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Research Article

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Control and Localization of Rat Adrenal Cyclic Guanosine 3′,5′-Monophosphate

COMPARISON WITH ADRENAL CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE

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ABSTRACT Cyclic AMP and cyclic GMP were measured in rat adrenal glands after either hypophysectomy alone or after hypophysectomy and treatment with ACTH. Adrenal cyclic GMP levels rise in acutely hypophysectomized rats to a maximum at 1 h of approximately 200% of control levels; there is a return to base line at 4–12 h after hypophysectomy. In contrast, adrenal cyclic AMP falls immediately to about 50% of control levels after hypophysectomy and remains at approximately 1 pmol per mg tissue. Doses of ACTH beyond the physiological range markedly suppress adrenal cyclic GMP while producing a 50-fold or greater rise in cyclic AMP in hypophysectomized rats. This pattern of adrenal cyclic GMP rise was unchanged in acutely hypophysectomized animals treated with dexamethasone. N^6-2′-0 dibutyryl cyclic AMP acted similarly to the effect of ACTH in bringing about a suppression of adrenal cyclic GMP levels.

Physiological i.v. pulse doses of ACTH produced a rapid dose related increase in adrenal cyclic AMP and a simultaneous suppression of adrenal cyclic GMP. In vitro incubation of quartered adrenal pairs with 500 mU ACTH produced elevated cyclic AMP levels and suppression of cyclic GMP.

Whereas adrenal cyclic AMP fell rapidly to 50% of control levels after hypophysectomy and remained at about 1 pmol per mg tissue for 7 days, adrenal cyclic GMP showed a biphasic rhythm in long-term hypophysectomized animals. After an initial peak at 1 h after hypophysectomy, adrenal cyclic GMP declined to baseline at 4–12 h but thereafter progressively rose with time, eventually reaching levels over 1 pmol per mg tissue.

Fluorescent immunocytochemical staining of rat adrenal zona fasciculata showed cyclic AMP largely confined to cytoplasmic elements with little fluorescence contained in nuclei. In contrast, cyclic GMP was found discretely positioned in nuclei with prominent fluorescence in nucleoli in addition to cytoplasmic localization.

It is concluded that in hypophysectomized rats ACTH, either directly or in conjunction with alteration of adrenal cyclic AMP, appears to be one factor which regulates adrenal cyclic GMP. The direction of cyclic GMP change and the different subcellular localization of the nucleotides suggest divergent roles for cyclic AMP and cyclic GMP in adrenocortical function. Furthermore, our observations suggest a role for adrenal cyclic GMP in nuclear directed events.

INTRODUCTION

The role of ACTH on adrenal steroidogenesis and adrenocortical growth capacity is well established. Considerable evidence has now accumulated that 3′,5′-cyclic AMP (cyclic AMP)[1] is an intracellular mediator of

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[1] *Abbreviations used in this paper:* cyclic AMP, 3′,5′-cyclic AMP; cyclic GMP, 3′,5′-cyclic GMP; dibutyryl cyclic AMP, N^6-2-0-dibutyryl cyclic AMP; PBS, phosphate-buffered saline; PDE, 3′,5′-beefheart cyclic nucleotide phosphodiesterase.

ACTH action on adrenal steroidogenesis (1–3). The acute effects of physiological doses of ACTH lead to a dose related accumulation of adrenal cyclic AMP and thereafter to parallel increases of rat adrenal corticosterone production.

The growth promoting effect of ACTH on the adrenal cortex is characterized by RNA and protein accumulation and then DNA replication (4–5). Masui and Garren (6) have shown that adrenal DNA polymerase and thymidine kinase activities increase concomitantly with DNA synthesis and are initiated at 24 h after ACTH administration. More recently, Fuhrman and Gill (7) have demonstrated the stimulation of the nucleolar localized RNA polymerase I by ACTH and N^6-2′-0 dibutyryl cyclic AMP (dibutyryl cyclic AMP) in isolated guinea pig adrenal nuclei. Therefore, it appears that ACTH and possibly cyclic AMP are major positive influences in the promotion of adrenal steroidogenesis and growth.

Recent evidence has suggested that 3′,5′-cyclic GMP (cyclic GMP), heretofore known to be present in most mammalian tissues (8), may be a biologically important regulator of growth in some tissues (9). The present studies were undertaken to examine the question of whether adrenal cyclic GMP is subject to hormonal regulation and to compare the regulation of adrenal cyclic GMP with that of cyclic AMP. Our observations suggest that adrenal cyclic GMP is suppressed by ACTH either directly or in conjunction with alterations in adrenal cyclic AMP. In addition, we have shown significant nuclear localization for adrenal cyclic GMP while cyclic AMP is predominantly found in the adrenocortical cytoplasm. The series of experiments described here show that ACTH exerts divergent effects on adrenal cyclic AMP and cyclic GMP and that the two nucleotides have differing intracellular localizations. The studies suggest that adrenal cyclic GMP does not play a role similar to that of cyclic AMP in the regulation of adrenocortical function. Adrenal cyclic GMP may be involved in nuclear directed events.

METHODS

Materials. Cyclic nucleotides used for radioimmunoassay standards were purchased from Sigma Chemical Co., St. Louis, Mo. Iodinated derivatives of the cyclic nucleotides and the antibodies used in the radioimmunoassay were prepared as described elsewhere (10). Rabbit gamma globulins and fluorescein-labeled goat antirabbit IgG (lot 16) were obtained from Miles Laboratories, Inc., Miles Research Div., Elkhart, Ind. Beef heart 3′,5′-cyclic nucleotide phosphodiesterase (PDE) (lot 120c-7,740) was purchased from Sigma Chemical Co. Porcine ACTH in 8% gelatin, employed for in vivo studies, was purchased from Armour Pharmaceutical Co., Chicago, Ill. ACTH powder refined (CI-305) dissolved in 1% acid-saline albumin, employed in both in vitro and in vivo studies, was obtained from Parke, Davis & Co., Detroit, Mich. Dibutyryl cyclic AMP, obtained from Boehringer Mannheim Corp., New York, was dissolved in 8% gelatin. Dexamethasone sodium phosphate (Decadron®) was purchased from Merck Sharp & Dohme (West Point, Pa.). Papaverine hydrochloride (lot 6VJ633) was obtained from Eli Lilly & Co., Indianapolis, Ind.

Male Sprague-Dawley rats weighing 160–180 g were utilized in these experiments. Preliminary experiments showed that minimal stress occurred, as determined by elevation of adrenal cyclic AMP concentration, if animals were killed quickly by blunt trauma instead of after anesthestic agents, cervical dislocation, etc. Therefore, some rats with intact pituitaries were killed quickly by blunt trauma. These animals served as controls. All other rats were hypophysectomized under light ether anesthesia by the transaural approach. At autopsy, rats with retained pituitary remnants were discarded. The adrenal glands were removed at specified times, trimmed free of adhering fat, and frozen immediately in liquid nitrogen. In some animals renal cortical tissue and skeletal muscle from the femoris muscle group were obtained and handled in the same manner. Tissue samples were stored at −80°C until processed for radioimmunoassay of cyclic AMP and cyclic GMP.

Studies in vivo. Groups of hypophysectomized rats, without further treatment, were sacrificed at various intervals for up to 7 days after hypophysectomy. A second group of acutely hypophysectomized animals was treated with single i.p. injections of either 20 U ACTH, 50 mg dibutyryl cyclic AMP, or 0.2 mg dexamethasone. Adrenals were removed at times specified in the text for assay of the cyclic nucleotides.

For studies examining the effects of physiological doses of ACTH, rats hypophysectomized 2 h earlier were anesthetized with pentobarbital (60 mg/kg) by i.p. injection. Pulse doses of ACTH, ranging from 0.05 to 1.0 mU, were injected into the femoral vein and the adrenals were removed 3 min later for assay of cyclic AMP and cyclic GMP. Control animals received i.v. injections of 1% acid-albumin [2] instead of ACTH.

Studies in vitro. The adrenal glands from rats hypophysectomized 2 h earlier were removed, trimmed free of adhering fat, weighed, and quartered. After a preincubation period of 1 h, paired adrenals were transferred to fresh medium and the study period started. Incubations were carried out in 25 ml Ehrlenmeyer flasks containing Krebs-Ringer bicarbonate solution with 200 mg of glucose per 100 ml (pH 7.4) in a Dubnoff metabolic shaker with a closed atmosphere of 95% O_2–5% CO_2 at 37°C. The final incubation volume was 2 ml with either 500 mU ACTH contained in 0.2 ml acid 0.1% albumin or 0.2 ml acid 0.1% albumin without ACTH serving as controls.

Adrenals were removed at 15 and 30 min, matted free of liquid with gauze, and frozen immediately in liquid nitrogen until processed for assay of the cyclic nucleotide content as described below.

Assay of cyclic AMP and cyclic GMP. Frozen tissue samples from 10–40 mg were homogenized in 1.0 ml ice cold 6% trichloroacetic acid (TCA). Protein precipitates were sedimented by centrifugation at 2,400 *g* for 20 min. 750 μl of the resulting supernate was aspirated and washed four times with 10 vol of diethyl ether saturated with water. The aqueous phase was evaporated to dryness under a nitrogen stream at 60–70°C. The resulting residues were then

[2] Vehicle in which ACTH powder was dissolved for i.v. injection consisted of 1% bovine serum albumin in 0.9% sodium chloride, pH 2.6.

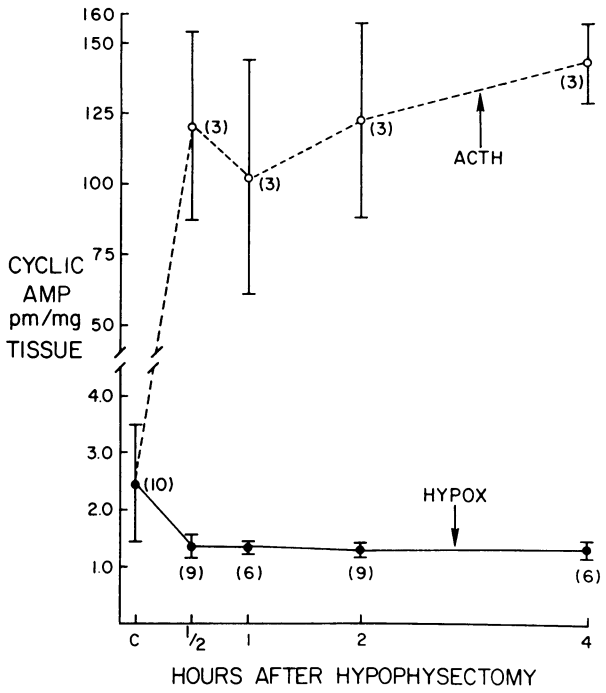

FIGURE 1 Acute effects of hypophysectomy and 20 U ACTH injected i.p. 15 min before sacrifice on rat adrenal cyclic AMP. Cyclic AMP is expressed as picomoles per mg of tissue. Each point represents the mean of the number of animals used, as shown in parentheses. The vertical bars indicate the SEM. O- - -O, cyclic AMP concentration after 20 U ACTH in hypophysectomized rat adrenals; ●——●, cyclic AMP concentration in hypophysectomized rat adrenals. Hypox indicates hypophysectomized only animals; ACTH designates those animals given ACTH after hypophysectomy.

reconstituted in 1 ml of 0.05 M sodium acetate buffer (pH 6.2) and samples used directly in the radioimmunoassay as described by Steiner, Pagliara, Chase, and Kipnis (10).

Confirmatory tests that the radioimmunoassay reflected valid measurements for both nucleotides were performed by adding increasing amounts of the tissue extracts in the assay and by incubation of samples with PDE (10). PDE treatment of tissue extracts were performed by adding to 300 μl of the buffer-tissue extract, 0.02 U PDE, and 15 μl 0.1 M $MgCl_2$. This mixture was incubated for 45 min at 37°C. The reaction was then stopped by boiling for 2 min. Tissue extracts increased linearly for both nucleotides with the quantity of tissue added to the immunoassay. PDE destroyed approximately 95–100% and 84–100% of cyclic AMP and cyclic GMP, respectively. Tissue blanks were negligible and constant for both cyclic nucleotides from sample to sample. Recovery of the cyclic nucleotides was monitored by adding known amounts of cyclic AMP and cyclic GMP to one equal quantity of tissue extract during deproteinization with TCA. Recovery was essentially 100% for both nucleotides.

Immunocytochemistry. Histochemical localization of the cyclic nucleotides in rat adrenal zona fasciculata was determined by an indirect immunofluorescent technique (11). Frozen, unfixed cryostat-cut sections 3–6 μm in thickness were treated with highly specific IgG from antisera raised in rabbits to either cyclic AMP or cyclic GMP as previously described (12). Rat adrenal zona fasciculata sections from untreated animals were dried in air on slides and treated in sequence for 30 min with each of the following: antibody to nucleotide (1:10 dilution of antibody to cyclic AMP, and 1:8 dilution of antibody to cyclic GMP) or control serum (1:10 dilution); phosphate buffered saline (PBS); and fluorescein-labeled goat antiserum to rabbit IgG in a 1:8 dilution. The slides were then washed for 10 min in PBS and mounted with 50% glycerine in PBS for examination under fluorescence microscopy. Individual slides were interpreted without regard to which antibody had been used for staining.

Adrenal cell fractions. Rat adrenal glands from untreated animals were homogenized in 0.88 M sucrose containing 3 mM $MgCl_2$ and 200 μM papaverine by hand with 10 strokes of a Teflon pestle in a glass homogenizing tube. The homogenization and all centrifugation steps were performed at 0–4°C. In some experiments no phosphodiesterase inhibitor was added. Although, this resulted in a lower content of total cyclic GMP without a change in total cyclic AMP, the relative distribution of both nucleotides was unaltered (see Results).

The homogenate was centrifuged at 1,000 *g* for 10 min in a refrigerated Sorvall RC2-B (Ivan Sorvall, Inc., Norwalk, Conn.). The crude pellet and cell debris was resuspended in 2.2 M sucrose containing 3 mM $MgCl_2$ and centrifuged at 45,000 *g* for 45 min in a model L3-50 ultracentrifuge (Beckman Instruments Inc., Fullerton, Calif.) to obtain the nuclear pellet fraction. This method of nuclei isolation, with only minor modification here, has been shown by electron microscopy to be free of cytoplasmic contami-

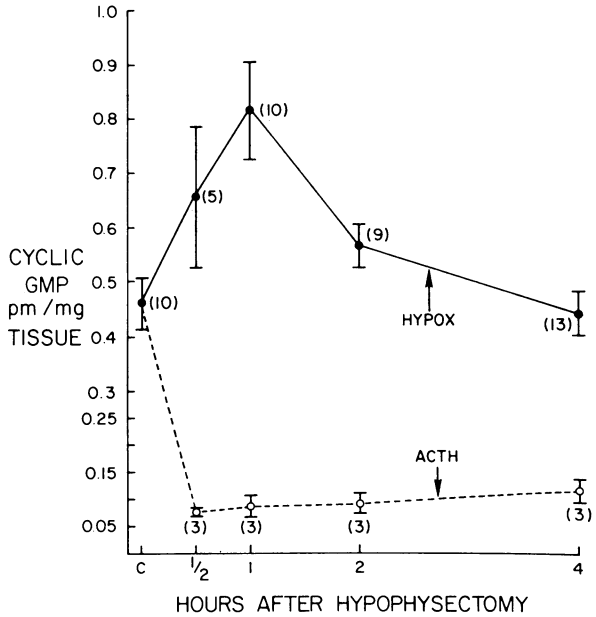

FIGURE 2 Acute effects of hypophysectomy and 20 U ACTH injected i.p. 15 min before sacrifice on rat adrenal cyclic GMP. Cyclic GMP is expressed as picomoles per mg of tissue. Points, number of animals and vertical bars (SEM) are as described in Fig. 1. O- - -O, cyclic GMP concentration after 20 U ACTH in hypophysectomized rat adrenals; ●——●, cyclic GMP concentration in hypophysectomized rat adrenals.

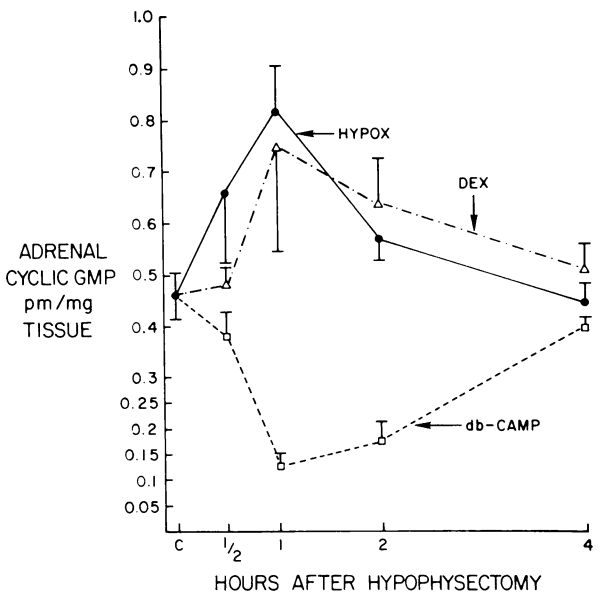

FIGURE 3 Effects of dexamethasone and dibutyryl cyclic AMP on adrenal cyclic GMP in acutely hypophysectomized rats. Cyclic GMP is expressed as picomoles per milligram tissue. Each point represents the mean of three pairs of adrenals. The vertical bars indicate the SEM; ●——●, cyclic GMP concentration in hypophysectomized rat adrenals; △-·-·△, cyclic GMP concentration in hypophysectomized rat adrenals after 0.2 mg dexamethasone injected i.p. 15 min before sacrifice; □- - -□, cyclic GMP concentration in hypophysectomized rat adrenals after 50 mg dibutyryl cyclic AMP injected i.p. 15 min before sacrifice. Dex indicates dexamethasone treated animals and db-C-AMP, animals treated with dibutyryl cyclic AMP.

nants (13). Crude mitochondrial and microsomal pellets were obtained from the 1,000 *g* supernate by differential centrifugation at 10,000 and 105,000 *g*, respectively. The postmicrosomal supernate was used as the cytosol fraction. Fractions were then treated with ice-cold TCA, extracted, and used in the radioimmunoassay as previously described. The protein content of TCA precipitates was measured by the method of Lowry, Rosebrough, Farr, and Randall (14) with bovine serum albumin as a standard.

Statistical analysis. Statistical evaluation of the data was performed using a one-way analysis of variance and a *f* distribution of the experimental groups.

RESULTS

Effects of acute hypophysectomy and ACTH, dexamethasone, and dibutyryl cyclic AMP on adrenal cyclic nucleotide levels in vivo. Adrenal cyclic AMP and cyclic GMP were measured in control, hypophysectomized, and hypophysectomized rats given 20 U ACTH 15 min before sacrifice. After hypophysectomy, adrenal cyclic AMP falls but not significantly ($P \geq 0.125$) to about 1 pmol per mg tissue (Fig. 1). As previously shown by Richman et al. (15), ACTH uniformly produces a striking 50-fold or greater rise in adrenal concentrations of cyclic AMP. The effects of hypophysectomy and of

TABLE I

Cyclic Nucleotide Levels in Rat Renal Cortex after Hypophysectomy and ACTH

Treatment	Cyclic nucleotide, mean±SEM		
	Cyclic AMP	Cyclic GMP	
	pm/mg tissue		
None	2.559±0.573	0.056±0.010	(3)*
Hypox (1 h)	2.134±0.479	0.044±0.008	(3)
Hypox + ACTH (1 h)	2.046±0.136	0.049±0.001	(4)
Hypox (4 h)	2.106±0.370	0.039±0.005	(3)
Hypox + ACTH (4 h)	2.804±0.772	0.031±0.006	(3)
Hypox (24 h)	2.027±0.751	0.057±0.029	(3)
Hypox + ACTH (24 h)	2.289±0.143	0.052±0.009	(3)

Hypox indicates hypophysectomy, time of sacrifice after hypophysectomy is shown in parenthesis. ACTH, 20 U administered i.p. 15 min before sacrifice.

* The number of observations is shown in parentheses.

20 U ACTH administered after hypophysectomy on adrenal cyclic GMP are opposite to those observed for cyclic AMP (Fig. 2). Thus, after hypophysectomy, adrenal cyclic GMP rises to a maximum at 1 h which is approximately 200% of control levels ($P < 0.005$). ACTH, 15 min before sacrifice, markedly lowered the cyclic GMP concentrations at each time interval ($P < 0.03$ at each time point).

To examine if the acute changes in adrenal cyclic GMP after hypophysectomy were due to the removal of a pituitary factor (ACTH or other hypophyseal factor) the following experiment was performed. Rat pituitaries ($n = 13$) were homogenized in 2 ml saline and 0.5 ml of the pituitary extract or 0.5 ml saline was injected subcutaneously without anesthesia into rats hypophysectomized 4 h earlier. Animals were sacrificed by blunt trauma 1 h after the injections. Adrenals from pituitary extract treated rats had a cyclic GMP concentration of 0.125±0.023 pm/mg tissue. Adrenal cyclic GMP was 0.232±0.015 pm/mg tissue in saline treated hypophysectomized rats ($P < 0.04$).

TABLE II

Cyclic Nucleotide Levels in Rat Skeletal Muscle after Hypophysectomy and ACTH

Treatment	Cyclic nucleotide, mean±SEM		
	Cyclic AMP	Cyclic GMP	
	pm/mg tissue		
None	0.880±0.320	0.017±0.005	(3)*
Hypox (1 h)	0.550±0.097	0.026±0.005	(3)
Hypox + ACTH (1 h)	0.914±0.049	0.028±0.010	(4)
Hypox (4 h)	0.747±0.236	0.023±0.003	(3)
Hypox + ACTH (4 h)	0.584±0.164	0.020±0.007	(3)
Hypox (24 h)	0.557±0.094	0.011±0.002	(3)
Hypox + ACTH (24 h)	0.588±0.067	0.015±0.006	(3)

* The number of observations is shown in parentheses.

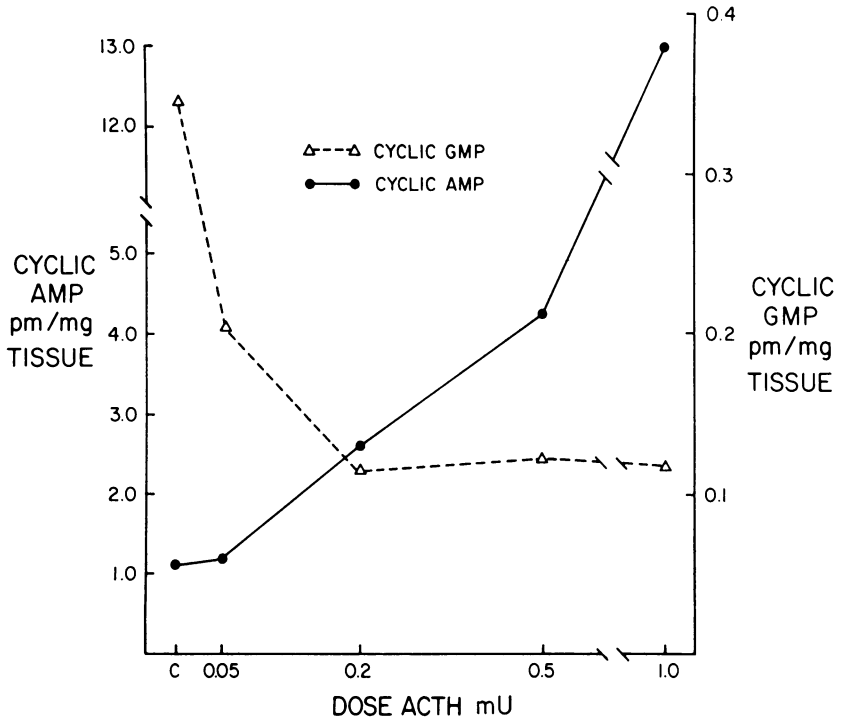

FIGURE 4 Effects of i.v. pulses of ACTH on adrenal cyclic AMP and cyclic GMP in rats hypophysectomized for 2 h. Each point represents the mean of two or three animals. The cyclic AMP concentration ●——●, on the left vertical axis is expressed in picomoles per mg of tissue; △- - -△, cyclic GMP concentration is shown on the right. The dose of ACTH from 0.05 to 1.0 mU is shown on the horizontal axis. Adrenals were excised 3 min after the i.v. injection of ACTH or the acid albumin vehicle control solution.

Inasmuch as glucocorticoids have been implicated as agents which can suppress cyclic GMP levels in some but not all tissues (10), experiments were performed to test the possibility that the observed suppression in adrenal cyclic GMP might be correlated with the stimulatory effect of ACTH on glucocorticoid levels. Rats were hypophysectomized, and some were given dexamethasone, 0.2 mg, 15 min before sacrifice. The pattern of adrenal cyclic GMP was unchanged in hypophysectomized rats treated with dexamethasone ($P > 1.0$) (Fig. 3).

A second group of hypophysectomized rats were treated with dibutyryl cyclic AMP, 50 mg, 15 min before sacrifice. Dibutyryl cyclic AMP acted similarly to the effect of ACTH bringing about a suppression of adrenal cyclic GMP ($P < 0.002$ at 1 and 2 h). Because this dibutyryl analogue of cyclic AMP lowered adrenal cyclic GMP, a rise in adrenal cyclic AMP may promote changes which lead to lower adrenal concentrations of cyclic GMP (Fig. 3).

To examine whether or not the changes in adrenal cyclic nucleotide levels observed under the present conditions were specific to adrenal tissue, skeletal muscle or renal cortical tissue from some animals were also assayed for cyclic AMP and cyclic GMP. Neither hypophysectomy or hypophysectomy plus 20 U ACTH, 15 min before sacrifice, changed basal levels of cyclic AMP or cyclic GMP in rat renal cortical or skeletal muscle tissue (see Tables I and II).

Relationship of adrenal cyclic nucleotide concentration to physiological graded doses of intravenous ACTH. Because it has been previously established that 0.05 mU of ACTH is the smallest i.v. dose of ACTH that will produce a detectable stimulation of steroidogenesis in the rat (3), we elected to study the relationship of adrenal cyclic AMP and cyclic GMP to graded doses of ACTH given i.v. over the physiological range. Rats were hypophysectomized and 2 h later were given pulses of ACTH in the femoral vein ranging from 0.05 to 1.0 mU. Adrenals were removed 3 min after either ACTH or the i.v. acid-albumin control injection for assay of cyclic AMP and cyclic GMP. The results are shown in Fig. 4.

It is apparent that ACTH produces a dose-related increase in adrenal cyclic AMP with a barely detectable rise in the nucleotide levels at the 0.05-mU dose. Stepwise increases of adrenal cyclic AMP follow with increasing amounts of ACTH. These results are quantitatively

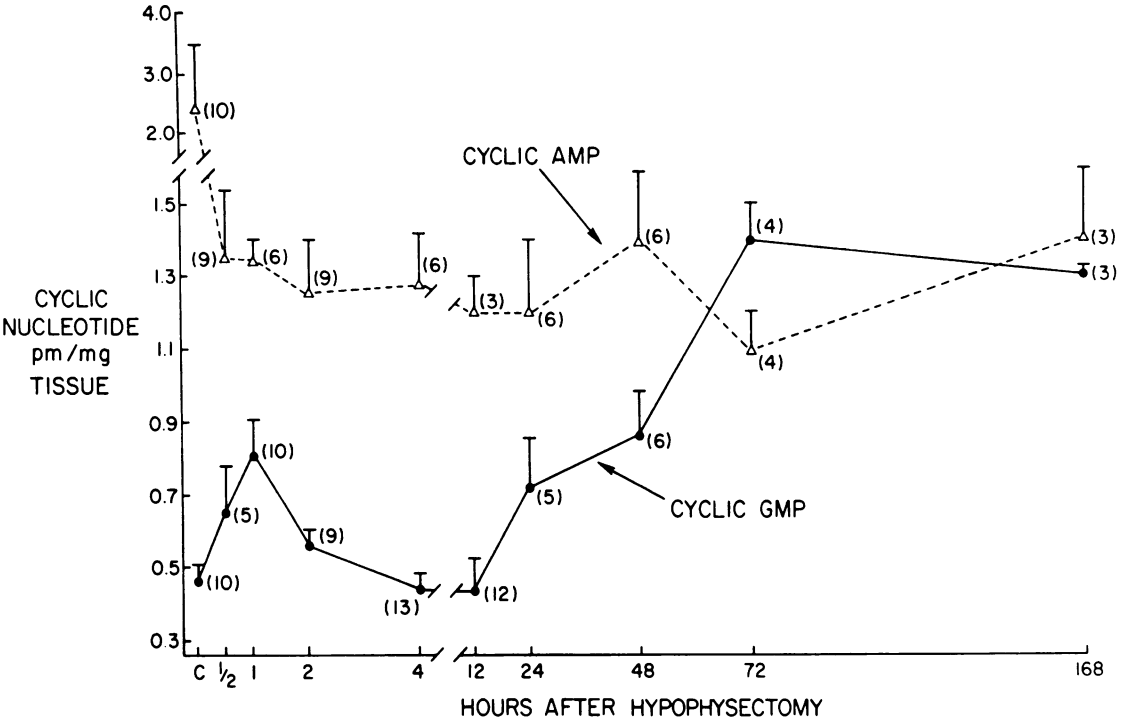

FIGURE 5 Effects of hypophysectomy on rat adrenal cyclic AMP and cyclic GMP over a 7-day period. Cyclic AMP, △---△, and cyclic GMP, ○——○, concentrations are expressed in picomoles per milligram of tissue. Each point represents the mean of the number of animals used, as shown in parentheses. The vertical bars indicate the SEM.

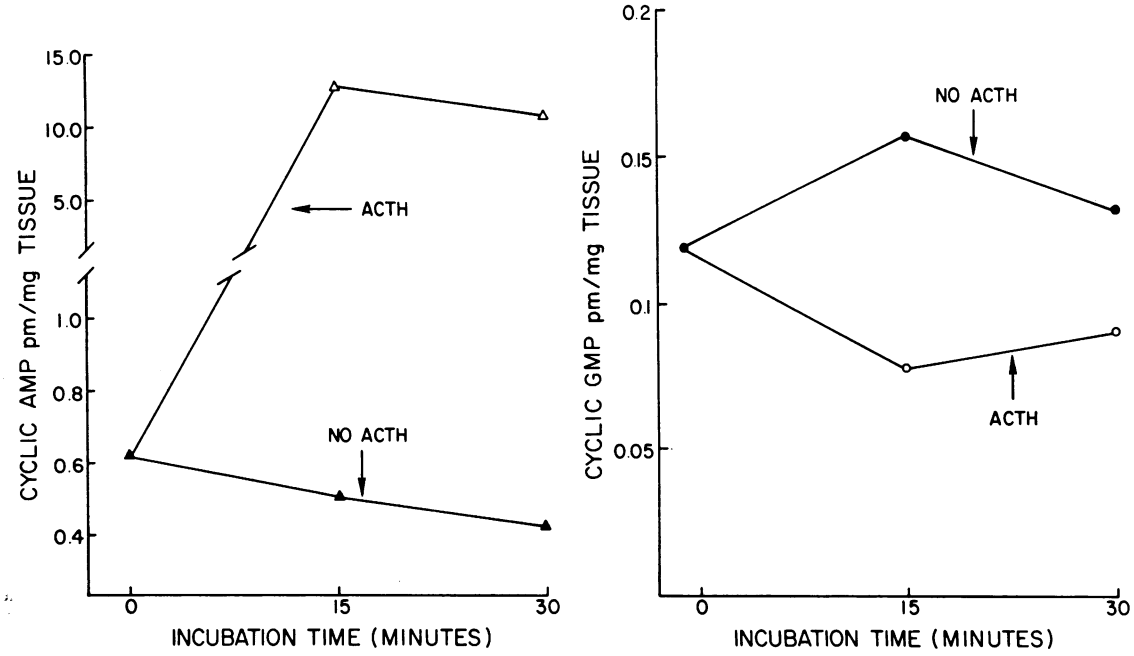

FIGURE 6 In vitro effect of 500 mU of ACTH on cyclic AMP and cyclic GMP in quartered adrenal pairs from 2-h hypophysectomized rats. Each point represents the mean of duplicate pairs. On the left, cyclic AMP concentration is in picomoles per milligram of tissue; △——△, ACTH treated; ▲——▲, without ACTH. On the right, cyclic GMP concentration is in picomoles per milligram tissue; ○——○, ACTH treated; ●——●, without ACTH.

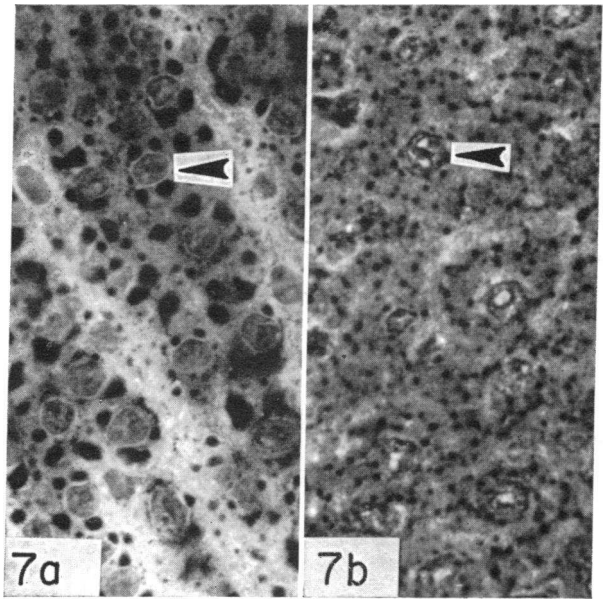

FIGURE 7 Cyclic nucleotide immunofluorescent photomicrographs (× 170) in rat adrenal zona fasciculata. (*a*) section treated with anticyclic AMP antibody showing predominant cytoplasmic staining with thin rim of staining along nuclear membrane. Arrow indicates cell with minimal nuclear staining and thin rim of fluorescence along nuclear membrane. (*b*) section treated with anticyclic GMP antibody, prominent staining occurs in nuclei, nuclear membrane, and nucleoli. In addition, diffuse fluorescence occurs in cytoplasm. Arrow shows cell with prominent nucleolar staining.

quite similar to those previously reported by Grahame-Smith, Butcher, Ney, and Sutherland (3). In contrast to the small rise of adrenal cyclic AMP, adrenal cyclic GMP is suppressed by approximately 40 and 70% with 0.05 mU and 0.2 mU ACTH, respectively. These experiments suggest that in physiological concentrations in vivo ACTH causes a small elevation of adrenal cyclic AMP as it submaximally stimulates steroidogenesis while at the same time adrenal cyclic GMP levels are being markedly suppressed.

Effect of long-term hypophysectomy on adrenal cyclic AMP and cyclic GMP. It is known that after hypophysectomy there is gradual diminution in both adrenal steroidogenesis and adrenal growth (3). After hypophysectomy, adrenal cyclic AMP falls rapidly and remains at about 1 pmol per mg tissue for up to 7 days (Fig. 5). In contrast, adrenal cyclic GMP appears to follow a biphasic rhythm, showing an initial peak at 1 h after hypophysectomy but declining at 4 and 12 h. Thereafter, cyclic GMP appears to rise progressively with time, eventually reaching levels over 1 pmol per mg tissue ($P < 0.00001$ at 72 and 168 h). Thus, after hypophysectomy, adrenal cyclic AMP and cyclic GMP eventually achieve comparable levels in long-term hypophysectomized animals.

Effect of ACTH on adrenal cyclic AMP and cyclic GMP in vitro. To rule out the possibility that alterations of adrenal blood flow or other factors in the in vivo system might account for changes of adrenal cyclic GMP previously observed, the following experiment was performed. Quartered rat adrenal pairs were incubated in duplicate with or without 500 mU ACTH added to the bathing medium. Adrenals were removed at 15 and 30 min for assay of cyclic AMP and cyclic GMP. The results of such an experiment are shown in Fig. 6.

As shown here, cyclic AMP had risen in the ACTH-treated adrenals from 0.628 pmol/mg tissue to 12.395 and 10.313 pmol/mg tissue at the 15 and 30 min points of incubation, respectively. On the other hand, adrenal cyclic GMP was suppressed by ACTH from 0.120 pmol/mg tissue to 0.079 and 0.091 pmol/mg tissue at these intervals of incubation time. Adrenal groups not containing ACTH showed opposite changes in the concentration of cyclic AMP and cyclic GMP.

Localization of cyclic nucleotides in rat adrenal zona fasciculata by fluorescence immunocytochemistry. When zona fasciculata sections are treated with the anticyclic AMP antibody, fluorescence is largely confined to the cytoplasm. Nuclei contain only minimal amounts of fluorescence with some staining along the nuclear membrane (Fig. 7A). Cyclic AMP consistently appeared in a streaked cytoplasmic distribution in adrenal zona fasciculata sections indicating perhaps that not all cells in this zone contain equal amounts of the nucleotide.

The pattern of cyclic GMP fluorescence, while showing some cytoplasmic staining, was localized predominantly to zona fasciculata nuclei. Prominent fluorescence is readily seen in nucleoli and along the nuclear membrane (Fig. 7B). In contrast to the streaked appearance of cyclic AMP, cyclic GMP staining was homogenously distributed in rat zona fasciculata.

Although not shown here, the patterns of fluorescence for cyclic AMP and cyclic GMP were the same in zona glomerulosa and zona reticularis as in zona fasciculata. Adrenal medulla contained areas of fluorescence for both nucleotides but to date has not been extensively studied.

Cyclic nucleotide levels in various adrenal cell fractions. To further substantiate the subcellular localization of the nucleotides, cyclic AMP and cyclic GMP were assayed in crude adrenal cell fractions obtained by differential centrifugation of whole rat adrenal homogenates as described above. An average of eight intact rats were killed by blunt trauma to minimize stress and the adrenals pooled for homogenization.

In some homogenates the initial sucrose solution contained 200 μM papaverine while others were processed

without phosphodiesterase inhibition. The presence of papaverine resulted in an approximate 350% increase of total cyclic GMP (65.56 pm/mg protein vs. 17.95 pm/mg protein without papaverine) without significant change in the total cyclic AMP (218.89 pm/mg protein vs. 210.94 pm/mg protein). However, papaverine did not alter the relative distribution of either nucleotide.[3]

Whereas 35% of the total cyclic AMP was present in particulate fractions (10% particulate nuclear fractions), twice as much of the total cyclic GMP (74%) was found in particles (46% particulate nuclear component). On the other hand, a substantially larger portion of cyclic AMP (54%) than cyclic GMP (18%) was located in the soluble fraction. These results lend support to the immunocytochemical findings and suggest that cyclic AMP is predominantly present in the cytosol whereas cyclic GMP is found in particulate fractions in rat adrenal cortex.

DISCUSSION

These studies indicate that the control of adrenal cyclic GMP is regulated at least partially by ACTH or by a cooperative mechanism involving ACTH and the endogenous level of cyclic AMP. The latter possibility is suggested by the observations that cyclic GMP levels fall as cyclic AMP concentrations increase after ACTH administration in acutely hypophysectomized animals. In addition, dibutyryl cyclic AMP mimicked the suppressive effect of ACTH on adrenal cyclic GMP; however, the physiological significance of this effect of dibutyryl cyclic AMP is difficult to interpret.

It is more likely that the control of cyclic GMP levels in rat adrenal is independent of cyclic AMP concentrations. Adrenal cyclic GMP levels follow a biphasic rhythm after hypophysectomy, progressively rising to concentrations comparable to cyclic AMP by 3 days; in contrast, adrenal cyclic AMP levels are reduced by 50% after hypophysectomy and remain constant throughout 7 days. In addition, physiological doses of ACTH significantly suppressed cyclic GMP while cyclic AMP concentrations showed a barely detectable rise. Therefore, these observations suggest that in vivo adrenal cyclic GMP is suppressible by ACTH and that a simple reciprocal relationship between cyclic AMP and cyclic GMP does not adequately explain the control of adrenal cyclic GMP. From the present experiments, we cannot exclude the possibility that other factors in addition to ACTH are important in the control of adrenal cyclic GMP after hypophysectomy.

The role of cyclic GMP in adrenal cortical function has not been established. The exogenous administration of cyclic GMP can promote adrenal steroidogenesis (16–18). However, the concentration of the nucleotide employed ranged from 1 to 10 mM and is as much as 10^6 times the concentration of cyclic GMP in rat adrenal. Our immunohistochemical studies revealed a prominent nuclear localization for cyclic GMP particularly in the nucleolus of zona fasciculata cells. These observations suggest binding sites for cyclic GMP in nuclear elements because the present histochemical studies were done on frozen, unfixed tissue sections and a free cyclic nucleotide might be expected to be lost during the treatment process. Because the activity of RNA polymerase I, a nucleolus located enzyme, is stimulated by ACTH (7), it seems reasonable to suggest that cyclic GMP may participate in the control of this or other nuclear enzymes.

In contrast, adrenal cyclic AMP was largely confined to cytoplasmic components in the immunohistochemical studies, and this localization is consistent with the previously demonstrated location of the cyclic AMP receptor protein (19). Thus, the different subcellular distribution of cyclic GMP and cyclic AMP shown here suggest divergent roles for the cyclic nucleotides in adrenal cortex.

Our studies suggest that adrenal cyclic GMP in vivo is likely functioning in a role dissimilar to that of adrenal cyclic AMP. This conclusion is based on: (*a*) the observations that cyclic GMP concentrations fall after ACTH administration in the hypophysectomized rat, and (*b*) the basal adrenal cyclic GMP/cyclic AMP ratio, higher than in most tissues (9), continues to rise in long-term hypophysectomized rats as the adrenal atrophies. The relationship of cyclic GMP to ACTH action is probably not a simple one, however, as evidenced by the biphasic rhythm in adrenal cyclic GMP after hypophysectomy.

These observations are in conflict with some recent data in cultured mouse fibroblasts in which cyclic GMP concentrations increased immediately after addition of serum (20, 21). The increase in cyclic GMP after the growth promoting stimulus is transient, and it is not clear from these experiments that cyclic GMP is a causal factor in fibroblast growth regulation. It seems likely that the concentrations of both cyclic AMP and cyclic GMP are critical at different stages of the cell cycle in both cultured cells and in tissues such as the adrenal in vivo, and no simple conclusions can be made at present on the role of the cyclic nucleotides in growth regulation.

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[3] Whitley, T. H., and A. L. Steiner. Unpublished observations.

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