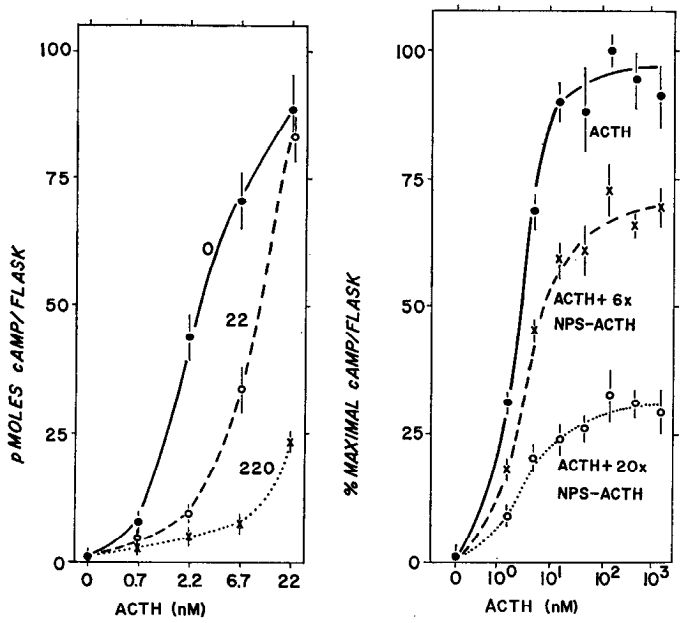


FIG. 6 (*left*). Inhibition of ACTH-induced cyclic AMP formation by NPS-ACTH. Adrenal cells (350,000 cells per flask) were incubated in duplicate. Incubations were terminated after 20 min and cyclic AMP was measured in duplicate for each flask. The nanomolar concentration of NPS-ACTH is indicated by the *number* above each curve. *Vertical bars* extend to the limits of S.E.M. of the picomoles of cyclic AMP per flask and are the result of four analyses per point.

FIG. 7 (*right*). Stimulation of cyclic AMP accumulation by mixtures of NPS-ACTH and ACTH at constant ratios. Adrenal cells (380,000 per flask) were incubated with varying concentrations of NPS-ACTH:ACTH mixtures for 15 min. The concentration of NPS-ACTH was adjusted such that it was 0, 6, or 20 times that of ACTH at each concentration of ACTH employed. Cyclic AMP was measured in the cells plus medium as described. Maximal cyclic AMP accumulation induced by ACTH alone was taken as 100%. *Vertical bars* extend to the limits of the S.E.M. for four determinations at each point. NPS-ACTH alone at the highest concentration (44 μM) stimulated cyclic AMP accumulation 1.5%.

quarters of the maximal amounts of steroid in response to NPS-ACTH. Thus, even low concentrations of ACTH stimulated production of more cyclic AMP than required for the synthesis of a given amount of steroid.

In order to ascertain that the apparent lack of correlation between the total cyclic AMP accumulation and steroid synthesis also holds for intracellular cyclic AMP, experiments were designed to estimate the intracellular and extracellular cyclic AMP concentrations. The intracellular cyclic AMP levels rose rapidly in response to graded concentrations of ACTH, reached maximal levels by 5 min, and then gradually declined (Fig. 10). On the other hand, the concentration of cyclic AMP in the medium increased steadily throughout the incubation period suggesting that cyclic AMP was leaving the cells. Similar results were obtained with NPS-ACTH as shown in Fig. 11.

In view of the observed leakage of cyclic AMP from the cells, the relationship of corticosterone synthesis to intracellular cyclic AMP levels following hormonal stimulation was investigated. The results given in Table IV show that there exists the same lack of correlation between corticosterone synthesis and intracellular cyclic AMP levels as was observed in the comparison of the total cyclic AMP (accumulated after 1 hour) with steroid production. Thus, concentrations of ACTH producing submaximal rates of corticosterone synthesis produced the same or

TABLE II

Effect of NPS-ACTH on ACTH-induced cyclic AMP synthesis

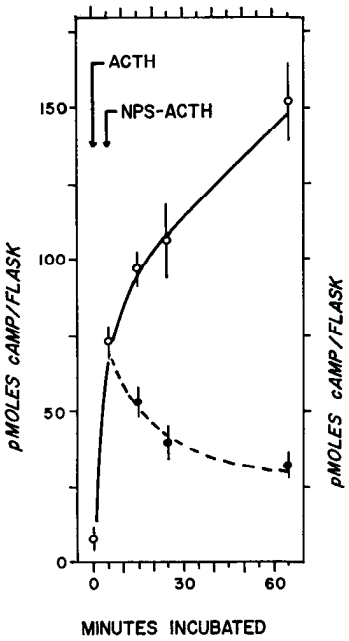
Isolated adrenal cells were incubated with ACTH, or NPS-ACTH, or both, at 37° for 5 min and cyclic AMP in the cells plus medium was measured as described under "Experimental Procedure." Values are expressed as picomoles of cyclic AMP per flask and are the mean ± S.E.M. for triplicate incubations. Dose-response curves for steroidogenesis for both ACTH and NPS-ACTH were also determined using cells from the same batch. The concentrations required for half-maximal stimulation of steroidogenesis were found to be 0.21 nM for ACTH and 12.0 nM for NPS-ACTH.

ACTH	NPS-ACTH			
	0 nM	110 nM	220 nM	440 nM
nM				
0	1.8 ± 0.20	1.83 ± 0.13	2.99 ± 0.63	2.35 ± 0.30
2.92	7.33 ± 0.78			
5.83	13.53 ± 1.23			
11.65	19.67 ± 1.87	6.72 ± 0.59 (72%)[a]	5.41 ± 0.40 (86%)	
23.3	29.27 ± 3.4		11.67 ± 0.64 (68%)	6.51 ± 0.59 (85%)
70.0	28.00 ± 0.83			

[a] The per cent inhibition due to NPS-ACTH is indicated in parentheses.

higher intracellular levels of cyclic AMP as concentrations of NPS-ACTH sufficient to stimulate steroidogenesis at maximal rates. For example, in Experiment 1 (Table IV) 0.44 nM ACTH induced a 44% increase in intracellular cyclic AMP at 5 min and a 61% increase at 15 min while stimulating steroidogenesis to three-quarters of the maximal rate; 66 nM NPS-ACTH, on the other hand, produced an 11% increase in intracellular cyclic AMP at 5 min and a 14% increase at 15 min while stimulating steroidogenesis to five-sixths of the maximal rate. NPS-ACTH (220 nM, which was the only concentration of NPS-ACTH producing a statistically significant increase in intracellular cyclic AMP over basal) induced the maximal rate of steroidogenesis while increasing cellular cyclic AMP to the same extent as 0.44 nM ACTH. In Experiment 2 of Table IV, it is seen that 220 nM NPS-ACTH produced a higher rate of steroidogenesis and a smaller increase in intracellular cyclic AMP levels than 1.1 nM ACTH. Even 660 nM NPS-ACTH, which produced a 3-fold higher rate of steroidogenesis than 1.1 nM ACTH did not increase the intracellular cyclic AMP level more than 1.1 nM ACTH. Although the responsiveness of different batches of isolated adrenal cells to the hormones varied as seen from the results in Tables I and IV, the relative potencies of ACTH and NPS-ACTH in terms of steroidogenesis as well as cyclic AMP stimulation remained the same. The variation in the responsiveness of different batches of cells are probably due to differences in the extent of digestion effected by different batches of collagenase.

The lack of correlation between the amount of cyclic AMP present at 5 or 8 min and the amount of steroid synthesized at 15 and 30 min (see Fig. 4 and Table IV) could be attributed to an earlier effect of NPS-ACTH or ACTH on cyclic AMP levels (*i.e.* prior to 5 min). According to this hypothesis, cyclic AMP generated in response to ACTH or NPS-ACTH at earlier time periods than those measured would have been responsible for the steroidogenesis observed later. If this were correct, the continued presence of ACTH, NPS-ACTH, and cyclic AMP would not be necessary for the continual stimulation of steroido-



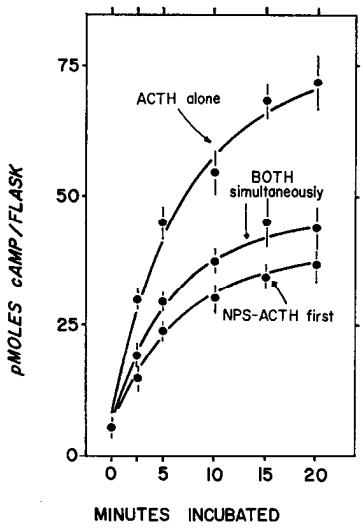


FIG. 8 (*left*). Effect of NPS-ACTH on cyclic AMP accumulation previously initiated by ACTH. Adrenal cells (360,000 cells per flask) were incubated in flasks containing 22 nM ACTH. After 5 min NPS-ACTH was added to half the flasks to produce a final concentration of 2,200 nM NPS-ACTH. Cyclic AMP was measured at the times indicated. *Vertical bars* represent the limits of the S.E.M. for duplicate determinations of cyclic AMP in each of two flasks per point.

FIG. 9 (*right*). Effect of ACTH on cyclic AMP synthesis in adrenal cells preincubated with NPS-ACTH. Adrenal cells (380,000 per flask) were incubated at 37° with 0 or 66 nM NPS-ACTH. After 10 min 6.6 nM ACTH was added to cells previously exposed to NPS-ACTH as well as those not exposed to the analog. In addition, a mixture of 6.6 nM ACTH and 66 nM NPS-ACTH was added to cells preincubated without NPS-ACTH. Cyclic AMP was measured at different times in each of the three groups. The values represent picomoles per flask and are the mean of four analyses at each point with *vertical bars* extending to the S.E.M.

genesis. That this is not the case may be readily seen from the results presented in Table V. When either ACTH or NPS-ACTH was removed from the medium by a simple dilution procedure, steroidogenesis ceased unless the hormone was added back to the medium. Thus, a short rise in cyclic AMP levels due to the peptides occurring at times earlier than those measured (Table IV) could not account for the lack of correlation between intracellular cyclic AMP levels and steroidogenesis measured concurrently and at a later time.

TABLE III

Relationship of cyclic AMP formation to steroidogenesis following stimulation by ACTH and NPS-ACTH

Adrenal cells (400,000 per flask: Experiment I; 800,000 per flask: Experiment II) were incubated with ACTH or NPS-ACTH in sextuplicate. After 1 hour corticosterone from three flasks and cyclic AMP from the remaining three flasks were measured as described under "Experimental Procedure." Values shown represent mean quantities of cyclic AMP or corticosterone per flask ± S.E.M. In both experiments cyclic AMP was analyzed in duplicate while corticosterone was measured in duplicate only in Experiment II. N refers to the total number of measurements used to calculate each mean. The significance of the differences between various means was calculated with the *t* test. Unless specified, *p* values refer to comparisons with basal values (no hormone). N.S., not significant.

Hormone	Cyclic AMP (N = 6)	*p* value	Corticosterone (N = 3)
nM	*pmoles/flask*		*% maximum*
Experiment I			
None	5.01 ± 0.15		0.2 ± 3.0
ACTH			
0.22	6.32 ± 0.39	0.005	9.7 ± 1.5
0.66	9.81 ± 0.33	0.001	47.6 ± 0.9
2.20	47.1 ± 2.6	0.001	90.7 ± 3.9
NPS-ACTH			
6.6	5.31 ± 0.33	N.S.	41.3 ± 3.7
22	7.55 ± 0.75	0.005	56.5 ± 3.8
66	8.65 ± 0.53	0.001	81.0 ± 0.6
2200	8.12 ± 0.60	0.001	96.6 ± 1.8
	9.81 > 8.65		81 > 47.6
	$p < 0.1$		$p < 0.001$
	(N = 6)		(N = 6)
Experiment II			
None	8.31 ± 0.18		0.2 ± 0.4
ACTH			
0.066	9.03 ± 0.25	0.05	2.1 ± 0.6
0.22	8.90 ± 0.41	N.S.	9.9 ± 0.4
0.66	12.30 ± 0.55	0.001	46.2 ± 1.3
2.20	24.63 ± 0.83	0.001	100.3 ± 4.5
NPS-ACTH			
6.6	9.57 ± 0.18	0.005	4.9 ± 1.2
22	9.69 ± 0.31	0.005	24.7 ± 0.7
66	9.19 ± 0.31	N.S.	53.3 ± 2.2
220	10.98 ± 0.37	0.001	79.1 ± 2.2
	12.3 > 10.98		79.1 > 46.2
	$p < 0.01$		$p < 0.001$

DISCUSSION

NPS-ACTH differs from ACTH in that an *o*-nitrophenyl sulfenyl group is attached to the indole group of the single tryptophan residue present in the hormone. The importance of the tryptophan residue in the lipolytic action of ACTH on isolated rat fat cells has been shown (6, 7). These studies also showed that NPS-ACTH is a very useful tool in distinguishing between receptors specific for polypeptide hormones of similar structure. Thus, NPS-ACTH was able to inhibit the ACTH-induced stimulation of adenylate cyclase in rat fat cell ghosts but was more potent than ACTH in stimulating rabbit fat cell ghost adenylate cyclase (7) as well as in stimulating melanophores in frog skins (15). Further studies[2] with a series of synthetic peptides related to ACTH and the melanotropins showed that the ability of these peptides to stimulate lipolysis in rat fat cells correlated well with their steroidogenic potencies whereas the melanophore-stimulating activities of the peptides paralleled their lipolytic activities in rabbit fat cells. Thus, rat fat cells and rat adrenal cells appeared to have similar structural requirements for favorable interaction with ACTH. In view of this and the inhibitory role of NPS-ACTH on lipolysis in rat fat cells, it was expected that NPS-ACTH would antagonize the actions of ACTH on rat adrenal cells also. Indeed, this expectation was realized when the effects of NPS-ACTH on cyclic AMP accumulation in rat adrenal cells were measured.

The effects of NPS-ACTH on ACTH-induced cyclic AMP synthesis are readily explained in terms of competitive inhibition.

[2] J. Ramachandran, S. Farmer, S. Lilcs, and C. H. Li, manuscript in preparation.

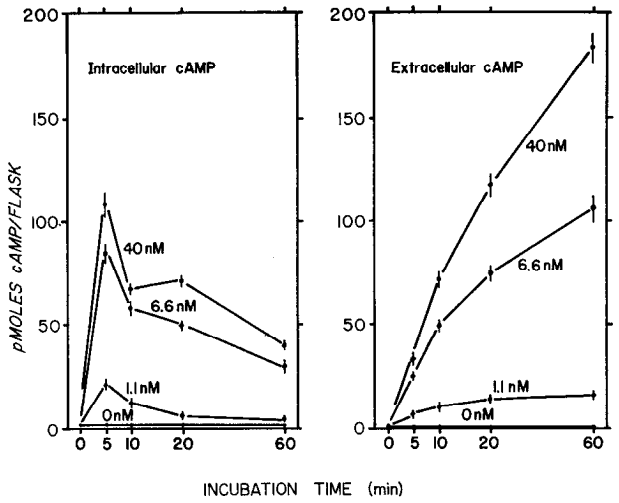


FIG. 10. Effect of ACTH on intracellular and extracellular levels of cyclic AMP as a function of time and concentration. Adrenal cells (250,000 cells per flask) were incubated with the concentration of ACTH indicated on the figure. After varying periods the cells were removed from the medium by filtration and the cyclic AMP retained in the cells on the filter (intracellular cyclic AMP) and that of the filtrate (extracellular cyclic AMP) were analyzed in duplicate as described under "Experimental Procedure." *Vertical bars* extend to the limits of the S.E.M. for six measurements at each mean.

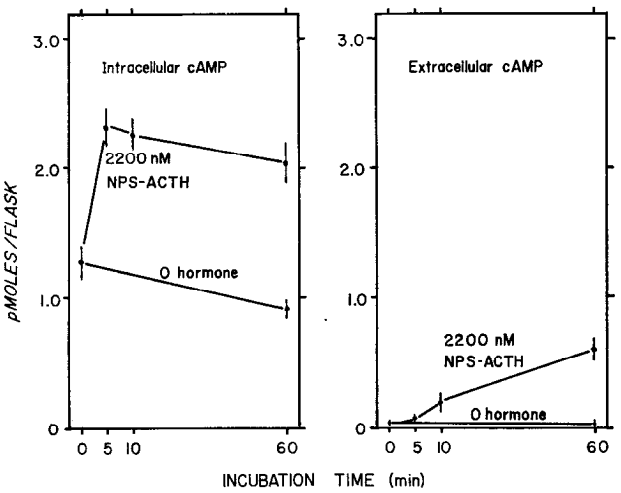


FIG. 11. Effect of NPS-ACTH on intracellular and extracellular cyclic AMP levels as a function of time. Adrenal cells (230,000 cells per flask) were incubated in triplicate as in Fig. 8 with NPS-ACTH. Extracellular and intracellular cyclic AMP levels were analyzed in duplicate. *Vertical bars* extend to the limits of the S.E.M. for six measurements at each mean.

However, quantitative analysis of the inhibition according to simple Michaelis-Menten kinetics was not possible owing to the fact that the interaction of ACTH with adrenal cells is complex. The concentration range over which ACTH stimulates cyclic AMP synthesis is too narrow to be described by the Michaelis-Menten model. This is true regardless of the time at which cyclic AMP synthesis was measured, be it 5 or 60 min. Thus, the concentration of ACTH required for half-maximal stimulation of cyclic AMP synthesis may not be a true measure of the affinity of the hormone for the adrenal receptors. Earlier studies of the adrenal-stimulating activities of synthetic peptides corresponding in sequence to various segments of the ACTH molecule suggested that the sequence Lys-Lys-Arg-Arg in positions 15 to 18 is important for attachment of the hormone to the target cell (16). Since NPS-ACTH appears to be a potent competitive inhibitor of the ACTH effect, the modification of the tryptophan residue must have reduced the ability of the hormone to stimulate cyclic AMP synthesis without drastic alteration of the ability of the hormone to bind to the adrenal cells.

The rates of association and dissociation of both ACTH and NPS-ACTH to and from the adrenal cells must be quite fast since addition of NPS-ACTH to cells already under maximal ACTH stimulation dramatically inhibits the ACTH effect causing cyclic AMP levels to fall rapidly. In addition, large doses of ACTH can stimulate cyclic AMP synthesis in cells previously exposed to NPS-ACTH with no measurable delay.

The observation that cyclic AMP levels in the adrenal cells can be lowered from the high levels induced by ACTH suggests that the cells must contain a mechanism for metabolizing cyclic AMP, probably a phosphodiesterase. That NPS-ACTH might be stimulating the phosphodiesterase and thus producing an apparent inhibition of the action of ACTH on cyclic AMP formation was ruled out by experiments in which theophylline was present along with NPS-ACTH (data not presented). NPS-ACTH was still able to inhibit the ACTH-induced stimulation of cyclic AMP formation. In these experiments the amount of cyclic AMP in the cells did not decrease after the addition of NPS-ACTH nearly as much as in the absence of theophylline. All these observations can be explained on the basis of an effect of NPS-ACTH counteracting the ACTH effect at the level of adenylate cyclase. Further support of the idea that NPS-ACTH is inhibiting the effect of ACTH on adenylate cyclase stimulation comes from studies of the action of NPS-ACTH on adenylate cyclase in adrenal homogenate fractions.[3] NPS-ACTH specifically inhibited the ACTH-induced stimulation of adenylate cyclase but had no effect on the stimulation due to fluoride ion.

In contrast to its marked antagonistic effect on cyclic AMP formation, NPS-ACTH proved to be 1.4% as potent as ACTH in stimulating steroidogenesis. This result suggested that although the integrity of the tryptophan residue in ACTH is essential for maximal cyclic AMP generation, it is not so for maximal steroidogenesis. In view of the fact that much higher concentrations of NPS-ACTH were required to produce maximum levels of steroidogenesis, the possibility that the observed NPS-ACTH effect might be due to ACTH contamination must be considered. However, this is unlikely for two reasons. First, several preparations of NPS-ACTH which were purified by column chromatography on carboxymethylcellulose (6) appeared to have similar steroidogenic potencies. Secondly, a synthetic peptide corresponding to the first 20 amino acid residues of ACTH, containing phenylalanine in place of tryptophan, was reported to be approximately one-fiftieth as potent as steroidogenic agent as the peptide containing tryptophan (17). Although this result was interpreted to mean that the substitution of the tryptophan reduced the affinity of the peptide for the target organ, subsequent studies (18) with bovine adrenal homogenates showed the synthetic peptide containing phenylalanine in place of tryptophan could bind to the adrenal tissue as well as the tryptophan-containing peptide. Thus, substitution of the tryptophan residue by a synthetic method which eliminates the problem of contamination produces effects essentially the same as observed when the tryptophan residue was chemically modified.

[3] J. Ramachandran, Eighth International Congress of Biochemistry, Lucerne, 1970.

TABLE IV

Relationship of intracellular cyclic AMP levels to steroidogenesis following stimulation with ACTH and NPS-ACTH

Adrenal cells (225,000 per flask: Experiment I; 400,000 per flask: Experiment II) were incubated with ACTH or NPS-ACTH in sextuplicate. At the times indicated cells from three flasks were removed from the medium by filtration for measurement of intracellular cyclic AMP in duplicate as described under "Experimental Procedure." Corticosterone was quantified at 15 min in both experiments and represents the steroid present in the cells plus medium. Values shown represent the mean ± S.E.M. for each treatment. N represents the number of measurements used to calculate each mean. The significance of differences between various means was calculated with the *t* test. Unless specified, *p* values refer to comparison with basal values (no hormone). N.S., not significant.

Hormone	cyclic AMP			*p*	Corticosterone
	5 min	*p*	15 min		
nM	*pmoles*				*% maximum*
Experiment I					
None	1.13 ± 0.09		1.19 ± 0.12		0.1 ± 0.2
ACTH					
0.22	1.49 ± 0.35	N.S.	2.14 ± 0.1	0.001	62.6 ± 4.3
0.44	1.63 ± 0.10	0.005	1.91 ± 0.14	0.005	75.6 ± 1.8
2.20	10.7 ± 0.45	0.001	14.6 ± 0.42	0.001	92.7 ± 3.1
NPS-ACTH					
6.6	1.23 ± 0.06	N.S.	1.28 ± 0.08	N.S.	24.3 ± 2.1
22	1.38 ± 0.06	0.1	1.37 ± 0.04	N.S.	74.0 ± 7.8
66	1.25 ± 0.07	N.S.	1.35 ± 0.03	N.S.	84.3 ± 3.3
220	1.60 ± 0.08	0.005	1.88 ± 0.11	0.005	99.9 ± 4.2
					84.3 > 75.6, $p < 0.05$; 99.9 > 75.6, $p < 0.05$
	8 min	*p*	16 min		
Experiment II					
None	2.11 ± 0.11		1.75 ± 0.10		−0.1 ± 6.5
ACTH					
0.44	3.00 ± 0.09	0.001	2.69 ± 0.11	0.001	0.1 ± 6.5
1.10	4.45 ± 0.15	0.001	3.33 ± 0.13	0.001	32.6 ± 3.8
2.20	29.8 ± 0.95	0.001	19.40 ± 1.69	0.001	95.9 ± 5.7
NPS-ACTH					
22	2.13 ± 0.10	N.S.	2.24 ± 0.08	0.01	0.0 ± 3.8
220	3.54 ± 0.17	0.001	3.07 ± 0.18	0.001	56.7 ± 2.2
660	3.79 ± 0.27	0.001	3.09 ± 0.10	0.001	99.8 ± 2.1
	4.45 > 3.79, $p < 0.1$; 4.45 > 3.54, $p < 0.005$				56.7 > 32.6, $p < 0.05$

NPS-ACTH produced a marked inhibition of ACTH-induced cyclic AMP synthesis even though high concentrations of NPS-ACTH stimulated steroidogenesis to the same maximum compared to ACTH. These results clearly suggest that the effects of ACTH on cyclic AMP synthesis and steroidogenesis must involve the interaction of the hormone with at least two types of receptors on the adrenal cell. The hypothesis involving stimulation of a single type of receptor by the hormone cannot account for the results presented here.

Even though a classical Michaelis-Menten analysis of the inhibition of ACTH-induced cyclic AMP synthesis by NPS-ACTH was not feasible for reasons stated earlier, the results clearly show that NPS-ACTH produced a 50% inhibition at a concentration 10 times that of ACTH. On the other hand, the average concentration of NPS-ACTH required for half-maximal stimulation of steroidogenesis was 80 times that of ACTH. These facts strongly support the hypothesis that at least two different affinity sites are involved in the interaction of ACTH with adrenal cells.

Perhaps the most surprising finding to emerge from the present investigation was that the total as well as the intracellular levels of cyclic AMP generated during submaximal stimulation of steroidogenesis were not the same for ACTH and NPS-ACTH. This observation and the finding that only a small fraction of the intracellular cyclic AMP synthesized in response to ACTH appeared to be involved in mediating the steroidogenic effect can be explained in two ways. (*a*) The steroidogenic effect of ACTH on adrenal cells is mediated by a small fraction of the total intracellular cyclic AMP which is in a separate compartment, or (*b*) the steroidogenic effect of ACTH is also mediated by an unknown factor other than cyclic AMP.

The first hypothesis requires that adrenal cells contain two types of affinity sites for ACTH which may be located on cells of different origin present in the adrenal cell suspensions or may be present in each cell in the population. The stimulation of one type of affinity site leads to the formation of small amounts of cyclic AMP in a special compartment. This "compartmentalized" cyclic AMP is responsible for the observed steroidogenic effect of the hormone. Stimulation of the other (presumably higher capacity) affinity site by ACTH results in the generation of the bulk of the cyclic AMP observed following stimulation with higher concentration of ACTH. The function of this large

TABLE V

Effect of washing ACTH and NPS-ACTH from adrenal cells on steroidogenesis

Isolated adrenal cells (400,000 per flask) were incubated with the peptides in a volume of 1 ml of KRB-BSA[a] for 5 min, at which time 4 ml of ice-cold KRB buffer were added and the cells were washed by centrifugation at 4°. The supernatant was removed by aspiration and 1 ml of the incubation medium with or without peptides was added to the pellet of cells for reincubation to give a 50-fold dilution. Reincubation was continued for ½ hour and corticosterone was measured as described. Values represent micrograms of corticosterone ± S.E.M. for triplicate incubations.

Hormone present during preincubation for 5 min	Hormone added after washing (reincubation for 30 min)	Corticosterone produced
nM	*nM*	
None	None	0.101 ± 0.025
ACTH		
1.1	None	0.208 ± 0.057
1.1	1.1	0.784 ± 0.067
1.1	Not incubated	0.089 ± 0.013
NPS-ACTH		
66	None	0.214 ± 0.055
66	66	0.847 ± 0.033
66	Not incubated	0.076 ± 0.017

[a] KRB-BSA, Krebs-Ringer bicarbonate buffer containing bovine serum albumin.

compartment of cyclic AMP is not evident. The existence of two affinity sites for ACTH in a mouse adrenal tumor cloned from a single cell *in vitro* has already been proposed by Lefkowitz *et al.* (19) on the basis of binding studies with ^{125}I-labeled ACTH.

In terms of this dual affinity site hypothesis, NPS-ACTH can stimulate only the affinity site mediating the steroidogenic effect, but apparently higher concentrations relative to ACTH are required. This may be due to lowering of the affinity of the hormone for this site as a result of the modification of the tryptophan residue of ACTH.

The second hypothesis invokes the involvement of a factor besides cyclic AMP in the steroidogenic action of ACTH. According to this, low concentrations of ACTH as well as high concentrations of NPS-ACTH would stimulate that affinity site on the adrenal cells which leads to the production of an unknown factor involved in steroid synthesis. Stimulation by higher concentrations of ACTH would produce this factor in addition to the large amount of cyclic AMP.

Whatever the mechanism, the results presented here strongly suggest that there is more than one type of receptor for ACTH on the adrenal cells and that ACTH and NPS-ACTH are interacting differently with these receptors. Further work in progress with other analogs of ACTH also suggest that these receptors can be distinguished in terms of function.

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