ELIMINATION OF GTP BIPHASIC REGULATION OF SYNAPTOSOMAL ADENYLATE CYCLASE BY MANGANESE AND SOLUBILIZATION

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Abstract—Effects of divalent cations and solubilization with Lubrol-PX were studied on guanine nucleotide regulation of synaptosomal adenylate cyclase activity of the rat caudate nucleus. In the presence of Mg\(^{2+}\), both GTP and Gpp(NH)p exerted biphasic actions on the membrane-bound adenylate cyclase activity. The \(K_{0.5}\) value for the GTP stimulation of the cyclase was 47 nM, and the value for the GTP inhibition was 4.5 \(\mu\)M. One hundred \(\mu\)M dopamine selectively enhanced the stimulatory phase of the GTP action, whereas 10 \(\mu\)M morphine selectively enhanced the inhibitory phase of the GTP action. When Mg\(^{2+}\) was replaced by Mn\(^{2+}\), the inhibition of the membrane-bound adenylate cyclase by these nucleotides and morphine was completely abolished; but the catalytic activity of adenylate cyclase was not impaired. These results suggest that the inhibitory action of GTP is responsible for the morphine inhibition of synaptosomal adenylate cyclase. Lubrol-solubilized adenylate cyclase preferred Mn\(^{2+}\) to Mg\(^{2+}\) for its activity. The stimulation of adenylate cyclase by either GTP or Gpp(NH)p was eliminated in the Sepharose 6B-fractionated solubilized preparation in the presence of either Mg\(^{2+}\) or Mn\(^{2+}\). Ten mM NaF also failed to activate the fractionated adenylate cyclase. In the fractionated solubilized preparation, GTP and Gpp(NH)p failed to inhibit adenylate cyclase. These results indicate that GTP and Gpp(NH)p are unable to inhibit the resolved catalytic unit of the synaptosomal adenylate cyclase.

Membrane-bound adenylate cyclase is stimulated or inhibited by hormones. It has been recently shown that GTP plays a crucial role in mediating the actions of both stimulatory and inhibitory hormones on adenylate cyclase activity (1, 2). Hormone-stimulable adenylate cyclase systems consist of at least three separate components: a hormone receptor, a guanine nucleotide regulatory protein (G protein), and a catalytic unit of adenylate cyclase (1–5). The catalytic unit separated from the G protein is active with Mn\(^{2+}\)-ATP, but it is inactive with the physiological substrate Mg\(^{2+}\)-ATP (6). The G protein is essential to constitute the Mg\(^{2+}\)-ATP-dependent activity of adenylate cyclase (6) and responsible for the activation of adenylate cyclase by guanine nucleotides and F\(^{-}\) (3, 7, 8). Stimulatory hormones increase the rate of GTP activation of adenylate cyclase (9, 10).

In addition to the G protein-mediated
stimulatory action of GTP, an inhibitory action of GTP on adenylate cyclase activity has been also demonstrated in a variety of tissues (11-15). In tissues in which GTP exerts both the stimulatory and the inhibitory action on adenylate cyclase activity, lower GTP concentrations (<0.1-1 μM) stimulate and higher GTP concentrations (≥0.1-1 μM) inhibit the cyclase (13-15). Hormones and neurotransmitters such as α-adrenergic agonists and adenosine inhibit adenylate cyclase in these tissues only in the presence of high GTP concentrations (13, 15-17). Cooper et al. (14) have shown that the inhibitory phase of the GTP biphasic action is linked to adenosine inhibition of rat adipocyte adenylate cyclase. These lines of evidence suggest that the inhibitory action of GTP is generally implicated in the action of inhibitory hormones on adenylate cyclase activity. However, the mechanism by which GTP and hormones inhibit adenylate cyclase has not yet been fully elucidated. It has been suggested that GTP inhibits adenylate cyclase through a component distinct from both the catalytic unit and the stimulatory G protein (18, 19). In tissues in which GTP inhibits adenylate cyclase, however, it has not been investigated whether GTP directly inhibits the resolved catalytic unit or not.

Experimental perturbation of an adenylate cyclase system is one of the useful approaches to investigate the roles of components of the cyclase system in regulation of the enzyme activity. It has been recently shown that Mn²⁺ impairs the GTP-dependent hormonal regulation of adenylate cyclase activity in frog erythrocytes (20), human platelets (19), and hamster adipocytes (21). The components of the adenylate cyclase system can be physically separated by solubilization of membranes with detergent and successive chromatography (22, 23). We demonstrated previously that opioids inhibit the synaptosomal adenylate cyclase of the rat caudate nucleus through a GTP-dependent process (24). In the present study, we have investigated effects of Mn²⁺ and solubilization with Lubrol-PX on the regulation of the synaptosomal adenylate cyclase activity in an attempt to elucidate the mechanism by which GTP and morphine inhibit the enzyme. The present results suggest that the inhibitory action of GTP on the adenylate cyclase activity is responsible for the morphine inhibition of the cyclase. In addition, we show that GTP no longer inhibited the resolved catalytic unit of the synaptosomal adenylate cyclase.

**MATERIALS AND METHODS**

**Enzyme preparations:** The crude synaptosomal fraction (8-12 mg protein) of rat caudate nucleus prepared as described previously (24) was lysed with 10 ml of 5 mM Tris-HCl (pH 7.4) for 30 min at 0°C. The crude synaptosomal lysates were precipitated by centrifugation at 15,000×g for 30 min (4°C). The lysates were suspended in ice-cold 1 mM EGTA, 5 mM Tris-HCl (pH 7.4) to yield a protein concentration of 0.8-1.0 mg/ml. This suspension was stored at −40°C until use.

The crude synaptosomal lysates (10-12 mg protein) were homogenized in 3 ml of ice-cold 1% (v/v) Lubrol-PX, 1 mM EGTA, 5 mM Tris-HCl (pH 7.4), and centrifuged at 100,000×g for 90 min (4°C). The supernatant was applied on a Sepharose 6B column (1.5×55 cm). The column was equilibrated and eluted with 0.1% (v/v) Lubrol-PX, 1 mM EGTA, 1 mM dithiothreitol, and 100 mM Tris-HCl (pH 7.4) at 4°C. Fractions of 0.8 ml were collected.

**Adenylate cyclase assay:** To avoid the complicating effects of the ATP-regenerating system on adenylate cyclase activity (25), the modified method (24) of Clement-Cormier et al. (26) was used. The total 500 μl of standard assay mixture contained 50 μl of
enzyme preparation, 0.12 mM ATP, 1.2 mM 3-isobutyl-1-methylxanthine, 50 mM Tris-HCl (pH 7.4), and 4.8 mM MgCl₂ or MnCl₂, unless otherwise indicated. The reaction was initiated by the addition of ATP. After the incubation for 5 min at 37°C, unless otherwise indicated, the reaction was terminated by boiling for 3 min. Cyclic AMP formed was assayed by the method of Tovey et al. (27) as described previously (24). The compounds including nucleotides employed in experiments did not significantly affect the cyclic AMP assay. Adenylate cyclase activity was linear with time for at least 10 min. Each experiment was performed in triplicate with results differing by less than 5% and repeated at least twice using different enzyme preparations. Data shown are the means of triplicate determinations of one representative experiment, unless otherwise indicated. The Student’s t-test was used for the statistical analysis.

Protein concentration was determined by the method of Bradford (28) using bovine gamma globulin as a standard.

Chemicals: Dopamine HCl was purchased from Wako Pure Chemical Ind. Morphine HCl was purchased from Takeda Chemicals. ATP (A 2383) and GTP were from Sigma Chemicals. Gpp(NH)p was obtained from P-L Biochemicals Inc. Lubrol-PX was from Nakarai Chemicals. Other chemicals were all of reagent grade.

RESULTS

Membrane-bound adenylate cyclase: Figure 1 shows that adenylate cyclase in the crude synaptosomal lysates was active in the presence of either Mg²⁺ or Mn²⁺. Since relatively high activities were obtained in the presence of either 4.8 mM MgCl₂ or MnCl₂, we used this concentration of the divalent cations exclusively in the following experiments.

In the presence of 4.8 mM MgCl₂, both GTP and Gpp(NH)p showed biphasic effects on adenylate cyclase activity in the lysates (Fig. 2). The K₀.₅ value for the GTP stimulation of the cyclase was 47 nM, and the value for the GTP inhibition was 4.5 µM. The effects of dopamine and morphine, which modulate adenylate cyclase activity through their receptors (24, 29), on the biphasic actions of these guanine nucleotides were studied. As shown in Fig. 2, 100 nM dopamine enhanced the stimulatory actions of these nucleotides. The K₀.₅ value for the GTP stimulation of adenylate cyclase was not altered, whereas the maximal stimulation by GTP was increased from 183±10% (mean±S.E.M., the number of separate experiments (n)=4) to 233±13% (n=4, P<0.05) by the addition of dopamine. Similar results were obtained with Gpp(NH)p as a substituent of GTP (Fig. 2). Dopamine did not affect the inhibitory actions of these nucleotides on the adenylate cyclase activity. Ten µM mor-
Fig. 2. Biphasic effects of GTP and Gpp(NH)p on membrane-bound adenylate cyclase activity in the presence of Mg$^{2+}$. Adenylate cyclase activity in the crude synaptosomal lysates of rat caudate nucleus was assayed in the presence of 4.8 mM MgCl$_2$ as described in the text. The assay was performed in the presence of increasing concentrations of GTP (left) or Gpp(NH)p (right) (●), plus 100 μM dopamine (∆) or 10 μM morphine (◇). Data shown are the means±S.E.M. of four experiments. Adenylate cyclase activity was expressed as a percentage of the control activity (176±12 pmol/mg protein/min). *: differed from the presence of only GTP or Gpp(NH)p (P<0.05, Student’s paired t-test).

Phrine enhanced the inhibitory action of GTP by reducing the $K_{0.5}$ value for the GTP inhibition from 4.5 μM to 2.7 μM and by increasing the maximal GTP inhibition from 29±2% (n=4) to 39±3% (n=4) (P<0.05). The Gpp(NH)p inhibition of adenylate cyclase was also enhanced slightly by morphine (Fig. 2). Morphine did not alter the stimulatory actions of these nucleotides. Thus, dopamine selectively enhanced the stimulatory actions of these guanine nucleotides, whereas morphine selectively enhanced their inhibitory actions.

When 4.8 mM MgCl$_2$ was replaced by the same concentration of MnCl$_2$, the adenylate cyclase activity in the lysates increased by approximately 200% (Fig. 1). Figure 3 shows the effects of GTP and Gpp(NH)p on the adenylate cyclase activity in the presence of Mn$^{2+}$. Mn$^{2+}$ completely abolished the inhibitory actions of these guanine nucleotides, but their stimulatory effects were still observed. Under these conditions where the GTP inhibition was eliminated, 10 μM morphine no longer inhibited adenylate cyclase. Thus, Mn$^{2+}$ impaired the function of the adenylate cyclase system that is responsible for the inhibition by guanine nucleotides and morphine, without disturbing the catalytic activity. Whether GTP was present or not, the addition of 100 μM dopamine resulted in inhibition of the adenylate cyclase activity in the presence of Mn$^{2+}$. It seems likely that dopamine was rapidly oxidized in the presence of the transitional metal Mn$^{2+}$. The oxidation product of dopamine might be responsible
Fig. 3. Effects of GTP and Gpp(NH)p on membrane-bound adenylate cyclase activity in the presence of Mn$^{2+}$. Adenylate cyclase activity in the crude synaptosomal lysates of rat caudate nucleus was assayed in the presence of 4.8 mM MnCl$_2$ with the indicated concentrations of GTP or Gpp(NH)p as described in the text. The assay mixture contained GTP (○), GTP plus 100 μM dopamine (△), GTP plus 10 μM morphine (●), or Gpp(NH)p (□).

Fig. 4. Effects of MgCl$_2$ and MnCl$_2$ on Lubrol-solubilized adenylate cyclase activity. Adenylate cyclase activity in the Lubrol-solubilized preparation of the crude synaptosomal lysates of rat caudate nucleus was assayed in the presence of the indicated concentrations of MgCl$_2$ (●) or MnCl$_2$ (○).

For the inhibition of adenylate cyclase mentioned above.

Lubrol-solubilized adenylate cyclase: To examine whether guanine nucleotides directly inhibit the catalytic unit or not, we studied the effects of GTP and Gpp(NH)p on the activity of Lubrol-solubilized adenylate cyclase. Lubrol-PX, when it was added to the lysate suspension, inhibited adenylate cyclase by 60% in the presence of 4.8 mM MgCl$_2$; but it activated the cyclase threefold in the presence of 4.8 mM MnCl$_2$. The 40% of Mg$^{2+}$-dependent and the 80% of Mn$^{2+}$-dependent activities were recovered as the solubilized adenylate cyclase. As shown in Fig. 4, the Lubrol-solubilized adenylate cyclase showed very low activities in the presence of Mg$^{2+}$, but it was still active in the presence of Mn$^{2+}$. The Lubrol-solubilized adenylate cyclase was applied on the Sepharose 6B column. Figure 5 shows the elution profile of adenylate cyclase activity from the column. The Mn$^{2+}$-dependent activity eluted as a single peak. The pooled peak fractions showed negligible adenylate cyclase activity in the presence of Mg$^{2+}$. Both GTP and Gpp(NH)p failed to activate adenylate cyclase in the pooled peak fractions in the presence of either 4.8 mM MgCl$_2$ or MnCl$_2$ (Fig. 6). Since the stimulation of adenylate cyclase by Gpp(NH)p is quasi-reversible, the extent of the Gpp(NH)p stimulation depends on the duration of incubation. Even after the preincubation at 23°C for 2 hr, however, Gpp(NH)p (10 nM-100 μM) did not show any effect on the
adenylate cyclase activity in the pooled peak fractions in the presence of either Mg$^{2+}$ or Mn$^{2+}$ (data not shown). The activation of adenylate cyclase by 10 mM F$^-$ was also abolished in the pooled peak fractions (Table 1). Thus, the adenylate cyclase activity eluted from the column was dependent on Mn$^{2+}$ and completely insensitive to the activation by either guanine nucleotides or F$^-$. As shown in Fig. 6, both GTP and Gpp

Table 1. Activation of adenylate cyclase by F$^-$. 

| Enzyme preparation                        | Fold activation by F$^-$ with Mg$^{2+}$ | Fold activation by F$^-$ with Mn$^{2+}$ |
|-------------------------------------------|----------------------------------------|----------------------------------------|
| Membrane-bound adenylate cyclase          | 7.1±0.8$^{1)$                           | 3.0±0.3$^{2)}$                         |
| Pooled peak fractions of Lubrol-solubilized adenylate cyclase | 1.0±0.2$^{3)$                           | 1.0±0.1$^{4)$                         |

Adenylate cyclase activity in the crude synaptosomal lysates and in the pooled peak fractions of the Lubrol-solubilized preparation was assayed in the presence of 4.8 mM MgCl$_2$ or 4.8 mM MnCl$_2$ with or without 10 mM NaF. Data expressed as the fold activation by F$^-$ are the means±S.E.M. of three experiments. The control activities without F$^-$ were $^{1)}$184±15, $^{2)}$405±35, $^{3)}$8±3 and $^{4)}$993±63 pmol/mg protein/min.
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Fig. 6. Effects of GTP and Gpp(NH)p on adenylate cyclase activity in the pooled peak fractions. Adenylate cyclase activity in the pooled peak fractions of the eluate from the Sepharose 6B column was assayed in the presence of 4.8 mM MgCl₂ (left) or 4.8 mM MnCl₂ (right) with the indicated concentrations of GTP (○) or Gpp(NH)p (□).

(NH)p could no longer inhibit adenylate cyclase in the pooled peak fractions in the presence of either Mg²⁺ or Mn²⁺.

DISCUSSION

GTP has been reported to exert not only a stimulatory but also an inhibitory (11, 12) or biphasic effect (14, 15, 30) on basal adenylate cyclase activity in other systems. Hydrolysis-resistant Gpp(NH)p has been known to strongly activate adenylate cyclase. The inhibitory or biphasic action of Gpp(NH)p on adenylate cyclase activity, however, has been recently shown in human platelets (11, 31), rat fat cells (32), and Chinese hamster ovary cells (33). In this paper, we demonstrated that both GTP and Gpp(NH)p had the biphasic effect on the activity of the synaptosomal membrane-bound adenylate cyclase of the rat caudate nucleus in the presence of Mg²⁺. Morphine enhanced the Mg²⁺-dependent inhibitory actions of these guanine nucleotides, but it did not affect the stimulatory action of GTP in the presence of Mg²⁺ or Mn²⁺. When the GTP inhibition of the membrane-bound adenylate cyclase was abolished by Mn²⁺, morphine no longer inhibited adenylate cyclase. Since Mn²⁺ rather increases the binding of [³H]dihydromorphine to opioid receptors in the rat brain (34, 35), it is unlikely that Mn²⁺ eliminates the morphine inhibition of adenylate cyclase by disturbing the binding of morphine to the receptors. Therefore, it is conceivable that Mn²⁺ functionally uncouples the opioid-inhibitable adenylate cyclase system in the synaptosomes. These results support our previous suggestions that the Mg²⁺-dependent inhibitory action of GTP on adenylate cyclase activity is responsible for the opioid inhibition of the synaptosomal enzyme and that opioids inhibit adenylate cyclase by enhancing the GTP inhibition of the cyclase (24). These suggestions are also supported by the observations in other systems that high inhibitory concentrations
(≥0.1–1 μM) of GTP are required for the inhibition of adenylate cyclase by α-adrenergic agonists in neuroblastoma-glioma hybrid cells (NG 108–15 cells) (15) and human platelets (11) and by adenosine analogs in rat adipocytes (13, 14) and rat cerebral cortical membranes (16).

Biochemical and genetic studies are revealing the molecular basis of the GTP activation of adenylate cyclase, which involves the G protein (2). However, little is known about the mechanism of the GTP inhibition of adenylate cyclase. In the present study, we investigated whether GTP directly inhibits the catalytic unit or not. Judging from both the requirement of Mn²⁺ for the catalytic activity and the insensitivity to the activation by either guanine nucleotides or F⁻ (6, 22, 23), the active catalytic unit was completely separated from the G protein activity by Sepharose 6B gel filtration. The inhibitory actions of GTP and of Gpp(NH)p were abolished in the separated catalytic unit. These results provide evidence that a structure(s) distinct from the catalytic site of adenylate cyclase is involved in the GTP inhibition of the synaptosomal adenylate cyclase. From our results and the findings that the binding of opioid agonists to brain opioid receptors is also regulated by GTP (35, 36), it is conceivable that GTP both regulates the opioid receptor and inhibits the catalytic unit of adenylate cyclase through a component discrete from both the receptor and the catalytic unit.

An inhibitory guanine nucleotide regulatory component distinct from the stimulatory G protein has been proposed in rat adipocytes on the basis of differential sensitivities of the GTP stimulatory and inhibitory action to Mn²⁺ (14), organic mercurials (14), trypsin digestion (18), and radiation inactivation (37). Our present results also show that Mn²⁺ reduced much more the GTP inhibition than the GTP stimulation of the synaptosomal adenylate cyclase. However, since the mechanisms underlying the differential modulations of the GTP stimulation and inhibition by these treatments are not known, these findings can not eliminate the possibility that GTP exerts both the stimulatory and inhibitory actions through a single population of G protein. One of the possible mechanisms mediating the GTP biphasic action through a single G protein is the hydrolysis of GTP to inactive or less active GDP by the GTPase associated with the G protein (38, 39). Assuming that high concentrations of GTP activate the GTPase leaving GDP bound to the G protein, the inactive or less active form of the G protein may accumulate in the presence of the high concentrations of GTP. This accumulation of the inactive or less active G protein may result in the inhibition of adenylate cyclase. The possibility that GTP and opioids inhibit adenylate cyclase by stimulating the GTPase has been recently suggested in NG 108–15 cells (40). However, since GTPase-resistant Gpp(NH)p also inhibited the synaptosomal adenylate cyclase, our results are inconsistent with the possibility mentioned above.

Elucidation of the mechanisms by which GTP exerts multiple regulatory actions on receptor-adenylate cyclase systems is essential to the understanding of the regulation of adenylate cyclase activity by hormones or neurotransmitters. Further study is required to clarify more fully the molecular basis of the GTP biphasic action on adenylate cyclase activity. The Lubrol-solubilized adenylate cyclase presented in this report may provide a useful tool for the investigation of functions of components in adenylate cyclase systems, and it is a material which can be used for further purification of the catalytic unit of the cyclase.

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