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Reversal of Persistently Stimulated Steroidogenesis by GTP and an Inhibitory Adrenocorticotropin Analogue in Adrenal Cells Pretreated with Adrenocorticotropin

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SUMMARY

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A single addition of $ACTH_{1-24}$ at concentrations of 0.1 to 10 nM to an *in vitro* suspension of isolated rat adrenal cells elicits a dose related steroidogenic response lasting from 150 to 240 min. The addition of fresh ACTH can initiate a new response after the spontaneous termination of the first, but persistent steroidogenesis does not require the continued presence of active hormone in the incubation medium. Biologically active hormone disappears from the extracellular medium within the first 90 min of incubation, while steroidogenesis can continue at an undiminished rate for an additional 120 min or more. Cells treated for 1 min with 100 nM ACTH, then rinsed and resuspended in hormone-free medium, produce as much cyclic AMP and corticosterone as do cells continually exposed to the same concentration of hormone. The persistent steroidogenesis seen in the ACTH-pretreated cells may be rapidly terminated by exposure of the cells to GTP or to the ACTH antagonist, $ACTH_{6-39}$. These agents can also greatly accelerate the slow dissociation of bound ^{125}I-$ACTH_{1-24}$ from the cells. These findings suggest that the persistence of steroidogenesis depends upon the presence of persistently bound biologically active hormone on the exterior surface of the adrenocortical cell. The rapid displacement of bound hormone by the competitive antagonist, $ACTH_{6-39}$, supports the hypothesis that there is negative cooperativity among ACTH receptors. The mechanism by which GTP increases the dissociation of bound hormone is unclear. Under the experimental conditions used, the addition of GTP to the medium does not appear to stimulate the production of cyclic AMP. GTP enhances the dissociation of the radiolabeled antagonist, ^{125}I-$ACTH_{6-39}$, as well as the dissociation of labeled agonist hormone. Thus it appears that GTP increases the dissociation of bound ACTH independently of adenylate cyclase activity, probably by interacting with sites on the cell surface and altering the ability of the receptors to bind and retain hormone.

INTRODUCTION

ACTH acts on the adrenal cell to stimulate the membrane bound enzyme, adenylate cyclase, raising intracellular levels of adenosine cyclic 3',5'-monophosphate and increasing the rate of steroid hormone synthesis. The initial event in the action of the

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hormone is believed to be a noncovalent interaction with specific receptors located on the external surface of the plasma membrane. A number of biochemical studies have focused on the initial binding event and the concomitant activation of adenylate cyclase (1–3). There is little information, however, about the factors that are required for the termination of the hormone-induced steroidogenic response. It is unclear, for example, whether ACTH must remain bound to its cell membrane receptor in order for steroidogenesis to persist. Traditionally, peptide hormones bound to their receptors are envisioned as being in rapid equilibrium with the surrounding extracellular medium. Thus a reduction in hormone concentration in the surrounding environment would lead to a prompt diminution in any hormonal effects requiring continued receptor occupancy. Under certain conditions, however, the effects of several different peptide hormones, including ACTH, have been shown to persist in target tissues despite removal of free hormone from the surrounding medium (4–7).

In studying the effects of ACTH on isolated rat adrenocortical cells we have found that stimulation of cyclic AMP and steroid production persists for considerable periods of time after removal of free hormone from the medium. The objective of the experiments described here has been to define the events required for the termination of hormonal effects and to investigate the ability of certain nucleotides and inhibitory ACTH analogues to terminate the state of persistent hormonal activation.

MATERIALS AND METHODS

ACTH peptides. Synthetic $ACTH_{1-24}$[1] (Synacthen), donated by Ciba, was used as a biological reference standard and for radioiodination. A similar $ACTH_{1-24}$ peptide, as well as $ACTH_{6-39}$, was also synthesized in our laboratory by a solid phase procedure and purified as described previously (8). The steroidogenic potencies of the Ciba $ACTH_{1-24}$ and of the solid phase product were identical when assayed in the isolated adrenal cell system described below.

Preparation of isolated rat adrenal cells. Female rats (250–300 g) were asphyxiated with CO_2. The adrenal glands were excised, decapsulated, quartered and placed in a siliconized glass flask containing Ham's F-10 medium (Grand Island Biological Co.), 2 ml per pair, supplemented with 10% fetal calf serum, penicillin (60 μg/ml) and streptomycin (135 μg/ml). Collagenase (Worthington), 2.5 mg/ml, and hyaluronidase (Sigma), 2.5 mg/ml, were added to the medium, and incubation was carried out without stirring at 37° for one hour in a 95% O_2–5% CO_2 water-saturated atmosphere. After decanting away the enzyme solution and rinsing once with fresh medium, the tissue fragments were taken up in enzyme-free medium and dispersed by drawing the suspension several times in and out of a siliconized Pasteur pipette. The medium containing the isolated cells was centrifuged at 900 × *g* for 10 min. The number of cells in an aliquot of the resuspended pellet was counted in a hemocytometer. The cells were stored in the medium at 24° until assays were performed.

For measurements of steroid or cyclic AMP production a final dilution of cells was made to give a cell count of 1 to 2.5 × 10^5 cells per ml. One milliliter aliquots of the diluted cells were added to polystyrene petri dishes (Falcon Plastics). Test solutions were added in a volume of 50 μl. During the assay the cells were incubated at 37° in a 95% O_2–5% CO_2 water-saturated atmosphere for the desired time. The assay sensitivity of different batches of cells, prepared for different experiments, showed considerable variability, with the minimum concentrations of $ACTH_{1-24}$ required for the stimulation of steroidogenesis ranging between 0.01 and 0.1 nM. This variability may have resulted in part from differences in the lots of enzyme preparations used to disperse the cells and in part from varying degrees of mechanical damage to the cells during dispersion and rinsing.

Steroid determination. A modification of the method of Silber *et al.* (9) was used. An

[1] The abbreviations used are: ACTH, adrenocorticotrophic hormone; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; cyclic AMP, adenosine cyclic 3′,5′-monophosphate. Synthetic analogues of human adrenocorticotrophic hormone ($ACTH_{1-24}$, $ACTH_{6-39}$) have been abbreviated by subscript notation to indicate the portion of the amino acid sequence included in the peptide fragment.

aliquot of cells and medium was added to 8 ml CH_2Cl_2 (Baker Chemical Co., spectral grade) and 0.05 ml 0.25 M NaOH. After mixing, the suspension was centrifuged for 10 min at 1500 × *g*. Five ml of the CH_2Cl_2 phase was removed and mixed with 2 ml of a chilled 65% H_2SO_4–35% absolute ethanol solution. After mixing, the CH_2Cl_2 phase was aspirated. The H_2SO_4–ethanol solution stood for 30 min prior to measurement of fluorescence using a Perkin-Elmer fluorometer with an exciting wave length of 470 nm and an emitting wave length of 530 nm. A standard curve was constructed using corticosterone (Mann Research Laboratories) as the reference steroid.

Cyclic AMP determinations. Extraction and measurement of cyclic AMP from incubated adrenal cell suspensions were performed using an immunoassay technique described by Steiner *et al.* (10). Due to low basal levels of cyclic AMP, both the samples and the cyclic AMP reference standard were acetylated to improve the sensitivity of the assay (11). To acetylate, 250 μl of sample or cyclic AMP reference solution was mixed with 15 μl of a solution containing one part triethylamine and two parts acetic anhydride. Triplicate aliquots of the acetylated samples were added directly to the radioimmunoassay tubes.

^{125}I-ACTH peptide binding to intact cells. $ACTH_{1-24}$ and $ACTH_{6-39}$ were iodinated with ^{125}I, using chloramine T as an oxidant as previously described (8). Free ^{125}I and damaged peptide were separated from the iodinated peptide using Quso G-23 (Philadelphia Quartz Co.). The final preparations of labeled peptides had specific activities of approximately 100 μCi/μg. Further details of the iodination method, as well as characterization of the biological activity of iodinated $ACTH_{1-24}$, have been published (8).

The measurement of binding of radioiodinated ACTH peptides to isolated adrenal cells was conducted as follows: To each 10 × 75 mm polystyrene assay tube was added (1) 2 to 5 × 10^4 cells suspended in 100 μl of 50 mM Tris, 236 mM sucrose, pH 7.5, (2) 100 μl of 1% bovine serum albumin (Sigma) in the Tris-sucrose buffer, (3) ^{125}I-$ACTH_{1-24}$ or ^{125}I-$ACTH_{6-39}$ in 100 μl of Tris-sucrose buffer, containing about 100,000 cpm, and (4) 100 μl of either buffer or buffer containing unlabeled peptide or nucleotide. The final volume of 400 μl was incubated at 4°. At the end of the desired incubation time, 200 μl of the incubation volume was layered over 200 μl of chilled Tris-sucrose buffer in a 400 μl polyethylene microcentrifuge tube (Beckman) and was centrifuged for five minutes in a Beckman microfuge. After aspiration of the supernatant fraction, the tips containing the cells were cut off and counted in a gamma counter.

Counts bound nonspecifically to the cells were determined in all experiments by adding an excess concentration (10 μM) of unlabeled $ACTH_{1-24}$ to some incubation tubes. This nonspecific binding, which was subtracted from the total counts bound in the incubation mixture, ranged from 1.0 to 5.0% of specific counts bound in different experiments.

RESULTS

Persistence of the effects of ACTH in adrenal cell suspensions. The adrenal cell suspensions responded to the addition of $ACTH_{1-24}$ in a dose dependent manner, as noted by other investigators (12). In our cell preparations, log-dose response curves were usually linear between 0.05 and 5 nM $ACTH_{1-24}$ with respect to steroidogenesis. The measurable response in cyclic AMP production occurred over a somewhat higher range of $ACTH_{1-24}$ concentration, from 1 to 100 nM. Due to this concentration discrepancy between steroid and cyclic AMP production, higher ACTH concentrations were used in experiments measuring steroid and cyclic AMP production than in those measuring steroidogenesis alone. Negligible quantities of corticosterone or cyclic AMP were produced by unstimulated cells over a 2-hour incubation period. The quantity of steroid produced was dependent upon the number of cells added. In response to $ACTH_{1-24}$, linear increases in steroidogenesis were observed at cell concentrations ranging from 0.8 to 2.5 × 10^5 cells per ml.

Of particular interest in the current studies was the dependence of steroid production upon incubation times. As shown in

Figures 1 and 2 a single addition of $ACTH_{1-24}$ stimulated corticosterone production which was detectable at 30 min, and which progressed in a linear manner for 150 to 240 min. At the end of this period, no further increase in steroid accumulation was seen, but a second addition of $ACTH_{1-24}$ to the incubation medium could cause an immediate resumption of steroidogenesis (Fig. 1).

To determine whether an accumulation of adrenal corticoids in the medium might exert a negative feedback and play a role in limiting the ACTH response, we added dexamethasone to the cells at the beginning of the incubation period. The results, as shown in Table 1, indicate that dexamethasone at a concentration of 10 mM (3.9 μg/ml) did not suppress cellular responsiveness. The concentration of dexamethasone used in this experiment should be sufficient to produce a glucocorticoid effect far greater than that of the corticosterone usually produced in response to a maximal dose of ACTH. Thus it appears that adrenal cells which have completed a response to a first exposure to ACTH are still viable and responsive, and that the accumulation of steroids in the medium does not necessarily impair their ability to respond to freshly added hormone.

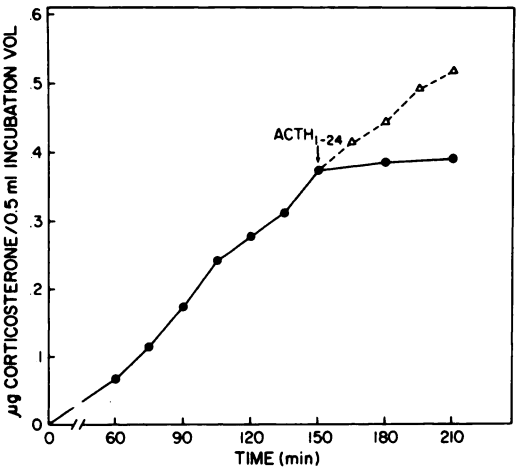

FIG. 1. *Time dependency of corticosterone production in response to an initial and second exposure to $ACTH_{1-24}$*

Cells, 1.88 × 10^5/ml, were exposed to 0.1 nM $ACTH_{1-24}$ at time zero and aliquots of the suspension were removed at subsequent time intervals for corticosterone analysis, ●——●. At 150 min after the initial $ACTH_{1-24}$ 10 nM $ACTH_{1-24}$ was added to an aliquot of cells and steroid production was evaluated, Δ-----Δ. Each point represents the mean of duplicate determinations.

We suspected that the duration of the steroidogenic response was limited not by cellular responsiveness but by the breakdown of the added ACTH to inactive products. The most readily measurable indicator of such breakdown would be the quantity of biologically active ACTH remaining in the medium throughout the period of incubation. A typical experiment relating duration of response and loss of ACTH activity is shown in Figure 2. A maximal dose of $ACTH_{1-24}$ was added initially to a suspension of cells and steroid accumulation was monitored in successive aliquots for 270 min. Other aliquots were centrifuged and the supernatant, containing unbound $ACTH_{1-24}$, was added to fresh cells and incubated for two hours. The biological activity remaining in the medium at various times after the beginning of the incubation is shown in the insert in Figure 2. With increasing incubation time there was a progressive loss of unbound ACTH activity, with only negligible amounts remaining after 90 min. In spite of the loss of all ACTH activity from the medium, steroidogenesis in the original cells proceeded at a stimulated rate for an additional 150 minutes before a plateau in steroid accumulation was reached.

It appeared that the cells need not be in continuous contact with free ACTH in order for steroidogenesis to persist. To test this possibility more directly, cells were exposed to a maximal dose of $ACTH_{1-24}$ for 1 min and then centrifuged. The cells were resuspended in 1 ml of fresh medium, allowed to stand for 10 min and recentrifuged. An aliquot of these rinsed cells was resuspended in ACTH-free medium, while another aliquot was suspended in medium containing a maximal stimulating concentration of $ACTH_{1-24}$. Cyclic AMP and steroid production were examined at intervals following the final resuspension. Figure 3 shows that both sets of cells, those continually exposed to ACTH and those exposed for only 1 minute produced cyclic AMP and

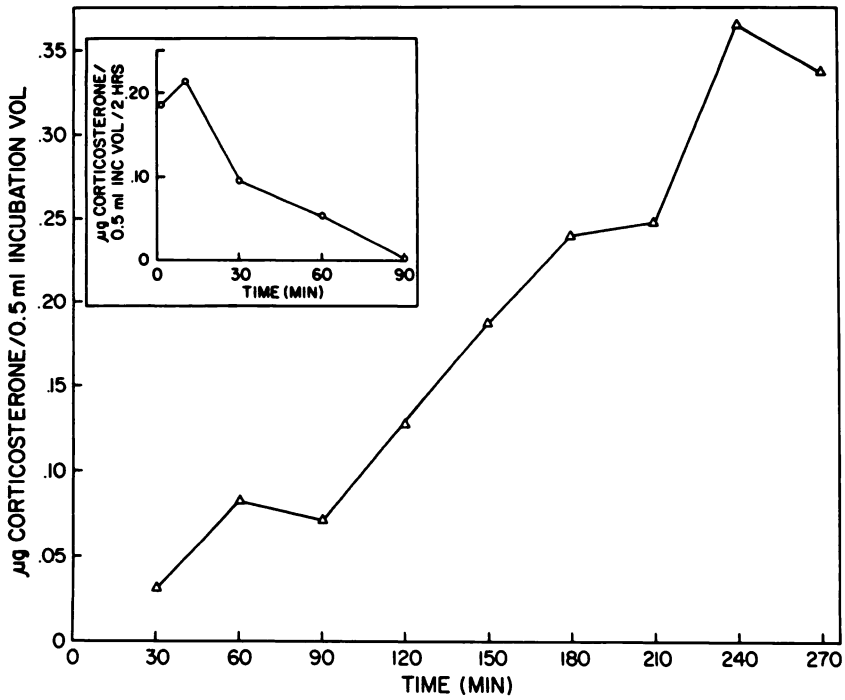

FIG. 2. *Steroid production in response to $ACTH_{1-24}$ with respect to time and ACTH activity remaining in the medium*

At time zero 10 nM $ACTH_{1-24}$ was added to the cells. At the times indicated by △——△ aliquots of cells and medium were taken for steroid determination. In the insert is shown the ACTH activity remaining in the medium after the addition of $ACTH_{1-24}$ at time zero. Determination of remaining ACTH activity was done in the following manner. At the times indicated on the abscissa of the insert, aliquots of cells exposed to $ACTH_{1-24}$ were centrifuged. The supernatant fractions, containing free ACTH were added to fresh cells, and these were allowed to incubate for two hours at which time corticosterone was measured. Subtracted from the steroid level after two hours of incubation was the basal level of unstimulated cells, 0.025 μg corticosterone, and the amount of steroid added to the fresh cells in the supernatant fraction from cells initially exposed to $ACTH_{1-24}$. Each point represents the mean of two determinations. There were 1.76×10^5 cells/ml.

TABLE 1

Effect of Dexamethasone on $ACTH_{1-24}$-induced steroidogenesis

Cells were incubated for two hours in the presence or absence of 10 μM dexamethasone. This concentration did not interfere with the fluorometric assay for cortisterone. Results are the means of duplicate steroid determinations. The subtraction or addition of the numbers in parenthesis gives the range of variation. There were 1.31×10^5 cells/ml.

	Corticosterone Production	
	Control	Dexamethasone
	μg/2 hr/0.5 ml	
Unstimulated cells	0.019 (±0.002)	0.022 (±0.003)
Cells + 0.1 nM $ACTH_{1-24}$	0.042 (±0.0005)	0.045 (±0.004)

corticosterone in an identical manner. To determine whether the persistence of hormonal stimulation in the briefly exposed cells might be due to inadequate washing of excess unbound ACTH from the cells, we employed various volumes of rinsing buffer up to 5 ml, and repeated the rinsing procedure up to 3 times. The same level of persistent stimulation was seen regardless of the volume of rinse or the number of rinses.

Persistence of the effects of dibutyryl cyclic AMP. At this point the evidence suggested that the persistence of hormone effects might be due to a tight association of active ACTH with the cells. Another less likely explanation might be that the 1 minute exposure to ACTH resulted in an initial

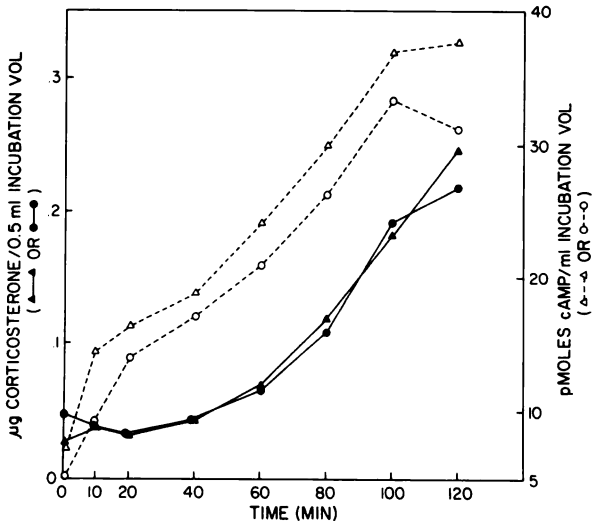

FIG. 3. *Persistence of steroidogenesis and cAMP formation after a brief exposure to $ACTH_{1-24}$*

Cells were exposed to $ACTH_{1-24}$, 0.1 μM, for one minute. The cells were centrifuged. The pellet was resuspended in one ml of fresh buffer and centrifuged again. This pellet was resuspended in one ml of fresh buffer. At varying intervals of time after resuspension, shown on the abscissa, aliquots of the suspension were taken for corticosterone, ▲——▲, and cAMP, △-----△, determinations. Another set of cells was treated in the same manner except that $ACTH_{1-24}$, 0.1 μM, was also added to the final resuspension. Cyclic AMP, ○-----○, and steroid ●——●, determinations were done after resuspension. There were 1.79×10^5 cells/ml. Each point represents the mean of triplicate determinations.

rise in intracellular cyclic AMP. Such an accumulation of cyclic AMP might persist in spite of washing and might be sufficient to maintain an accelerated rate of steroidogenesis for some time. To test this possibility we performed the experiment shown in Figure 4. A suspension of adrenal cells was exposed to a maximally stimulating concentration of dibutyryl cyclic AMP for 60 min. At the end of this time, when an increase in steroidogenesis had become apparent, an aliquot of the suspension was centrifuged, rinsed and resuspended in dibutyryl cyclic AMP-free medium, while another aliquot was left in contract with dibutyryl cyclic AMP. Constant exposure to dibutyryl cyclic AMP resulted in continuously stimulated steroid accumulation, as shown in Figure 4, while removal of dibutyryl cyclic AMP from the medium arrested further steroid production. Thus, unlike exposure to ACTH, initial exposure of the cells to a potent analogue of cyclic AMP did not result in persistent steroidogenesis. While natural cyclic AMP and dibutyryl cyclic AMP are different compounds which might be washed from the cells at different rates, the results in Figure 4 lend no support to the idea that accumulated intracellular cyclic AMP can account for prolonged steroidogenic activation.

Termination of persistent ACTH effects by $ACTH_{6-39}$ and GTP. If the persistence of ACTH effects is due to the continued presence of biologically active, bound hormone, why is the hormone not more easily removable upon repeated rinsing? The hormone might possibly remain associated with the cell simply because it is very tightly bound by external cell membrane receptors. Alternatively, the original hormone-receptor complex might have moved away from an external position on the membrane and become relatively inaccesible to the extracellular medium. To test these alternative possibilities we examined the effects of the hormone analogue $ACTH_{6-39}$ and the nucleotide GTP upon the persistence phenomenon.

$ACTH_{6-39}$ is a competitive antagonist of $ACTH_{1-24}$, having no detectable intrinsic agonist activity at the low calcium concentration (0.4 mM) present in Ham's F-10 medium. This inhibitory analogue is capa-

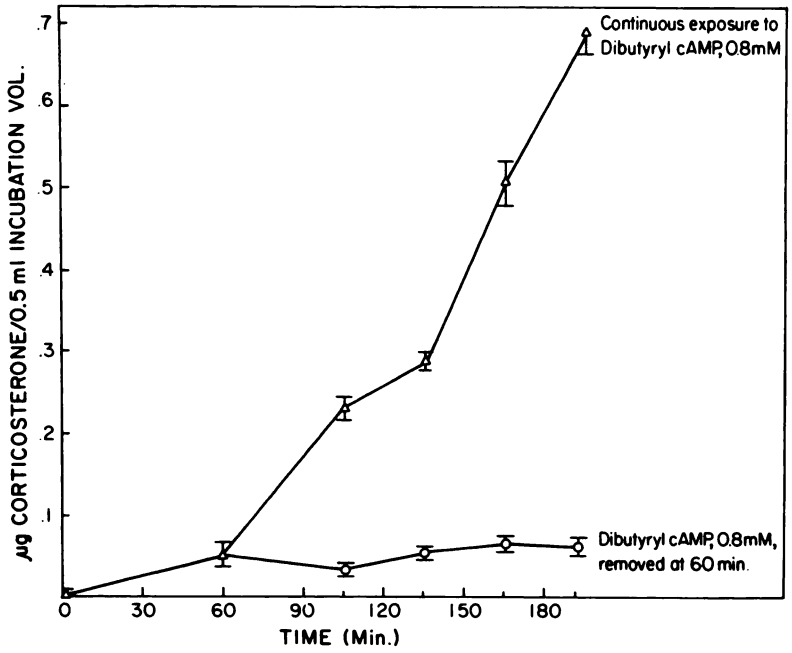

FIG. 4. *The lack of persistent steroidogenic activation in cells stimulated with dibutyryl cAMP*

Cells, 1.9×10^5/ml, were exposed to dibutyryl cAMP, 0.8 mM, for 60 min. At the end of this time an aliquot of cells was centrifuged, rinsed, recentrifuged and resuspended in 1 ml of fresh buffer. From the centrifuged cells, O——O, and the cells allowed to remain in contact with dibutyryl cAMP, Δ——Δ, aliquots were taken at various times, shown on the abscissa, for steroid determination. From each value, the amount of steroid present in the medium of unstimulated cells was subtracted. Each point represents the mean of triplicates. The vertical bars represent the S.E.M.

ble of blocking both the steroidogenic and cyclic AMP responses to hormone when added together with $ACTH_{1-24}$ in a 100-fold molar excess (13). GTP, when added in concentrations up to 3 mM to the cell suspension, does not affect basal levels of steroid or cyclic AMP production. As seen in Figure 5, GTP at a concentration of 1 mM does not impair ACTH-stimulated steroidogenesis. The dose response curve to ACTH in the presence of GTP indicates at least as much sensitivity to the hormone as the curve in the absence of the nucleotide.

In Figure 6 are shown the effects of both $ACTH_{6-39}$ and GTP on the persistence of steroidogenesis resulting from a brief initial exposure to $ACTH_{1-24}$. Cells were exposed to $ACTH_{1-24}$ for 1 min, centrifuged and resuspended for varying periods of time in medium alone or in medium containing either $ACTH_{6-39}$ or GTP. After the times of exposure indicated in Figure 6 the cells were centrifuged, resuspended in fresh medium and allowed to incubate for 2 hours to measure the residual steroidogenic effects of the original exposure to ACTH. Cells treated with either $ACTH_{6-39}$ or GTP demonstrated a time dependent decline in persistent steroidogenesis.

GTP and $ACTH_{6-39}$ were also examined for their ability to diminish persistent adenylate cyclase activation caused by a brief exposure to ACTH. As shown in Figure 7, addition of the inhibitory peptide analogue to activated, washed cells at times ranging from 0 to 60 min after hormonal activation caused prompt cessation of further production of cyclic AMP. These results suggest that the active hormone-receptor complexes which are presumably required for the maintenance of the activated state remain readily influenced by the addition of a hormone antagonist to the extracellular medium. This is true even at 60 min after the original exposure to the hormone has occurred.

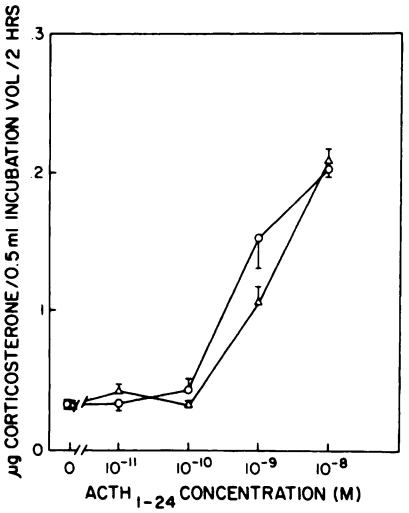

FIG. 5. *Effect of GTP on $ACTH_{1-24}$-induced steroidogenesis*

Cells, 1.9×10^5 ml, were incubated for two hours in the presence of varying concentrations of $ACTH_{1-24}$, shown on the abscissa, Δ——Δ. Other aliquots of the cells, also containing varying concentrations of $ACTH_{1-24}$, contained a fixed concentration of GTP, 1 mM, O——O.

GTP, unlike $ACTH_{6-39}$, is not an antagonist of ACTH in the present system, nor is it an inhibitor of adenylate cyclase activation by other peptide hormones. On the contrary, the nucleotide has been found to act synergistically with hormones in the activation of adenylate cyclase in certain other *in vitro* systems (14). The effects of the addition of GTP upon the accumulation of cyclic AMP by previously activated adrenal cells are shown in Figure 8. After initial treatment with $ACTH_{1-24}$ followed by rinsing, the cells were allowed to incubate and to generate cyclic AMP. At 20 min GTP was added to one aliquot of the cells, while another aliquot was allowed to continue to incubate in hormone free medium. Aliquots from both groups of cells were taken at frequent and early time intervals thereafter for measurement of cyclic AMP. No rise in cyclic AMP was seen in the GTP-treated cells relative to the controls. Instead, GTP produced an early and continuing decline in the total concentration of cyclic AMP.

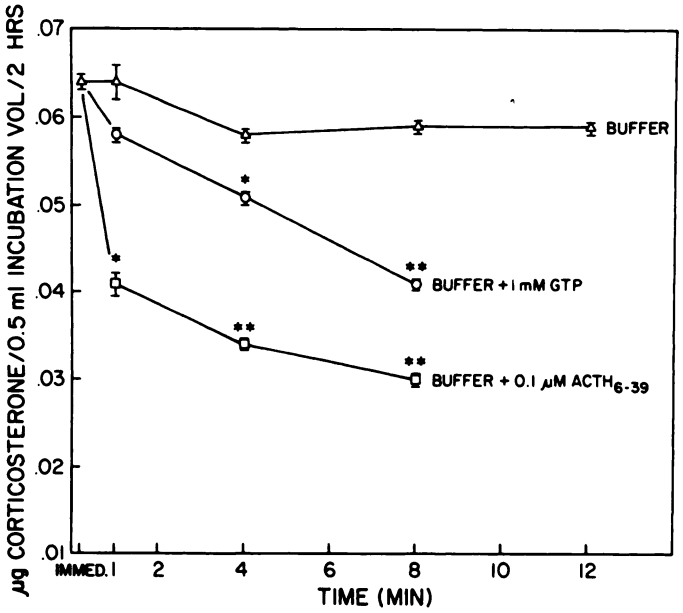

FIG. 6. *Decrease of persistent steroidogenesis in ACTH pretreated adrenal cells by GTP and $ACTH_{6-39}$.*

Cells, 1.98×10^5/ml, were first exposed to 0.2 nM $ACTH_{1-24}$ for one minute. The cells were centrifuged and resuspended in buffer, Δ——Δ, or buffer containing either GTP, 1 mM, O——O, or $ACTH_{6-39}$, 0.1 μM, □——□, and allowed to stand for varying periods of time, shown on the abscissa. At the end of this exposure, the cells were centrifuged and the pellet was resuspended in 1 ml of fresh buffer. These cells were incubated for two hours, after which time steroid determinations were done. 0.027 μg of corticosterone were produced by unstimulated cells. Each point represents the mean of three determinations. The bars indicate the S.E.M. * = $p < 0.05$, ** = $p < 0.01$ when compared to buffer alone using a Student's *t* test.

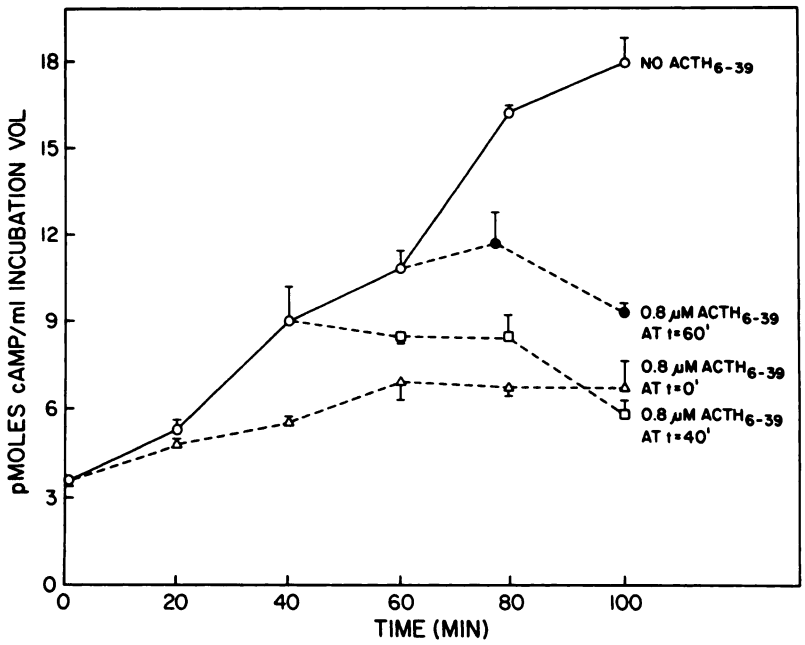

FIG. 7. *Termination of persistent activation of adenylate cyclase in ACTH pretreated cells by $ACTH_{6-39}$*

Cells, 2.0 × 10^5/ml, were exposed to $ACTH_{1-24}$, 0.1 μM, for one minute. These cells were centrifuged, resuspended in fresh buffer, recentrifuged and resuspended in 1 ml of fresh buffer. Cyclic AMP accumulation, measured at various times after resuspension as shown on the abscissa, O——O. Other aliquots of cells were resuspended before receiving $ACTH_{6-39}$, 0.8 μM, at the following. t = 0, Δ-----Δ; t = 40', □-----□; and t = 60', ●-----●. Unstimulated cells produced 3.68 pmoles of cAMP. Each point represents the mean of triplicate determinations. The bars indicate the S.E.M.

In similarly designed experiments we tested the ability of several other nucleotides to decrease the persistence of steroidogenesis in ACTH-treated cells. As shown in Table 2, GTP, at a concentration of 2 mM, had the greatest effect in decreasing steroidogenesis. GDP at the same concentration was somewhat less effective, while cyclic GMP was ineffective. ATP, instead of causing a decline, caused an increase in steroid production by the hormone pretreated cells.

The dissociation of radiolabeled ACTH peptides from adrenal cells. To further evaluate the possibility that persistent hormonal effects could be due to tightly bound, slowly dissociable ACTH on the cell membrane, we examined the interaction of radiolabeled ACTH with intact adrenal cells. In preliminary experiments we found that ^{125}I-$ACTH_{1-24}$ binding to the cells has characteristics quite similar to the binding of the same radioligand to broken adrenal cell membranes, as previously described in detail (8). Very little specific binding could be observed in various isotonic salt solutions, including the Ham's F-10 medium used in the steroid and cyclic AMP assays. As shown in Table 3, with isotonic buffers of lower ionic strength, such as the 50 mM Tris, 236 mM sucrose used in the present experiments, 10 to 20% of the total added counts were usually bound, and the amount of nonspecific binding was low relative to total binding. The attainment of steady state levels of binding was seen within 20 min at 4°, 5 min at 24° and 1 min at 37°. The maximal level of steady state binding was also temperature dependent, the highest levels being reached at 4°, followed by 24° and 37°. Regardless of the temperature, the total number of counts bound always declined with longer incubation times, and the unbound counts present in the medium after the decline could not be bound to fresh cells. The rate of decline was slowest

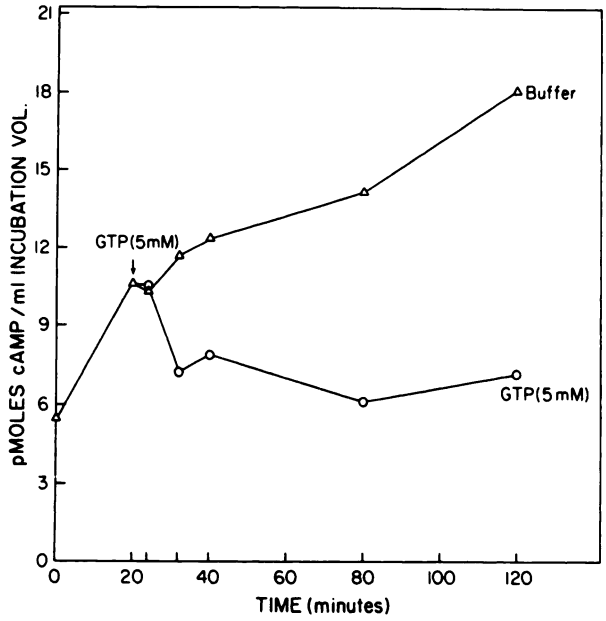

FIG. 8. *The effects of GTP upon persistent cyclic AMP accumulation by adrenal cells pretreated with $ACTH_{1-24}$*

Cells, 2.2×10^5/ml, were incubated for one minute with $ACTH_{1-24}$, 1 μM. After this exposure the cells were centrifuged, resuspended in fresh buffer, recentrifuged and resuspended in 1 ml of fresh buffer. Cyclic AMP determinations were made at various times after resuspension, shown on the abscissa, in the group of cells resuspended in buffer alone, Δ——Δ. An aliquot of cells received GTP, 5 mM, at 20 min after resuspension in fresh buffer. Cyclic AMP levels in this group, are shown by O——O. Each point represents the mean of three determinations. The means of the control and GTP treated group are significantly different from each other after a 10 minute exposure to GTP ($p < 0.01$ by a Student's *t*-test).

TABLE 2

Effect of nucleotides on steroidogenesis after pretreatment with $ACTH_{1-24}$

Cells, 1.51×10^5/ml, were incubated for one minute with $ACTH_{1-24}$, 0.25 nM. After this exposure the cells were centrifuged and resuspended in fresh buffer containing the nucleotides listed in the left hand column. The cells were incubated for five minutes with the nucleotides, at which time they were centrifuged and resuspended in fresh buffer. The cells were then allowed to incubate for two hours before steroid determinations were done. Steroid levels in unstimulated cells were 0.042 μg per 0.5 ml.

Nucleotide	Corticosterone Production
	(μg/0.5 ml incubation vol/2 hr)
Buffer Alone	0.41 ± 0.002 (S.E.M.)
GTP (2 mM)	0.31 ± 0.005*
GDP (2 mM)	0.36 ± 0.023**
cGMP (2 mM)	0.41 ± 0.018
ATP (2 mM)	0.46 ± 0.001*

* $p < 0.05$, when compared to buffer alone.

** $0.05 < p < 0.10$ when compared to buffer alone using a Student's *t*-test.

at 4° and we chose this temperature for our remaining experiments in order to minimize the rate of tracer damage. Binding was directly proportional to cell number in the range from 0.25 to 1.0×10^5 cells/400 μl. The binding process was also saturable, since the addition of unlabeled $ACTH_{1-24}$ caused a concentration dependent decrease in the binding of iodinated derivatives. In equilibrium experiments the effective range of concentration for the unlabeled hormone was from 0.01 to 1 μM. As was the case with broken adrenal membranes, the competition of ACTH analogues with ^{125}I-$ACTH_{1-24}$ for binding showed structural specificity. ACTH analogues, including $ACTH_{9-14}$, $ACTH_{25-39}$ and $ACTH_{1-8}$, which were inactive either as agonists or as antagonists in a broken adrenal cell adenylate cyclase assay (8) were unable to compete for binding sites on the intact cells.

The quantity of radiolabeled $ACTH_{1-24}$ specifically bound by the cells under vary-

TABLE 3

Effects of buffer composition on the binding of ^{125}I-ACTH$_{1-24}$ and the retention of biological effects from exposure to unlabeled ACTH$_{1-24}$

For measurement of the binding of radiolabeled hormone, cells, 6.47 × 10^4/400 μl were incubated in the indicated buffers with 1 nM ^{125}I-ACTH (100,000 cpm) for 20 min at 4° and then centrifuged to determine specifically bound counts, as described in the text. Each value represents the mean of triplicate determinations. For measurement of persistently bound unlabeled hormone, cells, 2 × 10^5/ml, were incubated in the same buffers with 1 nM ACTH$_{1-24}$ under the same conditions described above (20 min, 4°) and then centrifuged, rinsed, and resuspended in 1 ml Ham's F-10 medium. Cyclic AMP measurements were performed after incubation for 2 hours at 37°. Basal cyclic AMP levels for cells exposed to the different buffers without hormone have been subtracted to give the production rates in the right hand column. Each value represents the mean of triplicate determinations.

Buffer	^{125}I-ACTH$_{1-24}$ binding	Persistence of ACTH$_{1-24}$ effect
	(cpm specifically bound/10^5 cells)	*(pmoles cAMP/2 × 10^5 cells/2 hr)*
286 mM Tris	1888 ± 354 (S.E.M.)	1.29 ± 1.77
150 mM Tris, 136 mM Sucrose	4231 ± 487	4.69 ± 1.38
50 mM Tris, 236 mM Sucrose	19436 ± 619	16.01 ± 2.37

ing conditions correlated well with the retention of unlabeled hormone under the same conditions, as shown in Table 3. When cells were pretreated with 1 nM ACTH$_{1-24}$ in similar buffers of varying ionic strength, then centrifuged and resuspended in fresh Ham's F-10 medium for assay of cyclic AMP accumulation, the persistence of the biological effects of hormone pretreatment increased with decreasing ionic strength of the pretreatment buffer. The correlation between the specific binding of the tracer and the persistence of the biological effects of the unlabeled hormone supports the idea that both phenomena reflect an association of ACTH with membrane sites capable of transmitting a hormonal signal to the cells.

To examine the rate of dissociation of ^{125}I-ACTH$_{1-24}$, cells were incubated with tracer in a volume of 0.4 ml for 15 min at 4°, then centrifuged and resuspended at 4° in 15 ml of fresh buffer. At various times times after resuspension, aliquots were removed for the determination of bound counts. In preliminary experiments we tested the effects of varying volumes of resuspension buffer upon the dissociation rate. Aliquots of cells were first incubated with ^{125}I-ACTH$_{1-24}$, then centrifuged and resuspended for 30 minutes in volumes of buffer ranging from 1 ml to 90 ml, and finally, recentrifuged and counted. The number of retained counts was found to be identical in all cell aliquots regardless of dilution volume used for resuspension. We concluded from these data that little rebinding of the intact dissociated hormone was likely to occur, and that a resuspension volume of 15 ml was large enough to minimize the potential for rebinding.

As shown in Figure 9 ^{125}I-ACTH$_{1-24}$ dissociated slowly from the cells under these conditions, with approximately 50% remaining bound 85 min after dilution in hormone-free buffer. In contrast, the dissociation of bound counts from the cells was quite rapid when either unlabeled ACTH peptides or GTP were added to the resuspension buffer. The insert to Figure 9 depicts the results of an experiment in which dissociation was observed in the early time period between 0 and 15 minutes. During that time resuspension and dilution of the cells in buffer alone resulted in a negligible loss of bound counts. Resuspension of cells in the same volume of buffer containing 3 mM GTP, 30 μM ACTH$_{1-24}$ or 30 μM ACTH$_{6-39}$ caused a very rapid dissociation of labeled hormone within the first minute, followed by a slower phase lasting up to 15 min. At the high concentrations of GTP or peptides employed in these experiments, dissociation was virtually complete within 20 or 30 min.

The structural requirements for nucleotide-induced dissociation of ^{125}I-ACTH$_{1-24}$ from the cells were examined further with the results shown in Table 4. In the guanyl

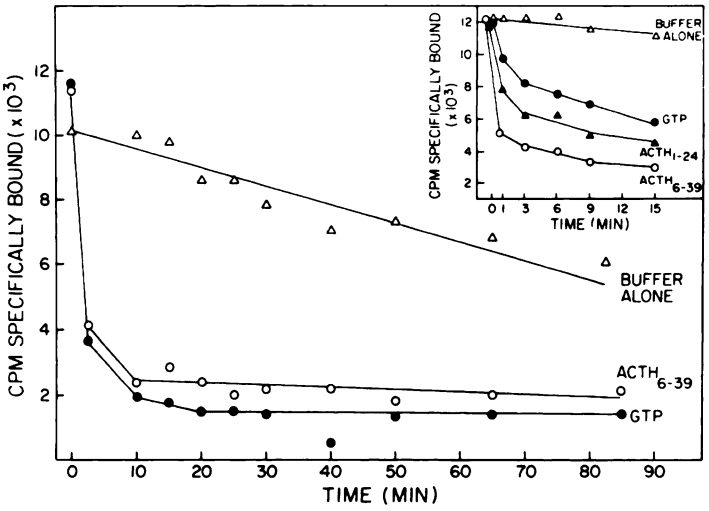

FIG. 9. *Dissociation of ^{125}I-ACTH$_{1-24}$ bound to intact adrenal cells*

The large graph depicts an experiment in which 8×10^6 cells were incubated with 133 pmoles of ^{125}I-ACTH for 15 min at 4°, as described in the text. After centrifugation, aliquots of these cells were resuspended in 15 ml of fresh buffer to give a 35-fold dilution when compared to the original incubation volume. Immediately after resuspension one set of aliquots received 10 μM ACTH$_{6-39}$ and another set 5 mM GTP. A third set was allowed to stand in buffer alone. At varying times after resuspension, shown on the abscissa, samples were taken from each group for determination of bound tracer after centrifugation. Nonspecific binding was determined by adding unlabeled ACTH$_{1-24}$ to other aliquots before the initial incubation with tracer and measuring bound counts after centrifugation. This value was subtracted from all points. The small graph in the upper right corner depicts a separate experiment in which the effects of GTP, 3 mM, ACTH$_{1-24}$, 30 μM, and ACTH$_{6-39}$, 30 μM, were compared at early time periods after resuspension. In this experiment 7×10^6 cells were initially incubated with 200 pmoles of ^{125}I-ACTH$_{1-24}$. Each point in both experiments is the mean of triplicate determinations.

nucleotide series the order of relative effectiveness was GTP > GDP > cGMP. The order of effectiveness among different nucleotide triphosphates was GTP > CTP > ITP ≫ TTP > ATP > UTP.

To determine whether the effects of GTP in promoting the dissociation of radiolabeled ACTH from the cell were specific for agonist peptides, we examined the effects of the nucleotide on the level of steady-state binding on an agonist, ^{125}I-ACTH$_{1-24}$, and an antagonist, ^{125}I-ACTH$_{6-39}$. As seen in Figure 10, increasing concentrations of GTP caused a progressive and similar decrease in the steady-state level of binding of both iodinated derivatives.

DISCUSSION

Steroid secretion by isolated adrenal cells *in vitro* persists for 150 to 240 min after a single addition of ACTH. There could be several possible reasons for termination of the hormonal effect, including the exhaustion of precursors for steroidogenesis, the depletion of cellular energy, or the accumulation in the medium of inhibitory concentrations of glucocorticoids or other metabolic products. None of these explanations are supported by our results. The addition of dexamethasone at the onset of incubation did not impair subsequent steroid production, and more importantly a second addition of ACTH could cause immediate resumption of steroidogenesis after the response to the first addition had ceased. It seemed likely from these preliminary experiments that it was not the state of responsiveness of the cells but the availability of active hormone which governed the duration of the response.

The concentration of active unbound hormone in the medium is undoubtedly of prime importance in the initiation of the activated state, but with continued incubation this concentration falls, reaching negligible levels after 90 min, while steroid production continues undiminished for a

TABLE 4

Dissocation rate of ^{125}I-ACTH$_{1-24}$ from intact cells after treatment with nucleotides

Cells, 9 × 10^6, were incubated with 188 pmoles ^{125}I-ACTH$_{1-24}$ for 20 min. After this time the cells were centrifuged and resuspended in fresh medium in the presence of the nucleotides indicated in the left hand column. After 20 min of incubation, bound ^{125}I-ACTH$_{1-24}$ was determined by centrifugation. Triplicate determinations were done.

Nucleotide added		Dissociation rate of ^{125}I-ACTH$_{1-24}$	Nucleotide concentration (mM) needed for a 25% increase in dissociation of ^{125}I-ACTH$_{1-24}$
(mM)		*(pmoles/10^6 cells/ 20 min)*	
None		2.9	—
GTP	3.0	11.3	0.1
	0.3	8.5	
	0.03	3.7	
GDP	5.0	9.8	1.68
	1.0	6.4	
	0.5	4.1	
cGMP	5.0	6.7	5.00
	1.0	5.8	
	0.5	5.5	
ATP	3.0	9.5	1.68
	1.0	5.1	
	0.3	3.4	
TTP	3.0	9.8	1.29
	1.0	6.2	
	0.3	4.5	
UTP	3.0	7.9	2.24
	1.0	4.9	
	0.3	4.7	
ITP	3.0	10.0	0.3
	1.0	8.6	
	0.1	4.7	
CTP	1.0	7.4	0.211
	0.1	6.9	
	0.03	4.5	

considerably longer period of time. It is unlikely that the persistence of steroidogenesis can be accounted for by the lingering effects of an initial accumulation of intracellular cyclic AMP, since the steroidogenic response to added dibutyryl cyclic AMP, unlike the response to hormone, ceases as soon as the stimulator is removed from the medium. Indeed, when both cyclic AMP concentration and steroid concentration are monitored after an initial pulse exposure to hormone, both continue to rise for up to 100 min, as shown in Figure 3. Taken

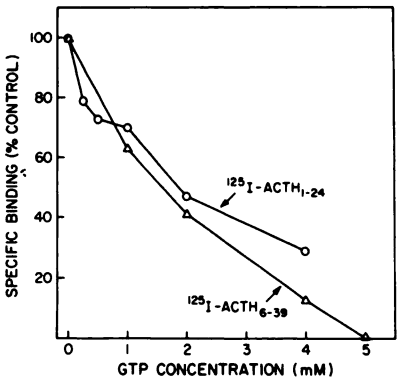

FIG. 10. *The effect of GTP on the steady state binding of ^{125}I-ACTH$_{1-24}$ and ^{125}I-ACTH$_{6-39}$ to intact adrenal cells*

To 2.6 × 10^4 cells, ^{125}I-ACTH$_{1-24}$ or ^{125}I-ACTH$_{6-39}$ was added with varying concentrations of GTP, shown on the abscissa. The incubation was carried out at 4° for 20 min. Specific binding of ^{125}I-ACTH$_{1-24}$, O——O, and ^{125}I-ACTH$_{6-39}$, Δ——Δ, is shown on the ordinate. Control binding in the absence of added nucleotide was 2.1 pmoles/10^6 cells and 19.1 pmoles/10^6 cells for labeled ACTH$_{1-24}$ and ACTH$_{6-39}$, respectively.

together, these results point to the existence of a reservoir of biologically active, cell-associated hormone which does not dissociate readily from the cell but continues to exert its effects for some time after the removal of free hormone from the extracellular medium.

Others have reported a prolonged effect of ACTH on adrenal tissues under different experimental circumstances. Taunton *et al.* originally reported that adrenal slices exposed to ACTH at 2° and washed, showed increased steroid production on subsequent incubation at 37°. The persistent effect could be reversed if the slices were treated with trypsin within 30 min of hormone exposure (15). Consistent with these findings are certain *in vivo* studies which have examined the temporal aspects of steroid secretion in response to injected ACTH. In the rat, ACTH given as a single supraphysiological i.v. dose has a half life in the plasma of less than 5 min (16) but steroid secretion, as measured in adrenal vein effluent can continue at a maximal rate for 60 to 90 min, indicating that under some circumstances the effects of ACTH can persist for some time after plasma ACTH val-

ues have declined to basal levels (17). Certain other peptide hormones also appear capable of remaining associated with their target tissues for relatively long periods of time. Behrman *et al.* have reported that steroidogenesis is stimulated in luteinized ovarian tissue removed from animals treated with luteinizing hormone 90 min before sacrifice. The addition of LH antiserum to this tissue *in vitro* can cause a rapid decrease in the rate of progesterone secretion (18).

In the case of insulin there is growing evidence that some of the biological effects are stimulated by hormone which is taken up into the cell, rather than by peptide remaining bound at sites on the external cell membrane (19). Evidence exists for intracellular localization of parathyroid hormone (20) and prolactin (21) in their respective target tissues. However, the function of these intracellular hormones is unknown. Internalized hormone might be expected to exert more prolonged effects that would not be rapidly reversible after removal of free hormone from the extracellular medium. In the adrenal, our studies suggest that bound ACTH, though slowly dissociable, is still readily influenced by changes in the extracellular medium. Both the hormone antagonist, $ACTH_{6-39}$, and the nucleotide, GTP, can cause rapid declines in accelerated rates of cyclic AMP and steroid formation, and prompt dissociation of ^{125}I-$ACTH_{1-24}$ from the cells. Thus, it seems likely that the hormone-receptor complexes which are responsible for the persistence of ACTH effects are still located on the exterior surface of the cell.

There are good reasons to believe that $ACTH_{6-39}$ interacts with the same receptor sites as ACTH itself, but forms receptor complexes which are ineffective in promoting adenylate cyclase activation. The $ACTH_{6-39}$ analogue behaves as a true competitive inhibitor of ACTH, both in broken adrenal membranes, where adenylate cyclase activity is measured (8), and in intact adrenal cells, where steroidogenesis can be followed (13). It is difficult to explain all of the observed effects of $ACTH_{6-39}$, however, on the basis of simple competition for the ACTH receptor. Our experiments with the effects of dilution on the dissociation of radiolabeled hormone from resuspended cells suggest that there is oridinarily very little rebinding of the hormone, once dissociation has occurred. Thus it seems unlikely that $ACTH_{6-39}$ terminates the persistent effects of $ACTH_{1-24}$ upon cells by a primary inhibition of rebinding. The increased dissociation rate of ^{125}I-$ACTH_{1-24}$ from the adrenal cell in the presence of $ACTH_{6-39}$ is similar to the effect of unlabeled insulin on the dissociation of ^{125}I-insulin from lymphocytes (22, 23) and probably is best explained in terms of negative cooperativity, a phenomenon in which the affinity of each receptor for hormone decreases as the number of occupied receptors increases. Scatchard analysis of ^{125}I-$ACTH_{1-24}$ binding to adrenal membranes has given a curvilinear plot which could also be consistent with the existence of such a phenomenon (8). If the occupied ACTH receptor is indeed able to influence the affinity of other receptors in a negative fashion, it is apparent that the occupying ligand need not be a biologically active ACTH molecule. The ligand must be capable only of interacting with the ACTH binding site and not of stimulating cyclic AMP or steroid production.

The mechanism by which GTP terminates the persistence of hormonal effects and facilitates the dissociation of ^{125}I-$ACTH_{1-24}$ from the cell is unclear. The order of effectiveness of several different nucleotides in producing both termination and dissociation is similar, with the exception of ATP, which enhances dissociation of labeled hormone but increases persistent steroidogenesis. We suspect that the latter stimulatory effect may be mediated by adenosine, a potential breakdown product of ATP which is known to have the ability to stimulate adrenal adenylate cyclase in the absence of ACTH (24). We initially supposed that GTP might be causing the dissociation of bound ACTH by facilitating the activation of adenylate cyclase in a manner analogous to that proposed for the β-adrenergic-adenylate cyclase system in erythrocytes (25). However, in cells pretreated with ACTH we were unable to detect even a transient rise in intracellular

cyclic AMP levels in response to the addition of GTP to the extracellular medium. In addition, GTP was effective in decreasing the steady-state level of binding of the labeled antagonist, $ACTH_{6-39}$, as well as the agonist, $ACTH_{1-24}$. In the β-adrenergic system described by Lefkowitz *et al.* (25) Gpp(NH)p, an analogue of GTP, increases the dissociation of agonists, presumably by promoting formation of an active adenylate cyclase complex, but the dissociation of antagonists, which are thought not to complex with the enzyme, is not affected. Since the $ACTH_{6-39}$ receptor complex is probably unable to interact with the enzyme, it seems unlikely that GTP could increase the dissociation rate of the peptide by causing activation of the enzyme.

Rodbell and co-workers have examined the effects of GTP on the interaction of glucagon with hepatic membranes, where the nucleotide can accelerate the dissociation of radiolabeled glucagon (26). These workers have proposed that there are two guanyl nucleotide binding sites in the glucagon system, one which modulates hormone-receptor interactions and another that regulates the catalytic activity of adenylate cyclase (27–29). Our data would be consistent with this proposal. By increasing the dissociation of bound ACTH independently of adenylate cyclase activity it would appear likely that in our experiments GTP is interacting with sites on the cell surface, thereby altering the ability of the receptor to bind and retain hormone.

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