Evidence in Intact Cells for an Involvement of GTP in the Activation of Adenylate Cyclase*

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The regulation of cAMP synthesis in response to a hormone requires the proper interaction between at least two components, a hormone receptor and the catalytic subunit. In early studies aimed at understanding adenylate cyclase control at a molecular level, Rodbell and co-workers (1, 2) demonstrated that guanine-containing nucleotides influence adenylate cyclase (EC 4.6.1.1; ATP pyrophosphate-lyase (catalyzing) activity (1) and hormone binding (2). Since this original observation, numerous reports have appeared describing effects of guanine nucleotides on adenylate cyclase (reviewed in Refs. 3 and 4). Although a thorough understanding of the receptor-adenylate cyclase system is not yet available, these studies have furnished much useful information about this complex function. It is now clear that guanine nucleotides (GTP) is presumably the physiologically important one) are essential for adenylate cyclase activation. The nucleotides stimulate catalytic activity, influence hormone binding to the receptor, and also probably modulate receptor-catalytic moiety interactions (3, 4).

Studies to date on molecular interactions in the adenylate cyclase system have been carried out using cell homogenates or purified membranes. An evaluation of a physiological role for GTP at the cellular level could be accomplished by manipulation of cells to decrease GTP. An analysis of the adenylate cyclase system in such cells could furnish information about hormonal response and GTP functions which cannot be obtained in broken cell systems. Any means used to lower GTP would have to alter GTP specifically without affecting ATP, the substrate for the adenylate cyclase reaction. A block in the de novo pathway for GMP biosynthesis would meet this criterion. GMP and AMP share IMP as a common intermediate in de novo biosynthesis (see Ref. 5 for recent review). IMP is converted to XMP by IMP dehydrogenase (EC 1.2.1.14; IMP:NAD oxidoreductase) and synthesis of GMP from XMP is catalyzed by guanylate synthetase (EC 6.3.5.2; XMP-L-glutamine amidohydrolase (AMP)). Thus specific inhibitors of these two enzymatic activities should decrease guanine nucleotide levels without appreciable effects on adenine nucleotides. Specific inhibitors of these enzymes are known. Virazole (6, 7), MPA (8-11), and thiazirole (12, 13) (see Fig. 1 for structures) are antiviral and antitumor agents whose primary site of action is inhibition of IMP dehydrogenase and guanylate synthetase. The aim of the present study is to verify a role for GTP activation of adenylate cyclase in intact cells. This was accomplished by the use of these inhibitors in intact, cultured NRK cells to lower GTP and to study the effects of decreased GTP on cAMP levels.

EXPERIMENTAL PROCEDURES

Cell Culture—Two clones of NRK cells were used. NRK 5W was obtained from D. Wallach and NRK S from E. Scolnick (both of The National Institutes of Health). Cells were grown as previously described (14). Cells were planted at 5 x 10^3 cells/cm^2 in 20-cm^2 tissue culture dishes. After 48 h media were changed and the next day additions were made without media changes and cAMP was extracted. Solutions (in H_2O) of virazole (40 mM), MPA (10 mM), thiazirole (200 mM), nucleosides (50 mM), and isotroproenol (20 mM) were made and aliquots were added to cultures. Nucleosides were not completely soluble under these conditions and care was taken to remove a homogeneous aliquot. Nucleosides quickly dissolved when diluted into culture medium. PGE, was dissolved in ethanol (6.7 mg/ml); 3 μl aliquots were added to culture (4 ml). Cholera toxin was dissolved in NaCl/Pi (20 μg/ml). Aliquots of water or ethanol did not alter cyclic AMP levels.

In all experiments, total cellular protein was determined in companion cultures, identically treated, by the method of Lowry et al. (15) with bovine serum albumin as standard. Cyclic AMP Analysis—The medium was removed by aspiration and 0.5 ml of cold 5% (w/v) trichloroacetic acid containing carrier [3H]cAMP was quickly added. The cAMP was analyzed, after purification through a Dowex 1-formate column, by the acetylation modification (16) of the radioimmunoassay technique (17) as previously described (14). In all experiments the contents from three cultures were pooled and cAMP was assayed in triplicate. The standard error of the mean of the triplicate assays was within 10% of the mean. ATP Quantitation by Enzymatic Analysis—Cultures were quickly rinsed twice with cold NaCl/Pi, and cold 0.3 N perchloric acid (0.6 ml)

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1 The abbreviations used are: virazole, 1-(β-D-ribofuranosyl)-1,2,4-triazole-3-carboxamide; mycophenolic acid, MPA, 6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-5-phenylalanoyl)-4-methyl-4-hexenoic acid; NRK, normal rat kidney; PGE, polyethyleneimine; NaCl/Pi, 0.01 M sodium phosphate, pH 7.4, 0.15 M NaCl; PGE, prostaglandin E2.
Nucleotides were identified by co-migration with authentic standards and quantitated by radioautographic localization followed by counting the appropriate spots.

**Adenylate Cyclase Assay**—Cells were treated for 2 h with MPA. The medium was removed and the culture was rinsed three times with ice-cold NaCl/Pi, drained, and cold 2 M Na2HP04 (pH 3.4) (20) or two-dimensional chromatography with step-formate followed by 1.5 M KHzPOd (pH 3.4) (20) or two-dimensional chromatography with step-formate followed by 1.5 M KHzPOd (pH 3.4) (20).

**2-Deoxyglucose Uptake**—Uptake of 2-deoxy[14C]glucose was done as previously described (23).

**Materials**—Virazole, MPA, and thiadiazole were obtained from the National Cancer Institute, Division of Cancer Therapy, Developmental Therapeutics Program, Drug Synthesis and Chemistry Branch; AMP antigen from Collaborative Research; PEI-cellulose TLC sheets from Brinkmann; ribonucleoside triphosphates, isoprotein (bitartrate salt), phosphoenol pyruvate, and pyruvate kinase from Sigma; [32P]orthophosphate and [a-32P]ATP from International Chemical and Nuclear Corp.; 2-deoxy[14C]glucose from New England Nuclear; enzymes for ATP analysis from Boehringer Mannheim; trichloroacetic acid and KH2PO4 from Baker; cholera enterotoxin from Schwarz/Mann; and PGF, was a generous gift from Dr. John Pike, Upjohn Co.

### RESULTS

#### Site of Action of Inhibitors in NRK Cells—Virazole, MPA, and thiadiazole are potentially useful agents to lower specifically GTP levels (8-11) in hormone-responsive cells. However, before such studies can be done, specificity of action must be established in the particular cell system to be studied. Also, since MPA is toxic to some cells with incubation longer than that required to lower GTP (10, 11), it must be determined that cells are viable during the period of analysis.

When virazole (0.1 to 0.5 mM), MPA (0.02 to 0.1 mM), or thiadiazole (2 to 4 mM) is added to NRK cells for 2 to 6 h and then removed, cells appear normal and continue dividing. Also after 2 h treatment with virazole or MPA, there is no nonspecific membrane damage; 2-deoxyglucose uptake into intact cells (data not shown), and adenylate cyclase activity in membrane fractions (see Table V) are not affected. Thus a short exposure to these agents does not irreversibly harm the cells. However, NRK cells show definite signs of toxicity after 24 h. Cells are very granular, and normerat cells, which are probably dead, are floating in the media. If the primary site of action of these agents is inhibition of IMP dehydrogenase, exogenous guanine-containing compounds should prevent toxicity. To test this we added purine ribonucleosides along with the inhibitor. Cells appear normal after 24 h if guanosine (0.5 mM) is included with either of the three inhibitors. Adenosine, inosine, or xanthosine do not protect cells treated with virazole. The effects of the other two inhibitors have not been tested.

We then measured ribonucleoside triphosphates. ATP is easily measurable in small quantities by enzymatic analysis (24) (see "Experimental Procedures"). However, similar assay procedures for quantitating the other triphosphates in small amounts have not been developed. For their determinations we used [32P]orthophosphate labeling. Virazole or MPA treatment decreases GTP by about 80% in 2 h, with only a small additional decrease by 6 h (Table I). The ATP levels fall only about 15% in 2 h, with a much larger decrease by 6 h (Table I). The fall in ATP is probably secondary to the fall in GTP since GTP is required in the biosynthesis of AMP from IMP (5). At 2 h UTP is increased 20 to 25% and CTP is slightly increased (Table II). These relative changes in nucleotide triphosphate levels are in agreement with previous studies with mouse lymphoma (10) and neuroblastoma (11) cells. As with toxicity described above, the decrease in GTP is prevented by guanosine but not by other purine ribonucleosides (Table II). The nucleosides do not have an appreciable effect on ATP levels (Table II).

These experiments demonstrate that the agents act through inhibition of IMP dehydrogenase in NRK cells, and that short inhibitor treatments are not toxic. Thus these agents could be useful for the study of IMP dehydrogenase in NRK cells.
TABLE I
Time course for effect of virazole and MPA on ATP and GTP levels
NRK 5W cells were labeled for 16 h with [32P]orthophosphate. ATP and GTP were extracted at the indicated times and quantitated using one-dimensional TLC as described under "Experimental Procedures." Virazole was present at 0.2 μM and MPA at 0.05 mM. Results are expressed as the mean ± S.E. of separate determinations from three cultures.

<table>
<thead>
<tr>
<th>Condition</th>
<th>ATP (cpm/μg protein, ×10^6)</th>
<th>GTP (cpm/μg protein, ×10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 30 min</td>
<td>18.8 ± 0.3</td>
<td>2.62 ± 0.02</td>
</tr>
<tr>
<td>2 h</td>
<td>19.8 ± 0.5</td>
<td>2.69 ± 0.15</td>
</tr>
<tr>
<td>6 h</td>
<td>19.9 ± 0.9</td>
<td>2.55 ± 0.07</td>
</tr>
<tr>
<td>Virazole 30 min</td>
<td>20.4 ± 0.66</td>
<td>1.85 ± 0.12</td>
</tr>
<tr>
<td>2 h</td>
<td>17.0 ± 0.11</td>
<td>0.47 ± 0.07</td>
</tr>
<tr>
<td>6 h</td>
<td>12.3 ± 1.17</td>
<td>0.39 ± 0.05</td>
</tr>
<tr>
<td>MPA 30 min</td>
<td>21.3 ± 0.1</td>
<td>2.01 ± 0.09</td>
</tr>
<tr>
<td>2 h</td>
<td>18.7 ± 1.0</td>
<td>0.56 ± 0.09</td>
</tr>
<tr>
<td>6 h</td>
<td>12.6 ± 0.2</td>
<td>0.44 ± 0.02</td>
</tr>
</tbody>
</table>

TABLE II
Effect of inhibitors and purine ribonucleosides on purine and pyrimidine ribonucleoside triphosphate levels
NRK 5W were treated for 2 h with nucleosides (0.5 mM), virazole (0.1 mM), or MPA (0.05 mM) as described in Table I. One experiment, all four triphosphates were determined using two-dimensional chromatography. In two other experiments, ATP and GTP were analyzed with one-dimensional chromatography. This procedure does not separate CTP and UTP. CTP and UTP values are the average of two determinations from two separate cultures. ATP and GTP are expressed as the mean ± S.E. for the number of determinations indicated.

<table>
<thead>
<tr>
<th>Condition</th>
<th>ATP (cpm/μg protein, ×10^6)</th>
<th>GTP (cpm/μg protein, ×10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>19.0 ± 0.4 (6)</td>
<td>2.63 ± 0.08 (6)</td>
</tr>
<tr>
<td>Guanosine</td>
<td>18.6 ± 0.7 (6)</td>
<td>3.04 ± 0.15 (6)</td>
</tr>
<tr>
<td>Xanthosine</td>
<td>27.6 ± 0.3 (3)</td>
<td>3.09 ± 0.35 (3)</td>
</tr>
<tr>
<td>Inosine</td>
<td>23.5 ± 0.3 (3)</td>
<td>3.06 ± 0.35 (3)</td>
</tr>
<tr>
<td>Adenosine</td>
<td>30.0 ± 1.26 (3)</td>
<td>2.57 ± 0.17 (3)</td>
</tr>
<tr>
<td>Virazole + guano-</td>
<td>16.0 ± 0.46 (6)</td>
<td>0.49 ± 0.04 (6)</td>
</tr>
<tr>
<td>sine</td>
<td>18.4 ± 1.13 (4)</td>
<td>2.24 ± 0.09 (4)</td>
</tr>
<tr>
<td>Virazole + xantho-</td>
<td>17.1 ± 0.89 (6)</td>
<td>0.50 ± 0.04 (6)</td>
</tr>
<tr>
<td>sine</td>
<td>20.6 ± 0.68 (5)</td>
<td>2.69 ± 0.11 (5)</td>
</tr>
<tr>
<td>MPA + guanosine</td>
<td>19.3 ± 0.64 (3)</td>
<td>0.61 ± 0.07 (3)</td>
</tr>
<tr>
<td>MPA + xanthosine</td>
<td>17.5 ± 0.23 (3)</td>
<td>0.59 ± 0.01 (3)</td>
</tr>
<tr>
<td>MPA + inosine</td>
<td>22.5 ± 0.50 (3)</td>
<td>0.68 ± 0.07 (3)</td>
</tr>
</tbody>
</table>

used to lower specifically GTP levels in NRK cells and cAMP metabolism could be studied in intact cells in which GTP has been decreased by 80%.

**PGE Response**—Virazole (Fig. 2) and MPA (Fig. 3) in a dose-dependent manner decrease the ability of PGE to elevate cAMP in NRK 5W cells. Basal cAMP levels are not affected. Two hours after virazole addition, inhibition is maximal with no further decrease for up to 6 h (Fig. 4). Similar time dependence is observed following addition of MPA (0.05 mM) (data not shown). If this inhibition is due to a decrease in GTP through an inhibition of IMP dehydrogenase, guanosine or xanthosine, but not inosine or adenosine, should prevent and reverse the inhibition. When added along with the inhibitor guanosine (0.5 mM) completely overcomes the inhibition with MPA or virazole (Table III). In cells treated with virazole for 2 h guanosine addition for an additional 1 h restores 77% of the PGE response (data not shown). Xanthosine prevents the inhibition with virazole, but it is ineffective in conjunction with MPA (Table III). A lack of an effect with xanthosine could be explained by an inhibition of guanylate synthetase. MPA inhibits this enzyme in addition to IMP dehydrogenase in mouse lymphoma cells (10). The effects of the drugs on guanylate synthetase from NRK cells have not been tested. Inosine does not prevent the decreased PGE,

![Fig. 2. Virazole concentration dependence. NRK 5W cells were incubated for 2 h with virazole at the indicated concentration. Cultures were then treated with PGE, (5 μg/ml) for 15 min and the cyclic AMP extracted. Each point is the average of three assays of a sample from three pooled dishes (see "Experimental Procedures").](http://www.jbc.org/)

![Fig. 3. MPA concentration dependence. NRK 5W cells were incubated for 2 h with MPA at the indicated concentration and treated as described in Fig. 2. The results from two experiments are shown.](http://www.jbc.org/)

![Fig. 4. Time dependence for virazole treatment. NRK 5W cells were incubated at the indicated times with virazole (2 × 10^-4 M) and treated as described in Fig. 2.](http://www.jbc.org/)
response. Adenosine by itself slightly inhibited the PGE₁ response; therefore its effects on preventing the actions of the inhibitors cannot be evaluated. Interestingly, adenosine raises basal cAMP levels about 25%. This increase with adenosine is apparent in cells treated with virazole or MPA. This small increase is in contrast to the large (100-fold) stimulation by adenosine in cultured astrocytoma cells (25). Further analysis should be done in more responsive cells such as astrocytoma cells to determine whether the response to adenosine is independent of GTP.

MPA and virazole were also tested in NRK-S, another clone of NRK cells. The inhibitor concentration dependence and time dependence for decrease in PGE₁ response (data not shown) are essentially identical with that observed with NRK 5W cells (Figs. 2 to 4).

Treatment of NRK 5W with thiadiazole (2 mM) (Fig. 1) for 6 h decreases the response to PGE₁ (5 μg/ml) from 58 to 30 pmol/mg of protein, without affecting basal values.

Isoproterenol Response—The response of NRK-S cells to isoproterenol, a β-adrenergic agonist, was also tested. The increase in cyclic AMP is very rapid (Fig. 5). In two experiments optimal times of 60 s were detected; in two other experiments, maximum levels of cyclic AMP were observed at 30 s (Fig. 5). Thus to determine accurately, under optimal conditions, the inhibition of the isoproterenol response in cells treated with MPA or virazole, time dependence must be accurately measured in each experiment. As shown in Fig. 5, the response to isoproterenol is decreased in cells treated for 2 h with MPA. In this experiment the decrease was 70%; in three other experiments in which optimal times were determined, the inhibition was 60%, 50%, and 30%. Thus there is some variation in different experiments.

As with PGE₁, the isoproterenol response is not decreased by MPA if guanosine, but not inosine or xanthosine, is added (data not shown). The inhibition with 2 h MPA treatment is reversible if the MPA-containing medium is replaced by guanosine-containing medium for 5 min. In separate experiments 65% and 95% of the isoproterenol response during a 30-s treatment was restored.

Cholera Toxin Activation—Cholera toxin is another agent that elevates cAMP in mammalian cells (26). In cell homogenates, GTP stimulates the activation of adenylate cyclase by this agent (27, 28). Furthermore, it has been proposed that its activation of adenylate cyclase may be due to an inhibition of a specific GTPase activity (29). Treatment of NRK cells with virazole or MPA decreases slightly the amount of cAMP in cells treated with cholera toxin (Table IV); however, the inhibition is not significant (p = 0.2 for both virazole and MPA inhibition). The response with isoproterenol under identical conditions is shown for comparison (Table IV).

Desensitization—The fall in cAMP after reaching a maximum (30–52), a process termed "desensitization," is a function which is also influenced by GTP (33–35). We therefore studied desensitization in cells treated with virazole or MPA. Desensitization is observed in MPA- or virazole treated cells following addition of isoproterenol (Fig. 5) or PGE₁ (Fig. 6). In fact the desensitization to PGE₁ in treated cells may be faster than in untreated cells (Fig. 6).

Adenylate Cyclase Activity—Adenylate cyclase activities in membranes from MPA-treated and control cells are similar (Table V). Also MPA (0.05 mM) does not inhibit basal or PGE₁ plus GTP-stimulated adenylate cyclase activities. Thus, the inhibition of hormone response is not observed at the membrane level. However, when supernatant effects on membrane adenylate cyclase are compared, it is clear that MPA supernatants are less active. This decrease is presumably due
GTP and Adenylate Cyclase Activation in Intact Cells

The decreased response can be explained by current models for the role of GTP in the activation of adenylate cyclase. Guanine nucleotides decrease affinity of receptor for hormone (2, 4, 33-37), but yet increase activity of hormone-stimulated adenylate cyclase. To explain this apparent paradox, it has been suggested (35) that the receptor can exist in two conformational states; hormone binding to one state is of high affinity, the other of low affinity. The high affinity site is unable to activate adenylate cyclase when occupied by hormone; binding of hormone to the low affinity state results in activation of adenylate cyclase. Guanine nucleotides shift the equilibrium between the two states toward the low affinity state, thus activating the system.

Recently Lad et al. (38) proposed an alternative explanation. They observed in liver membranes that both glucagon receptor and the adenylate cyclase catalytic subunit bind GTP. They suggested that both units require bound GTP for proper coupling and that this complex is activated by the hormone. They further proposed that the receptor without bound GTP has a high affinity for the hormone but in this form, it cannot couple to the catalytic unit. Thus, under conditions of low GTP, adenylate cyclase cannot be activated by hormone.

In other studies on the effects of MPA on stimulation by β-adrenergic agonists it has been reported that MPA treatment of mice bearing Ehrlich ascites tumor cells reduced by 50% the response of these cells to epinephrine (39) and MPA treatment of cultured glioma cells reduced the isoproterenol response by about 35% (36).

**TABLE V**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Adenylate cyclase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control membranes</td>
</tr>
<tr>
<td></td>
<td>pmol CAMP formed/mg protein/15 min</td>
</tr>
<tr>
<td>None</td>
<td>23 ± 1.0</td>
</tr>
<tr>
<td>Fluoride (10 mM)</td>
<td>1052 ± 10.0</td>
</tr>
<tr>
<td>GTP (0.1 mM)</td>
<td>100 ± 6.0</td>
</tr>
<tr>
<td>PGE1 (10 μg/ml)</td>
<td>39 ± 4.3</td>
</tr>
<tr>
<td>PGE1 + GTP</td>
<td>362 ± 15.0</td>
</tr>
<tr>
<td>PGE1 + control supernatant</td>
<td>141 ± 5.7</td>
</tr>
<tr>
<td>PGE1 + MPA supernatant</td>
<td>79 ± 3.7</td>
</tr>
</tbody>
</table>

Recent studies with mutant mouse lymphoma S49 cells deficient in adenylate cyclase activity or receptor-catalytic coupling (37, 40) have suggested that an interaction between receptor and catalytic unit is necessary for desensitization. Our results are consistent with this proposal but also indicate that desensitization requires less GTP than does activation of adenylate cyclase. This is supported by the fact that desensitization of NRK adenylate cyclase to isoproterenol stimulation in membrane studies shows an absolute requirement for GTP (41).

**GTP May Exist in Separate Compartments within the Cell**—Under conditions where total GTP is decreased by 80%, the ability of isoproterenol or PGE1 to elevate cAMP is decreased by about 50%. If one considers the concentration of GTP in the cell it is surprising that adenylate cyclase response decreases. Assuming a value of 3 to 5 mM for ATP (42), the cellular GTP is about 0.5 mM. GTP activates adenylate cyclase in homogenates of NRK cells at 1 μM to 0.1 μM (43). Therefore, if GTP is uniformly distributed within cytoplasm, it is present in sufficient amounts to fully activate hormonal response even if decreased 80%. This suggests the existence of a separate pool of GTP for activation of adenylate cyclase, and that this pool is rapidly turning over and is susceptible to depletion during treatment with inhibitors. A functional compartmentalization of GTP has also been proposed in glioma cells (39).

There is evidence that GTP is not uniformly distributed in the cell. GTP tightly bound to the N site of tubulin turns over slower than the total cellular GTP in Chinese hamster cells (44). Neuroblastoma cells treated with MPA elongate normally after serum removal, although GTP levels are decreased by 70% (11). Cell elongation is a process which involves tubulin polymerization and hence GTP (45). This normal elongation could be explained by the existence of a GTP pool for tubulin polymerization, possibly bound to tubulin, which is not rapidly degraded. MPA treatment of skin cells does not lower cGMP levels (46). Although GTP levels were not measured in this...
study (46), MPA treatment decreases GTP levels in other cells (Table I) (10, 11). These studies taken together suggest that a separate GTP pool for guanylate cyclase exists. There is no evidence at this time to link this pool with tubulin.

The concept of compartmentalization of GTP raises the question of regulation of GTP available to adenylate cyclase. Could a GTP pool be subject to specific regulation? And could agents which increase hormonal response such as picolinic acid (14), hydrocortisone (47), or possibly colchicine (48) alter this pool? Our current studies are aimed at evaluating this possibility.

Acknowledgments—We would like to thank Wayne Anderson and Cynthia Jaworski for help with the CAMP assay and Prabhavatia Mukku for help with the cAMP assay.

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