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The Role of Adrenocorticotropin and Calcium in Adenosine Cyclic 3′, 5′-Phosphate Production and Steroid Release from the Isolated, Perfused Cat Adrenal Gland

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SUMMARY

Isolated cat adrenal glands were perfused *in situ* with Locke's solution. The addition of synthetic adrenocorticotropin (0.4–400 μunits/ml) to the perfusion solution for 40 min caused a sustained rise in both tissue adenosine cyclic 3′,5′-phosphate levels and corticosteroid release. After a 5-min exposure to ACTH, tissue cyclic AMP levels fell to control values within 30 min, while corticosteroid release was still near maximum. Theophylline increased both basal and ACTH-stimulated tissue cyclic AMP levels but did not further enhance corticosteroid release. Perfusion with calcium-free or potassium-free Locke's solution increased tissue cyclic AMP levels 3–6 fold but did not augment steroid output. Under these ion-deficient conditions, ACTH produced only a very small additional increment in tissue cyclic AMP concentrations but caused a marked increase in steroid release. The addition of cyclic AMP or its dibutyryl derivative to the perfusion solution for up to 30 min did not increase corticoid output. Cyclic AMP was detected in the adrenal perfusate, and its rate of release was augmented by ACTH and by calcium deprivation. These observations are discussed in light of the concept that an increase in tissue cyclic AMP is not sufficient to trigger steroid release, but that a redistribution of cell calcium produced by ACTH is also required.

INTRODUCTION

Substantial evidence has appeared to implicate adenosine cyclic 3′,5′-monophosphate as a mediator in the action of adrenocorticotropin on adrenal corticosteroid biosynthesis. Haynes and his associates first showed that the addition of ACTH to incubated adrenal slices resulted in the accumulation of tissue cyclic AMP (1), and that cyclic AMP stimulated steroid production when added to the incubation medium (2). These data prompted Haynes to elaborate the hypothesis that ACTH action results in the accumulation of cyclic AMP which itself can mimic the steroidogenic effect of ACTH. More recently, Grahame-Smith *et al.* (3) have shown that upon the addition of ACTH to adrenal slices, the rise in adrenal cyclic AMP levels could be temporally and quantitatively correlated

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with the increasing steroidogenic response. From such evidence Sutherland and his colleagues have developed the "second messenger" concept, whereby the specific hormone (first messenger) activates a membrane-bound adenyl cyclase which leads to an increase in cyclic AMP production within a given cell (4). The cyclic AMP (second messenger), in turn, triggers the series of molecular events leading to hormone release.

Calcium is another intracellular mediator which has been shown to play an important role in the responses of the adrenal gland to ACTH. Calcium deprivation depresses steroid production *in vitro* (5, 6) and corticosteroid release from the isolated, perfused adrenal gland induced by low concentrations of ACTH (7). Furthermore, more recent studies from our laboratory have shown that adrenal stimulation by ACTH is associated with a redistribution of cellular calcium (8).

In light of the apparent importance of both cyclic AMP and calcium in the mode of action of ACTH, the present experiments were carried out on the isolated, perfused cat adrenal gland to help elucidate the relative roles of these two agents in the complex series of events which are triggered by the action of ACTH and terminate in the extrusion of corticosteroid from the cell.

MATERIALS AND METHODS

Isolated cat adrenal glands were perfused *in situ* at room temperature according to the method of Douglas and Rubin (9). In certain experiments only the left gland was perfused after removal of the right gland and kidney. In other experiments both adrenals were perfused simultaneously. This method approximates the situation *in vivo* yet eliminates the influence of a number of factors, including the anterior pituitary. Furthermore, the intact adrenal preparation does not have the disadvantages of many systems *in vitro*, in which there is tissue damage and loss of cellular integrity and compartmentalization.

All perfusions were carried out with normal or modified bicarbonate-buffered Locke's solution, equilibrated with 95% oxygen and 5% carbon dioxide, with a pH of 7.0. The rate of flow was maintained between 1.0 and 1.5 ml/min by regulating the perfusion pressure. Synthetic ACTH was added to the perfusion solution for various time intervals when required. After an appropriate period of perfusion, the left or right gland was quickly removed from the cat, trimmed of extraneous tissue, and plunged into liquid nitrogen. The glands were then weighed, homogenized in 0.6 N perchloric acid, and centrifuged at low speed to remove cell debris. The supernatant fluid was adjusted to pH 7.5 with 2 N KOH and then assayed for cyclic AMP by the enzymatic isotopic displacement method of Brooker *et al.* (10). All samples were assayed in sextuplicate, and mean values obtained for each sample were expressed in nanomoles per gram of gland, wet weight. The medulla was not separated from the cortex; however, its contribution to the total cyclic AMP concentration, if any, must be regarded as negligible since the medulla represents only 5% of the total wet weight of the cat adrenal gland. In certain experiments samples of perfusate were assayed for cyclic AMP in a similar manner. In three additional experiments, cyclic AMP was determined by the radioimmunoassay method of Steiner *et al.* (11).

To identify the nucleotides after column chromatography and lyophilization, extracts of adrenal homogenates and perfusates were applied to Whatman No. 40 paper and an ascending chromatogram was developed for 18 hr in a mixture of 1-butanol, acetic acid, and water (50:20:30 by volume). By comparing the R_f values of the homogenates and perfusates with those of known standard nucleotides, cyclic AMP was the sole nucleotide which could be identified in the test sample.

The perfusate was analyzed for 11-hydroxycorticosteroids by a previously described acid-fluorescence method (7).

Chemicals were obtained from the following sources: ethylene glycol bis(β-aminoethyl ether)-N,N'-tetracetic acid, J. T. Baker Chemical Company; theophylline, Nutritional Biochemicals Corporation; adenosine 3′5′-monophosphate, Boehringer-Mannheim; N^6,O^2-dibutyryl adenosine monophosphoric acid (monosodium salt) and snake venom (*Ophiophagus* Hannah), Sigma Chem-

ical Company; ^{3}H-adenosine 3′,5′-cyclic phosphate (16.3 Ci/mole) and goat anti-rabbit γ-globulin, Schwarz/Mann; anti-cyclic AMP antiserum and ^{125}I-tyrosine methyl ester of 2′-0 succinyl adenosine 3′,-5′-monophosphate, Collaborative Research; and synthetic β1,24-ACTH, a generous gift from Dr. J. J. Chart of Ciba Pharmaceuticals.

RESULTS

Basal cyclic AMP levels. Glands were initially perfused for 90 min with Locke's solution to mitigate the residual effects of endogenous ACTH which might have been released during preparation of the adrenal glands for perfusion. After this time period, when the spontaneous rate of steroid release was very low (Table 1), the adrenal cyclic AMP concentrations were also generally low. However, these basal cyclic AMP concentrations varied from one cat to another, ranging from 5.2 to 74.5 nmoles/g of tissue, with a mean value of 35.7 (Table 1). On the other hand, the cyclic AMP levels of similarly treated paired glands from the same animal were strikingly similar. Under such conditions, the average cyclic AMP concentration of the right gland was 110 ± 11% of the left (mean of six paired glands). Therefore, in certain experiments the effects of ACTH were analyzed on the basis of relative increase in cyclic AMP levels, with the left gland used as the unstimulated control and the right gland exposed to ACTH.

TABLE 1

Effect of ACTH and theophylline on adrenal cyclic AMP levels and corticosteroid release

Glands were perfused with Locke's solution for 120 min. In certain experiments theophylline was added to the perfusion medium during the final 40 min of perfusion; in other experiments theophylline was present during the final 60 min of perfusion and ACTH was added during the final 40 min. The corticosteroid output was determined by collecting sequential samples of perfusate during the last 40 min of perfusion, before the glands were frozen and assayed for cyclic AMP. The results represent mean values ± standard errors.

ACTH	Theophylline (0.5 mM)	No. of experiments	Cyclic AMP	Corticosteroid output
μunits/ml			nmoles/g tissue	μg/40 min
0	−	27	35.7 ± 3.6	<0.5
0	+	10	52.0 ± 6.0[a]	<0.5
4	−	4	63.3 ± 16.5[a]	10.5 ± 1.4
4	+	3	449 ± 104[b]	7.4 ± 1.5
40	−	4	238 ± 32[a]	15.4 ± 3.4
40	+	3	220 ± 14[b]	19.9 ± 1.8
400	−	6	507 ± 77[a]	10.2 ± 2.5

[a] $p < 0.02$ compared to group of control glands perfused with Locke's solution in the absence of theophylline.

[b] $p < 0.01$ compared to group of control glands perfused with Locke's solution in the presence of theophylline.

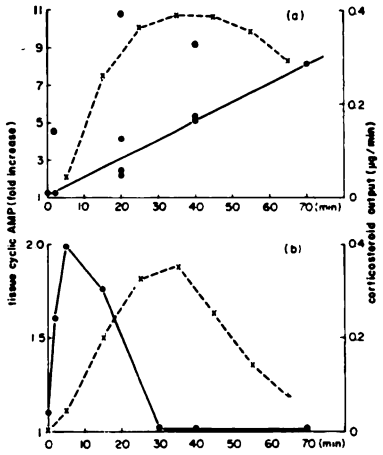

FIG. 1. *Effect of ACTH on adrenal cyclic AMP levels and steroid release*

Both glands were perfused with Locke's solution for 90 min. The control, left gland was removed and analyzed for cyclic AMP. One of the following procedures was then carried out. a. ACTH (0.4 μunit/ml) was added to the perfusion medium at zero time, and at the designated time intervals perfusion was stopped and the right glands was assayed for cyclic AMP. b. ACTH (4 μunits/ml) was added at zero time for up to 5 min. Perfusion with an ACTH-free solution was continued for various intervals up to 70 min before cyclic AMP determinations were made. Each cyclic AMP value (●) was calculated from a different set of paired glands (see RESULTS). The steroid outputs (×) are mean values obtained from the perfusate of right glands. Cyclic AMP levels during constant exposure to ACTH were determined in the presence of 0.5 mM theophylline (⊙).

The basal cyclic AMP levels are quite high in comparison to those reported in rat adrenals (3); however, this difference may be due to the time required for excision and trimming of the cat adrenal prior to freezing.

Effect of ACTH on cyclic AMP concentrations and steroid release. During constant exposure to a low concentration of ACTH (0.4 μunit/ml), no increase in cyclic AMP levels was observed during the first 2 min of stimulation. By 20 min an increase was observed which continued up to 70 min of stimulation (Fig. 1a). During this prolonged period of stimulation, steroid output began to increase during the first 10 min and was reasonably well maintained over the 70-min period (Fig. 1a). When 4 μunits/ml of ACTH were added to the perfusion medium, adrenal cyclic AMP levels increased up to 2-fold during the first 5 min (Fig. 1b). When the perfusion medium was changed to ACTH-free Locke's solution, cyclic AMP levels declined rapidly and fell to control levels 30 min after exposure to ACTH. By contrast, corticosteroid release continued to increase during perfusion with the ACTH-free medium, and reached a maximum 30–40 min after exposure to ACTH (Fig. 1b). Even 70 min after ACTH was removed, corticosteroid secretion rates were still perceptibly increased despite the fact that the cyclic AMP concentrations in the adrenal gland had been at control levels for at least 30 min.

During a 40-min exposure to ACTH, the cyclic AMP levels could be correlated with the ACTH concentration in the range of 4–400 μunits/ml (Table 1). Despite the fact that at these dose levels of ACTH there was a graded increase in the tissue cyclic AMP concentration, there was no significant difference in the amount of steroid secreted during a 40-min exposure to the three concentrations of stimulating hormone (Table 1).

In order to confirm the validity of the adrenal cyclic AMP values obtained by the isotopic displacement method, a second series of cyclic AMP levels were determined using the radioimmunoassay method Steiner *et al.* (11). Paired glands were perfused with Locke's solution. After the left gland was removed, ACTH (40 μunits/ml) was added for an additional 40 min. The average cyclic AMP values (± standard error) for three control and three stimulated glands were 42.3 ± 15.0 and 257.7 ± 51.9 nmoles/g of tissue, respectively. These values are very similar to those obtained using the method of Brooker *et al.* (Table 1), and verify the reliability of the latter method, which was used in all subsequent experiments. In addition, these values are comparable to those obtained with ACTH in the rat adrenal *in vivo* (3).

Effect of theophylline. Theophylline inhibits the degradation of cyclic AMP by tissue phosphodiesterase in many systems. The addition of theophylline (0.5 mM) to the perfusion medium increased the average basal cyclic AMP levels by more than 30%, although there was no discernible increase in spontaneous corticosteroid secretion (Table 1). Moreover, theophylline dramatically increased the accumulation of cyclic AMP during exposure to low ACTH concentrations. In the presence of theophylline ACTH (0.4 μunit/ml) produced a 4–5-fold increase in cyclic AMP during the first 2 min of stimulation, whereas in the absence of theophylline this short stimulation resulted in no perceptible increase in cyclic AMP levels (Fig. 1a). During more prolonged stimulation periods with ACTH (0.4 μunit/ml), 9–11-fold increases in tissue cyclic AMP levels were obtained in the presence of theophylline (Fig. 1a). Theophylline also strikingly increased tissue cyclic AMP concentrations induced by 4 μunits/ml of ACTH, but not by 40 μunits/ml of ACTH (Table 1).

Just as theophylline did not increase spontaneous corticosteroid release, it did not enhance steroid release elicited by 4 μunits/ml of ACTH, although cyclic AMP levels were augmented approximately 7-fold by theophylline (Table 1). Corticosteroid release induced by 40 μunits/ml of ACTH was also not significantly enhanced by 0.5 mM theophylline (Table 1); in one experiment, when 5 mM theophylline was employed, the secretory response to this same ACTH concentration was depressed by almost 80%.

Effect of ion deprivation. Glands which

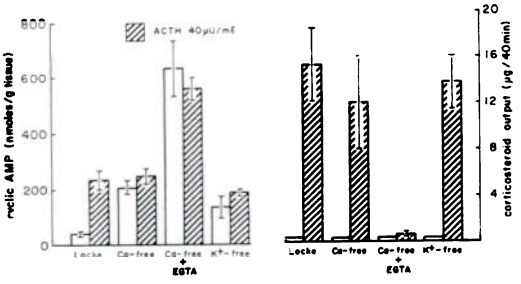

FIG. 2. *Effect of cation deprivation on tissue cyclic AMP concentration and steroid release*

Glands were perfused with normal Locke's solution or ion-deprived Locke's solution for 40 min, in the presence or absence of ACTH (40 μunits/ml), and assayed for cyclic AMP. During the perfusion period the perfusate was collected and assayed for corticosteroids. Each vertical bar represents mean values (± standard errors) for tissue cyclic AMP concentrations and steroid output obtained from at least three different preparations. Standard errors are not given for the average spontaneous rates of corticosteroid release, since these values usually fell below the sensitivity of the assay method.

were perfused with calcium-free Locke's solution showed approximately a 6-fold increase in the tissue concentration of cyclic AMP, although there was no increase in spontaneous steroid release (Fig. 2). The addition of ACTH (40 μunits/ml) caused only a very small additional increment in cyclic AMP levels in the absence of calcium, but a large increase in steroid output (Fig. 2). The increase in steroid release in the absence of calcium may appear paradoxical in light of our previous findings indicating a calcium-dependent secretory mechanism in the adrenal cortex (7); however, this calcium requirement was most clearly observed when low ACTH concentrations were given for short periods of time. The ACTH concentration employed in the present experiments (40 μunits/ml for 40 min) was greatly in excess of that required for the maximal response.

When the calcium-chelating agent EGTA[3] (0.1 mM) was added to the calcium-deprived perfusion medium, tissue cyclic AMP concentrations were further increased (Fig. 2). The addition of ACTH did not produce any additional enhancement of cyclic AMP levels (Fig. 2). No corticoid secretion was observed during perfusion with EGTA in either the presence or absence of ACTH (Fig. 2). The lack of steroid secretion in the presence of EGTA despite the very high tissue levels of cyclic AMP may be explained by the severe calcium deprivation produced by this chelating agent. However, when calcium was restored to a gland which had been perfused with a calcium-free solution plus EGTA, there was no increase in steroid release, but when ACTH was added together with the calcium, there was a prompt and striking augmentation in corticosteroid secretion (Fig. 3).

Perfusion with a potassium-free (K^+-free) medium for 30 min also increased the basal cyclic AMP concentration almost 4-fold, and under these conditions ACTH increased the cyclic AMP concentration merely by an additional 30%; however, only in the presence of ACTH was steroid output increased during potassium deprivation (Fig. 2).

Effect of exogenous cyclic nucleotide. Perfusion with cyclic AMP or its dibutyryl derivative produced no increase in the rate of corticosteroid release into the perfusate (Fig. 4). In one experiment, cyclic AMP (0.6 mM) was constantly perfused through a gland for 20 min with no perceptible effect

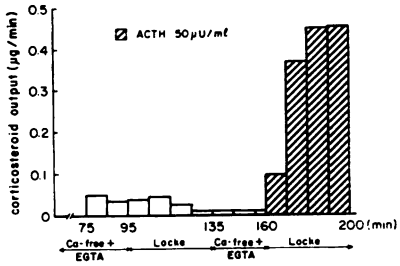

FIG. 3. *Corticosteroid release after severe calcium deprivation*

A gland was perfused for two 30-min periods with calcium-free Locke's solution plus EGTA (0.1 mM). After each period of calcium-free perfusion, calcium was added, first in the absence and then in the presence of ACTH. Each vertical bar depicts the average rate of steroid release during a 10-min collection period.

[3] The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

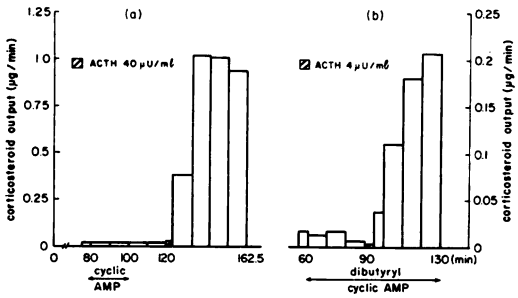

FIG. 4. *Effects of exogenous cyclic nucleotide on corticosteroid release*

Left glands were perfused with Locke's solution in two separate experiments. a. Cyclic AMP (0.6 mM) was added to the perfusion fluid from 80 to 100 min of perfusion, and ACTH (40 μunits/ml) was added from 120 to 122.5 min of perfusion. b. Dibutyryl cyclic AMP (0.5 mM) was present from 60 to 130 min of perfusion, and ACTH (4 μunits/ml) was present from 90 to 95 min of perfusion.

on steroid output, although a subsequent 2.5-min exposure to ACTH (40 μunits/ml) caused a prompt enhancement of secretion (Fig. 4a). In other experiment, dibutyryl cyclic AMP (0.5 mM) was added to the perfusion fluid for 30 min with no effect on release, although a 5-min exposure to ACTH (4 μunits/ml) resulted in a striking increase in steroid release (Fig. 4b). Over all, six experiments were carried out with cyclic AMP or its dibutyryl analogue in the concentration range of 0.05–1.0 mM, and in every experiment these nucleotides failed to augment steroid output, although in the same preparations ACTH proved a potent stimulus.

Cyclic AMP release. Cyclic AMP was also detected in the adrenal perfusate. The spontaneous rate of cyclic AMP release was fairly constant over time in a given preparation (Fig. 5), but the basal rate of release from gland to gland ranged from 0.02 to 0.18 nmole/min, with a mean value of 0.09 ± 0.03 nmole/min (six experiments). Exposure to ACTH for 40 min caused a large fluctuation in the rate of cyclic AMP release, which varied with the ACTH concentration (Fig. 5). Increases in cyclic AMP output were usually observed within the first 10 min of exposure to ACTH. The mean rate of cyclic AMP release during a 40-min exposure to 400 μunits/ml of ACTH was 0.52 ± 0.11 nmoles/min (three experiments), which was 6 times higher than the spontaneous rate of release. Perfusion with theophylline did not strikingly increase the basal or ACTH-induced cyclic AMP output (Table 2), despite the fact that theophylline produced a 7-fold increase in cyclic AMP tissue levels

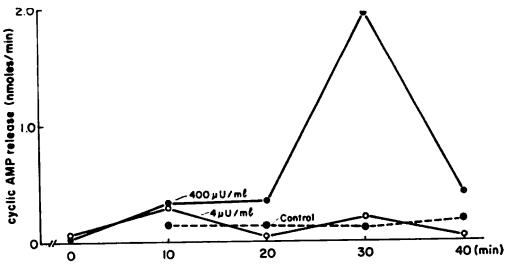

FIG. 5. *Cyclic AMP release into adrenal perfusate*

Glands were perfused with Locke's solution, with and without ACTH. The perfusate was collected at 10-min intervals for 40 or 50 min and assayed for cyclic AMP. The values for zero time were obtained from samples collected just prior to the 40-min exposure to ACTH. Each of the three experiments shown was performed with a different preparation.

TABLE 2

Cyclic AMP release into adrenal perfusate

Glands were perfused for 30–40 min with normal Locke's solution or various modifications of it. ACTH was then added, and perfusion was continued for an additional 40 min. Samples of perfusate were collected sequentially every 10 min and assayed for cyclic AMP. Control samples were collected during the 10 min prior to the addition of ACTH. Each experiment was performed with a different preparation.

Medium	ACTH	Cyclic AMP release	
		Control	ACTH[a]
	μunits/ml	*nmoles/min*	
Locke's	4	0.08	0.13
Locke's + theophylline	4	0.13	0.20
Locke's	40	0.09	0.19
Ca^{++}-free	40	0.57	0.63
Ca^{++}-free + EGTA	40	1.20	0.28

[a] Average rate of release over a 40-min period.

with the same ACTH concentration (4 μunits/ml) (Table 1).

Perfusion with a calcium-free medium increased the spontaneous release of cyclic AMP in both the presence and absence of EGTA (Table 2), just as calcium deprivation increased basal tissue cyclic AMP levels (Fig. 2). During calcium deprivation ACTH (40 μunits/ml) produced no striking increase in cyclic AMP release above the already enhanced basal levels (Table 2); this same ACTH concentration did not clearly raise tissue cyclic AMP levels during calcium deprivation (Fig. 2).

DISCUSSION

The hypothesis that the action of ACTH is mediated through an increase in the intracellular concentration of cyclic AMP has received much experimental support. Grahame-Smith *et al.* (3) have shown from studies *in vitro* and *in vivo* that increases in adrenal cyclic AMP induced by ACTH occurred before increases in the rate of adrenal steroidogenesis, that increasing ACTH concentrations produced increasing tissue cyclic AMP concentrations, and that tissue cyclic AMP levels remained elevated while the rate of steroidogenesis was maintained. The present experiments, which were carried out on the intact gland perfused *in situ*, confirm all these observations, which are compatible with the hypothesis that cyclic AMP is somehow involved in the steroidogenic action of ACTH.

However, when all the present results are correlated, one is forced to question seriously the hypothesis that cyclic AMP is the sole mediator of ACTH action. If an increase in cyclic AMP were singularly responsible for the effect of ACTH, one might expect a correlative rise in tissue cyclic AMP concentrations and corticosteroid release. But corticosteroid output was not enhanced by increasing ACTH concentrations or by theophylline, despite the fact that both increased adrenal cyclic AMP levels. One may argue that there is no reason why cyclic AMP cannot attain levels in excess of the concentrations required for maximal steroid output. However, other examples of the dissociation between cyclic AMP concentrations and steroid release were observed. Although constant stimulation with ACTH caused a continuously increasing level of tissue cyclic AMP and a fairly well-maintained steroid output, brief exposures to a low ACTH concentration caused only a small, transient rise in tissue cyclic AMP levels and yet steroid release persisted long after cyclic AMP concentrations had returned to control levels. Farese *et al.* (12) also reported a lack of temporal correlation between ACTH-stimulated tissue cyclic AMP levels and steroidogenesis *in vitro*; although their results are quantitatively similar to our own, steroid production rather than steroid release was measured.

The calcium deprivation studies lend further support to the idea that an increase in tissue cyclic AMP is not sufficient to trigger corticosteroid release. Perfusion with a calcium-free solution increased basal levels of tissue cyclic AMP; yet steroid output was enhanced only when ACTH was added. It has been shown from studies *in vitro* that calcium is required for the steroidogenic action of exogenous cyclic AMP (5); however, in the present investigation, when adrenal cyclic AMP levels were strikingly increased by calcium deprivation plus EGTA, restoring calcium to the perfusion medium enhanced steroid release only when accompanied by ACTH. In addition, during potassium deprivation—in the presence of the normal calcium concentration—cyclic AMP levels were significantly augmented, although steroid release was observed only when ACTH was present. These observations confirm that an increase in tissue cyclic AMP, in the presence or absence of calcium, is not sufficient to elicit steroid release, but that an additional effect of ACTH is also required.

The observation that prolonged perfusion with cyclic AMP or its dibutyryl derivative did not increase corticosteroid release from the perfused gland—despite the fact that this preparation is exquisitely sensitive to brief exposures to microunit concentrations of ACTH—further supports the hypothesis that cyclic AMP cannot mimic all the effects of ACTH in the intact gland. Cyclic nucleotides augment adrenal steroidogenesis in

tissue sections (2), cell fractions (13), cell cultures (14), and even steroid release from preparations *in vivo* (15), although very high concentrations may be required in the whole animal (16). The inability of cyclic nucleotide to mimic the action of ACTH in the isolated, intact gland could be ascribed to poor penetration of the agent into the cortical cell because of its size, charge, and molecular structure. On the other hand, the amount of cyclic nucleotide presented to the perfused gland was grossly in excess of the intracellular tissue concentration, so that only small amounts of the nucleotide would have to penetrate to elicit a response. Actually, the observed effect which we were investigating in all these studies was steroid release into the perfusate, and so it is possible, in light of the studies alluded to above, that perfusion with cyclic AMP or its dibutyryl analogue did, in fact, increase steroid production within the gland. Similarly, the increase in tissue cyclic AMP observed with cation deprivation may also be associated with increased amounts of steroid within the gland (7). Corroboration of this idea will have to await further investigation. In any event, the inability of exogenous cyclic nucleotide to increase corticosteroid release is consistent with our other data, which indicate that a rise is tissue cyclic AMP content is not sufficient to trigger the physiological release response from the intact gland.

If cyclic AMP cannot mimic the action of ACTH, it follows that ACTH must have one or more effects in addition to merely increasing the level of cyclic nucleotide in the tissue. Previous reports from our laboratory have shown that the stimulatory action of ACTH on the adrenal cortex is associated with a redistribution of cellular calcium from a more readily exchangeable to a less readily exchangeable fraction (8). This translocation of cell calcium is temporally correlated with corticosteroid release and persists well after exposure to ACTH. Thus, it is proposed that among its protean actions ACTH causes (a) an increase in tissue cyclic AMP which augments steroidogenesis within mitochondria (6, 13) through a persistent change in some protein component of the cell (12), and (b) a redistribution of calcium to some active site—possibly the endoplasmic reticulum or mitochondria—from which steroid release is initiated.

Since calcium deprivation augments adenyl cyclase activity in a number of preparations *in vitro* (17–20), including the adrenal cortex (21), it is tempting to speculate further that ACTH and calcium deprivation enhance cyclic AMP levels by a similar mechanism. The present finding that the stimulatory effects of ACTH and calcium deprivation are not additive and the previous observation on broken-cell preparations that calcium is required for ACTH stimulation of adrenocortical adenyl cyclase (22) are both consistent with this concept. ACTH may modulate cyclic AMP formation by releasing calcium from a membrane-bound cyclase binding site, and the translocation of this calcium fraction to the cell interior may be responsible for triggering the secretory process. However, more direct evidence is needed to confirm the hypothesis that the release of calcium and the activation of adenyl cyclase are initiated by the action of ACTH at the same receptor. It is of interest that Rasmussen (23) has recently proposed a similar interaction between calcium and cyclic AMP after reviewing data obtained from various biological systems.

The conclusions drawn from the present study are based upon the supposition that the observed changes in cyclic AMP concentrations occur within cortical cells. However, since the adrenal gland represents a heterogeneous group of tissue components, one cannot rule out the possibility that extracortical tissue may also contribute to the alterations in cyclic AMP levels. Alternatively, cellular compartmentalization of cyclic AMP may also account for at least some of the apparent dissociation between cyclic AMP levels and steroid release.

Cyclic AMP release. The release of cyclic nucleotide into the extracellular medium has been observed with pigeon erythrocytes (24) and from the perfused rat liver (25), and the present studies demonstrate the release of cyclic AMP into the adrenal perfusate. These data, together with the detection of cyclic nucleotide in blood (26) and urine (27), indicate that cyclic nucleotide release from cells

may be a rather general phenomenon and may provide another means whereby the cell maintains cyclic AMP concentrations within physiological limits.

Although the present investigation does not provide detailed information concerning the mechanism of cyclic AMP release from cortical cells, a few general conclusions can be drawn. First, the rate of cyclic AMP output appeared to correlate with the amount of cyclic AMP in the gland, which suggests that release may involve simple diffusion, secondary to cyclic AMP accumulation in the tissue. Second, cyclic AMP release into the perfusate, unlike steroid release, is not a calcium-dependent phenomenon. Even in the presence of EGTA, high rates of cyclic AMP efflux were observed although steroid release was abolished.

Finally, the average amount of cyclic AMP release per minute approximates 1–2% of the total tissue cyclic AMP. Thus, over a prolonged perfusion period, cyclic AMP output may accumulate to a considerable degree relative to the cyclic nucleotide in the gland. These findings suggest that turnover of cyclic AMP may be more important than absolute tissue concentrations in modulating physiological activity. Whenever possible, therefore, future studies should consider cyclic AMP efflux from cells, as well as its synthesis and degradation.

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