Regulation of Hormone-Receptor Coupling to Adenyl cyclase

EFFECTS OF GTP AND GDP*

(Received for publication, October 15, 1979, and in revised form, July, 18, 1980)

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GDP and GTP regulation of receptor-mediated stimulation of adenyl cyclase in membranes of S49 murine lymphoma cells (S49), NS-20 murine neuroblastoma cells (NS-20), rabbit corpora lutea (CL), and turkey erythrocytes. GTP and GDP affected equally well isoproterenol and prostaglandin El (PGE1), by PGE1 and the adenyl cyclase systems, GDP was clearly not inactive. Concentration effect curves for active hormone in the presence of GDP had higher apparent K values than in the presence of GTP.

In spite of differences between the effects of GTP and GDP on hormonal stimulation of adenyl cyclase activities, GTP and GDP affected equally well isoproterenol binding, regardless of whether or not its receptor could be shown to stimulate adenyl cyclase in the presence of GDP. Determination of transphosphorylation of GDP to GTP showed that at saturating concentrations, the proportion of GDP converted to GTP is negligible and unaffected by hormonal stimulation. Concentrations giving 50% inhibition were determined for GTP- and GDP-mediated inhibition of guanylyl-5'-yl imidodiphosphate stimulation in the absence and presence of stimulatory hormones. In all four systems studied, GTP and GDP interacted with about equal potency and hormonal stimulation was not accompanied by a selective decrease in affinity for GDP.

One way to explain all of the results obtained is to view hormonally sensitive adenyl cyclase systems as two-state enzymes whose activities are regulated by GDP and GDP through an allosteric site related to the catalytic moiety, and receptors as entities that are inactive and hence unable to couple unless occupied by hormones and activated by any guanine nucleotide through a distinct receptor-related process.

Some of us have reported that the liver plasma membrane-bound adenyl cyclase can be stimulated by glucagon to near maximal levels in the presence of GDP (1). This GDP-mediated stimulation occurred under conditions where transphosphorylation to GDP was less than 1%, and where activity stimulated by GMP-P(NH)P was fully blocked. K values for GDP- and GDP-mediated inhibition of GMP-P(NH)P-stimulated activity were similar, as were apparent K values for GDP- and GDP-mediated activation of glucagon-stimulated activity. Glucagon-stimulated activity in the presence of GDP was about 80 and 60% of that observed in the presence of GTP and GMP-P(NH)P, respectively. In contrast to hormone-stimulated activity, basal activity was unaffected by GDP. These results suggested that while the GTPase putatively associated with membrane-bound adenyl cyclases (2–6) may play a key role in the deactivation of adenyl cyclase, hormonal stimulation of adenyl cyclase may occur independently of its existence. Indeed, it would appear that adenyl cyclase is under two interacting types of regulatory controls: one consisting of a nucleotide-dependent regulation of stimulation by the hormone-occupied receptor, and the other of a hormone-independent regulation by the GTPase postulated to be associated with the guanine nucleotide-binding component of adenyl cyclases.

In contrast, Cassel, Selinger, and co-workers (4–9) have, on the basis of work with turkey erythrocyte adenyl cyclase, proposed that hormonal stimulation involves an obligatory exchange of GTP (or GTP analog) for GDP and that the primary effect of the hormone-occupied receptor is the facilitation of this reaction (7). It has been suggested that such an exchange mechanism facilitating displacement of GDP by GTP (8–10) may be operative not only in the turkey erythrocyte system, but also in mammalian systems such as the rat liver adenyl cyclase (9). According to this hypothesis, the
data showing glucagon stimulation upon GDP addition could be accounted for by assuming that hormonal stimulation decreases GDP affinity and increases GTP affinity for the regulatory site, so as to allow the 1% of GTP formed by transphosphorylation from added GDP to act positively and become an allosteric nucleotide responsible for the effect seen.

Since the intrinsic mechanism by which hormones act would be different if it were one of altering the adenylyl cyclase-guanylate nucleotide interaction as opposed to activation of adenylyl cyclase regardless of what nucleotide happens to be occupying it, the resolution as to whether or not hormone can activate adenylyl cyclases known to be occupied by GDP becomes of central importance in the elucidation of the mechanism of hormone and receptor action. To decide whether, in the liver system, glucagon alters the affinities for GDP and GTP in the manner indicated above, it would be necessary to determine their respective apparent K's, values in the presence of glucagon. Technically, this is possible for GDP by evaluating the competitive (partial) inhibition of GTP-P(NH)P stimulation by GDP, because glucagon-stimulated activity in the presence of GDP-P(NH)P is seen in the absence of GMP-P(NH)P. However, it is not possible to determine the apparent K for GTP because, under the assay conditions used, the difference between glucagon-stimulated activity in the presence of GMP-P(NH)P and that in the presence of GDP (-15%) is too small to allow a meaningful assessment of GMP-P(NH)P versus GTP competition.

To obtain independent information on the mechanism of hormone action, we explored the possibility that GDP would promote hormonal stimulation in other adenylyl cyclase systems. We also sought to gain information as to whether hormones may act. However, the overall picture is complex.

Receptor Coupling to Adenylyl Cyclases by GDP

The results indicate that hormones do not differentially affect the potencies of GDP and GTP, and that GDP is capable of promoting hormonal stimulation. This provides key information on the possible kinetic mechanism by which hormones may act. However, the overall picture is complex. GDP promotes hormonal activation in some, but not all adenylyl cyclases tested. In systems where GDP supports activation by one hormone, it does not necessarily do so with respect to a second hormone affecting the same adenylyl cyclase.

The data are discussed in terms of a recently proposed two-state model of adenylyl cyclase and strongly suggest that the role of guanine nucleotide regulation of hormone action on adenylyl cyclases constitutes a GTP-mediated amplification and GDP-mediated dampening system whose degree of amplification and dampening varies with the receptor type as well as from tissue to tissue.

**EXPERIMENTAL PROCEDURES**

**Materials**—Inorganic $^{32}$P (carrier-free) and Na$^{32}$P (carrier-free) were purchased from Union Carbide (Tuxedo, NY). High specific activity [$\alpha$-$^{32}$P]GTP (specific activity, 1,000 to 2,000 Ci/mmol) was from International Chemical and Nuclear Co. (Irving, CA). Plastic-backed polyethyleneimine-cellulose plates for thin layer chromatography were from Bio-Rad Laboratories (Richmond, CA). Hydrocortisone (HYP) was a gift from Sandzio (East Hanover, NJ). (-)-Isoproterenol was a gift from Dr. Nachod, Sterling-Winthrop Laboratories (Rensselaer, NY), and prostaglandin E$_1$ (PGE$_1$) was a gift from Dr. J. E. Pike, the Upjohn Company (Kalamazoo, MI). GDP was a generous gift from Dr. Fritz Eckstein, Max Planck Institut, Göttingen, W. Germany. ATP and AMP-P(NH)P were purified similarly according to Walseth and Johnson (12) and was purified by DEAE-Sephadex-A-25 chromatography as described below. [$\alpha$-$^{32}$P]GTP (specific activity > 50 Ci/mmol) was synthesized according to Gallop et al. (14) and purified by high pressure liquid chromatography as described elsewhere (15). 125I-HYP was synthesized following the procedure of Maguire et al. (13) and purified by high pressure liquid chromatography as described elsewhere (14). Membrane Preparation—Membrane particles from S49 mouse lymphoma cells were prepared according to Ross et al. (15). S49 cells were grown in 6-liter spinner flasks in Dulbecco's modified Eagle's medium (Grand Island Biological Company, Garden City, NY) containing 10% heat-inactivated horse serum (Grand Island Biological Company, Garden City, NY) as described elsewhere (16). Clonal line NS-20 neuroblastoma cells C1300 (17) were grown and membrane particles were prepared as described elsewhere (18).

Membrane particles from corpora lutea (19) were prepared from 7-day pseudopregnant New Zealand white rabbits.

Untreated and isoproterenol plus GMP-treated turkey erythrocyte membranes were prepared and purified according to Abramowitz et al. (20).

Membrane particles were kept frozen at -70°C suspended in the following media: S49 cell membranes in 1 mM dithiothreitol, 2.5 mM MgCl$_2$, 1 mM EDTA, and 10 mM Hepes-Ne, pH 8.0; 10% horse serum, 1 mM MgCl$_2$, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.5; and turkey erythrocyte membranes in 20 mM dithiothreitol, 10 mM KCl, 8% sucrose, 1 mM MgCl$_2$, and 10 mM Tris-HCl, pH 7.5. Except for turkey erythrocyte membranes, which were diluted in the same medium, all other membranes were diluted at least 10-fold in H$_2$O immediately prior to their use.

Adenylyl Cyclase Assays—Unless indicated otherwise, adenylyl cyclase activities were determined by monitoring the conversion of [$\alpha$-$^{32}$P]ATP to [$\beta$-$^{32}$P]cAMP in 5 min at 25°C. Incubations contained in 150 ml of a solution of 50 mM KCl, 50 x 10$^{-6}$ M cAMP, 1 mM EDTA, 1 mM [H]cAMP (approximately 10,000 cpm), 25 mM Tris-HCl, pH 7.5, and 10 ml of either NS-20 neuroblastoma membranes (2 to 4.5 mg of protein) or corpus luteum membranes (1.5 to 1.8 mg of protein). Activity in 10 ml of S49 membrane suspension (2.4 mg of protein) was determined under the same conditions except that MgCl$_2$ (10 mM) and Tris-HCl buffer was replaced with 25 mM Hepes-Tris pH 8.0. Activity in 10-$\mu$l aliquots of isoproterenol plus GMP-treated turkey erythrocyte membranes (10 mg of protein) were determined also under the same conditions with the exception that ethylene glycol bis(2-aminoethyl) ether N,N',N'-tetraacetic acid (EGTA) was substituted for EDTA and 10 mM KCl was added. Other additions (hormones and guanine nucleotides) are denoted in the tables and legends to figures describing the experiments performed. Reactions were stopped by addition of 100 ml of a "stopping solution" containing 10 mM cAMP, 40 mM ATP, 10 mM AMP, 10 mM ADP, and 1% sodium dodecyl sulfate followed by cooling to 0-4°C until they were processed further. [$\beta$-$^{32}$P]cAMP formed was isolated and quantitated by double chromatography over Dowex 50 and alumina according to a modification (21) of the method of Solomon et al. (22). This modification, which consists only of increasing the size of the Dowex 50 column by 50% and that of the alumina columns by 100%, leads to significantly lower blanks, especially when more than 10$^4$ cpm are used per assay, and allows for indefinite re-use of the Dowex columns and for re-use of the alumina columns for between 25 to 50 times.

Results are expressed as $5 	imes 10^{-5}$ of [$\beta$-$^{32}$P]cAMP formed during the 5-min incubations per mg of membrane protein added. Since, unless indicated otherwise, the assays were carried out at a specific radioactivity of total adenine nucleotides (AMP-P(NH)P plus ATP)
of 10^6 counts/5 min/pmol, the numerical values reported are also
equivalent to the picomoles of cAMP formed/min/mg of protein if it
is assumed that the accumulation rates were constant with time and
both ATP and AMP-P(NH)P serve as substrates with equal efficacy.

Test for Maintenance of Nucleotide Levels Throughout Incubations—To assess the proportion of initially added [α-32P]ATP re-
maining as such at the end of incubation for adenylyl cyclase activity,
0.5- to 1.0-μl aliquots of the stopped and cooled reaction mixtures
were spotted upon polyethyleneimine-cellulose plates. After chro-
matography (ascending with 1 M LiCl as developing solvent), the
areas containing ATP, ADP, and AMP-P(NH)P serve as substrates with equal efficacy.

Distribution of radioactivity between the various nucleo-
tides—To assess the proportion of initially added [α-32P]ATP re-
matographing with GTP, GDP, and GMP by liquid scintillation
counting. Results are expressed as the percentage of
initially added [α-32P]ATP that co-chromatographs with ATP.

To test for maintenance of GTP and GDP and for their intercon-
versions, parallel incubations were carried out in which either
[α-32P]GTP or [α-32P]GDP (–10^4 cpm/50 μl of incubation mixture)
were substituted for the labeled ATP and reactions were stopped
with 100 μl of solution containing 10 mM GTP, 10 mM GDP, 10 mM
GMP, and 1% sodium dodecyl sulfate followed by immediate cooling
to 0-4°C. Distribution of radioactivity between the various nucleo-
tides was determined by thin layer chromatography of 1- to 2-μl
aliquots of the stopped reaction mixtures on polyethyleneimine-cellu-
lose plates using 1 M LiCl as developing solvent. The percentage of
initially added [α-32P]GTP or [α-32P]GDP migrating as GTP and
GDP were calculated after determination of radioactivity co-chro-
matographing with GTP, GDP, and GMP by liquid scintillation
counting.

125I-HYP Binding Assays—The binding reactions were carried out
in a final volume of 500 μl containing 0.1 mM 125I-HYP (120,000 cpm/
assay tube), 2 mM MgCl2, 1.0 mM EDTA, 0.1% bovine serum albumin,
0.1 mM ascorbic acid, and 25 mM of the indicated buffer. When
present, the concentrations of guanine nucleotides were 0.5 mM. Incubations
were carried out in glass test tubes (12 × 75 mm) at 32.5°C. After 30 min, the binding reactions were stopped by addition
of 1.5 ml of ice-cold 0.1% bovine γ-globulin (Schwarz/Mann, Catalogue
No. 903004) in 0.1 M NaCl. All subsequent steps were carried out in
the cold. After mixing, 1.0 ml of 20% polyethylene glycol (Fisher,
Catalogue No. P156) was added to each tube; the contents of the
tubes were mixed and centrifuged at 4°C for 10 min at 3,000 rpm. The
supernatant fluids were discarded by careful aspiration, and the
pellets were resuspended in 2.0 ml of 0.1 M NaCl and reprecipitated
by addition of 1.0 ml of 20% polyethylene glycol. After a second
centrifugation for 10 min at 3,000 rpm and separation of the super-
natant fluid by aspiration followed by careful wiping of the walls of the tubes with cotton-tipped applicators (Scientific Products, Cata-
logue No. A5002-1), the 125I-HYP retained in the tubes on the
membranes that had been co-precipitated with the γ-globulin was deter-
mined by scintillation counting in a Searle Autogamma analyzer,
nuclide counter. Proteins were determined by the method of Lowry et al. (23) using
bovine serum albumin as standard.

RESULTS

Effects of Guanine Nucleotides on Adenylyl Cyclase Activ-
tivities in S49, NS-20, and Corpus Luteum Membranes—Activities
were determined in the absence of nucleoside triphosphate-
regenerating system using 0.5 mM AMP-P(NH)P and
10 μM [α-32P]ATP as substrates. Under these conditions (Ta-
ble I), GTP was found to stimulate basal activities in all three systems
2- to 3-fold. Addition of 100 μM GDP did not alter
basal activities in S49 and corpus luteum membranes, and
inhibited NS-20 adenylyl cyclase between 50 and 70%.

No hormonal stimulation could be elicited unless a guanine
nucleotide was added. In S49 lymphoma membranes, GTP promoted coupling of
isoproterenol and PGE1 receptors, with PGE1-stimulated ac-
tivity being 65 to 70% of that seen with isoproterenol. GDP
addition also promoted coupling of both receptors, but with
less efficacy: isoproterenol- and PGE1-stimulated activities
were 60 and 20%, respectively, of that seen with isoproterenol
in the presence of GTP.

In NS-20 neuroblastoma membranes, GTP promoted coupling
of PGE1 and adenosine receptors, with phenylisopro-
pyladenosine-stimulated activity being 35 to 45% of that seen
with PGE1. GDP addition promoted coupling of the more
active PGE1 receptor, but not of adenosine receptor. PGE1-
stimulated activity sustained by GDP was between 50 and 70% of that seen with
GTP.

In corpus luteum membranes, GTP promoted coupling of
PGE1 and isoproterenol receptors, with isoproterenol-stimu-
lated activity being about 50% of that seen with PGE1. GDP
addition promoted coupling only to a minor degree of the
more effective receptor. PGE1-stimulated activity with GDP
was 12 to 20% of that seen with GTP.

As shown in Table II, stimulation of S49 cell and NS-20
neuroblastoma adenylyl cyclases by isoproterenol and PGE1,
and by PGE1 and phenylisopropyladenosine, respectively, are
not additive, indicating that in each of these membranes,
a single adenylyl cyclase system is stimulated by more than one
hormone receptor. Combinations of PGE1 and isoproterenol
elicited a partially additive response in corpus luteum mem-

Table I

| Hormones added to assays | Nucleotide added to adenylyl cyclase assays* |
|-------------------------|---------------------------------------------|
| None                    | None                                        |
| None                    | 100 μM GDP                                  |
| None                    | 100 μM GTP                                  |
| A. S49 murine lymphoma cell membranes (1.6 μg/assay) | 10 mM AMP × 10–15/5 min/mg |
| None                    | 7.8 ± 1.2                                   |
| PGE1 (10 μg/ml)         | 6.4 ± 0.4                                   |
| Isoproterenol (10–4 M)  | 9.1 ± 0.8                                   |
| B. NS-20 murine neuroblastoma cell membranes (2.0 μg/assay) | 10% LiCl as substrate |
| None                    | 10.6 ± 0.9                                  |
| PIA (10–4 M)            | 9.6 ± 0.7                                   |
| PGE1 (10 μg/ml)         | 11.3 ± 1.2                                  |
| C. Corpus luteum membranes (1.5 μg/assay) | 2.0% NaCl |
| None                    | 13.7 ± 1.2                                  |
| Isoproterenol (10–4 M)  | 11.3 ± 2.3                                  |
| PGE1 (10 μg/ml)         | 10.1 ± 2.2                                  |

* Assays were carried out in the absence of a nucleoside triphosphate-
regenerating system and with 0.5 mM AMP-P(NH)P plus 10 μM [α-32P]ATP as substrate. Incubations were for 5 min. Values are
means ± S.D. of triplicate determinations. For the rest of the condi-
tions, see under "Experimental Procedures."
TABLE II

| Additions to assays        | Adenylyl cyclase activity<sup>a</sup> |
|---------------------------|---------------------------------------|
|                           | 20 μM GTP | 20 μM GDP-P(NH)p |
|                           | pmol cAMP/min/mg |
|---------------------------|------------|------------------|
| A. S49 murine lymphoma membranes (3.4 μg/assay) | | |
| None                      | 9.1 ± 0.8 | 34.7 ± 1.9 |
| PGE<sub>1</sub>           | 31.5 ± 0.7 | 59.8 ± 1.7 |
| Isoproterenol             | 61.4 ± 1.5 | 74.1 ± 1.4 |
| PGE<sub>1</sub> plus isoproterenol | 63.9 ± 0.4 | 75.5 ± 1.7 |
| B. NS-20 neuroblastoma membranes (5.0 μg/assay) | | |
| None                      | 6.4 ± 0.5 | 49.6 ± 1.0 |
| PGE<sub>1</sub>           | 87.2 ± 0.7 | 91.6 ± 1.8 |
| PIA                       | 14.6 ± 1.0 | 65.4 ± 1.8 |
| PGE<sub>1</sub> plus PIA  | 82.8 ± 1.8 | 92.3 ± 3.3 |
| C. Rabbit corpus luteum membranes (3.8 μg/assay) | | |
| None                      | 38.8 ± 0.2 | 128.6 ± 2.2 |
| PGE<sub>1</sub>           | 109.0 ± 5.5 | 204.7 ± 7.2 |
| Isoproterenol             | 75.3 ± 2.0 | 195.8 ± 2.8 |
| PGE<sub>1</sub> plus isoproterenol | 137.8 ± 3.9 | 236.4 ± 4.6 |

<sup>a</sup> When present, PGE<sub>1</sub> was 10 μg/ml, isoproterenol was 10<sup>-3</sup> M, and PIA was 10<sup>-5</sup> M. Each of these concentrations elicits a maximal response in the systems tested.

<sup>b</sup> Adenylyl cyclase activities were assayed under conditions described under “Experimental Procedures” except that 0.1 mM [γ-<sup>32</sup>P]ATP (2,000 cpm/pmole) was used as substrate, and the nucleoside triphosphate-regenerating system consisting of 20 μM creatine phosphate, 0.2 mg/ml of creatine phosphokinase, and 0.02 mg/ml of myokinase was included. Incubations were for 10 min at 32.5°C. Values are means ± S.D. of triplicate determinations.

Conversion of GTP to GDP and of GDP to GTP. This was accomplished, in part, by using 0.5 mM AMP-P(NH)p as the bulk of substrate (4, 24), by reducing membrane concentrations (and hence nucleotide-metabolizing activities) as much as possible, and by carrying out incubations for only 5 min. Under the conditions chosen (see under "Experimental Procedures"), more than 80% of the [γ-<sup>32</sup>P]ATP initially added as well as more than 75% of initially added GTP are found at the end of the incubation period still to co-chromatograph upon thin layer chromatography on polyethyleneimine-cellulose with ATP (not shown) and with GTP (left panels of Fig. 1), respectively. In fact, nucleoside triphosphate levels were maintained over a wide variation of concentrations ranging from 0.01 to 100 μM and above (Fig. 1).

Incubations were also carried out over a wide range of GDP concentrations in the absence and the presence of stimulatory hormones and the chromatographic behavior of trace amounts of initially added [α-<sup>32</sup>P]GDP was monitored. As shown in the right panels of Fig. 1, S49, NS-20, and corpus luteum membranes have a transphosphorylation mechanism. This system, which converted GDP to GTP, was found to be of low capacity and high affinity, such that at concentrations in excess of 1 μM added GDP, the proportion of GDP converted to GTP decreased substantially. This decrease in the percentage of GDP appearing as GTP at the end of incubations was not only observed up to 100 μM, as shown in Fig. 1, but continued beyond that and up to 1,000 μM added GDP, the highest concentration tested (not shown). When incubations with [α-<sup>32</sup>P]GTP or [α-<sup>32</sup>P]GDP were performed without membranes, 90 to 92% of the added guanine nucleotide chromatographed as the added nucleotide. No attempt was made to subtract these background distributions in the experiments shown in Fig. 1.

FIG. 1. Effect of incubation of varying concentrations of GDP and GTP with S49 cells, NS-20, and corpus luteum membranes under adenylyl cyclase assay conditions. Incubations were for 5 min at 32.5°C. Compositions of the incubation media were those described under "Experimental Procedures," using the indicated concentrations of unlabeled GDP or GTP, 0.5 mM AMP-P(NH)p, 10 μM ATP and, per 50-μl assay, either 1.25 × 10<sup>6</sup> cpm of [α-<sup>32</sup>P]GTP (2,000 Ci/mmol), 7.5 nM, left panels; or 0.95 × 10<sup>6</sup> cpm of [α-<sup>32</sup>P]GDP (1,000 Ci/mmol), 11 nM, right panels. Incubations contained, per 50-μl assay, 1.75 μg of S49 cell membranes and 10<sup>-4</sup> M isoproterenol (top panels); 2.2 μg of NS-20 membranes and 10 μg/ml of PGE<sub>1</sub> (middle panels); or 1.8 μg of rabbit corpus luteum membranes and 10 μg/ml of PGE<sub>1</sub> (lower panels). For the rest of the conditions, see under "Experimental Procedures." Results are expressed as percentage of initially added radioactivity that co-chromatographed with GDP or GTP after the 5-min incubations. When incubations were carried out without membranes, 90% to 92% of the added radioactivities chromatographed as the added nucleotide. These background values were not subtracted from the distributions shown.
Although it would be possible to determine apparent \( K_\text{m} \) values for the effects of GTP in the absence and the presence of hormonal stimulation, such a determination is not possible for GDP, because it does not affect basal activity in either S49 or corpus luteum membranes. Further, due to the extent of transphosphorylation at low concentrations of added GDP, hormonal stimulation observed at these concentrations would be heavily biased by formation of GTP. We therefore compared interactions of GTP and GDP by testing for their effectiveness in inhibiting stimulation by GMP-P(NH)P. Table III presents IC\(_{50}\) values for GTP- and GDP-mediated inhibition of GMP-P(NH)P stimulation of S49, NS-20, and corpus luteum membrane adenylyl cyclases, as assayed in the absence of hormones and under conditions of membrane concentration, AMP-P(NH)P, and ATP identical with those used in the experiments shown in Table I and Fig. 1. The results indicate that in the absence of hormone, GDP and GTP are about equipotent in all three systems. Addition of hormones in the presence of GMP-P(NH)P resulted in activities such that although IC\(_{50}\) values could be obtained for GDP using the same incubation conditions, it was not possible to determine a competitive effect of GTP. So we determined IC\(_{50}\) values for GDP in the absence and presence of hormone under the conditions used in the experiments of Fig. 1 and Table I, and used different conditions that allowed us to measure IC\(_{50}\) values for GTP both in the absence and presence of hormone in S49 cell membranes and corpus luteum membranes.

The results of one set of these experiments is shown in Fig. 2. It can be seen that addition of stimulatory hormone did not alter the potency of GDP in any of the systems studied (right panels of Fig. 2). Neither did hormonal stimulation affect significantly the potencies with which either the S49 cell system or the corpus luteum system interact with GTP (top and bottom left panels of Figure 2). We were unable to determine the effect, if any, of hormonal stimulation on the potency of GTP in NS-20 membranes (middle left panel of Fig. 2), for we were faced with a situation similar to that seen in liver membranes in which hormone-stimulated activities in the presence of GMP-P(NH)P were too close to those seen in presence of GTP to allow us to study a competitive interaction.

The experiments shown in Fig. 2 were repeated several times and showed unequivocally that hormonal stimulation has no selective effect on the IC\(_{50}\) value with which GTP interacts with the S49 and corpus luteum adenylyl cyclases when compared to those with which GDP interacts with the same systems. Thus, GDP and not GTP formed during the incubation, was responsible for the stimulation of hormonal response in S49 membranes and for the small but significant prostaglandin response in corpus luteum membranes. The finding that both control and hormone-stimulated activities were constant from 16 to 500 \( \mu \text{M} \) added GDP (Fig. 2) is consistent with this contention, since over this range of concentration, the percentage of GTP formed is constantly decreasing. If hormonal responses were absolutely dependent on GTP, then one should have observed a continuous decrease in hormonal stimulation with increasing concentration of added GDP, since GDP is a competitive ligand at the guanine

![Fig. 2](image-url)

**Fig. 2.** Inhibition of GMP-P(NH)P stimulation of adenylyl cyclase activities by GTP (left panels) and GDP (right panels) as determined in the absence (□) and presence (●) of hormonal stimulation. Incubations with GTP were for 10 min at 32.5°C using 0.2 mM ATP (2,000 cpm/pmol) in the presence of a nucleoside triphosphate-regenerating system (RS) consisting of 20 mM creatine phosphate, 0.2 mg/ml of creatine phosphokinase, 0.02 mg/ml of myokinase, 10 \( \mu \text{M} \) GMP-P(NH)P, and either 5.3 \( \mu \text{g} \)/assay of S49 membranes, 4.2 \( \mu \text{g} \)/assay of NS-20 membranes, or 2.8 \( \mu \text{g} \)/assay of corpus luteum membranes. Incubations with GDP were for 5 min at 32.5°C using 0.5 mM AMP-P(NH)P, 10 \( \mu \text{M} \) [\( ^{32} \)P]ATP (10\(^5\) cpm/pmol) in the absence of a nucleoside triphosphate-regenerating system, 20 mM GMP-P(NH)P, and either 1.8 \( \mu \text{g} \)/assay of S49 cell membranes, 2.4 \( \mu \text{g} \)/assay of NS-20 neuroblastoma membranes, or 1.8 \( \mu \text{g} \) of corpus luteum membranes. When present, isoproterenol was 10\(^{-5}\) M and PGE\(_1\) was 10\(^{-5}\) M. Activities obtained in the absence of GMP-P(NH)P are shown as open symbols: □, no hormone addition; □, plus isoproterenol (top panels) or PGE\(_1\) (middle and bottom panels). For the rest of the conditions, see the figure and under "Experimental Procedures." Vertical lines and numbers next to them denote IC\(_{50}\) values, i.e., the concentrations of added GTP or GDP at which 50% inhibition was obtained.

### Table III

**Inhibition of GMP-P(NH)P stimulation of adenylyl cyclase**

| System          | GTP (\( \mu \text{M} \)) | GDP (\( \mu \text{M} \)) |
|-----------------|--------------------------|--------------------------|
| S49 lymphoma    | 12.3                     | 9.5                      |
| NS-20 neuroblastoma | 4.5                    | 6.3                      |
| Corpus luteum   | 3.1                      | 4.0                      |

*IC\(_{50}\)* concentration of GTP or GDP required to obtain 50% inhibition of the stimulation of activity due to 20 \( \mu \text{M} \) GMP-P(NH)P in the assays. These values were obtained from concentration effect curves for GDP and GTP between 2 and 200 \( \mu \text{M} \) assayed in the absence and presence of GMP-P(NH)P as described under "Experimental Procedures."
nucleotide regulatory site(s), both in the absence and in the presence of hormone, as shown in the right panels of Fig. 2. Although an IC\textsubscript{50} value for the interaction of GTP with the NS-20 neuroblastoma system could not be obtained in the presence of hormone, the following two arguments are in favor of GDP being a positive, though less effective, mediator of hormone stimulation in this system as well: 1) as shown above for other systems, such as turkey erythrocyte membranes, prostaglandin stimulation did not decrease when the added GDP was increased from 16 to 500 \mu M; and 2) GDP\textsubscript{S} an analog of GDP, was also effective in promoting PGE\textsubscript{1} stimulation (Table IV). It has been argued (9) that GDP\textsubscript{S} may act in some systems as a “GTP-like” nucleotide. However, the data shown in Table IV indicate that GDP\textsubscript{S} behaves as an analog of GDP inasmuch as both inhibit basal activity. A GTP-like effect of GDP\textsubscript{S} would have led to an increase in basal activity.

The data can be summarized by stating that while GTP and GDP are almost equipotent in the presence and absence of hormone, the extent of coupling of hormone-occupied receptors to adenylyl cyclases is much greater in the presence of GTP than GDP. GDP couples only the effective receptors and fails to do so for receptors that show rather small responses in the presence of GTP.

**Inhibition of the Turkey Erythrocyte Adenylyl Cyclase System by GDP:** Lack of Effect of Isoproterenol—Cassel and Selinger (8) described that in contrast to “untreated” turkey erythrocyte membranes, the adenylyl cyclase system in membranes subjected to previous treatment with isoproterenol and GMP is stimulated by GMP-P(NH)P. The “appearance” or “unmasking” of the GMP-P(NH)P effect was ascribed to a “clearing” of the enzyme system of GDP during the isoproterenol treatment effect curve in “Experimental Procedures.” Vertical lines and numbers next to them represent IC\textsubscript{50} values, i.e., concentrations of added GDP at which 50\% inhibition was obtained.

**TABLE IV**

| Nucleotide addition | Adenylyl cyclase activities\textsuperscript{a} | Basal Activity due to GDP, \textsuperscript{a} | Cpm of \[^{32}\text{P}]	ext{ATP} × 10\textsuperscript{-5} min/mg |
|---------------------|-----------------------------------------------|---------------------------------|-----------------------------------------------|
| None                | Adenylyl cyclase activities\textsuperscript{a} | 15.2 ± 1.0                      | 0.2 ± 0.2                                      |
| GDP (100 \mu M)     | Adenylyl cyclase activities\textsuperscript{a} | 3.0 ± 0.4\textsuperscript{d}    | 58.4 ± 1.2\textsuperscript{d}                 |
| GDP\textsubscript{S} (100 \mu M) | Adenylyl cyclase activities\textsuperscript{a} | 3.9 ± 0.5\textsuperscript{d}    | 14.8 ± 1.2\textsuperscript{d}                 |

\textsuperscript{a} Values are means ± S.D. of triplicate determinations (basal activities) or the difference ± S.D. between the means of triplicate determinations of activities assayed in the presence of 10 \mu M of PGE\textsubscript{1} and their respective basal activities. Assays were carried out in the presence of 0.5 mM AMP-P(NH)P and 10 \mu M \[^{32}\text{P}]	ext{ATP} as substrate. Incubations were for 5 min. For the rest of the conditions, see under “Experimental Procedures” and Table I.

\textsuperscript{b} Activity in the presence of PGE\textsubscript{1}, minus activity in its absence.

\textsuperscript{c} Not different from zero: p > 0.5.

\textsuperscript{d} Significantly different from control (no nucleotide added): p < 0.01.

**Fig. 3.** Inhibition of GMP-P(NH)P-stimulated adenylyl cyclase activity by GDP in turkey erythrocyte membranes as seen in the absence (\(\square\)) and presence (\(\bullet\)) of isoproterenol. Isoproterenol plus GMP-treated membranes (10 \mu g/assay), washed free of isoproterenol (30), were used. Incubations were for 5 min in the presence of 0.5 mM AMP-P(NH)P, 10 \mu M \[^{32}\text{P}]	ext{ATP} (50 × 10\textsuperscript{6} cpm/assay), and 20 \mu M GMP-P(NH)P in the absence of a nucleoside triphosphate-regenerating system (RS). When present, isoproterenol was 10\% M. For the rest of the conditions, see under “Experimental Procedures.” Vertical lines and numbers next to them represent IC\textsubscript{50} values, i.e., concentrations of added GDP at which 50\% inhibition was obtained.

**Fig. 4.** Effect of guanine nucleotides on hormone concentration effect curves in S49 cell, NS-20 neuroblastoma, and corpus luteum membranes. Effects of varying the concentrations of hormone on adenylyl cyclase activities were measured without (\(\square\)) and with addition of 200 \mu M GTP (\(\mathbin{\bigcirc}\)) and 200 \mu M GDP (\(\mathbin{\bigtriangleup}\)). In addition to all the components specified under “Experimental Procedures,” the assays also contained 0.02\% bovine serum albumin in the experiments shown in A and B. For further details of assay procedures, see under “Experimental Procedures.”
Receptor Coupling to Adenylyl Cyclases by GDP

... enol plus GMP treatment. We investigated the effect of isoproterenol on the potency with which GDP inhibits GMP-P(NH)P stimulation in treated turkey erythrocyte membranes and found that isoproterenol stimulation is associated with a slight increase in the overall affinity of the system for GDP (Fig. 3). As shown, at 1000 µM added GDP, this nucleotide did not sustain hormonal stimulation of the turkey erythrocyte adenylyl cyclase system (Fig. 3). Similarly, no hormone effect was seen in the presence of 1 to 100 µM GDP/βS (not shown).

Effect of Type of Nucleotide Used on the Concentration of Hormonal Stimulation—Fig. 4 shows representative experiments in which the apparent Kₐ for activation of three adenylyl cyclase systems and the extent of stimulation obtained were determined as a function of the type of guanine nucleotide added. In agreement with results shown in Table I and Fig. 2, GTP and GDP promoted coupling of PGE₁ receptor to the corpus luteum and the NS-20 neuroblastoma adenylyl cyclase systems, and of isoproterenol receptor to the S49 cell system. In all the cases, when maximal receptor-mediated stimulation was lower (presence of GDP), the apparent Kₐ was higher.

Effect of GDP on Isoproterenol Binding to S49 Cell, Corpus Luteum, and Turkey Erythrocyte Membranes—[¹²⁵I]HYP bound specifically to membranes containing β-adrenergic receptors. Binding was prevented by simultaneous addition of propranolol or isoproterenol and showed stereoselectivity. Between 10- and 100-fold higher concentrations of (+) isomers than of (−) isomers were required to effect 50% inhibition of [¹²⁵I]HYP binding (not shown). The effect of GTP and GDP on inhibition of [¹²⁵I]HYP by varying concentrations of (−)-isoproterenol was tested on S49, corpus luteum, and turkey erythrocyte membranes. As shown in Fig. 5 for S49 and corpus luteum membranes, and in Fig. 6 for untreated and isoproterenol plus GMP-treated turkey erythrocyte membranes, both GTP and GDP (at 500 µM) caused an increase in the IC₅₀.

The data reported here indicate that: 1) GTP and GDP are approximately equipotent in their interaction with adenylyl cyclases; 2) in none of the systems examined, including that of turkey erythrocytes, did hormonal stimulation result in a value for isoproterenol. The effect was more marked in S49 cell membranes than in corpus luteum membranes, and was approximately equal in untreated and treated erythrocyte membranes. The guanine nucleotide effect on isoproterenol binding was mimicked by both the GTP analog GMP-P(NH)P and the GDP analog GDP/βS (not shown for S49 and corpus luteum membranes, but shown for turkey erythrocyte membranes in Fig. 6). Transphosphorylation of GDP to GTP under the conditions of incubation used for the binding experiments of Figs. 5 and 6 was unaffected by isoproterenol addition (10⁻⁴ M), and was less than 0.5% with S49 and turkey erythrocyte membranes and less than 1% with corpus luteum membranes (not shown).

Discussion

The Interactions of GDP with Adenylyl Cyclases: Mediation of Hormonal Stimulation—Since the discovery of a hormone-activated GTPase thought to be intimately associated with the regulation of adenylyl cyclase (2-9, 25), it has been postulated that GTP occupancy of the regulatory site would be required for further hormonal stimulation of adenylyl cyclases. This hypothesis has gained credence since Cassel and Selinger (8) reported that in turkey erythrocyte membranes, isoproterenol increases the off-rate of bound GDP, thus facilitating the binding of GTP and subsequent activation of adenylyl cyclase. Such a mechanism, if generally applicable, would preclude the possibility of hormonal stimulation in the presence of excess GDP, provided that in the presence of hormone, GDP interacts with the system.

The data reported here indicate that: 1) GTP and GDP are approximately equipotent in their interaction with adenylyl cyclases; 2) in none of the systems examined, including that of turkey erythrocytes, did hormonal stimulation result in a...
significant decrease in the apparent potency of GDP; and 3) in the two systems where the potency of GTP in the presence and absence of hormone has been susceptible to examination, no major difference is observed due to the presence of hormone. It therefore seems safe to conclude that hormonal effects seen in the presence of a known 10-fold excess of GDP over GTP are occurring predominantly due to GDP. The data show that GDP is clearly not inactive in all cases, even though it is less effective than is GTP in promoting coupling of hormone receptors to adenylyl cyclases. Further, GDP sustains stimulation only by rather effective receptors (see below). Thus, we have defined situations where hormone-receptor coupling is mediated truly by GDP and not by contaminating GTP. Since there appears to be no experimental basis for assuming that hormonal stimulation results in a selective alteration of potency of either GDP or GTP, we feel that the coupling of glucagen receptors to liver adenylyl cyclase observed by some of us earlier under circumstances of only minimal transphosphorylation was indeed due to GDP and not to the less than 1% contaminating GTP (1).

The above conclusions imply that interaction of GTP (or a GTP analog) with adenylyl cyclase is not an obligatory requirement for hormonal stimulation and that increased nucleotide exchange rates observed (or assumed) to occur upon hormone addition are a consequence of the stimulatory effect of active hormone receptor on the guanine nucleotide-binding site as opposed to being the cause for stimulation of adenylyl cyclase by GTP (20). The fact that GTP is not an obligatory requirement for hormonal stimulation is further underscored by the finding that in the NS-20 neuroblastoma system (Table IV), the liver glucagon-sensitive adenylyl cyclase (9) and the catecholamine-stimulated parotid adenylyl cyclase (9) are hormonally stimulated in the presence of the GDP analog GDPαS. This analog was shown not to be susceptible to transphosphorylation (9).

Differential Effects of Guanine Nucleotide on Binding and Coupling: An Interpretation—There seems to be no apparent correlation between the capacity of a nucleotide to affect binding and promote positive coupling. GTP and GDP affect binding equally in corpus luteum, S49, and turkey erythrocyte membranes. Both nucleotides are equipotent in interacting with the guanine nucleotide-binding site on the regulatory component as measured by adenylyl cyclase activities. However, GDP promotes coupling of isoproterenol receptor to adenylyl cyclase in S49 cell membranes but not in corpus luteum or turkey erythrocyte membranes. We have recently proposed a two-state model (20, 26-28) based on the hysteretic enzyme concept (29-31) that satisfactorily accounts for the known features of GTP as well as GDP regulation of hormone stimulation of several mammalian adenylyl cyclases, primarily that from rat liver but also those of rabbit corpus luteum, kitten heart, S49 cells (28), and turkey erythrocytes (20). We find that the data presented here can also be satisfactorily explained by a two-state model for the guanine nucleotide-modulated adenylyl cyclase which is further regulated by active hormone-receptor complex. It has been proposed that an active hormone-receptor complex stimulates adenylyl cyclase by increasing the rate of transition from the inactive to the active form, this would result in amplification of the hormonal response (28). If a nucleotide-sensitized hormone-receptor complex is considered to be the active state of the receptor, i.e. one that can stimulate adenylyl cyclase, it would be predicted that potency of hormonal stimulation depends on the type of nucleotide that occupies the guanine nucleotide site on the regulatory component associated with the catalytic subunit. Such an argument tacitly includes the presence of two distinct guanine nucleotide effects: one affecting hormone-receptor interaction, rendering the hormone-receptor complex active, and another regulating the catalytic moiety of adenylyl cyclase, such as has been proposed on kinetic (32, 33) and biochemical (34) grounds for the rat liver system.

While we have no evidence in any of the systems studied here that there are two structurally different guanine nucleotide sites involved in hormonal stimulation of adenylyl cyclase activity, there are evidences that guanine nucleotide-binding sites behave differently, depending on whether adenylyl cyclase regulation or receptor regulation is examined. It has been shown that, in liver membranes preactivated with GMP-P(NH)P and then washed, a subsequent stimulatory effect of glucagon requires the co-addition of further guanine nucleotide, be it GMP-P(NH)P itself, GTP, or GDP (1, 33). Ross et al. (15) reported that while the effect of GMP-P(NH)P on S49 cell membrane adenylyl cyclase is of the apparently irreversible type being resistant to washing, that on isoproterenol binding is readily reversible. Strosberg and co-workers (35) have recently shown that pre-exposure of turkey erythrocyte membranes to GMP-P(NH)P, known to lead to "persistent" wash-resistant activation of the adenylyl cyclase in these membranes, does not result in a persistent effect in terms of protecting against isoproterenol-mediated sensitization of a N-ethylmaleimide-affected —SH group whose alkylation results in a decrease in measurable binding sites. Involvement of interacting nucleotide sites is also suggested by Cassel and Selinger's data (8), which showed that dissociation of [3H]-GDP from turkey erythrocyte membranes under the influence of isoproterenol is stimulated by free guanine nucleotide addition. Although Cassel and Selinger's data may indicate the existence of two nucleotide sites related in such a manner that in the presence of hormone, occupancy of one may result in "tight" binding and occupancy of the other may result in increased nucleotide exchange rates at both sites (homotropic negative cooperativity), there are currently no data to indicate that such interactions occur in the absence of hormone. The data in this communication do not address the question of homotropic interactions between guanine nucleotide-binding sites. Further experimentation is required to determine whether such interactions do exist. Regardless of whether two sites are involved, it seems clear that at least two distinct guanine nucleotide effects are involved in the overall guanine nucleotide regulation of receptor-mediated stimulation of adenylyl cyclase activity. If so, the following points can be made.

1) Based on the data obtained, we shall ascribe an efficacy or intrinsic activity factor to the active hormone-receptor complex. This factor increases with increasing capacity of the active receptor to stimulate the basic adenylyl cyclase system. By doing so, we can assign orders of efficacy to the catecholamine, prostaglandin, and adenosine receptors as seen in the presence of GDP in the systems studied here. Thus, under the assay conditions used in the S49 system, the isoproterenol receptor has higher intrinsic activity than does the PGE1;
Receptor Coupling to Adenylyl Cyclases by GDP

receptor (Table I), and in the NS-20 neuroblastoma system, the PGE receptor has a higher intrinsic activity than does the adenosine receptor (Table I). The difference between the two receptors in the NS-20 system is more marked than in the S49 cell system, and in the corpus luteum, the PGE receptor has higher efficacy than does the isoproterenol receptor (Table I).

2) In all three systems, hormone-stimulated activities were less in the presence of GDP than GTP. One explanation for this finding is that although both GTP and GDP alter receptor behavior equally, they act differently on the basic adenyl cyclase system where GTP promotes activation by stabilizing the active conformation, but where GDP either does not modify basal activity (S49, corpus luteum) or actually promotes inhibition by shifting the equilibrium between the active and inactive conformations towards the inactive form in a manner that is barely detectable (S49, corpus luteum) or clearly evident NS-20 neuroblastoma, turkey erythrocytes.

3) If the extent of transition from the inactive to active form of the enzyme adenyl cyclase induced by the active receptor is directly proportional to the efficacy factor of the hormone, it follows from points 1 and 2 above that: (a) adenyl cyclase should be stimulable by an active hormone-receptor complex with or without a nucleotide bound to the regulatory site associated with the catalytic moiety; (b) the stimulation of the enzyme would be facilitated by a nucleotide that by itself tends to activate (such as GTP; see Fig. 4); (c) the stimulation of the enzyme could occur but to a lesser extent also in the presence of an inhibitory nucleotide, provided that the receptor acting on the system has a sufficiently high efficacy so as to overcome the nucleotide effect (e.g. less than optimal stimulation of S49 by isoproterenol and NS-20 and corpus luteum by PGE); and (d) stimulation of the enzyme in the presence of inhibitory nucleotide would not be obtained if inhibition by the nucleotide is extensive, such as seen in the turkey erythrocyte adenyl cyclase, where basal activity is more than 99% inhibited by GDP (cf. Ref. 20), or if the enzyme is coupled to a low efficacy receptor even if the receptor is active, such as observed for the catecholamine-sensitive adenyl cyclase of corpus luteum adenyl cyclase, where no stimulation by isoproterenol is seen with GDP even though the binding effect is obtained and receptor is presumably active.

Thus, in the adenyl cyclase systems studied here, as well as in the rat liver adenyl cyclase, the recently proposed hypothesis (26–28) that adenyl cyclase behaves as a hysteretic two-state enzyme system in which the state transitions are regulated independently by the active hormone-receptor complex and guanine nucleotide (GTP-stimulating and GDP-inhibiting to various degrees) seems to be a reasonable one. In addition, the data and reasonings presented here indicate that receptors are affected and ultimately activated by the concerted interactions with guanine nucleotides and hormones. We have previously shown that a two-state guanine nucleotide-regulated adenyl cyclase, upon which hormones act, serves as a signal amplifier when the guanine nucleotide site on the regulatory moiety associated with the catalytic component is occupied by a stimulatory nucleotide, and as a signal dampener if the regulatory moiety is occupied by an inhibitory nucleotide. As shown by the data of Fig. 4 and corroborated by simulations (28), amplification and dampening results in corresponding changes in both sensitivity and magnitude of responses. Although the data shown here demonstrate that under defined conditions of assay, hormones activate some adenyl cyclases in the presence of GDP, it should be emphasized that in vivo, the relative GTP-GDP ratios are such that it is most likely that the system is occupied by GTP. Hence, GTP should be considered the main intracellular physiological effector of the system. However, dampening effects due to GDP, such as shown here, may become important if 1) a GTPase such as proposed by Cassel and Selinger (8) is associated with the adenyl cyclase, and 2) local compartmentalization phenomena exist in the environment (membranes) that surround the nucleotide-binding components. There exist no data at the present time to support or dispute the second suggestion. Clearly, it remains to be demonstrated whether hormonal regulation of adenyl cyclase in the intact cells is under the influence of GDP.

On the Interpretation of the Data: Some Limitations—Models and interpretations are not always unique. There may, therefore, be alternative explanations for the data presented above. An alternative explanation would have to account, just as our interpretation does, for the following findings: 1) stimulation of the rate of activation of GMP-P(NH)P by hormones independent of increases of nucleotide exchange rates in some but not all systems; 2) GDP-mediated hormonal stimulation in some adenyl cyclases even though both GTP and GDP act equally well at the level of hormone-receptor interaction; 3) inhibitory guanine nucleotide mediation of positive coupling of a nucleotide-dependent, hormone-occupied receptor; 4) a concentration effect curve for hormones obtained in the presence of a guanine nucleotide that stimulates basal adenyl cyclase activity more effectively lies to the left of that obtained in the presence of a less effectively stimulating guanine nucleotide (28); and 5) signal-dampening consequences observed upon substituting GDP for GTP.

In spite of being able to account for all of the above experimental findings, the model set forth by us is limited, for it is based solely on kinetic arguments. Other approaches, especially biochemical ones, should prove useful in elucidating the molecular mechanism of how hormone receptors couple to adenyl cyclase.

Acknowledgments—We are grateful to Professor Fritz Eckstein, Max Planck Institut, Göttingen, Germany, for his generous gift of GDP/$. We thank Mr. Philip L. Mints for excellent technical assistance and Mr. Mick Scheib for efficient secretarial assistance.

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