MORPHINE-INDUCED CHANGES IN CYCLIC AMP METABOLISM AND PROTEIN KINASE ACTIVITY IN THE BRAIN

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Abstract—The effects of consecutive oral administration of morphine on the cyclic AMP synthesizing system and cyclic AMP dependent protein kinase activity in the cerebral cortex of mice were examined. The administration of morphine (2-4 weeks) induced an increase of the cyclic AMP formation by activating adenylate cyclase, whereas responses of the cyclic AMP synthesizing system to biogenic amines (norepinephrine, dopamine and histamine) added in vitro was found to be significantly attenuated in these animals. Cyclic AMP dependent protein kinase activity in the cerebral cortex was also increased following a consecutive oral administration of morphine. These changes in the activities of adenylate cyclase and protein kinase were found mainly in crude mitochondrial and/or synaptosomal fractions. Morphine induced decrease in the response of the cyclic AMP synthesizing system to biogenic amines was rapidly reversed, and a significant increase of the cyclic AMP formation in the presence of added norepinephrine compared with that found in morphinized animals was observed following the administration of levallorphan, a narcotic antagonist. On the other hand, the changes in adenylate cyclase and cyclic AMP dependent protein kinase activities were not affected significantly by levallorphan administration. These results suggest that alterations in activities of cyclic AMP synthesizing system and of cyclic AMP dependent protein kinase may be involved in processes of the formation of morphine dependence. Possible involvement of abrupt increments in the sensitivity of “norepinephrine receptor-adenylate cyclase” system and a subsequent increase in cerebral cyclic AMP is also suggested as a cause of morphine withdrawal syndrome.

It is well known that adenosine 3',5'-monophosphate (cyclic AMP) acts as an intracellular mediator of the action of various biogenic amines and polypeptide hormones (1), and may play an important role in the function of the central nervous system (2). Recently, it has also been suggested that cyclic AMP-dependent protein kinase, which is selectively stimulated by a low concentration of cyclic AMP, may play an important role in regulating cell functions (3).

We reported previously that consecutive administrations of morphine lead to an increase in the activity of adenylate cyclase without affecting phosphodiesterase activity in the mouse cerebral cortex (4). Similarly, it has been reported recently that alterations in the cyclic AMP-adenylate cyclase system may be involved in the occurrence of narcotic dependence (5-11). Pharmacological significance of these changes in morphine abstinence, however, was not fully examined. Collier and Francis (8) found that oral administration of drugs
inhibiting phosphodiesterase as well as intracerebroventricular injection of cyclic AMP intensifies the expression of morphine abstinence and suggested the association of increased brain cyclic AMP with morphine abstinence. Similarly, a direct association of brain cyclic AMP content and the intensity of the precipitated abstinence syndrome in morphine-dependent rats was reported (12). In contrast, Singhal et al. (10) reported that withdrawal of morphine treatment from addicted rats significantly decreased the activity of cerebral adenylate cyclase without affecting that of phosphodiesterase. These results strongly suggest that the increased brain cyclic AMP during morphine abstinence (8, 12) is not explainable by changes in activities of adenylate cyclase and phosphodiesterase, enzymes responsible for the formation and degradation of cerebral cyclic AMP respectively.

The present investigation was designed to examine alterations in the response of the cerebral adenylate cyclase system to biogenic amines as a possible cause of the increased brain cyclic AMP during morphine abstinence. In addition, the effect of consecutive administrations and subsequent withdrawal of morphine on the synaptosomal cyclic AMP dependent protein kinase activity was also evaluated to determine whether or not changes in the metabolism of cyclic AMP may affect the activity of this enzyme.

MATERIALS AND METHODS

Male albino mice weighing 25-30 g were used except for the experiments examining the effect of adrenalectomy on morphine-induced changes in cerebral adenylate cyclase activity. In latter experiments, adrenalectomized and sham operated male Wistar rats weighing 150-200 g were used. These animals were maintained in a thermally regulated room. In acute experiments, animals were sacrificed 90 min after the intraperitoneal injection of morphine (morphine hydrochloride, 25 mg/kg). In chronic experiments, morphine was administered continuously for 1-4 weeks in a liquid diet method as previously described by Shuster et al (13). The daily dose of morphine hydrochloride was 55-98 mg/kg. Control animals were subjected to the same experimental conditions without morphine administration. The assessment of the antinociceptive effect of morphine was determined by a hot plate method (31). The test was carried out immediately before and 60 min after the intraperitoneal injection of morphine hydrochloride (10 mg/kg).

Assay of adenylate cyclase activity: Adenylate cyclase activity in crude mitochondrial (P2) fraction from the cerebral cortex separated by the procedure of Gray and Whittaker (14) was measured as described by Krishna et al (15). The incubation medium contained 40 mM Tris (hydroxymethyl) aminomethane (Sigma)-HCl buffer (pH 7.3), 3.3 mM MgSO4, 0.5 mM isobutylmethylxanthine, 20 mM theophylline, 1 mM [3H]-ATP (specific activity: 26 mCi/mmol) and the enzyme preparation (1-2 mg of crude mitochondrial (P2) protein) in a final volume of 1.8 ml. Incubations were carried out at 37°C for 5 min and were terminated by immersing test tubes in a boiling water bath for 2 min after the addition of 0.3 ml of the solution of carrier cyclic AMP (1.5 mg). Recoveries of the carrier cyclic AMP in all incubations were determined spectrophotometrically by measuring the absorption at 260 nm after chromatographic separation and ZnSO4-Ba(OH)2 precipitation (15), and used to correct
the experimental value of [3H]-cyclic AMP formed in each of these fractions. The radioactivity of [3H]-cyclic AMP formed was measured using a Packard liquid scintillation spectrometer (model 3375) after separation by column chromatography (Dowex 50W x 4, 200-400 mesh, 0.4 × 3.3 cm).

Measurement of cyclic AMP formation in cerebral cortical slices: The formation of cyclic AMP in slices from cerebral cortex was measured by a modification of the method of Shimizu et al (16). Following the pulse labeling of 14C-ATP in slices from the cerebral cortex by incubating for 40 min with the Krebs-Ringer bicarbonate solution containing 2.4-2.8 μM of [14C]-8-adenine (specific activity 60 mCi/mmol), the slices from both morphine-treated and control animals were transferred respectively to flasks containing the Krebs-Ringer Tris HCl (pH 7.4) solution and incubated in the presence or absence of various drugs. Twenty mM of theophylline and 0.5 mM isobutylmethylxanthine were added to prevent the metabolic degradation of formed [14C]-cyclic AMP by phosphodiesterase. Following 5 min incubation, the reaction was stopped by the addition of 1.5 mg of carrier cyclic AMP, followed by boiling for 3 min and homogenization. The homogenate was brought to near dryness by a cool air blower and the residue was suspended and centrifuged. The formed [14C]-cyclic AMP was separated by column chromatography (Dowex 50W x 4, H+ form) from other labeled nucleotides in an aliquot of the supernatant. The absorption at 260 nm of each fraction was determined after the chromatographic separation and ZnSO4-Ba(OH)2 precipitation (15), and used to correct the experimental value of [14C]-cyclic AMP formed. In preliminary experiments, it was confirmed that neither acute nor consecutive administration of morphine modify significantly the uptake of 14C-adenine as well as the rate of pulse labeling of 14C-ATP in slices from the cerebral cortex. The results were expressed as the percentage of total [14C]-compounds present as [14C]-cyclic AMP after the incubation (% conversion).

Assay of protein kinase activity: Protein kinase activity was measured according to the radiometric method of Miyamoto et al (17). The incubation medium contained 100 mM sodium acetate buffer (pH 6.0), 1 mM magnesium acetate, 10 mM sodium fluoride, 2 mM theophylline, 0.3 mM EGTA, 0.5 mg histone (Sigma Type II-A), 5 μM [γ-32P]-ATP (specific activity 0.4 Ci/mmol) and enzyme preparation (15-30 μg of particulate protein). Reaction was carried out in the presence or absence of 1 μM cyclic AMP at 30°C for 60 sec. The reaction was terminated by the addition of 2 ml of the solution containing 5% (W/V) trichloroacetic acid, 0.25% phosphotungstic acid and 0.06N sulphuric acid. After adding 0.1 ml of 0.68% (W/V) bovine serum albumin to promote the precipitation, the tubes were centrifuged. After washing the pellet twice, by dissolving in 0.1 ml of 1N NaOH and reprecipitating by the addition of 2 ml of the solution used for the termination of the reaction, the precipitate was finally dissolved in 0.1 ml of 1N NaOH. Each sample was placed in 15 ml of scintillator (consisted of 500 ml of toluene, 500 ml of ethylene glycol monoethyl ether, 4 g of 2,5-diphenyloxazol and 0.2 g of 1,4-bis [2-(5-phenyloxazoyl)]-benzene), and the radioactivity of 32P incorporated into protein was measured with a Packard Tri-Carb liquid scintillation spectrometer (model 3390). The activity of cyclic AMP-dependent protein
kinase was expressed as pmoles of $^{32}$P incorporated into histone (calculated by the difference between the rate of phosphorylation in the presence and absence of cyclic AMP) per mg protein in 1 min.

Protein content was determined by the method of Lowry et al (18).

Data analysis: Statistical analyses of results were made by application of Student’s $t$-test for paired samples. Changes were considered significant when $P$ was 0.05 or less.

Chemicals used: Morphine hydrochloride and levallorphan (Lorfan) (Takeda Chemical Co., Osaka); histamine dihydrochloride; 1-norepinephrine (1-arterenol) hydrochloride; dopamine (3-hydroxytyramine) hydrochloride (Sigma Chemical Co., St. Louis, Mo.). All dosages of morphine are expressed as the free base. Morphine and levallorphan were dissolved in isotonic saline unless otherwise stated in the methods, and injected intraperitoneally. Control animals received an equivalent volume of isotonic saline.

RESULTS

Effect of consecutive oral administration of morphine on cyclic AMP synthesis

The effect of consecutive oral administration of morphine for 1–4 weeks on adenylate cyclase activity and cyclic AMP formation in the mouse cerebral cortex measured in the absence of added biogenic amines is shown in Fig. 1. Adenylate cyclase activity as well as basal rate of cyclic AMP formation increased significantly in the cerebral cortex; these increases were statistically significant in animals given morphine for 2 and 4 weeks. In contrast, a single administration of an analgesic dose of morphine (25 mg/kg, i.p.) did not modify either adenylate cyclase activity or cyclic AMP formation in the mouse cerebral cortex. The increase of cerebral cyclic AMP formation found in consecutively morphine-treated animals was not reversed by the administration of 5 mg/kg, i.p. of levallorphan, a dose capable of inducing typical signs of morphine withdrawal in these animals, but disappeared following the discontinuance of morphine administration for 1 week (Fig. 2). It was also found that the time courses of the recovery of decreased analgesic response for

![Graph](image)

Fig. 1. Effects of consecutive oral administration of morphine on cyclic AMP formation and adenylate cyclase activity in mouse cerebral cortex. Each value is the mean±S.E. obtained from 4–7 separate experiments. $^*$P<0.01, $^{**}$P<0.05, compared with each control value.
a challenging dose of morphine (10 mg/kg) and the decrement from the enhanced cyclic AMP formation due to morphine have an inverse relationship and these changes returned simultaneously to normal ranges 8 days after discontinuation of morphine (Fig. 3). These results suggest that the observed increase in cyclic AMP synthesizing system due to morphine may be a transient phenomenon and reversed easily by discontinuance of the administration but may not be directly related to the well-known withdrawal syndrome.

Fig. 2. Effect of levallorphan and discontinuance of morphine administration on morphine induced increase of basal cyclic AMP formation in mouse cerebral cortex. Animals used in this experiment were treated continuously with morphine for 2 weeks before subjection to levallorphan treatment or the discontinuance of morphine administration. Each value represents the mean ± S.E. obtained from 4–6 separate experiments except that in experiments employing the discontinuance of morphine administration. In the latter case, the mean of 3 experiments is presented. *Levallorphan (5 mg/kg, i.p.) was given 10 minutes prior to sacrifice. **Measured at 1 week after initiation of the discontinuance of morphine administration. *P<0.01, compared with each control value.

Fig. 3. Time-course of recoveries of increased basal cyclic AMP formation and decreased analgesic response in mice following discontinuance of morphine administration. Each value in this figure represents the mean of 5 separate determinations. Morphine was given continuously for 2 weeks before subjection to discontinuance experiments. Ten mg/kg of morphine was administered i.p. on each experimental day to assess antinociceptive effects.
Effects of consecutive oral administration of morphine on sensitivity of cyclic AMP synthesizing system to biogenic amines

Effects of consecutive administration of morphine on the response of cyclic AMP synthesizing system to exogenously added biogenic amines are shown in Fig. 4. In the absence of added amines, the formation of cyclic AMP in mouse cerebral cortical slices was significantly increased by consecutive oral administrations of morphine and this increase was not antagonized by levallorphan administration. In the presence of 1 mM of norepinephrine, dopamine and histamine, by which the formations of cyclic AMP in cerebral cortical slices from control animals were increased 167, 73 and 60% respectively, such a significant increment in the formations of cyclic AMP by the consecutive morphinization was not detected. Similarly, the disappearance of accentuating effects of consecutive morphinization on the formation of cyclic AMP was observed invariably when 0.01–5 mM of norepinephrine were added (Fig. 5). In contrast, ten minutes after the administration of levallorphan when the withdrawal signs were observed in most animals, morphine induced decrease in the response of the cyclic AMP synthesizing system to norepinephrine, dopamine and histamine was rapidly reversed, and a significant increase of the cyclic AMP formation in the presence of added norepinephrine compared with morphinized animals was observed (Fig. 4). In comparison with the norepinephrine-stimulated cyclic AMP formation found in cerebral cortical slices from consecutively morphinized animals, 57% increase was induced within 10 min after the levallorphan administration. On the other hand, no such a change

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**Fig. 4.** Effect of levallorphan and discontinuance of morphine administration on morphine induced changes in sensitivity of cyclic AMP synthesizing system of mouse cerebral cortex to biogenic amines. Morphine-treated animals were given morphine continuously for 2 weeks before use in each experiment. The concentration of each amine added in vitro was 1 mM. *Levallorphan (5 mg/kg, i.p.) was given 10 minutes prior to sacrifice. **Measured at 1 week after the initiation of discontinuance of morphine administration (2 weeks). Mean ± S.E. obtained from 4–5 separate experiments is shown except experiments determining the effect of discontinuance of morphine administration. In latter experiments, averages obtained from 3 separate experiments are presented. *P<0.01, compared with each control value. **P<0.01, compared with the value obtained in consecutively morphinized animals.
in cyclic AMP formation or in the response to biogenic amines was detected following a single administration of morphine. These results suggest that this rapid increase in the sensitivity of cerebral cyclic AMP synthesizing system to norepinephrine at the time of withdrawal may be an important factor involved in the occurrence of morphine withdrawal syndrome.

**Effect of consecutive administration of morphine on cyclic AMP-dependent protein kinase activity**

Since consecutive administration of morphine induces alterations in cyclic AMP metabolism in the mouse brain, cyclic AMP-dependent protein kinase activity, which is assumed to be involved in the regulation of functional states of neuronal membranes (19), was determined in the cerebral cortex from these animals. As shown in Fig. 6, synaptosomal (P2-B) cyclic AMP-dependent protein kinase activity was significantly activated following consecutive administration of morphine for 2 weeks. This activation disappeared at 1 week after the discontinuance of morphine administration, but was not reversed by treatment with levallorphan. The pattern of these changes was similar to that of adenylate cyclase. On
the other hand, no alteration in synaptosomal cyclic AMP-dependent protein kinase activity was observed in acutely morphine-treated animals.

**Effect of adrenalectomy on morphine-induced changes in cerebral adenylate cyclase activity**

It is well known that chronic administration of morphine induces an alteration of the function of pituitary-adrenal axis (20). Since corticosteroid hormones have a regulatory role in the metabolism of cerebral cyclic AMP (21), we examined the effect of consecutive administration of morphine on cerebral adenylate cyclase activity in adrenalectomized rats to see whether or not alterations in adrenal functions may be involved in morphine-induced activation of adenylate cyclase activity. As shown in Table 1, adrenalectomy induced a significant fall in cerebral adenylate cyclase activity without affecting that of phosphodiesterase. Consecutive morphine administration, however, induced a significant increase of cerebral adenylate cyclase activity in adrenalectomized animals, suggesting that alterations in the adrenal function may not be involved in processes of morphine-induced activation of cerebral adenylate cyclase.

**DISCUSSION**

The present study clearly demonstrates that when continuously administered, morphine induces a significant increase of cerebral cyclic AMP formation via the activation of adenylate cyclase. Although the biochemical mechanisms underlying this activation of adenylate cyclase remain to be elucidated, the present results suggest that this activation is not due to morphine-induced alterations in the outflow of corticosteroid hormones (20), which have a modulating role in the cerebral metabolism of cyclic AMP (21). In contrast to the elevation of adenylate cyclase activity, the response of cyclic AMP synthesizing system to biogenic amines (norepinephrine, dopamine and histamine) was significantly decreased in cerebral cortical slices from continuously morphine-treated animals. In addition, the former action of morphine was not reversed by a narcotic antagonist, levallorphan, whereas the latter effect was rapidly restored by levallorphan administration. These findings suggest that morphine may have two distinct effects on cyclic AMP generating systems in the brain. It has been recently reported that in neuroblastoma x glioma hybrid cells, narcotics induce

**TABLE 1. Effect of adrenalectomy on morphine induced changes in adenylate cyclase and phosphodiesterase activities of rat cerebral cortex**

| Enzyme activity | Adenylate Cyclase (pmol/mg protein/min) ± S.E. | Phosphodiesterase (nmol/mg protein/min) ± S.E. |
|-----------------|---------------------------------------------|-----------------------------------------------|
| 1) Sham Operated | 201±10                                      | 35.7±0.9                                      |
| 2) 1)+ Morphine  | 347±54**                                    | 37.0±1.3                                      |
| 3) Adrenalectomized | 120±5                                      | 35.9±2.7                                      |
| 4) 3)+ Morphine  | 234±32*                                     | 32.6±3.5                                      |

Morphine (55–98 mg/kg/day) was administered orally in a liquid diet for 2 weeks (see ‘Methods’ for details). **Compared with 1), P<0.02. *Compared with 3), P<0.01.
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a readily reversible inhibition as well as a late compensatory increase in adenylate cyclase activity (6, 22). Our findings in mouse cerebral cortex also indicated that consecutive morphine administration for 2–4 weeks induces an increase of adenylate cyclase activity which requires discontinuance of the administration for 7–8 days before obtaining full recovery. These results suggest that the increase of adenylate cyclase activity observed in this study may be identical or similar to the late compensatory increase of the enzyme activity, possibly recognized as a compensatory response to decreased intracellular cyclic AMP, as reported previously in morphinized neuroblastoma × glioma hybrid cells (6, 22).

One of the interesting findings in this study is that consecutive administration of morphine induces a significant attenuation of the sensitivity of the cerebral cyclic AMP synthesizing systems to biogenic amines such as norepinephrine, dopamine and histamine. This change was reversed rapidly by a narcotic antagonist, levallorphan, suggesting that it may be mediated by the opiate receptor. In addition, a significant and rapid increase of the cyclic AMP formation in the presence of added norepinephrine compared with morphinized animals was observed following the administration of levallorphan. These results suggest that such a rapid increase in the norepinephrine sensitivity and subsequent increment of cerebral cyclic AMP synthesis may play an important role in inducing the morphine withdrawal syndrome. Among the biogenic amines that have been considered to be involved in the effects of morphine, the most prominent have included norepinephrine, dopamine and 5-hydroxytryptamine. Following the withdrawal of morphine, depletions in brain norepinephrine and dopamine (23) without changes in the levels of 5-hydroxytryptamine (24) have been found, although some conflicting results were also reported (See ref. (25) for pertinent references). In addition to these changes in the cerebral concentration of biogenic amines, rapid alterations of cerebral cyclic AMP, an intracellular mediator of actions of these amines, subsequent to the changes in sensitivity of the synthesizing system to these amines may have some relevance to the morphine withdrawal syndrome.

Biochemical properties of cerebral protein kinase activated by intracellular cyclic AMP have been examined intensively (3) and possible roles of this enzyme in regulating synaptic transmission were considered (26). In this study it was found that cyclic AMP-dependent protein kinase activity in synaptosomes is significantly activated following the consecutive administration of morphine. A similar increase in microsomal protein kinase activity was reported in the abstinent rats (27). These results suggest that, in addition to previously described alterations in the cyclic AMP generating system, changes in the activity of synaptic membraneous protein kinase may also play an important role in the formation of morphine dependence and/or the occurrence of abstinence symptoms possibly by changing the functional states of synaptic membranes.

The data obtained in the present study indicate that consecutive morphine administration induces the activation of cerebral adenylate cyclase and cyclic AMP dependent protein kinase activities in addition to the decreased sensitivity of cyclic AMP synthesizing system to biogenic amines. The specificity of these phenomena for morphine dependence, however, is questioned by the following facts: chronic ethanol administration capable of
inducing ethanol-dependent conditions also induces the activations of cerebral adenylate cyclase (28) and cyclic AMP dependent protein kinase (29), as well as the attenuation of norepinephrine sensitivity of the cyclic AMP synthesizing system (30). These results indicate that the observed changes in cyclic AMP generating systems of the brain are not specific for morphine, but may be a basic biochemical change for inducing or maintaining drug dependent conditions.

Although molecular mechanisms underlying the activations of adenylate cyclase and protein kinase, and the attenuation of sensitivity of the cyclic AMP synthesizing system to biogenic amines remain to be elucidated, we wish to propose the following working hypothesis concerning the role of cyclic AMP in the process of drug dependence. During a consecutive intake of certain drugs, intracellular levels of cyclic AMP in the brain increase due to the activation of adenylate cyclase. In fact, cerebral cyclic AMP levels in chronically ethanol treated animals showed a significant increase (28). This may lead to a compensatory shift in the enzyme synthesis and/or degradation and the sensitivity of biogenic amine receptor-adenylate cyclase system, thus intracellular cyclic AMP levels are kept within normal ranges. Soon after withdrawal of the drug, rapid recovery of the decreased sensitivity of biogenic amine receptor-adenylate cyclase systems and the increased sensitivity of norepinephrine receptor-adenylate cyclase system occurs in the brain. These changes lead to a rapid increase in intracellular cyclic AMP since the recovery of increased activity of adenylate cyclase is rather slow and the basal level of cyclic AMP synthesizing system may well be kept high. We hypothesize that such a rapidly induced supersensitivity of the norepinephrine receptor-adenylate cyclase system in brain cells may play an important role in the occurrence of withdrawal signs in drug dependent subjects.

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