The Requirement of Guanine Nucleotides for Glucagon Stimulation of Adenylate Cyclase in Rat Liver Plasma Membranes

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Previously we showed that commercially available ATP contains some substance(s) acting like GTP on liver adenylate cyclase and that using purified ATP as substrate, GTP enhances the glucagon-stimulated activity even in the presence of a millimolar concentration of ATP. In the present work on the precise role of guanine nucleotide in the action of glucagon, we purified commercial crystalline ATP by three successive column chromatographies on DEAE-cellulose using ammonium bicarbonate, pH 8.6, and 7 M urea, 0.02 M Tris/HCl, pH 7.4 as buffer systems.

One of the substances separated in this way from commercial ATP was identified as GTP by its ultraviolet spectra and by two-dimensional thin layer chromatography on polyethyleneimine-cellulose.

When adenylate cyclase activity was determined using a 1 mM concentration of this highly purified ATP as substrate, glucagon over the concentration range of 0.1 nM to 10 µM caused only minimal stimulation (1.5- to 2.0-fold) in the absence of added GTP. In the presence of 10 µM GTP, however, 1 µM glucagon increased the activity 15-fold. In the presence of 100 µM GTP, 5 nM glucagon caused half-maximal stimulation. In the presence of glucagon, the effect of GTP was dose-dependent and 1.0 µM GTP caused about half-maximal stimulation. GTP at a concentration of 10 to 100 µM caused about 2-fold increase in the enzyme activity in the absence of glucagon, but did not increase NaF-stimulated activity significantly.

Glucagon-stimulated adenylate cyclase activity was also increased by GDP or 2',deoxy-GTP and, to a lesser extent, by GMP. The enzyme activity in the absence of glucagon was stimulated by 2',deoxy-GTP but not by GDP, GMP, or guanosine. GDP seemed to have a direct action on the glucagon-stimulated activity because the incubation mixture did not contain a nucleotide-regenerating system and because the affinity of the glucagon-stimulated activity for GDP was similar to that for GTP. Guanyl-5'-yl-imidodiphosphate enhanced the enzyme activity in both the presence and absence of glucagon.

The ratio of glucagon-stimulated activity to basal activity, which seems to reflect the degree of coupling of glucagon binding with enzyme activation, increased as a function of the concentration of GTP or GDP, but not guany1-5'-yl imidodiphosphate. GDP had more effect on the ratio than GTP over the concentration range of 1 to 100 µM nucleotide.

These results show that commercial ATP may be contaminated with GTP and other GTP-like substances and that guanine nucleotide is essential for the action of glucagon on the liver adenylate cyclase system. It is suggested that the observed effect of GTP may actually be the effects of both GTP itself and GDP formed from it during incubation; GDP rather than GTP may actually be involved in the coupling process of hormone receptor to adenylate cyclase.

GTP is known to be important for the action of hormones on adenylate cyclase systems of various tissues and the cyclase system in eukaryotic cells has been shown to contain a nucleotide regulatory site as well as hormone receptor and catalytic sites (1). Rodbell et al. first demonstrated the nucleotide regulatory site and thought it to interact preferentially with GTP in studies on the glucagon-stimulated cyclase system in rat liver (2). In their system, GDP seemed to be essential for the action of glucagon, but its effect was only apparent when the ATP concentration was low. Subsequent studies on other systems (3-5) showed that millimolar concentrations of ATP could replace GTP in stimulating the action of hormones. Therefore, it was uncertain whether GTP was absolutely essential and whether it actually plays a physiological role in the action of hormones on adenylate cyclase systems.

Recently, we reported that the effect of GTP on the glucagon-stimulated cyclase system in rat liver plasma membranes varied depending on the lot of commercial ATP employed as substrate (6). When ATP purified by DEAE-cellulose column chromatography was used, even at a millimolar concentration, GTP consistently enhanced the glucagon-stimulated cyclase activity (6). Thus, some unknown substance(s) in commercial ATP has GTP-like activity and, contrary to previous conclusions, ATP itself, even at high concentration, cannot replace GTP in stimulating the action of hormones. Accordingly, the difficulty in determining the significance of GTP in the action of hormone seems to be due to the presence of a GTP-like substance(s) in the ATP added to the cyclase assay mixture.

However, previous studies using purified ATP as substrate showed that glucagon stimulated adenylate cyclase even in the absence of added GTP (6). Therefore, it is still uncertain whether GTP is actually required for the action of glucagon on the liver adenylate cyclase system.

In the present study we purified ATP further by three successive column chromatographies on DEAE-cellulose and...
using this highly purified ATP we showed that guanine nucleotide is essential for the action of glucagon on adenylate cyclase in purified rat liver plasma membranes. We also showed that one of the effective contaminants in commercial ATP is guanine nucleotide.

**EXPERIMENTAL PROCEDURES**

Materials—Cyclic 3'-5'AMP (27.5 C/mmol) was purchased from the Radiochemical Centre, Amersham. ATP, GTP, GDP, GMP, guanosine, 2'-deoxy-GTP, and glucagon were obtained from Sigma Chemical Co. Gpp(NH)p was purchased from Boehringer. Plastic sheets of polyethyleneimine (PEI)-cellulose, were from Merck, cellulose sheets of DE-52 from Whatman, and neutral alumina from ICN Pharmaceuticals.

**Preparation of Liver Plasma Membranes—** Liver plasma membranes were obtained essentially according to the method of Berman et al. (7). The livers (50 to 80 g) of male Wistar rats, weighing 200 to 300 g, were minced and homogenized in 9 volumes of 0.25 M sucrose, 0.5 mM CaCl₂, 10 mM Tris/HCl, pH 7.4, by 40 gentle strokes of a loose-fitting Dounce homogenizer. The homogenate was then centrifuged twice at 150 x g for 5 min and the supernatant was further centrifuged at 2200 x g for 15 min. The precipitate was resuspended in the same buffer and centrifuged at 30,000 x g for 15 min. The resulting pellet was homogenized gently in a small volume of the buffer and aliquots were layered on a discontinuous sucrose gradient, prepared using 8 ml of 45% (d = 1.90) sucrose, 10 ml of 41.5% (d = 1.18) sucrose, and 10 ml of 37% (d = 1.16) sucrose. These sucrose solutions were made in a solution of 0.5 mM CaCl₂ and 10 mM Tris/HCl, pH 7.4. The tubes were then centrifuged at 25,000 x g for 90 min in a Spinco SW 27 rotor. The plasma membrane fraction at the interface between 37 and 41.5% sucrose was collected and dialyzed against the same buffer and then centrifuged at 2200 x g for 15 min. The supernatant was further centrifuged at 150 x g for 5 min and the supernatant was further centrifuged at 150,000 x g for 1 h. The membrane fraction was then homogenized again in a small volume of the same buffer and recentrifuged. The resulting pellet was resuspended in 25,000 ml of 0.05 M NaCl in 7 M urea, 0.02 M Tris/HCl, pH 7.4, and dialyzed at 4°C against the same buffer for 48 h. The solution was then centrifuged at 48,300 x g for 1 h. The resulting supernatant was then dialyzed and stored at -70°C.

**Ultraviolet Spectra—** Ultraviolet spectra were measured in a Cary 118 spectrophotometer. The following buffers were used to examine the effect of pH on the spectral properties: 0.1 N HCl for pH 1, 20 mM NaH₂PO₄/Na₂HPO₄ for pH 7, and 250 mM Na,HPO₄/NaOH for pH 11.

**RESULTS**

**Purification of ATP—** The chromatogram of ATP on the first DEAE-cellulose column is shown in Fig. 1. Fractions in the large peak of ATP (Fractions 290 to 360) were collected for further purification. A small but significant amount of an unknown substance(s), detected by its absorbance at 280 nm, was eluted after the large peak of ATP. The fractions (Fractions 372 to 435) containing this unknown substance(s) were collected and the material was purified further by three successive chromatographies on DEAE-cellulose columns.

The ATP obtained from the first column was purified further by DEAE-cellulose column chromatography in the presence of glucagon.
obtained on the third DEAE-cellulose column with a linear gradient of 0.12 to 0.24 M ammonium bicarbonate, pH 8.6, at a flow rate of 250 ml/h and 18-ml fractions were collected. Fractions of eluate containing ATP (Fractions 117 to 125 and 206 to 215) were pooled and used for further purification. The unknown substance(s) in Fractions 371 to 435, shown with a 100-fold larger scale on the right ordinate, was purified further as described in the text.

Fig. 2 (right). Chromatograms of ATP on the second (A) and third (B) DEAE-cellulose columns. A, ATP (about 1600 absorbance units at 260 nm) obtained by the first column chromatography was dissolved in 7 M urea, 0.02 M Tris/HCl, pH 7.4, and applied to a DEAE-cellulose column (2.6 × 40 cm). The flow rate was 54 mll/h and 8-ml fractions were collected. B. Fractions of ATP from the second column (Fractions 144 to 172 shown in A) were combined, diluted with distilled water, and applied to the column (2.6 × 40 cm). The column was eluted with a linear gradient of 0.1 to 0.23 M ammonium bicarbonate, pH 8.6. The flow rate was 120 ml/h and 12-ml fractions were collected.

Influence of Purity of Added ATP on Adenylate Cyclase Activity—Previously we reported that the basal activity, as well as the glucagon- and NaF-stimulated activities of the cyclase measured with unpurified ATP, was much greater than those measured with purified ATP, and that GTP did not have any effect on the activities with unpurified ATP (6). In contrast, GTP greatly increased glucagon-stimulated enzyme activity when highly purified ATP was used as substrate (Table I). When highly purified ATP was used, glucagon caused a 2-fold increase in the activity over the basal level in the absence of added GTP, and a 16-fold increase in the presence of 10 μM GTP. The basal and fluoride-stimulated activities were only affected slightly by added GTP. Thus, the difference in the effects of GTP on the respective activities was clearly shown as a ratio of the activities in the presence and absence of GTP, and the ratio for glucagon-stimulated activity using highly purified ATP was much greater than that using ATP purified by the method reported previously (purified ATP) (6). This fact indicates that the step of DEAE-cellulose column chromatography in the presence of 7 M urea was very effective for purification of ATP and that there must be some active contaminant(s) in ATP besides U-I or U-II, which was removed by this DEAE-cellulose column chromatography in the presence of 7 M urea.
FIG. 3. Chromatograms of the unknown substance(s) on DEAE-cellulose columns. A, the unknown substance(s) separated from ATP by two successive DEAE-cellulose column chromatographies (see the text) was applied to the third column (0.9 x 26.5 cm) and the column was eluted with a linear gradient of 0.12 to 0.18 M ammonium bicarbonate, pH 8.6. The flow rate was 30 ml/h and 6-ml fractions were collected. B, the unknown substance(s) recovered in a peak from the third column (Fractions 26 to 33 shown in A) was dissolved in 0.05 M NaCl in the presence of 7 M urea, 0.02 M Tris/HCl, pH 7.4, and applied to the column (0.9 x 24.5 cm). The column was eluted with a linear gradient of 0.05 to 0.2 M NaCl in 7 M urea, 0.02 M Tris/HCl, pH 7.4. The flow rate was 24 ml/h and 4.3-ml fractions were collected.

FIG. 4. Thin layer chromatography of U-I on PEI-cellulose. U-I (0.2 absorbance unit at 260 nm) was applied to a PEI-cellulose sheet and co-chromatographed with authentic compounds (0.02 pmol each) in two dimensions as indicated. The details of the method are described in the text.

Effects of U-I and U-II on Adenylate Cyclase Activity—When U-I, which was identified as GTP as described above, was added with highly purified ATP to give a concentration comparable to that on addition of unpurified ATP, it greatly increased glucagon-stimulated adenylate cyclase activity (Table II). Glucagon-stimulated activity was also increased slightly by U-II. These substances did not exert consistent effects on the basal activity, and the fluoride-stimulated activity was decreased by the simultaneous additions of U-I and U-II.

Effect of Concentration of GTP on Adenylate Cyclase Avcivity—

TABLE I

<table>
<thead>
<tr>
<th>ATP</th>
<th>GTP</th>
<th>Adenylate cyclase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pmoi/mg protein/min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Basal Glucagon NaF</td>
</tr>
<tr>
<td>Highly purified</td>
<td>-</td>
<td>25.6 56.3 1900</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>54.0 872 1420</td>
</tr>
<tr>
<td></td>
<td>+GTP/-GTP</td>
<td>2.1 15.5 11.1</td>
</tr>
<tr>
<td>Purified</td>
<td>-</td>
<td>31.2 240 1040</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>44.9 1000 1190</td>
</tr>
<tr>
<td></td>
<td>+GTP/-GTP</td>
<td>1.4 4.2 1.1</td>
</tr>
</tbody>
</table>
ity - Fig. 6 shows the effect of GTP concentration on the basal and the glucagon-stimulated adenylate cyclase activities with highly purified ATP as substrate. The basal activity was enhanced about 2-fold by 10 to 100 μM GTP. The glucagon-stimulated activity was enhanced significantly by as little as 0.01 μM GTP and maximally by 10 to 100 μM GTP, and about 0.1 μM GTP caused half-maximal stimulation.

The ratio of glucagon-stimulated activity to basal activity increased with an increase in GTP concentration, indicating clearly that activation of the adenylate cyclase system by glucagon is a function of the GTP concentration in the incubation medium.

Effect of Concentration of Glucagon on Adenylate Cyclase Activity - The adenylate cyclase activities measured with and without 100 μM GTP in the presence of various concentrations of glucagon are shown in Fig. 7. Unlike in previous studies, concentrations of 0.1 nM to 10 μM glucagon had scarcely any stimulatory effect in the absence of added GTP. In the presence of 100 μM GTP, however, glucagon greatly stimulated adenylate cyclase, stimulation being half-maximal with about 5 nM and maximal with 1 μM glucagon. These results indicate that GTP is absolutely required for glucagon-stimulated adenylate cyclase activity.

Effects of Various Guanine Derivatives on Adenylate Cyclase Activity - Other guanine derivatives also increased glucagon-stimulated adenylate cyclase activity (Table III). GDP and 2'-deoxy-GTP stimulated the enzyme to the same extent as GTP at concentrations of 0.1 mM, whereas GMP caused less stimulation and guanosine had no effect.

The basal adenylate cyclase activity, which was enhanced 2-fold by the presence of 100 μM GTP or 2'-deoxy-GTP was not affected by the presence of GDP, GMP, or guanosine. Gpp(NH)p, an analogue of GTP, enhanced both the glucagon-stimulated activity and the basal activity, as reported for other systems (13, 14). The ratio of glucagon-stimulated activity to basal activity was increased more by GDP than by GTP, and was not affected by Gpp(NH)p.

The observed effect of GDP on the glucagon-stimulated activity is probably not due to GTP formed from GDP because no ATP regenerating system was present in the incubation medium. Moreover, since the affinities of glucagon-stimulated adenylate cyclase for GDP and GTP were similar (Fig. 8A), the effect of GDP was probably not due to GTP contaminating the GDP preparation.

The fact that GDP enhanced the glucagon-stimulated adenylate cyclase activity raised the question of whether the effect of GDP was actually due to GTP or to GDP derived from it. To test this we compared the concentration dependence of the effects of GDP and Gpp(NH)p, which is thought to be resistant

![Fig. 6. Effect of the concentration of GTP on adenylate cyclase activity. Adenylate cyclase activity was determined in the absence () and presence (●) of 1 μM glucagon using 1 mM highly purified ATP as substrate. The ratio of glucagon-stimulated activity to basal cyclase activity is shown by the dotted line.](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Additions</th>
<th>Basal</th>
<th>Glucagon</th>
<th>NaF</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>35.3</td>
<td>50.4</td>
<td>1010</td>
</tr>
<tr>
<td>U-I</td>
<td>35.7</td>
<td>644</td>
<td>1160</td>
</tr>
<tr>
<td>U-II</td>
<td>39.1</td>
<td>104</td>
<td>394</td>
</tr>
<tr>
<td>U-I + U-II</td>
<td>41.9</td>
<td>656</td>
<td>866</td>
</tr>
</tbody>
</table>

**TABLE II**

**FIG. 7.** Effect of the concentration of glucagon on adenylate cyclase activity. Adenylate cyclase activity was determined in the absence () and presence (●) of 100 μM GTP using 1 mM highly purified ATP as substrate.

**TABLE III**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Adenylate cyclase activity</th>
<th>Activity ratio (glucagon/basal)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Glucagon</td>
</tr>
<tr>
<td>None</td>
<td>22.8 ± 4.8</td>
<td>34.3 ± 3.1</td>
</tr>
<tr>
<td>GDP</td>
<td>42.0 ± 1.6</td>
<td>519 ± 35</td>
</tr>
<tr>
<td>GMP</td>
<td>21.0 ± 2.7</td>
<td>612 ± 28</td>
</tr>
<tr>
<td>Guanosine</td>
<td>31.2 ± 4.4</td>
<td>157 ± 8</td>
</tr>
<tr>
<td>dGTP</td>
<td>16.5 ± 4.0</td>
<td>48.1 ± 7.7</td>
</tr>
<tr>
<td>Gpp(NH)p</td>
<td>45.2 ± 5.2</td>
<td>520 ± 53</td>
</tr>
<tr>
<td>268 ± 2</td>
<td>744 ± 45</td>
<td>2.8</td>
</tr>
</tbody>
</table>
tions tested (Fig. 8B). It only increased the ratio of glucagon-than the latter at high nucleotide concentrations (1 to 100
dose-related effect of GTP shown in Fig. 6 but was greater
basal activity increased with an increase in GDP concentra-
0.1 slighly by 0.1 to 1
slow displacement of the tightly bound ligands, GDP or GTP.
These conflicting results on the action of guanine nucleotide
must be due in part to the introduction into the incubation mixture of GTP or another GTP-like contaminant(s) of the
ATP or enzyme preparations. Thus, these results must be examined employing a system free from possible contamin-
with GTP or another GTP-like substance(s).
The effects of GDP on the cyclase system are also uncertain.
The effect of GDP must be examined in the absence of a nucleotide-regenerating system which converts GDP to GTP.
Rodbell et al. (2) formerly concluded that GDP as well as GTP enhanced adenylate cyclase activity in the presence of 5 m
glucagon from studies using adenyl-5'yl imidodiphosphate,
an analogue of ATP, as substrate. However, recently they
reported the different observation that GDP inhibits the en-
zeyme activities obtained in the absence and presence of 1 m
glucagon but does not affect the activity in the presence of 1
µm glucagon (21). In the present study we showed that in the
presence of 1 µm glucagon, GDP enhanced liver adenylate
cyclase with a similar affinity for GTP and stimulated it to the
same extent as GTP or Gpp(NH)p. No nucleotide-regenerating
system was present, and so GDP probably acted directly on the
adenylate cyclase system. Consistent with our find-
ing, Hanpune et al. (17) recently demonstrated that GDP
stimulated both the epinephrine- and glucagon-stimulated
adenylate cyclase systems in liver membranes in the absence
of a nucleotide-regenerating system.

Adenylate cyclase activity in the absence of glucagon was
stimulated by Gpp(NH)p. As the ATP used as substrate was
highly purified and free from guanine nucleotide, the
Gpp(NH)p probably activated the enzyme by itself, rather
than inhibiting the hydrolysis of natural guanine nucleotide.
GTP also activated the enzyme in the absence of glucagon; the
triphosphate group of the nucleotide may activate adenylate
cyclase in the absence of glucagon. The fact that the effect
of GTP was much less than that of Gpp(NH)p may be explained

DISCUSSION
In this work we examined the role of GTP in the glucagon-
stimulated adenylate cyclase system in liver plasma membr-
anes using highly purified ATP as substrate. Previously (6),
we reported that glucagon stimulated the enzyme 6- to 8-fold
in the absence of added GTP, even when ATP purified by two
successive DEAE-cellulose column chromatographies was
used as substrate. The present study shows that additional
purification of ATP by DEAE-cellulose column chromatogra-
phy in the presence of 7 m urea, in which separation of the
nucleotides depends on the net negative charge of their phos-
phate residues (9), was essential for removing a contami-
nant(s) having a GTP-like effect. Using this highly purified
ATP as substrate, we found that guanine nucleotide was es-
sential for the action of glucagon on adenylate cyclase in liver
plasma membranes. This confirmed our previous conclusion
that a high concentration of ATP cannot replace GTP in
stimulation of the action of glucagon (6). The nucleotide regu-
larly site seems to interact specifically with guanine nucleo-
tide, not with ATP, indicating the possible physiological sig-
nificance of intracellular guanine nucleotide in regulating
the action of glucagon at the membrane level.

We identified one of the substances contaminating commer-
cial crystalline ATP as GTP by its ultraviolet spectra and
mobilities on two dimensional thin layer chromatography in
different solvent systems. The contamination of ATP with
GTP explains previous findings (1-5) that GTP often failed to
enhance adenylate cyclase activity stimulated by hormones
in the presence of millimolar concentrations of ATP. We also
found that commercial ATP contained some substance(s) other
than U-I or U-II that had more or less the same activity as
GTP on adenylate cyclase. We are now attempting to purify
and identify this separated from ATP by the DEAE-cellulose
column chromatography in the presence of 7 m urea.

Previous reports on the mode of action of GTP on adenylate
cyclase systems stimulated by hormones are conflicting, indi-
cating either a change in the apparent Km for ATP (15) or in
the Vmax of the enzyme (5), or both (16, 17). Rodbell et al. (18)
reported that GTP induced a shift in the affinity of the liver
adenylate cyclase system for glucagon but this was not ob-
erved by others in other adenylate cyclase systems (17, 19,
20). Recently, Rodbell et al. (21-23) proposed the three states
model in which the cyclase system responds to Gpp(NH)p
slowly, resulting in isomerization of the enzyme to a highly
active state. In the cyclase system of neuroblastoma (24),
however, this slow activation by Gpp(NH)p is attributed to
slow displacement of the tightly bound ligands, GDP or GTP.

GTP explains previous findings (1-5) that GTP often failed to
enhance adenylate cyclase activity stimulated by hormones
in the presence of millimolar concentrations of ATP. We also
found that commercial ATP contained some substance(s) other
than U-I or U-II that had more or less the same activity as
GTP on adenylate cyclase. We are now attempting to purify
and identify this separated from ATP by the DEAE-cellulose
column chromatography in the presence of 7 m urea.
by the difference in stabilities of the terminal phosphates of these two nucleotides at the membrane (25).

The ratio of glucagon-stimulated activity to basal activity, which is assumed to reflect the degree of transmission of information of glucagon binding to adenylate cyclase, was found to be a function of the concentration of GDP or GTP, but not of Gpp(NH)p. At high concentrations (10 to 100 μM) GDP increased the ratio about twice as much as GTP. At this high concentration Gpp(NH)p did not have any effect on the ratio. Although Gpp(NH)p is known to be hydrolyzed rapidly by membrane preparations (25, 26), most of the Gpp(NH)p bound to membranes remains in an intact form (25). On the other hand, GTP was undoubtedly converted to GDP by nucleotidases in the membranes (25). Thus, the effect of GTP that we observed was probably a combination of the effects of GTP itself and GDP derived from it during incubation. GTP itself may activate the basal activity, as Gpp(NH)p does, and GDP derived from GTP may contribute to enhancement of the action of glucagon on the cyclase system.

The present study shows that guanine nucleotide is essential for stimulation of adenylate cyclase by glucagon; the nucleotide acts as a modulator in the coupling of glucagon binding and adenylate cyclase activation. Thus, the nucleotide may well have a physiological role in determining the response of adenylate cyclase to hormones, as suggested in the case of desensitization induced by prolonged exposure of a tissue to a homologous hormone (27, 28). A guanine nucleotide-free system like that used in this work is essential in all further studies on the action of glucagon and probably of other hormones on the adenylate cyclase system.

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N Kimura and N Nagata


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