MOLECULAR PHARMACOLOGY, 23:369–377

Activation of Adrenal Adenylate Cyclase by Guanine Nucleotides

Promotion of Nucleotide Binding by Calcium but Not by Adrenocorticotropic Hormone

DARIEN D. MAHAFFEE AND DAVID A. ONTJES

Departments of Medicine and Pharmacology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514

Received May 21, 1982; Accepted October 26, 1982

SUMMARY

Calcium ion is essential for normal stimulation of adrenal cortical adenylate cyclase by adrenocorticotropic hormone (ACTH). Both ACTH and Ca^{2+} act to promote the activation of adenylate cyclase by guanine nucleotides such as guanyl-5′-yl imidodiphosphate [Gpp(NH)p]. To define further the mechanisms by which Ca^{2+} and ACTH interact with guanine nucleotides, we have correlated the binding of [^{3}H]Gpp(NH)p to adrenal membranes and solubilized membrane proteins with activation of membrane-bound and solubilized adenylate cyclase. Ca^{2+} increases both the rate of reversible nucleotide binding and the rate of adenylate cyclase activation by nucleotide. This effect is accompanied by the appearance of binding sites having an 8- to 10-fold higher affinity for [^{3}H]Gpp(NH)p. In contrast to Ca^{2+}, ACTH increases the rate of enzyme activation but has no significant effect on nucleotide binding. In Ca^{2+}-depleted membranes, measured nucleotide binding is low, and ACTH has no effect on enzyme activation. Once nucleotide is initially bound, both divalent cations and hormone can promote the transition of the enzyme to an activated state. Mg^{2+} is more effective than Ca^{2+} in promoting this transition, while Ca^{2+} is more effective than Mg^{2+} in promoting initial nucleotide binding. When membranes containing bound [^{3}H]Gpp(NH)p are solubilized with Lubrol PX, adenylate cyclase activity elutes on Sepharose 4B with an apparent molecular weight of 160,000. The major fraction containing bound nucleotide elutes with an apparent molecular weight of 40,000–50,000. Nucleotide bound to this fraction is increased by pretreatment of the membranes with Ca^{2+} but is not affected by pretreatment with ACTH. Nucleotide bound to solubilized membrane components dissociates after treatment with EDTA. These findings suggest that Ca^{2+} promotes the initial binding of Gpp(NH)p to a biologically effective site that may involve a guanine nucleotide regulatory protein. ACTH activates adenylate cyclase by promoting a step subsequent to the binding of guanine nucleotide.

INTRODUCTION

Many peptide hormones, including ACTH,[1] activate adenylate cyclase in target tissues by a mechanism involving guanine nucleotides (1–3). In several well-characterized tissues, GTP and its analogues are known to bind to a guanine nucleotide regulatory subunit. The GTP-subunit complex can interact in turn with the catalytic subunit of adenylate cyclase, leading to increased catalytic activity (4–7). The activation of the enzyme is believed to be reversed rapidly by the hydrolysis of bound GTP to GDP (8). Thus in the intact cell the rate of binding of fresh GTP to the regulatory subunit may be envisioned as a possible rate-limiting step in maintaining the catalytic subunit in a stimulated state. There is evidence that some hormones may act at this step by increasing the availability of GTP binding sites and hence the rate of GTP binding (9, 10). Secondary to this effect there may be an increased rate of GTP degradation at the binding site by an associated GTPase (11). One can also envision other possible rate-limiting steps distal to guanine nucleotide binding, including a bimolecular interaction between the activated regulatory subunit and the catalytic subunit or a final conformational change in the catalytic subunit leading to increased activity. Little is known about the possible effects of hormones upon such steps.

This research was supported by National Institutes of Health Grant AM198288.

[1] The abbreviations used are: ACTH, adrenocorticotropic hormone; $ACTH_{6-39}$, a synthetic analogue of human ACTH containing the portion of the peptide sequence indicated by subscript notation; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; App(NH)p, adenyl-5′-yl imidodiphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

0026-895X/83/020369-09$02.00/0

The activation of adenylate cyclase in the adrenal cortex is dependent upon calcium ion as well as ACTH (12). We have found previously that the permissive effect of Ca^{2+} upon ACTH action is exerted not through the binding of ACTH to the hormone receptor but through the interaction of the enzyme complex with guanine nucleotides (3). ACTH or calcium ion (or both) might facilitate activation either by promoting nucleotide binding or by promoting subsequent events in the activation process. We have attempted here to define more precisely the mechanisms of action of ACTH and calcium by correlating their effects on enzyme activation with their effects on the binding of a stable GTP analogue, Gpp(NH)p, to adrenal membranes.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]cyclic AMP and [α-^{32}P]ATP were obtained from New England Nuclear Corporation (Boston, Mass.); App(NH)p, Gpp(NH)p, and [^{3}H]Gpp(NH)p (6–30 Ci/mmole) from ICN (Cleveland, Ohio); pyruvate kinase and phosphoenolpyruvate from Calbiochem; Sepharose 4B from Pharmacia (Piscataway, N. J.); Lubrol PX from Sigma Chemical Company (St. Louis, Mo.); and porcine ACTH powder (62 units/mg) from Parke-Davis (Morris Plains, N. J.). $ACTH_{6-39}$ peptide was synthesized in this laboratory as previously described (13).

Tissue preparation. Adrenal membranes were prepared from homogenates of adrenal glands from 160- to 240-g female Sprague-Dawley rats by methods previously described in detail (3, 14) and suspended in buffer containing 1 mM dithiothreitol, 15 mM theophylline, and 50 mM Tris (pH 7.5) (Tris buffer), using 5 ml of buffer per original adrenal pair unless otherwise indicated. In experiments utilizing Ca^{2+}- or Mg^{2+}-depleted membranes, 5 mM EGTA or EDTA was added to the membrane suspension and the mixture was allowed to stand for 30 min at 0° before final centrifugation at 10,000 × *g* for 20 min. The pellet was washed twice with Tris buffer and resuspended in Tris buffer containing either 0.1 mM EGTA or EDTA.

Pretreatment of membranes with guanine nucleotide. After incubation at 30° with varying combinations of Gpp(NH)p, [^{3}H]Gpp(NH)p, ACTH, and metal ions as described under Results, membrane suspensions were routinely chilled and centrifuged for 3 min at 0° in 1.5-ml polyethylene tubes in a Beckman Microfuge. After removal of the supernatant fraction, the pellets were resuspended in the desired buffer and recentrifuged. The rinsing and resuspension process was repeated three times before the membranes were resuspended for assay of enzyme activity or determination of bound [^{3}H]Gpp(NH)p. Preliminary experiments had shown that the content of bound nucleotide was not reduced appreciably by additional rinses, nor were more than 10% of bound counts dissociated by allowing the membranes to stand after rinsing for up to 2 hr in fresh buffer at 0°. Unbound [^{3}H]Gpp(NH)p, recovered from the original supernatant fractions after incubation, retained full capacity to activate and bind to fresh membranes, indicating stability under the conditions employed.

In preparing membranes pretreated with both ACTH and Gpp(NH)p for adenylate cyclase assay, we added the competitive ACTH inhibitor, $ACTH_{6-39}$, at a concentration of 1 μM to the first rinse. Previously published experiments had shown this concentration of the inhibitor to be effective in displacing all biologically active ACTH from the membranes (3, 15). Thus, no active hormone was carried over into the enzyme assay.

Measurement of enzyme activity. Adenylate cyclase was assayed by measuring the formation of [α-^{32}P]cyclic AMP from [α-^{32}P]ATP as previously described (3, 14). The assay mixture routinely contained 50 mM Tris (pH 7.5), 8 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM dithiothreitol, 1 mM 3-isobutyl-1-methylxanthine, 1 mM cyclic AMP, 1 mM ATP, 8 mM phosphoenolpyruvate and 0.42 unit of pyruvate kinase in a total volume of 0.1 ml. Manganese was included because previous observations in our laboratory had shown that a concentration of 2 mM was sufficient to overcome the direct inhibitory effects of Ca^{2+} on the catalytic activity of adenylate cyclase (3). Reaction times of 5 min at 30° were used to obtain linear initial rates. Reactions were stopped by addition of 1 ml of cold solution containing 0.16 mM ATP and 0.16 mM cyclic AMP. All assays were run in triplicate and were corrected for blank assay tubes kept at 0°. Protein in assay samples was measured by the method of Lowry *et al.* (16).

Measurement of nucleotide binding. Bound [^{3}H]Gpp(NH)p was measured by liquid scintillation counting after solubilization of 50-μl aliquots of membrane suspension in 0.5 ml of NCS solution (Amersham Corporation, Des Plaines, Ill.). In all binding studies, 100 μM unlabeled nucleotide was added to sets of control tubes to determine "nonspecific" binding. This was always less than 5% of the binding in the presence of tracer alone.

Solubilization of membranes with detergent. Membranes pretreated with nucleotides, ACTH, and metals were suspended in buffer containing 10 mM Tris (pH 7.5), 1 mM dithiothreitol, and 0.5% (w/v) Lubrol PX at a protein concentration of 1 mg/ml. After standing for 30 min at 0°, the mixture was centrifuged at 27,000 × *g* for 60 min, and the solubilized components contained in the supernatant fractions were chromatographed directly on columns of Sepharose 4B.

RESULTS

Effects of Ca^{2+} and ACTH on nucleotide binding and adenylate cyclase activation. To correlate binding of guanine nucleotide with activation of adenylate cyclase, we incubated EGTA-pretreated adrenal membranes for varying periods of time with 50 nM [^{3}H]Gpp(NH)p (5 μCi/nmole) at 30° in the presence and absence of added $CaCl_2$ (5 mM) and ACTH (0.1 μM). The results are shown in Fig. 1. There was generally a positive correlation between enzyme activation and [^{3}H]Gpp(NH)p specifically bound. In the absence of calcium, little [^{3}H]Gpp(NH)p was bound, and virtually no activation of adenylate cyclase occurred. At any given time of incubation Ca^{2+} increased the extent of both specific [^{3}H]Gpp(NH)p binding (A) and enzyme activation (B). ACTH itself had no effect on the rate of binding of the nucleotide, but in the presence of Ca^{2+} it promoted dramatically the rate of enzyme activation. In membranes lacking added Ca^{2+}, ACTH had no effect on either

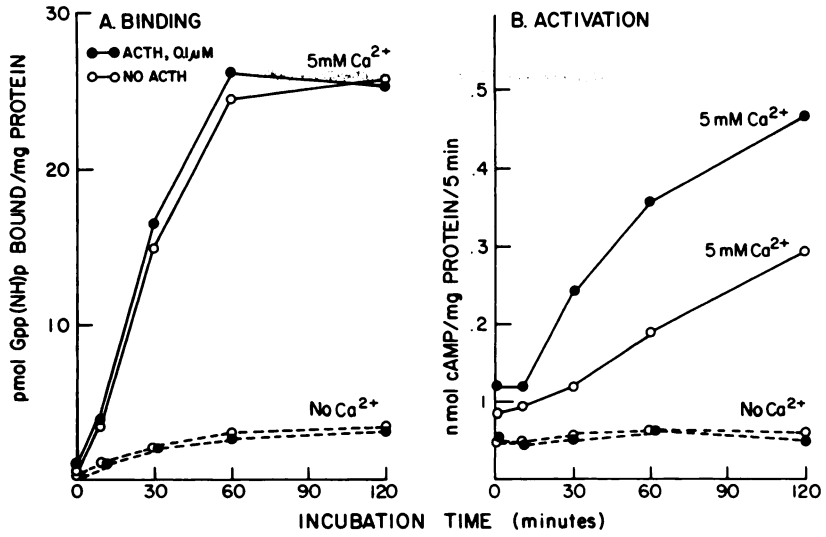

FIG. 1. *Effect of ACTH on [^{3}H]Gpp(NH)p binding and adenylate cyclase activation*

Adrenal membranes which had been depleted of Ca^{2+} by pretreatment with EGTA as described under Experimental Procedures were incubated with [^{3}H]Gpp(NH)p in the presence or absence of ACTH and Ca^{2+}. Specific binding (A) and adenylate cyclase activity (B) were determined as described in the text.

binding or activation, and enzyme activity remained at near basal levels. These results suggested that binding of guanine nucleotide to the membranes, as measured by [^{3}H]Gpp(NH)p uptake, was essential for the activation of membrane adenylate cyclase. While Ca^{2+} seemed to be essential for nucleotide binding, the mechanism of action of ACTH was more likely to involve the promotion of steps subsequent to binding.

Effects of Ca^{2+} and ACTH on dissociation of bound nucleotide and loss of adenylate cyclase activity. We wished to test whether binding of nucleotide was reversible and whether the presence of Ca^{2+} or hormone might affect dissociation as well as association. After first incubating membranes with 50 nM [^{3}H]Gpp(NH)p in the presence of 1 mM $CaCl_2$, 8 mM $MgCl_2$, and 0.1 μM ACTH for 30 min at 30° to obtain both nucleotide binding and enzyme activation, we rinsed the membranes with cold Tris buffer containing 1 μM $ACTH_{6-39}$ to remove bound ACTH. Aliquots of the membrane suspension were then incubated at 30° in Tris buffer containing either 1 mM EDTA, 1 mM $CaCl_2$, or 1 mM $CaCl_2$ + 0.1 μM ACTH. Control aliquots were kept at 0° in Tris buffer without further additions. After varying periods of incubation time, membrane suspensions were chilled and rinsed at 0° with Tris buffer containing 1 mM EGTA and 1 μM $ACTH_{6-39}$. Rinsed membranes were assayed immediately for adenylate cyclase activity and were counted for residual binding of [^{3}H]Gpp(NH)p with typical results as shown in Fig. 2. There was a positive correlation between the dissociation of nucleotide from the membranes and the loss of enzyme activity regardless of the incubation conditions. The binding of [^{3}H]Gpp(NH)p was clearly reversible, but the rates of dissociation were generally slower than the rates of association shown in Fig. 1. In the presence of EDTA, both Gpp(NH)p dissociation and enzyme deactivation proceeded with a half-time of approximately 1 hr at 30°. The presence of free Ca^{2+} slowed both the nucleotide dissociation rate and the enzyme deactivation rate markedly, increasing the half-time to 7–10 hr. ACTH had no effect on dissociation whether added alone (data not shown) or together with Ca^{2+} (Fig. 2). Both dissociation and enzyme deactivation proceeded very slowly at 0°, showing 80–85% retention of initial values after 20 hr.

Effects of Ca^{2+} and ACTH on nucleotide binding at equilibrium. To characterize further the effects of ACTH and Ca^{2+} on [^{3}H]Gpp(NH)p binding, we incubated nonchelated membranes with varying concentrations of nucleotide (2 nM–10 μM) for 60 min at 30°. Preliminary experiments had shown that the binding of tracer reached a stable maximum after 60 min over this range of ligand concentrations. Binding data from a typical experiment are shown in Fig. 3. Curves have been fitted to the experimentally determined points by a computer modeling method for quantitative analysis of radioligand binding data described by De Lean *et al.* (17). Scatchard plots of the same data are shown in Fig. 4. In incubations without added Ca^{2+} (Fig. 3A and C), computer analysis of the binding data indicated apparent K_d values of 760 ± 80 nM in the absence of ACTH and 920 ± 140 nM in the presence of ACTH. The data were most appropriately described by a model having a single class of binding sites (Fig. 3A and C). The same data appear to give a linear plot by Scatchard analysis (Fig. 4). The calculated number of sites in the absence and presence of ACTH were 57 ± 5 and 57 ± 7 pmoles/mg of membrane protein, respectively.

In the presence of 5 mM Ca^{2+} (Fig. 3B and D), binding curves were most appropriately described by a model having two classes of binding sites. The two-site system suggested by computer modeling of the data correlates with the curvilinear Scatchard plot in Fig. 4. In the presence of added Ca^{2+} the calculated K_d values for the high-affinity sites were 100 ± 49 nM and 94 ± 64 nM in the absence and presence of ACTH, respectively, while the corresponding numbers of sites were 43 ± 21 and 38

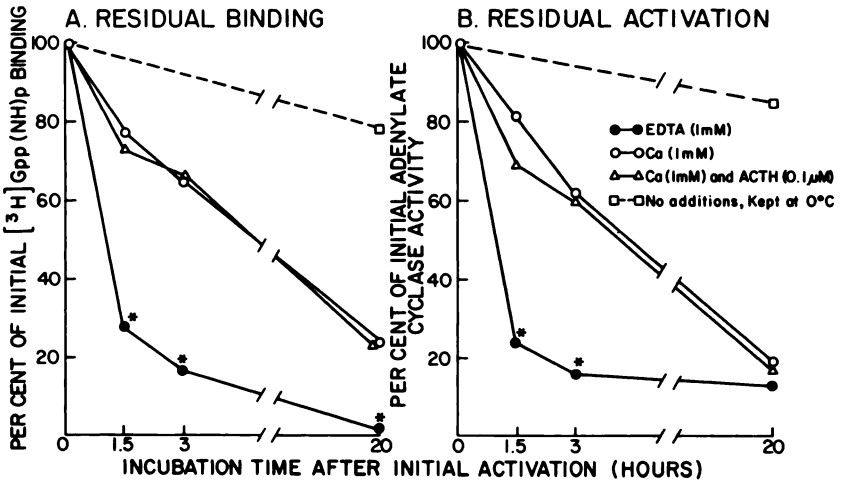

FIG. 2. *Time-dependent dissociation of bound [^{3}H]Gpp(NH)p and loss of adenylate cyclase activity*

Activated membranes containing bound [^{3}H]Gpp(NH)p were prepared as described in the text and incubated for varying periods of time at 30° in Tris buffer containing either 1 mM EDTA (●), 1 mM $CaCl_2$ (○), or 1 mM $CaCl_2$ + 0.1 μM ACTH (△). Control samples with no additions were kept at 0° (□). At indicated times samples were removed, centrifuged at 0°, rinsed once with Tris buffer containing 1 μM $ACTH_{6-39}$ and 1 mM EGTA, and once with Tris buffer before resuspension and assay for [^{3}H]Gpp(NH)p binding and adenylate cyclase activity. The data represent the mean results of triplicate assay determinations, where the initial value (100%) for bound [^{3}H]Gpp(NH)p was 6.6 ± 0.2 pmoles/mg of protein and initial cyclase activity was 158 ± 5 pmoles of cyclic AMP/mg of protein/5 min. *Asterisks* indicate $p < 0.05$ as compared with $CaCl_2$ alone or $CaCl_2$ + ACTH.

± 28 pmoles/mg of membrane protein. Thus, the presence of Ca^{2+} was accompanied by the appearance of sites with an 8- to 10-fold increase in affinity for the guanine nucleotide, but ACTH itself had no significant effect on binding affinity. The low-affinity sites in the presence of 5 mM Ca^{2+} gave calculated K_d values of 1540 ± 1250 and 1160 ± 990 nM in the absence and presence of ACTH, while the numbers of sites were 82 ± 18 and 87 ± 24 pmoles/mg of protein, respectively.

The results of these experiments indicating that Ca^{2+}, but not ACTH, is necessary for high-affinity binding of Gpp(NH)p agree essentially with the binding data of Fig. 1, where the time of binding was varied instead of the ligand concentration.

Effects of App(NH)p on [^{3}H]Gpp(NH)p binding in the presence of calcium and ACTH. The ATP analogue, App(NH)p, has been reported to allow more accurate measurement of biologically significant binding sites for guanine nucleotides in membranes by competing for binding to nonspecific or nonfunctional Gpp(NH)p bind-

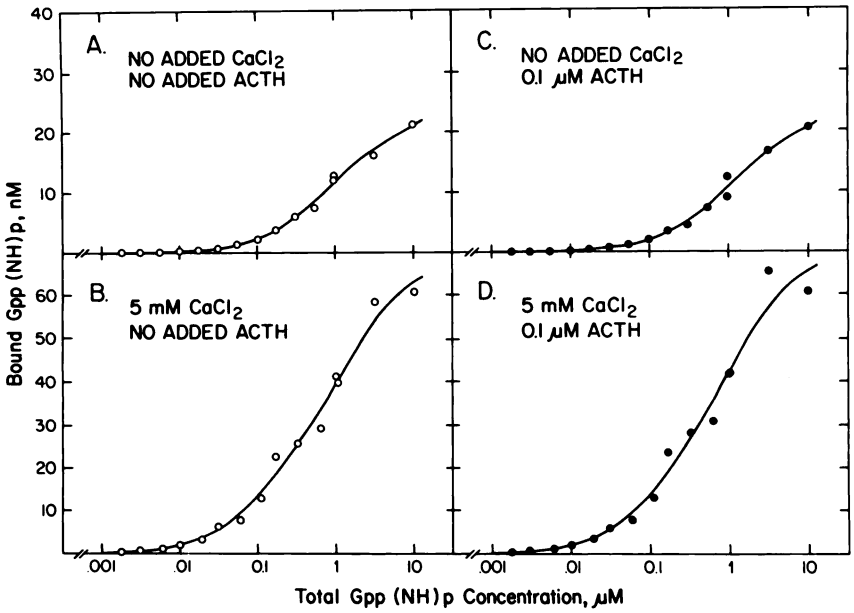

FIG. 3. *[^{3}H]Gpp(NH)p binding saturation curves*

Adrenal membranes untreated with EGTA or EDTA were incubated with varying concentrations of [^{3}H]Gpp(NH)p (specific activity 3.5 Ci/mmole) at 30° for 60 min in the presence or absence of ACTH (0.1 mM) and $CaCl_2$ (5 mM). Specific binding was determined as described in the text. The binding data were analyzed by a weighted nonlinear least-squares computer curve-fitting procedure (17). Protein concentration was 0.356 mg/ml in the absence of $CaCl_2$ (A and C) and 0.556 mg/ml in the presence of $CaCl_2$ (B and D).

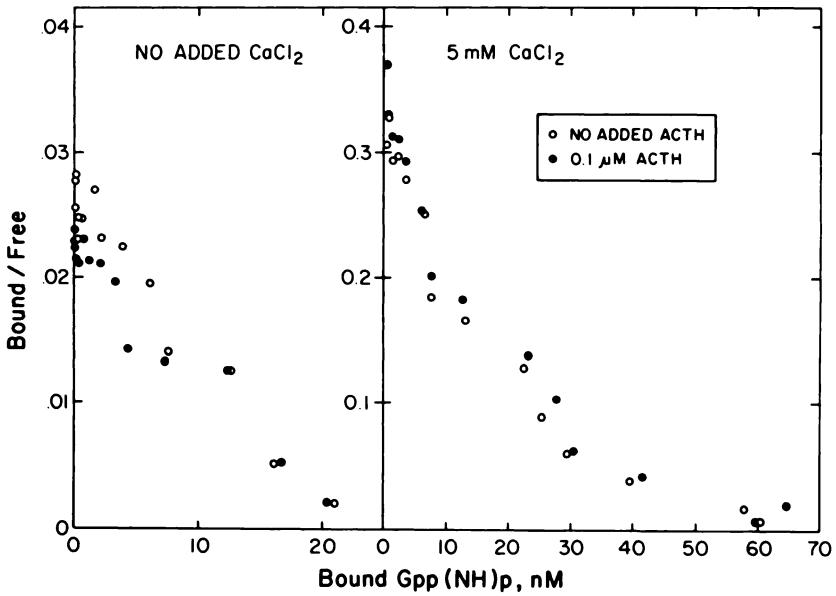

FIG. 4. *Scatchard analysis of [^{3}H]Gpp(NH)p binding*

[^{3}H]Gpp(NH)p binding data from Fig. 3 were replotted for Scatchard analysis. Data from Fig. 3A and C are shown in the *left panel* (no added $CaCl_2$). Data from Fig. 3B and D are shown in the *right panel* (5 mM $CaCl_2$). Analysis of the data by the computer curve-fitting procedure described in Fig. 3 provided a statistical analysis which compared goodness of fit between one- and two-site binding affinity models. A two-site model for the curve in the presence of Ca^{2+} (with or without ACTH present) yielded a highly significant improvement in the computer fit when compared with a one-site model (for two-site model $p < 0.05$). In the absence of Ca^{2+} (with or without ACTH present), a one-site model was most appropriate (for two-site model $p > 0.4$).

ing sites (10, 18). Wishing to test whether this compound might reduce the number of apparent [^{3}H]Gpp(NH)p binding sites under our experimental conditions, we incubated EGTA-pretreated membranes with 50 nM [^{3}H]Gpp(NH)p for 60 min at 30° in the presence of $CaCl_2$ (5 mM), ACTH (0.1 μM), and App(NH)p (100 μM). After rinsing, aliquots were counted for bound [^{3}H]Gpp(NH)p and assayed for adenylate cyclase activity. Typical results are shown in Table 1. At a large molar excess, App(NH)p did reduce the binding of [^{3}H]Gpp(NH)p to membranes in the presence of Ca^{2+} and ACTH. The quantity of Gpp(NH)p bound was reduced by approximately 33%. At the same time, the adenylate cyclase activity of membranes similarly treated with App(NH)p was reduced by approximately 25%. It appeared, therefore, that App(NH)p was capable of competing with Gpp(NH)p for a portion of the high-affinity (Gpp(NH)p binding sites. Rather than being nonspecific or nonfunctional, these sites appeared to be functionally related to the activation of the enzyme.

TABLE 1

Effects of App(NH)p on guanine nucleotide binding and adenylate cyclase activation induced by calcium and ACTH

Incubations of EGTA-pretreated adrenal membranes were carried out for 60 min at 30° in the presence of 50 nM [^{3}H]Gpp(NH)p. After rinsing as described in the text, aliquots of the membranes were counted for bound [^{3}H]Gpp(NH)p and assayed for adenylate cyclase activity.

Additions during incubation	Gpp(NH)p bound	Enzyme activity
	pmoles/mg protein	*pmoles cyclic AMP/ mg protein/5 min*
None	3.01 ± 0.13	63.3 ± 2
ACTH, 0.1 μM	2.26 ± 0.13	61.7 ± 1.6
Ca^{2+}, 5 mM	24.46 ± 2.1	190.2 ± 9
ACTH + Ca^{2+}	26.41 ± 1.16	358 ± 23
ACTH + Ca^{2+} + App(NH)p, 100 μM	17.58 ± 0.06[a]	269 ± 6[a]

[a] Significantly lower than ACTH + Ca^{2+} ($p < 0.05$).

Commercially available App(NH)p may be contaminated with ATP (19). To rule out the possibility that the effects of App(NH)p on nucleotide binding could have been due to ATP, we measured the effects of varying concentrations of ATP (10 nM–1 mM) together with ACTH and Ca^{2+} under the same conditions employed in Table 1. At concentrations of 100 μM or less, ATP had no effect, either positive or negative, on the binding of [^{3}H]Gpp(NH)p. The effects seen with 100 μM App(NH)p were therefore unlikely to have been due to contamination with ATP.

Distinction between metal effects on nucleotide binding and on postbinding enzyme activation. Metal ions such as Ca^{2+} or Mg^{2+} might affect not only nucleotide binding but also steps in adenylate cyclase activation following binding. To test the effects of these metals on the activation process subsequent to the initial binding of nucleotide, we pretreated membranes with EDTA to deplete both Ca^{2+} and Mg^{2+}. After resuspension in Tris buffer the membranes were incubated briefly with [^{3}H]Gpp(NH)p (3 μM) for 10 min at 30° in the absence of metals. Under these conditions approximately 5 pmoles of [^{3}H]Gpp(NH)p were bound per milligram of protein, but there was no measurable activation of the enzyme. The washed membranes, now containing suboptimal concentrations of bound [^{3}H]Gpp(NH)p, were then incubated with ACTH (0.1 μM) and varying concentrations of $CaCl_2$ or $MgCl_2$ at 30° for 30 min to cause enzyme activation. As seen in Fig. 5, Mg^{2+} was a strong promoter

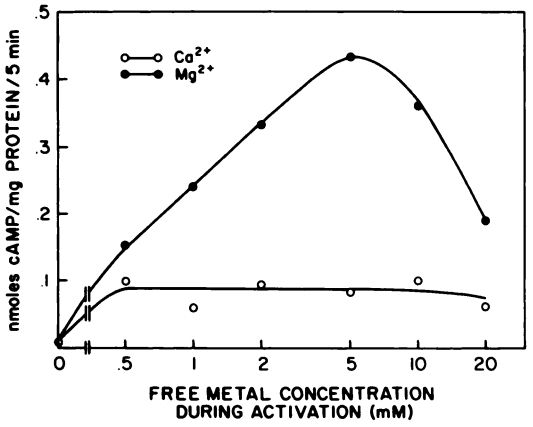

FIG. 5. *Effect of metals on adenylate cyclase activation after initial binding of [^{3}H]Gpp(NH)p*

Adrenal membranes which had been depleted of metals by pretreatment with EDTA as described under Experimental Procedures were incubated with [^{3}H]Gpp(NH)p (0.6 Ci/mmole) at 30° for 10 min. The membranes were then rinsed and resuspended, and aliquots were incubated in the presence of ACTH and varying concentrations of Ca^{2+} or Mg^{2+}. After centrifugation the samples were rinsed with the ACTH inhibitor, $ACTH_{6-39}$ (1 μM), in Tris buffer to remove bound ACTH prior to assay of adenylate cyclase activity.

of activation under these conditions. Maximal activation was obtained at Mg^{2+} concentrations of 5–10 mM. Ca^{2+}, although a strong promoter of [^{3}H]Gpp(NH)p binding in previous experiments, was less effective than Mg^{2+} in promoting postbinding activation.

To compare the effects of the same metals on the initial nucleotide binding steps, we incubated EDTA-pretreated membranes again with [^{3}H]Gpp(NH)p (3 μM) for 10 min at 30°, this time in the presence of varying concentrations of Ca^{2+} or Mg^{2+}. After termination of binding by centrifugation at 0° and rinsing with Tris buffer, we counted aliquots of the resuspended membranes for bound [^{3}H]Gpp(NH)p. The results are shown in Fig. 6A. Other aliquots of the rinsed membranes, containing variable quantities of bound nucleotide, were incubated at 30° for 30 min after the addition of ACTH (0.1 μM) and $MgCl_2$ (8 mM) to promote optimal enzyme activation. These results are shown in Fig. 6B. Ca^{2+} was a much more effective promoter of binding than Mg^{2+}, as seen in Fig. 6A. Maximal binding was obtained at Ca^{2+} concentrations of 5 mM. The nucleotide binding occurring in the presence of Ca^{2+} could ultimately bring about a greater degree of enzyme activation, as seen in Fig. 6B. Thus at least a part of the added binding measured in the presence of Ca^{2+} was functional binding, probably related to the site of interaction between the nucleotide and the adenylate cyclase complex.

Solubilization of bound nucleotide and adenylate cyclase activity from adrenal membranes. To learn more about the nature of [^{3}H]Gpp(NH)p binding sites and their relationship to the catalytic unit of adenylate cyclase, we preincubated EGTA-treated membranes with [^{3}H]Gpp(NH)p in the presence or absence of Ca^{2+} and ACTH before solubilization with Lubrol PX. Solubilized proteins from variously pretreated membrane aliquots were applied simultaneously to identical columns of Sepharose 4B. Fractions were counted for [^{3}H]Gpp(NH)p and assayed for adenylate cyclase. The elution patterns are compared in Fig. 7.

The major peaks of bound [^{3}H]Gpp(NH)p were found in Fractions 30–32 of all columns, having a K_{av} of 0.72. When related to the elution from the same columns of a series of standard proteins, this fraction appeared to have a molecular weight of approximately 40,000–50,000. Smaller peaks of [^{3}H]Gpp(NH)p were recovered from the void volume (Fractions 14–16) and in the salt volume (Fractions 38–42) of each column. Adenylate cyclase activity eluted in a peak distinct from bound [^{3}H]Gpp(NH)p (Fractions 24–27), with a K_{av} of 0.53 and an apparent molecular weight of 160,000.

The recovery of enzyme activity and bound nucleotide was dependent on the presence of Ca^{2+} and ACTH during treatment of the membranes with [^{3}H]Gpp(NH)p. In the absence of both Ca^{2+} and hormone, little adenylate cyclase or bound nucleotide was recovered (Fig. 7A). With Ca^{2+} present the recovery of both enzyme activity and bound nucleotide was increased (Fig. 7B). With both Ca^{2+} and ACTH present there was a further increase in the recovery of enzyme activity, but no change in the recovery of bound nucleotide (Fig. 7C).

The asymmetry of the major peak of bound [^{3}H]Gpp(NH)p (Fig. 7B and C) suggested again that the nucleotide binding sites were probably heterogeneous in nature. The fractions (Tubes 30–32) containing the high-

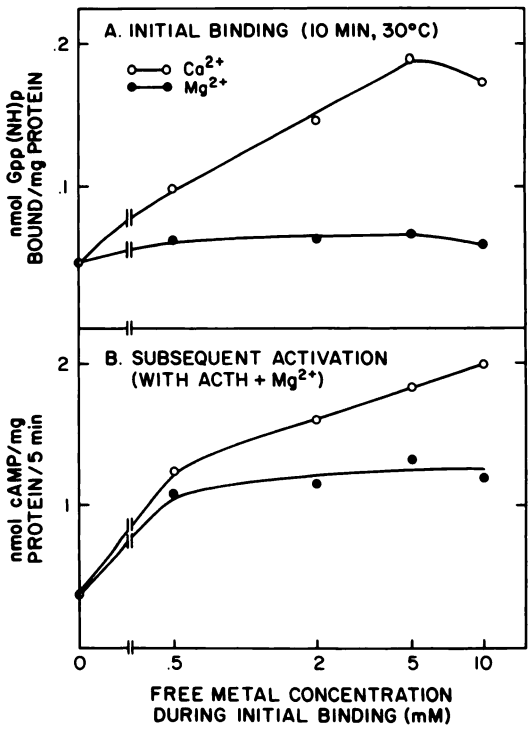

FIG. 6. *Effects of metals on initial nucleotide binding*

Adrenal membranes, depleted of metals by pretreatment with EDTA, were incubated with 3 μM [^{3}H]Gpp(NH)p at 30° for 10 min in the presence of varying concentrations of free Ca^{2+} or Mg^{2+}. After centrifugation and rinsing, specific binding was determined on aliquots as shown in A. Additional aliquots were subsequently incubated at 30° for 30 min in the presence of ACTH (0.1 mM) and Mg^{2+} (8 mM) to cause optimal enzyme activation. After centrifugation and rinsing with the ACTH inhibitor, $ACTH_{6-39}$ (1 μM), these samples were assayed for adenylate cyclase activity as shown in B.

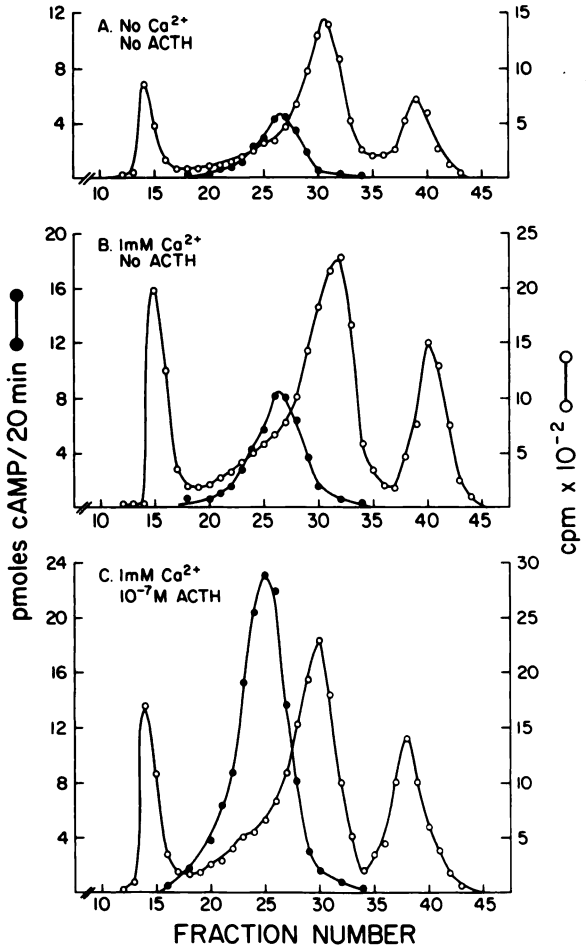

FIG. 7. *Sepharose 4B gel filtration of solubilized adenylate cyclase and bound [^{3}H]Gpp(NH)p*

Adrenal membranes, depleted of Ca^{2+} by pretreatment with EGTA, were incubated with 3 μM [^{3}H]Gpp(NH)p at 30° for 60 min with (A) no additions, (B) 1 mM Ca^{2+}, or (C) 1 μM Ca^{2+} + 0.1 μM ACTH. After centrifugation and rinsing, membranes were solubilized with Lubrol PX as described under Experimental Procedures. Aliquots (1.5 ml) of the supernatant fractions were applied to Sepharose 4B columns (1.4 × 42 cm) and eluted with (A) 10 mM Tris (pH 7.5), 1 mM dithiothreitol, and 1 mM $MgCl_2$, or (B and C) a similar buffer also containing 0.2 mM $CaCl_2$. Fractions, collected at a flow rate of about 2.5 ml/hr, were assayed for adenylate cyclase activity and counted for tritium. Calibration of the columns gave the following K_{av} values for standard proteins: myoglobin (M_r 18,000), 0.89; bovine albumin (M_r 68,000) 0.61; aldolase (M_r 158,000), 0.46; catalase (M_r 240,000), 0.34. The void volume was 119 ml and the total volume 50.5 ml in each column.

est concentrations of bound nucleotide contained insignificant enzyme activity, while fractions on the leading edge of the same peak (Tubes 23–26) still contained significant quantities of bound nucleotide together with maximal enzyme activity. We were interested in the possibility that the [^{3}H]Gpp(NH)p associated with the enzyme peak might be bound to a different class of sites than those eluting distinctly from the enzyme peak. As an initial approach in attempting to characterize these sites further, we pooled the eluates associated with enzyme activity (Tubes 23–26) and those not associated with enzyme activity (Tubes 30–32). We incubated aliquots of the two pools for 20 min at 30° in Tris buffer containing either 1 mM $CaCl_2$ or 5 mM EDTA. After

TABLE 2

Recovery of counts after rechromatography of bound [^{3}H]Gpp(NH)p

Membranes were pretreated with [^{3}H]Gpp(NH)p, Ca^{2+}, and ACTH, rinsed, and solubilized with Lubrol PX. The supernatant fraction was applied to a column of Sepharose 4B as described for Fig. 7C. Fractions containing nucleotide eluting either together with adenylate cyclase (Tubes 23–26) or separate from adenylate cyclase (Tubes 30–32) were pooled and incubated with either 1 mM $CaCl_2$ or 5 mM EDTA before reapplication to identical columns to separate bound and free [^{3}H]Gpp(NH)p. For further details see the text.

Fractions pooled from original column	Incubation conditions	% Total counts recovered from	
		Bound fractions	Nonbound fractions
Tubes 23–26	$CaCl_2$, 1 mM	>95%	None
Tubes 23–26	EDTA, 5 mM	35%	65%
Tubes 30–32	$CaCl_2$, 1 mM	>95%	None
Tubes 30–32	EDTA, 5 mM	33%	67%

incubation, the aliquots were reapplied to similar columns and eluted at 0° with Tris buffer that contained either 1 mM $CaCl_2$ (for the Ca^{2+}-treated aliquots) or 1 mM EDTA (for the EDTA-treated aliquots). Fractions were counted for content of [^{3}H]Gpp(NH)p. Results are summarized in Table 2. When untreated with EDTA the [^{3}H]Gpp(NH)p from both the "enzyme-associated" and "non-enzyme-associated" pools rechromatographed in its original position, remaining bound to a substance of higher molecular weight. When treated with EDTA, the nucleotide in both pools was largely dissociated, the major portion rechromatographing in fractions (Tubes 40–42) coinciding with the salt volume of the column. Thus both the nucleotide bound to higher molecular weight binding sites and that bound to sites of lower molecular weight were readily dissociated upon removal of free calcium. These results showing chelator-induced dissociation from solubilized binding sites were consistent with the effects of chelator on the dissociation of nucleotide from membrane-associated sites (Fig. 2).

DISCUSSION

These results extend our earlier findings that calcium ion promotes activation of adenylate cyclase by guanine nucleotides. Calcium-dependent activation by Gpp(NH)p can be demonstrated in adrenal membranes either pretreated or untreated with chelating agents (3). Our present experiments suggest that guanine nucleotide activation may be divided into several steps including nucleotide binding and one or more postbinding interactions. Ca^{2+}, Mg^{2+}, and ACTH all appear to have qualitatively different effects upon these steps.

Calcium ion is clearly most effective in promoting the binding of guanine nucleotide to its initial site of action. The affinity of the most avid Gpp(NH)p binding sites in adrenal membranes is markedly increased in the presence of Ca^{2+}, causing the rate of nucleotide binding to increase. The high-affinity sites in our adrenal membrane system have an apparent K_d and capacity for Gpp(NH)p similar to binding sites described by others in liver (20), avian erythrocytes (21), pancreatic acinar cells (22), and Leydig cells (10). Moreover, the majority of the adrenal binding sites appear to be associated with a protein fraction of molecular weight range 40,000–50,000 in solubilized mem-

brane preparations. This fraction is distinct from the catalytic unit of adenylate cyclase.

In spite of the measurable changes in high-affinity specific binding of [^{3}H]Gpp(NH)p in the presence of Ca^{2+} and the positive correlation between nucleotide binding and adenylate cyclase activation, additional information is needed to interpret the biological significance of these changes. In separating solubilized membrane components by sucrose density centrifugation (4) or by gel filtration (5), others have concluded that less than 5% of the total [^{3}H]Gpp(NH)p binding protein is associated with the catalytic component of adenylate cyclase. Our own observations on solubilized binding components indicate that a minority of the bound [^{3}H]Gpp(NH)p coelutes with the peak of adenylate cyclase activity. Whether this component is physically associated with the catalytic unit of the enzyme is unknown. Both this binding component and the more abundant binding component eluting in a lower molecular weight range appear to require the continued presence of Ca^{2+} to maintain an association with the guanine nucleotide.

Reliable methods for the quantitative estimation of functional guanine nucleotide regulatory subunits in intact membranes are not generally available. As mentioned under Results, some investigators (10, 18) have used the ATP analogue App(NH)p in an effort to reduce the number of nonspecific binding sites for Gpp(NH)p. In one report (18) there was a 40% reduction of Gpp(NH)p binding sites when cardiac membranes were preincubated with 0.15 mM App(NH)p, a concentration which had no effect on adenylate cyclase activity. In our own test system, comparable concentrations of App(NH)p also reduced Gpp(NH)p binding, but enzyme activation was reduced as well. Thus App(NH)p appeared to offer little advantage.

Other investigators have attempted to estimate the number of guanine nucleotide binding sites by measuring incorporation of [^{32}P]ADP-ribose under the influence of cholera toxin. These estimates, ranging from 150 fmoles (23) to 8 pmoles/mg of protein (24), while generally lower than those obtained by measurement of [^{3}H]Gpp(NH)p binding, may still exceed the number of nucleotide binding sites associated with the catalytic unit at a given time. The biological role of the apparent surplus of guanine nucleotide binding sites is unknown.

In spite of these uncertainties regarding the interpretation of measurements of total [^{3}H]Gpp(NH)p binding, our enzyme activation experiments suggest that the changes in nucleotide binding promoted by Ca^{2+} have functional significance. The increased Gpp(NH)p bound to membranes during a brief exposure to Ca^{2+} can later promote increased activation of the enzyme when the membranes are incubated under standardized conditions.

Magnesium ion is less effective than Ca^{2+} in promoting nucleotide binding, but is much more effective in promoting activation of adenylate cyclase, once binding has occurred. For optimal activation, then, both Ca^{2+} and Mg^{2+} are required. Mg^{2+} is known to be important, if not essential, to adenylate cyclase activation by guanine nucleotides in several other tissues, including avian erythrocytes (25), rat brain (26), and cardiac muscle (27). In these systems the precise mechanism of Mg^{2+} action is still unknown.

ACTH, unlike either metal ion, has no significant effect on the rate of [^{3}H]Gpp(NH)p binding or the apparent affinity of [^{3}H]Gpp(NH)p binding sites. The important effects of this hormone appear to be exerted entirely upon steps subsequent to the binding of nucleotide. In this respect ACTH may differ in its action from that of luteinizing hormone on Leydig cell membranes from the testis. Dufau *et al.* (10) have reported that luteinizing hormone increases the number of binding sites for [^{3}H]Gpp(NH)p in its target tissue, where it acts together with guanine nucleotides to augment adenylate cyclase activity. The action of ACTH may also differ from the actions of β-adrenergic agonists in avian erythrocytes, where the degradation rate of GTP is increased in the presence of catecholamine (11). Here it has been proposed that increased GTP degradation is due to increased availability of fresh GTP to the regulatory GTP binding site and hence an increased rate of binding of GTP.

Our experiments support the hypothesis that Ca^{2+} is essential for ACTH action because it promotes the initial binding of guanine nucleotides to sites involved in adenylate cyclase regulation. When Gpp(NH)p is used as the nucleotide ligand, the metal appears to exert a major effect on the rate of nucleotide association. The addition of Ca^{2+} is associated with the appearance of greater heterogeneity in nucleotide binding sites and a marked increase in the affinity of the most avid sites. It remains to be determined whether the Ca^{2+}-dependent nucleotide binding sites solubilized with Lubrol PX from adrenal membranes are similar to the subunits of the regulatory protein as characterized in other tissues (4, 28). It will also be important to learn whether the effects of Ca^{2+} are exerted directly upon the nucleotide binding protein, implying a metal binding site on the protein itself, or indirectly through effects on membrane properties such as fluidity or permeability (29).

ACKNOWLEDGMENTS

We would like to thank Susan Price for preparation of the manuscript, and Drs. John P. Perkins, H. Shelton Earp, T. Kendall Harden, and McHardy M. Smith for helpful suggestions. We are indebted to Dr. Gary L. Stiles, Division of Cardiology, Department of Medicine, Duke University Medical Center, for the analysis of [^{3}H]Gpp(NH)p binding data by computer modeling.

REFERENCES

1. Rodbell, M. The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature (Lond.)* **284**:17–22 (1980).
2. Glossman, H., and H. Gips. Adrenal cortex adenylate cyclase: comparison between the action of GTP and 5′-guanylyl-imidodiphosphate on the particulate enzyme from bovine adrenal cortex and rat adrenals. *Naunyn-Schmiedebergs Arch. Pharmacol.* **286**:239–249 (1974).
3. Mahaffee, D. D., and D. A. Ontjes. The role of calcium in the control of adrenal adenylate cyclase: enhancement of enzyme activation by guanyl-5′-yl imidodiphosphate. *J. Biol. Chem.* **255**:1565–1571 (1980).
4. Pfeuffer, T. GTP-binding proteins in membranes and the control of adenylate cyclase. *J. Biol. Chem.* **252**:7224–7234 (1977).
5. Welton, A. F., P. M. Lad, A. C. Newby, H. Yamamura, S. Nicosia, and M. Rodbell. Solubilization and separation of the glucagon receptor and adenylate cyclase in guanine nucleotide-sensitive states. *J. Biol. Chem.* **252**:5947–5950 (1977).
6. Ross, E. M., A. C. Howlett, K. M. Ferguson, and A. G. Gilman. Reconstitution of hormone-sensitive adenylate cyclase activity with resolved components of the enzyme. *J. Biol. Chem.* **253**:6401–6412 (1978).
7. Strittmatter, S., and E. J. Neer. Properties of the separated catalytic and regulatory units of brain adenylate cyclase. *Proc. Natl. Acad. Sci. U. S. A.* **77**:6344–6348 (1980).
8. Cassel, D., and Z. Selinger. Mechanism of adenylate cyclase activation by cholera toxin: inhibition of GTP hydrolysis at the regulatory site. *Proc. Natl. Acad. Sci. U. S. A.* **74**:3307–3311 (1977).

9. Cassel, D., and Z. Selinger. Mechanism of adenylate cyclase activation through the β-adrenergic receptor: catecholamine-induced displacement of bound GDP by GTP. *Proc. Natl. Acad. Sci. U. S. A.* **75**:4155–4159 (1978).
10. Dufau, M., A. J. Baukal, and K. J. Catt. Hormone-induced guanyl nucleotide binding and activation of adenylate cyclase in the Leydig cell. *Proc. Natl. Acad. Sci. U. S. A.* **77**:5837–5841 (1980).
11. Cassel, D., and Z. Selinger. Activation of turkey erythrocyte adenylate cyclase and blocking of the catecholamine-stimulated GTPase by guanosine 5′-(γ-thio)triphosphate. *Biochem. Biophys. Res. Commun.* **77**:868–873 (1977).
12. Bar, H. P., and O. Hechter. Adenyl cyclase and hormone action. III. Calcium requirement for ACTH stimulation of adenyl cyclase. *Biochem. Biophys. Res. Commun.* **35**:681–686 (1969).
13. Ways, D. K., C. F. Zimmerman, and D. A. Ontjes. Inhibition of adrenocorticotropin effects on adrenal cell membranes by synthetic adrenocorticotropin analogues: correlation of binding and adenylate cyclase activation. *Mol. Pharmacol.* **12**:789–799 (1976).
14. Mahaffee, D. D., and D. A. Ontjes. Properties of adenylate cyclase solubilized from rat adrenal membranes: effects of ACTH and other stimulators on solubilization. *J. Cyclic Nucleotide Res.* **3**:325–334 (1977).
15. Ways, D. K., D. D. Mahaffee, and D. A. Ontjes. An adrenocorticotropin analog [ACTH (6–39)] which acts as a potent *in vitro* adrenocorticotropin antagonist at low calcium concentration and as a weak agonist at high calcium concentration. *Endocrinology* **104**:1028–1035 (1979).
16. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
17. De Lean, A., A. A. Hancock, and R. J. Lefkowitz. Validation and statistical analysis of a computer modeling method for quantitative analysis of radioligand binding data for mixtures of pharmacological receptor subtypes. *Mol. Pharmacol.* **21**:5–16 (1982).
18. Baker, S. P., and L. T. Potter. A minor component of the binding of [^{3}H] guanyl-5′-yl imidodiphosphate to cardiac membranes associated with the activation of adenylate cyclase. *J. Biol. Chem.* **256**:7925–7931 (1981).
19. Penningroth, S. M., K. Olehnik, and A. Cheung. ATP formation from adenyl-5′-yl imidodiphosphate, a nonhydrolyzable ATP analog. *J. Biol. Chem.* **255**:9545–9548 (1980).
20. Salomon, Y., and M. Rodbell. Evidence for specific binding sites for guanine nucleotides in adipocyte and hepatocyte plasma membranes. *J. Biol. Chem.* **250**:7245–7250 (1975).
21. Spiegel, A. M., and G. D. Aurbach. Binding of 5′-guanylyl-imidodiphosphate to turkey erythrocyte membranes and effects on β-adrenergic-activated adenylate cyclase. *J. Biol. Chem.* **249**:7630–7636 (1974).
22. Svoboda, M., P. Robberecht, J. Camus, M. Deschodt-Lanckman, and J. Christophe. Association of binding sites for guanine nucleotides with adenylate cyclase activation of rat pancreatic plasma membranes. *Eur. J. Biochem.* **83**:287–297 (1978).
23. Johnson, G. L., H. R. Kaslow, and H. R. Bourne. Genetic evidence that cholera toxin sustrates are regulatory components of adenylate cyclase. *J. Biol. Chem.* **253**:7120–7123 (1978).
24. Cassel, D., and T. Pfeuffer. Mechanism of cholera toxin action: covalent modification of the guanyl nucleotide-binding protein of the adenylate cyclase system. *Proc. Natl. Acad. Sci. U. S. A.* **75**:2669–2673 (1978).
25. Spiegel, A. M., E. M. Brown, S. A. Fedak, C. J. Woodard, and G. D. Aurbach. Holocatalytic state of adenylate cyclase in turkey erythrocyte membranes: formation with guanylyl-imidodiphosphate plus isoproterenol without effect on affinity of β-receptor. *J. Cyclic Nucleotide Res.* **2**:47–56 (1976).
26. Sahyoun, N., C. J. Schmitges, H. LeVine, and P. Cuatrecasas. Molecular resolution and reconstitution of the Gpp(NH)p and NaF sensitive adenylate cyclase system. *Life Sci.* **21**:1857–1864 (1977).
27. Alvarez, R., and J. J. Bruno. Activation of cardiac adenylate cyclase: hormonal modification of the magnesium ion requirement. *Proc. Natl. Acad. Sci. U. S. A.* **74**:92–95 (1977).
28. Northup, J. K., P. C. Sternweis, M. D. Smigel, L. S. Schleifer, E. M. Ross, and A. G. Gilman. Purification of the regulatory component of adenylate cyclase. *Proc. Natl. Acad. Sci. U. S. A.* **77**:6516–6520 (1980).
29. Hirata, F., and J. Axelrod. Phospholipid methylation and biological signal transmission. *Science (Wash. D. C.)* **209**:1082–1090 (1980).

Send reprint requests to: Dr. Darien D. Mahaffee, Department of Medicine, University of North Carolina School of Medicine, Chapel Hill, N. C. 27514.