INHIBITION OF ADENYLATED CYCLASE BY GTP AND ITS MODULATION BY OPIATE RECEPTOR IN RAT CAUDATE NUCLEUS

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Abstract—Morphine inhibited the adenylate cyclase activity of the crude synaptosomal fraction of the rat caudate nucleus in the presence of GTP, GDP, Gpp(NH)p or ITP. The purine nucleotides themselves had an inhibitory action on the enzyme. β-Endorphin and Met-enkephalin also inhibited the enzyme in the presence of GTP. The GTP-dependent inhibitory action of morphine was blocked by naloxone. Various opiates and opioid peptides inhibited the enzyme by up to approximately 20 per cent in the presence of GTP. The relative potency was in higher order of levorphanol > β-endorphin > Met-enkephalin > morphine > pentazocine. Levorphanol was about 50,000 times as potent as its biologically inactive enantiomer, dextrophan. Morphine enhanced the inhibitory actions of GTP and GTPase-resistant Gpp(NH)p on the adenylate cyclase activity. These results suggest that GTP plays an important role in the regulation of adenylate cyclase activity in the rat caudate nucleus and that the occupation of opiate receptor by agonists inhibits the enzyme through an actual increase in the inhibitory action of GTP, rather than a suppression of the enzymatic degradation of GTP.

Opiates have been reported to inhibit the basal, PGE1-stimulated or dopamine-stimulated adenylate cyclase activity in many brain preparations (1-6). However, there are negative observations concerning the inhibitory effect of opiates on brain adenylate cyclase (7-10). It is well documented that opiates and opioid peptides inhibit basal and PGE1-stimulated adenylate cyclase activities in neuroblastoma-glioma hybrid cells (NG108-15) (11-15). Guanosine 5'-triphosphate (GTP) which has a slight stimulatory effect on the adenylate cyclase activity is essential to the stimulation of the enzyme by hormones. Hormones enhance the stimulatory effect of GTP, resulting in an increase of the enzyme activity (16). GTP is reported to reduce the binding of several hormones to receptors (17). These investigations into the roles of GTP in hormonal stimulation of adenylate cyclase have facilitated studies of the mechanism of hormonal inhibition of the enzyme. Blume and coworkers have reported recently that guanine nucleotides reduce the binding of opiates to opiate receptors and that opiates and opioid peptides require GTP for the inhibition of adenylate cyclase in NG108-15 cells (18-20). We also found that GTP decreases the specific binding of opiate
agonists to membranes of the rat caudate nucleus (unpublished). In this paper we will show that the binding of opiate agonists to receptors enhances the inhibitory action of GTP on the adenylate cyclase activity to result in inhibition of the enzyme.

MATERIALS AND METHODS

Tissue preparation: After the decapitation of male Wister rats (200–250 g), caudate nuclei were rapidly removed and homogenized in 20 vol. (w/v) of ice-cold 0.32 M sucrose. The homogenate was centrifuged at 1,000×g for 10 min (4°C) and the supernatant was centrifuged again at 10,000×g for 30 min (4°C). The supernatant was discarded and the pelleted crude synaptosomal fraction was suspended in ice-cold 1 mM EGTA, 5 mM Tris-HCl, pH 7.4. This suspension was used immediately for adenylate cyclase assay.

Adenylate cyclase assay: Adenylate cyclase activity was assayed by a modification of the method of Clement-Cormier et al. (21) in the final 0.5 ml of reaction mixture containing 0.12 mM ATP, 4.8 mM MgCl₂, 1.2 mM 3-isobutyl 1-methylxanthine, 0.1 mM EGTA and 45.5 mM Tris-HCl, pH 7.4. The crude synaptosomal suspension (75–100 μg protein) was added to the reaction medium lacking ATP and MgCl₂ with other additions (opiate, GTP etc.) and equilibrated at 0°C for 10 min. After initiating the reaction by the addition of ATP and MgCl₂, the mixture was incubated at 37°C for 5 min. The reaction was terminated by denaturing the enzyme in boiling water for 3 min. Protein precipitates were removed by centrifugation (2,000×g, 5 min), and the supernatant was diluted with 10 mM EDTA, 50 mM Tris-HCl, pH 7.4. The concentration of cyclic AMP in the diluted supernatant was determined by the method of Tovey et al. (22) in duplicate which differed by less than 3%. The compounds used (nucleotides, opiates etc.) did not interfere with the diluted cyclic AMP assay at the concentration used. Each adenylate cyclase activity was assayed in duplicate which differed by less than 5%. The enzyme activity was corrected by using a boiled preparation as an enzyme blank. Non-enzymatic formation of cyclic AMP was not detectable under these conditions. All experiments were repeated at least twice with different preparations.

The protein concentration was determined by the method of Bradford (23) using bovine gamma globulin as a standard. The concentration of Na⁺ was determined by the sodium-electrode method.

Chemicals: The following compounds were used in this experiment. Levorphanol HCl and (±)pentazocine HCl (provided by Prof. M. Hori) Dextrorphan HCl (provided by Prof. E.J. Simon) (±)Naloxone HCl (provided by Sankyo Co., Ltd.) Met-enkephalin and β-endorphin (provided by Takeda Chem. Ind., Ltd.) R(-)Morphine HCl (purchased, Takeda Chem. Ind., Ltd.) ATP (A-2383, prepared by phosphorylation of adenosine). GTP, GDP and CTP (purchased, Sigma) Gpp(NH)p and ITP (purchased, P-L Biochemicals).

RESULTS

Effects of GTP and morphine: As shown in Table 1, 10 μM GTP inhibited the adenylate cyclase activity of the crude synaptosomal fraction of the rat caudate nucleus either in the presence or absence of morphine. In the absence of GTP, morphine showed no effect on the enzyme activity, however, the enzyme activity was 17% inhibited by morphine in the presence of GTP. These results indicate that the inhibition of adenylate cyclase by morphine is dependent on GTP. The inhibition of the enzyme by β-endorphin and Met-enkephalin was also dependent on GTP (1 μM β-endorphin: 1% and 19%, 1 μM Met-enkephalin: 2% and 21% inhibition,
Table 1. Effects of GTP and morphine on adenylate cyclase activity of rat caudate nucleus

The crude synaptosomal fraction of the rat caudate nucleus was incubated at 37°C for 5 min as described in the text. The concentration of GTP and morphine was 10 μM.

| Addition       | Adenylate cyclase activity (pmol/mg protein/min) |
|----------------|--------------------------------------------------|
| None           | 124±20                                           |
| GTP            | 101±9*                                           |
| Morphine       | 123±20                                           |
|                | 84±10***                                         |

Data represent means±SD of five separate experiments. *: differed from the absence of GTP (P<0.025). **: differed from the absence of GTP (P<0.01). +: differed from the absence of morphine (P<0.01). Student's paired t-test.

Effects of opiates and opioid peptides: In the presence of GTP, various opiates and opioid peptides inhibited adenylate cyclase in a dose-dependent manner (Fig. 2). The maximal inhibition by each agent was much the same in degree (≈20%). The concentration of these agents required for half-maximal inhibition (IC50), and Hill coefficients of these inhibitions are summarized in Table 2. Levorphanol was the most potent inhibitor tested with an IC50 of 0.2 nM, approximately 50,000 times more potent than its biologically inactive enantiomer, dextrorphan. This suggests that these opiates inhibit the enzyme stereospecifically. As shown in Fig. 3, naloxone, an opiate antagonist, blocked the GTP-dependent inhibition by morphine, while naloxone itself did not affect the enzyme activity. All these results strongly suggest that occupation of the opiate receptors by agonists is coupled to the inhibition of adenylate cyclase activity in the rat caudate nucleus. Table 3 shows the effects of levorphanol, β-endorphin and Met-enkephalin on the GTP-dependent inhibition of adenylate cyclase by morphine. Each of these compounds, including morphine, at the concentration of 0.1 μM, showed maximal inhibition (≈20%) of the enzyme. These agents exhibited no additive effect on the maximal inhibitory action of morphine, therefore, it is conceivable that the same population of adenylate cyclase molecules or molecules regulating the enzyme is affected.

Effects of nucleotides, NaF and morphine: The effects of GTP at various concentrations with and without morphine on the adenylate cyclase activity are shown in Fig. 4. GTP partially inhibited the enzyme and this
Inhibitory effect was enhanced by morphine. The concentration of GTP required for half-maximal inhibition (5 μM) could be decreased to 0.8 μM by the addition of 10 μM morphine. The maximal inhibitory action of GTP on the enzyme was increased to 35% from 25% by morphine. Thus, morphine intensified the action of GTP, resulting in inhibition of adenylate cyclase. This inhibition was dependent on the concentration of GTP (apparent Km of 0.2 μM for GTP). The inhibitory action of morphine decreased in the presence of GTP at high concentrations (>10 μM). As shown in Table 4, several nucleotides inhibited the adenylate cyclase activity; the relative inhibitory potencies of these nucleotides on the enzyme were in order of Gpp(NH)p > GDP > GTP = GDP > GDP irrespective of the presence of morphine. Morphine induced an increase in the inhibition of the enzyme by guanyl 5′-yl imidodiphosphate [Gpp(NH)p], a GTPase-resistant analog of GTP. Therefore, it seems that the hydrolysis of the terminal (γ) phosphate bond of guanine nucleotides by GTPase is independent of the inhibition of adenylate cyclase by these nucleotides and the action of morphine on the adenylate

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![Graph](image-url)

**Fig. 2.** Effects of opiates and opioid peptides on adenylate cyclase activity. Adenylate cyclase activity was assayed in the presence of 1 μM GTP. The adenylate cyclase activity without opioid compounds in the presence of 1 μM GTP was expressed as 100%. Each point is the mean of duplicates of one representative experiment.

**Table 2.** Concentrations of opioid compounds required for half-maximal inhibition of adenylate cyclase and Hill coefficients of the inhibition

| Compound        | IC50 (nM) | nH |
|-----------------|-----------|----|
| Levorphanol     | 0.2       | 0.8|
| β-endorphin     | 0.7       | 1.0|
| Met-enkephalin  | 2         | 0.7|
| (-) Morphine    | 3         | 0.7|
| (+) Pentazocine | 9         | 0.5|
| Dextrophan      | ≥10,000   | 0.6|

Adenylate cyclase activity was assayed at 37°C for 5 min in the presence of 1 μM GTP. IC50 represents the concentration of compound required for half-maximal inhibition. nH represents Hill coefficient of the inhibition.
cyclase activity. NaF (10 mM) stimulated the adenylate cyclase activity about three-fold. Inhibition of the F- stimulated enzyme by morphine was not apparent.

Effects of NaCl: Since Na+ reduces the binding of opiate agonists to receptors independently of the presence of GTP (24), we examined the effect of Na+ on the GTP-dependent inhibitory action of morphine on the adenylate cyclase activity. As shown in Table 5, the activity of adenylate cyclase was increased exclusively by the addition of NaCl in a dose-dependent manner. In the absence of GTP, morphine did not inhibit the Na+-stimulated enzyme activity. In the presence of 10 µM GTP, NaCl had no

![Fig. 3. Effect of naloxone on the inhibition of adenylate cyclase by morphine. The crude synaptosomal fraction of the rat caudate nucleus was incubated in the presence of 10 µM GTP with (■) or without (○) 10 µM morphine. Each point is the mean of duplicates of one representative experiment.](image1)

![Fig. 4. Effects of GTP and morphine on adenylate cyclase activity. Assay was performed in the presence (■) or absence (○) of 10 µM morphine with indicated concentrations of GTP. △ represents per cent inhibition of adenylate cyclase activity by 10 µM morphine. Each point is the mean of duplicates of one representative experiment.](image2)

Table 3. Effects of levorphanol, β-endorphin and Met-enkephalin on inhibition of adenylate cyclase by morphine

| Compound        | Adenylate cyclase activity (pmol/mg protein/min) | % Inhibition by Morphine |
|-----------------|-----------------------------------------------|--------------------------|
| None            | 129 ± 2                                         | 100                      |
| Levorphanol     | 128 ± 2                                         | 82                       |
| β-endorphin     | 127 ± 2                                         | 81                       |
| Met-enkephalin  | 126 ± 2                                         | 81                       |

Assay was performed in the presence of 10 µM GTP. The concentration of each opioid compound was 0.1 µM. The control activity (100%) was 129 pmol/mg protein/min. Data are the means of duplicates of one representative experiment.
Table 4. Effects of nucleotides and NaF on adenylate cyclase activity in the presence or absence of morphine

| Addition | Adenylate cyclase activity (pmol/mg protein/min) | % Inhibition by morphine |
|----------|-----------------------------------------------|--------------------------|
| None     | 108±7                                         | 105±8                    | NS           |
| GTP      | 88±3*                                         | 72±3**                   | 18           |
| GDP      | 88±3*                                         | 71±3**                   | 19           |
| Gpp(NH)p | 75±5*                                         | 66±2**                   | 12           |
| ITP      | 96±4*                                         | 85±3**                   | 11           |
| CTP      | 104±6                                         | 98±5                     | NS           |
| NaF      | 317±17                                        | 318±20                   | NS           |

The concentration of each nucleotide and morphine was 10 μM. The concentration of NaF was 10 mM. GDP: guanosine 5'-diphosphate, Gpp(NH)p: guanyl 5'-yl imidodiphosphate, ITP: inosine 5'-triphosphate, CTP: cytidine 5'-triphosphate. Each value is the mean±SD of four determinations of two experiments. *: differed from the absence of nucleotide (P<0.05). **: differed from the absence of morphine (P<0.05). NS: not significantly differed from the absence of morphine. Student's t-test.

Table 5. Effects of NaCl on the inhibition of adenylate cyclase by morphine

| NaCl added (mM) | Adenylate cyclase activity (pmol/mg protein/min) | % Inhibition by morphine |
|-----------------|-----------------------------------------------|--------------------------|
|                 | with GTP                                       | without GTP               |
|                 | Morphine % Inhibition by morphine              | Morphine % Inhibition by morphine |
| 0               | 110                                           | 138                       | 138                      | 1                       |
| 10              | 108                                           | 140                       | 140                      | 0                       |
| 20              | 119                                           | 145                       | 143                      | 1                       |
| 50              | 131                                           | 152                       | 156                      | 2                       |
| 100             | 166                                           | 165                       | 169                      | 2                       |
| 200             | 187                                           | 193                       | 189                      | 2                       |

The crude synaptosomal fraction of the rat caudate nucleus was equilibrated with or without 10 μM GTP and various concentrations of NaCl in the presence and absence of 10 μM morphine at 0°C for 10 min. When NaCl was not added, the Na+ concentration of the mixture was less than 1 mM. Adenylate cyclase activity was assayed at 37°C for 5 min. Data are the means of duplicates of one representative experiment.

marked influence on the GTP-dependent inhibitory action of morphine.

**DISCUSSION**

The stimulation of adenylate cyclase by hormones and neurotransmitters is generated by at least three distinct membrane components including the receptor, catalytic unit and a protein, referred to here as G component, which mediates the multiple regulatory effects of guanine nucleotides. G component is responsible for the stimulation of adenylate cyclase by guanine nucleotides and by F-(25, 26) and is closely related to GTPase activity (27). Hormonal stimulation of the enzyme activity appears to be the result of stimulation of the interaction between GTP and G component (28, 29). However, little is known of the mechanism of hormonal inhibition of adenylate cyclase.
The binding of opiate agonists to opiate receptors resulted in inhibition of the adenylate cyclase activity in the rat caudate nucleus. GTP was required for the inhibition by opioids. Taken together with the fact that GTP reduces the receptor binding of opioids (18, 19), GTP is probably essential for the coupling of opiate receptors to adenylate cyclase. Hill coefficients of the enzyme inhibition for the compounds examined were equal to or smaller than 1. In NG108-15 cells, the coefficient is reportedly larger than or equal to 1 (11, 20). These ligands are considered to act indirectly on adenylate cyclase through opiate receptors. The exact meaning of these Hill coefficients remains obscure unless the interactions of all molecules involved in the enzyme inhibition are elucidated.

In NG108-15 cells Na⁺ is also required for the inhibitory action of D-Ala-Met-enkephalinamide on adenylate cyclase and enhances the GTP-dependent inhibitory action of the peptide (20). In the rat caudate nucleus, Na⁺ itself was not required for the action of morphine and had no marked influence on the maximal GTP-dependent inhibitory action of morphine. Why Na⁺ was not required is unknown, however, the impurity of our preparation which consisted of heterogeneous neurons might make obscure the enhancing effect of Na⁺.

Purine nucleotides such as guanine and inosine nucleotides were more effective in the coupling of opiate receptors to adenylate cyclase than pyrimidine, CTP (Table 4). These results are compatible with the specificity for purine nucleotides reported by Blume and coworkers regarding the coupling in NG108-15 cells (20) and in the inhibition of opiate receptor binding by nucleotides (18). These nucleotide specificities in the coupling of opiate receptors are similar to those in the coupling of glucagon receptor to adenylate cyclase which is stimulated by glucagon (30). In the latter case, GTP is also known to reduce the receptor binding of glucagon (31). Thus, there is some analogy between opiate receptors and other hormonal receptors with regard the regulatory effects of guanine nucleotides. The analogy suggests that a similar mechanism is involved in the stimulatory and inhibitory couplings of receptors to adenylate cyclase.

Guanine nucleotides inhibited adenylate cyclase in the rat caudate nucleus. This finding has been verified by other workers (9, 10, 21). Clement-Cormier et al. (21) reported that the inhibitory action of GTP on the enzyme activity was independent of the concentration of substrate, ATP. Competitive inhibition, therefore, does not seem to be a suitable mechanism for the enzyme inhibition by GTP. The chelation of Mg²⁺ by guanine nucleotides hardly would explain the mechanism of the inhibition for the following two reasons: 1) the concentration of MgCl₂ used was fairly high compared to concentrations of nucleotides, 2) GDP which has about one-tenth of the chelating activity of GTP for divalent cations (32) was similar to GTP in inhibitory activity against adenylate cyclase. Morphine enhanced the inhibitory action of GTP on the adenylate cyclase activity by reducing the GTP concentration required for half-maximal inhibition and by increasing the maximal inhibition. The enhancing effects of morphine on the maximal inhibitory activity of GTP and the inhibitory activity of GTPase-resistant Gpp(NH)₃ can hardly be explained by the possibility that morphine elicits an apparent increase in the concentrations of guanine nucleotides by inhibiting GTPase. Therefore, it appears that the occupation of opiate receptors by agonists results in inhibition of adenylate cyclase by enhancing the inhibitory effect of GTP on the enzyme. Havemann and Kuschinsky (6)
demonstrated that morphine inhibited the basal activity of the enzyme in the rat striatum, but other workers (8, 9) reported that such action was not seen with morphine. The discrepancy in the action of morphine may partly be attributed to the GTP-dependency of the inhibitory action of morphine. In our results, not only low concentrations (<1 μM) but also high concentrations (>10 μM) of GTP reduced the inhibitory effect of morphine on the enzyme activity (Fig. 4). The concentration of GTP in the assay mixture without additional GTP depends on the endogenous GTP and on the contaminated GTP in commonly used ATP (33). Using a low concentration of synthetic ATP, we could lower the GTP concentration in the assay mixture to the extent that the effect of added 0.1 μM GTP was evident in the presence of morphine.

GTP stimulates adenylate cyclase of diverse tissues through the G component. Recent studies revealed the fact that guanine nucleotides inhibit the enzyme activity in fat cells (34), platelets (35), NG108-15 cells (36) and ovaries (37). Available evidence indicates that α-adrenergic agents and opioid compounds exert GTP-dependent inhibitory actions on adenylate cyclase present in both platelets (35, 38) and NG108–15 cells (36) and NG108-15 cells (20) respectively. Cooper et al. showed that the inhibition of fat cell adenylate cyclase by acenoline analogs is attributable to the inhibitory action of GTP on the enzyme activity (39). Rodbell and coworkers and Steer et al. have recently proposed that the inhibitory G component is distinct from the common stimulatory G component (17, 35). Apart from the direct evidence for the theory of two G components, our results together with the observations by other workers suggests that the inhibitory action of the GTP-G component complex on adenylate cyclase activity is implicated in the inhibition of the enzyme by hormones and neurotransmitters.

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