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ACCELERATED COMMUNICATION

A Novel Action of Morphine in the Rat Locus Coeruleus: Persistent Decrease in Adenylate Cyclase

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SUMMARY

This study describes a novel action of morphine on adenylate cyclase activity in the rat locus coeruleus (LC). We have previously shown that acute *in vitro* morphine inhibits adenylate cyclase activity in isolated LC membranes, whereas chronic *in vivo* morphine treatment increases enzyme activity in this brain region. We now report that acute *in vivo* morphine treatment produces a 25–30% decrease in adenylate cyclase activity in the LC, which persists in *in vitro* assays in the absence of opiates. This *in vivo* effect is clearly distinct from the acute inhibition of adenylate cyclase observed during exposure of isolated LC membranes to opiates *in vitro*. The *in vivo* effect was not reversed by the inclusion of naloxone, an opiate receptor antagonist, in the assay, and acute *in vitro* opiate inhibition of the enzyme was the same in LC membranes isolated from control and morphine-treated rats. Thus, the *in vivo* effect does not appear to be due to residual morphine retained in the membrane preparation. This persistent decrease in adenylate cyclase was found to occur in a dose-dependent manner and to be mediated through the actions of morphine at opiate receptors, inasmuch as the inhibition was prevented by concomitant *in vivo* administration of naltrexone, a long-acting opiate receptor antagonist. This effect was also specific to the LC, in that it was not observed in the other brain regions examined, which included the dorsal raphe, neostriatum, and frontal cortex. Acute *in vivo* clonidine, an α_2-adrenergic receptor agonist known to have actions in the LC similar to those of morphine, produced a similar persistent decrease in adenylate cyclase activity in this brain region. In contrast, other drugs with different actions on the LC failed to produce this effect. This decrease in adenylate cyclase activity induced by acute *in vivo* morphine or clonidine, which persists in isolated membranes after the removal of the drugs, may be an early step in the sequence of events that leads to the development of opiate or clonidine addiction in the LC.

Although the biochemical mechanisms by which opiates mediate their acute and chronic effects remain uncertain, one of the best known actions of opiates is their effect on adenylate cyclase activity. Acute *in vitro* inhibition of adenylate cyclase activity by opiates has been demonstrated in neuroblastoma × glioma (NG108) cells (1) and in a number of regions of the central nervous system, including rat neostriatum, frontal cortex, dorsal raphe, and LC and mouse spinal cord-dorsal root ganglion explants (2–8). This *in vitro* opiate inhibition of adenylate cyclase activity is thought to be mediated by the inhibitory G protein, G_i, because it can be blocked by pertussis toxin (4, 6–9). Furthermore, chronic opiate treatment has been shown to increase adenylate cyclase activity in NG108 cells (1), in LC (7), and in dorsal root ganglion explants (8). These findings have provided a basis for a model of the development of opiate addiction, namely, that increases in adenylate cyclase activity, and hence in intracellular cAMP levels, represent biochemical correlates of opiate tolerance and/or dependence (1, 7, 8, 10).

The LC is a brain region that has served as a useful model to study the acute and chronic actions of opiates from behavioral, physiological, and biochemical perspectives. It has been shown that regulation of LC neuronal activity plays an important role in mediating certain characteristic behavioral actions of both acute and chronic opiates in primates (11). Physiologically, acute morphine administration inhibits LC cell firing in the rat but, as the animal becomes tolerant to morphine, LC cell firing rates return to normal levels (12–14). LC neurons also become dependent on opiates, in that administration of an opiate receptor antagonist increases LC firing rates several fold above control levels *in vivo* (12). Furthermore, the onset of increased LC neuronal activity, and its recovery to normal

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ABBREVIATIONS: LC, locus coeruleus; G protein, guanine nucleotide-binding protein; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; DADLE, D-Ala^2-D-Leu-enkephalin; GABA, γ-aminobutyric acid.

levels, proceed with a time course that parallels the onset and recovery of behavioral withdrawal symptoms[1] (12). Chronic morphine treatment also produces a number of biochemical changes specifically in the LC that correlate with these behavioral and physiological changes. Thus, in addition to the increase in adenylate cyclase activity mentioned above (7), chronic morphine treatment has also been shown to increase levels of certain G proteins (15), cAMP-dependent protein kinase activity (16), and a number of phosphoprotein substrates of the protein kinase (17) specifically in the LC.

In previous studies characterizing the regulation of adenylate cyclase activity by morphine in the LC, it was observed that, in addition to the acute *in vitro* inhibition and the chronic *in vivo* increase in adenylate cyclase activity, acute *in vivo* morphine treatment decreased adenylate cyclase activity in this brain region (7). In the present study, we have further characterized this latter phenomenon and have shown that it represents a novel regulation of adenylate cyclase activity. Acute morphine treatment appears to induce a persistent decrease in adenylate cyclase activity, which is not dependent upon the continuous presence of the opiate.

Materials and Methods

***In vivo* drug treatments.** Male Sprague-Dawley rats (150–200 g) were used in these studies and housed under a 12-hr light/dark cycle, with food and water *ad libitum*. For acute treatment, all drugs were administered via subcutaneous injection, except chloral hydrate, which was injected intraperitonealy; control animals were injected with saline. Animals were used 30 min after the various injections, at which time the behavioral effects are maximal (18). In some experiments, pellets that contained 75 mg of morphine base (from the National Institute on Drug Abuse) were implanted subcutaneously, and the rats were then sacrificed from 1 to 24 hr after pellet implantation.

Morphine (0.3 to 100 mg/kg, morphine sulfate; National Institute on Drug Abuse) and clonidine (0.1 to 1.0 mg/kg, Sigma Chemical Co., St. Louis, MO) both produced partial sedation at the maximal doses administered. These are doses that, although high for an acute dose, are used standardly to produce morphine and clonidine addiction in rats (19–21). Diazepam (10 to 30 mg/kg; Hoffmann-La Roche) produced a similar degree of sedation. Chloral hydrate (400 mg/kg, a dose routinely used to anesthetize rats; Sigma) produced unconscious animals. Dextromethorphan (Sigma), which was given at a dose of 100 mg/kg, the maximum morphine dose administered, produced no noticeable sedation or other behavioral effects.

Concomitant treatment of rats with naltrexone (DuPont, Wilmington, DE) and morphine [or yohimbine (Sigma) and clonidine (Sigma)] was accomplished by administering the antagonist, via subcutaneous injection, 5 min before morphine (or clonidine) administration. Antagonist doses chosen were those that blocked the behavioral effects of the agonists.[1]

Adenylate cyclase assays. Rats were sacrificed by decapitation, and the brains were removed rapidly and placed in ice-cold buffer (126 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM D-glucose, pH 7.4). The LC and dorsal raphe were isolated by obtaining coronal cross-sections of brainstem, 0.7 mm thick, with a tissue slicer and by excising the nuclei from the sections by taking 15-gauge punches with a syringe needle, as described previously (16). Initially, protein levels per punch were assayed by the method of Lowry *et al.* (22), and were found to be very consistent; therefore, subsequent data were normalized per punch rather than per mg of protein. Frontal cortex and neostriatum (caudate/putamen) were isolated by gross dissection and the data were normalized per mg of protein.

The isolated brain regions were sonicated (1 mg of tissue/200 μl) immediately in 20 mM Tris, pH 7.4, 1 mM dithiothreitol, 1 mM EGTA, and 200 μl of each sample were centrifuged at 10,000 × *g* for 10 min at 4°. The pellet was then resuspended in 10 mM Tris, pH 7.4 (350 μl) for use in the assay. The adenylate cyclase assays were performed as described by Jakobs *et al.* (23), in a final volume of 100 μl that contained 50 mM triethanolamine HCl, pH 7.4, 50 μM [α-^{32}P]ATP (2×10^6 cpm, New England Nuclear, Boston, MA), 0.1 mM EGTA, 1.0 mM dithiothreitol, 1.0 mM isobutylmethylxanthine (Sigma), 0.1 mM cAMP, 2 mM $MgCl_2$, 5 mM creatine phosphate, 0.4 mg/ml creatine phosphokinase, and 0.2% (w/v) bovine serum albumin. The membranes, assayed as duplicates that contained 5 μg of protein/tube, were preincubated with various drugs for 12 min at 30°. The reaction was then initiated with the addition of [α-^{32}P]ATP and terminated after 12 min by the addition of 0.8 ml of 0.1% sodium dodecyl sulfate containing 100 μM cAMP. The double-column method of Salomon (24) was used to analyze levels of [α-^{32}P]cAMP.

In some experiments, adenylate cyclase activity was assayed as described above but under "enzyme-activating" conditions[2] (25, 26), which substituted 2 mM $MnCl_2$ for 2 mM $MgCl_2$ and included 50 μM forskolin (Sigma), which was the maximum concentration that could be attained due to its limited solubility.

Results

The effect of acute *in vivo* morphine treatment on adenylate cyclase activity was examined in the LC and several other regions of rat brain. Such treatment was found to decrease enzyme activity by 25–30%, compared with control animals, in the LC. This effect was specific to the LC among the brain regions studied, which included the dorsal raphe, frontal cortex, and neostriatum (Fig. 1).

The decrease in the LC was seen in both basal and forskolin-stimulated adenylate cyclase activity (Table 1). This persistent decrease in adenylate cyclase activity following acute *in vivo* morphine treatment is an effect distinctly different from the acute *in vitro* inhibition of the enzyme by opiates described previously in the LC and in other cell types, for two reasons. First, the inclusion of 100 μM naloxone, a specific opiate receptor antagonist, in the assay did not reverse the effect (Table 1). Second, the degree of acute *in vitro* inhibition by 100 μM DADLE of adenylate cyclase activity was similar in LC membranes isolated from control rats versus rats treated acutely with morphine (Table 2). [This concentration of DADLE was used because it has been shown to maximally inhibit adenylate cyclase activity in the LC in a naloxone-reversible manner (7).] These two observations are in contrast to what one would expect if the acute *in vivo* decrease in adenylate cyclase was merely due to residual morphine retained in the membranes, namely, that the effect would be naloxone-reversible and that the ability of *in vitro* DADLE to inhibit adenylate cyclase activity would be attenuated. In addition to inhibition by DADLE, adenylate cyclase activity was also inhibited to the same degree by 100 μM morphine in isolated LC membranes from control and acutely treated rats. Furthermore, the inclusion of both morphine and DADLE together in the assay produced an inhibition equivalent to that seen with either agonist alone, indicating that they are both acting on the same population of receptors to maximally inhibit adenylate cyclase activity (data not shown).

[1] E. J. Nestler, K. Rasmussen, J. Krystal, and G. Aghajanian, unpublished observations.

[2] K. Seamon, personal communication.

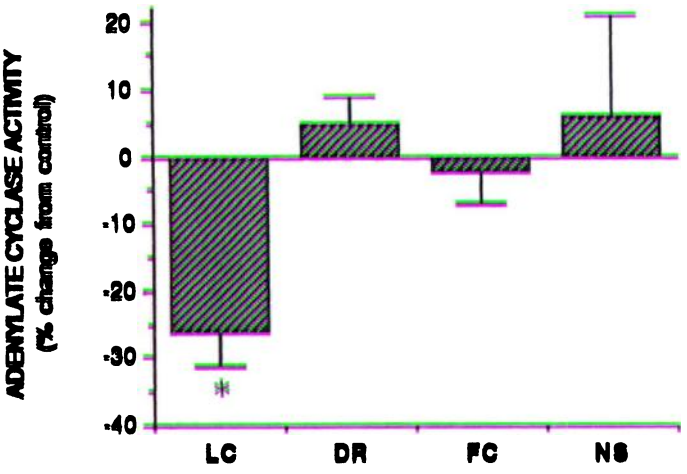

Fig. 1. Regional specificity of acute *in vivo* morphine regulation of adenylate cyclase. Treated rats received 100 mg/kg morphine via subcutaneous injection. Control rats received saline injections. The animals were sacrificed 30 min after the injections, and the LC nuclei and other brain regions were removed and assayed for adenylate cyclase activity as described in Materials and Methods. The data shown represent forskolin (5 μM)-stimulated adenylate cyclase activity and are expressed as per cent change from control ± standard error. The number of animals used ranged from 7 to 11. Specific activities (pmol/min/mg) of adenylate cyclase ± standard error in control animals were: LC 139 ± 7; dorsal raphe (*DR*), 137 ± 18; frontal cortex (*FC*), 354 ± 40; neostriatum (*NS*), 1110 ± 20. *$p \leq 0.025$ by χ^2 test.

TABLE 1

Regulation of adenylate cyclase activity in the rat LC by acute *in vivo* morphine treatment

Treated rats received 30 mg/kg morphine via subcutaneous injection. Control rats received saline injections. The animals were sacrificed 30 min after the injections, and the LC nuclei were isolated. Adenylate cyclase activity was assayed under basal and 5 μM forskolin-stimulated conditions in the presence or absence of 100 μM naloxone. The assay was carried out as described in Materials and Methods. The data represent the means of values obtained from four animals in each group (control and morphine-treated) ± standard error.

	Adenylate Cyclase Activity		
	Basal	Forskolin	Forskolin + naloxone
	pmol/min/mg		
Control	26.1 ± 2.6	120 ± 6	116 ± 6
Morphine-treated	16.0 ± 1.3[a]	74.8 ± 8.3[a]	76.3 ± 8.0[a]

[a] $p \leq 0.005$ by Student's *t* test.

TABLE 2

Lack of effect of acute *in vivo* morphine on DADLE inhibition of adenylate cyclase

Rats received acute morphine treatment (100 mg/kg), and isolated LC nuclei were assayed for adenylate cyclase activity as described under Materials and Methods. Included in the assay were 5 μM forskolin, 100 μM GTP, and 100 mM NaCl, in the presence or absence of 100 μM DADLE. Data are expressed as the mean ± standard error, with the number of rats in parentheses.

	Adenylate Cyclase Activity		Inhibition by DADLE
	−DADLE	+DADLE	
	pmol/mg/min		%
Control	109 ± 7.0 (4)	85.0 ± 6.0 (4)[a]	22% ± 6%
Acute morphine	74.9 ± 8.5 (4)	54.5 ± 5.6 (4)[a]	24% ± 6%

[a] DADLE inhibition of adenylate cyclase activity in both control and morphine-treated animals was statistically significant at $p \leq 0.05$ by Student's *t* test.

Acutely, treatment with clonidine, an α_2-adrenergic receptor agonist, has been shown to inhibit LC cell firing through mechanisms similar to those of morphine (27). It was, therefore, of interest to investigate whether this drug could produce similar effects on adenylate cyclase activity in the LC. It was found that acute *in vivo* clonidine treatment decreased activity of the enzyme approximately 15% in this brain region (Table 3). In addition, the clonidine-induced decrease was persistent, in that it was not reversed by the inclusion of 100 μM yohimbine, an α_2-adrenergic receptor antagonist, in the assay (data not shown). This is by analogy to the inability of naloxone to reverse the acute *in vivo* morphine-induced decrease in enzyme activity.

In order to study whether the effects of morphine and clonidine on adenylate cyclase were mediated through their specific receptors, opiate or α_2-adrenergic receptor antagonists were co-administered with morphine or clonidine (Table 3). The morphine-induced decrease in adenylate cyclase activity was reversed completely by systemic co-administration of naltrexone, an opiate receptor antagonist, but not by yohimbine. Conversely, co-administration of yohimbine reversed the effect of clonidine on adenylate cyclase, whereas naltrexone did not. These results demonstrate that clonidine and morphine exert similar acute effects on adenylate cyclase activity, but through the activation of their distinct receptors. The effect of clonidine on adenylate cyclase activity tended to be smaller than that produced by morphine; clonidine decreased enzyme activity approximately 15%, compared with the 25–30% decrease observed with morphine treatment. Interestingly, the effects of

TABLE 3

Receptor-specific regulation of adenylate cyclase activity by acute *in vivo* morphine or clonidine treatment

Rats received subcutaneous injections of drugs at the doses indicated. In the cases where rats were concomitantly treated with an antagonist, the antagonist was administered 5 min before the agonist. The data shown represent forskolin (5 μM)-stimulated adenylate cyclase activity, although similar effects were seen under basal conditions. Data are expressed as per cent of control ± standard error, with the number of rats in parentheses. Specific activities of adenylate cyclase were similar to those reported in Tables 1 and 2.

Drug Treatment	Adenylate Cyclase Activity
	(percent of control)
Morphine (100 mg/kg)	74 ± 5 (11)[a]
Morphine (100 mg/kg) + naltrexone (100 mg/kg)	97 ± 5 (8)
Naltrexone (100 mg/kg)	95 ± 2 (3)
Morphine (30 mg/kg)[b] + yohimbine (15 mg/kg)	84 ± 6 (8)[a,c]
Clonidine (1.0 mg/kg)	84 ± 6 (4)[a]
Clonidine (1.0 mg/kg) + yohimbine (15 mg/kg)	95 ± 7 (4)
Yohimbine (15 mg/kg)	102 ± 7 (4)
Clonidine (1.0 mg/kg) + naltrexone (100 mg/kg)	77 ± 8 (4)[a,d]
Morphine (30 mg/kg) + clonidine (1.0 mg/kg)	73 ± 9 (4)[a,e]
Dextromethorphan (100 mg/kg)	100 ± 5 (3)
Chloral hydrate (400 mg/kg)	99 ± 7 (11)
Diazepam (30 mg/kg)	98 ± 6 (11)

[a] $p \leq 0.05$ by χ^2 test.
[b] Morphine was reduced to 30 mg/kg for co-administration with yohimbine, because at higher doses the combination of the two drugs was toxic.
[c] Not significantly different from morphine (30 mg/kg); $p \geq 0.2$ by Student's *t* test.
[d] Not significantly different from clonidine (1.0 mg/kg); $p \geq 0.2$ by Student's *t* test.
[e] Not significantly different from either morphine (30 mg/kg) or clonidine (1.0 mg/kg); $p \geq 0.2$ by Student's *t* test.

the co-administration of maximal doses (see below) of clonidine and morphine were not additive, in that together the drugs produced an effect equivalent to that seen in response to morphine alone (Table 3).

Acute morphine treatment was found to decrease adenylate cyclase activity in a dose-dependent manner. It can be seen in Fig. 2 that there was a tendency for this decrease to occur at 10 mg/kg morphine, with a maximal decrease in enzyme activity achieved at a dose of 30 to 100 mg/kg. These are the doses that are used to induce states of profound morphine addiction in rats (19). The inhibitory effect of clonidine on adenylate cyclase activity also occurred in a dose-dependent manner (data not shown). The clonidine-induced decrease was observed at 0.1 mg/kg and appeared maximal at 0.3 to 1.0 mg/kg, which are doses used to suppress morphine withdrawal in rats (20) and to induce clonidine addiction in LC neurons (21).

It was of interest to investigate whether other drugs with physiological actions distinct from those of morphine and clonidine in the LC could alter adenylate cyclase activity after *in vivo* administration (Table 3). Diazepam, which inhibits LC cell firing (28), but via mechanisms different from those of morphine and clonidine (i.e., through a GABAergic chloride conductance and not through G proteins and cAMP), did not produce a decrease in adenylate cyclase activity. Dextromethorphan, which is structurally similar to morphine but without pharmacological activity at opiate receptors (29), also had no effect on adenylate cyclase activity. Finally, chloral hydrate, which produces sedation as does morphine but through a non-opiate mechanism, did not affect enzyme activity.

The time course of acute morphine regulation of adenylate cyclase was examined by the implantation of subcutaneous morphine pellets (75 mg), which produce a steady state condition of continuous morphine administration over a period of 24 hr or more (30), an effect that cannot be achieved with single injections. The maximal decrease in adenylate cyclase activity observed in response to single pellet implantations was similar in magnitude to that observed with subcutaneous injections. The decrease in adenylate cyclase activity was found to be rapid in onset and short in duration. Maximal decrease occurred 2–4 hr after pellet implantation, but by 6 hr enzyme activity had returned to normal levels (Fig. 3). It is of interest to note that the time at which adenylate cyclase activity is maximally decreased corresponds to the time at which LC neuronal firing is maximally inhibited under these conditions (12).

A change in adenylate cyclase activity could be achieved by alterations in G proteins, by alterations in the intrinsic catalytic activity of the enzyme itself, or by both. The addition of high levels of forskolin and the replacement of magnesium with manganese are conditions reported to activate adenylate cyclase near maximally and, thereby, decrease the contributions of G protein regulation to enzymatic activity[2] (25, 26). Therefore, it was of interest to examine the effects of acute *in vivo* morphine and clonidine administration on LC adenylate cyclase activity under these assay conditions. It was found that enzyme-activating conditions greatly increased the specific activity of adenylate cyclase [1100 ± 180 versus 180 ± 25 pmol/min/min (mean ± SE, four animals)] and that morphine and clonidine decreased adenylate cyclase activity to similar degrees under both the standard and the high forskolin/manganese conditions. Adenylate cyclase activity in response to acute morphine was 77 ± 10% (four animals) of control levels under enzyme-activating conditions, compared with 74 ± 5% (11 animals) under standard conditions. Similarly, enzyme activity in response to clonidine was 85 ± 6% (four animals) and 87 ± 5% (11 animals) under enzyme-activating and standard conditions, respectively.

Discussion

This study reports the initial characterization of a novel action of morphine, a stable decrease in adenylate cyclase activity that is induced by acute *in vivo* morphine treatment in the rat LC. This decrease in adenylate cyclase activity is not due to residual morphine being retained in the membranes; it is not antagonized by the inclusion of the opiate receptor

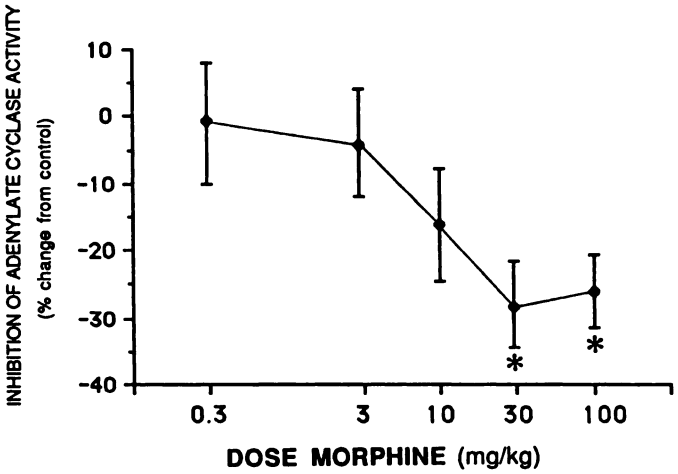

Fig. 2. Dose dependence of acute *in vivo* morphine inhibition of adenylate cyclase activity in rat LC. Morphine was administered subcutaneously at the doses indicated 30 min before the animal was sacrificed. LC nuclei were isolated and adenylate cyclase activity was assayed as described under Materials and Methods. The data shown represent forskolin (5 μM)-stimulated adenylate cyclase activity, and data are expressed as per cent change from control ± standard error. The number of animals used ranged from 4 to 11. $^*p < 0.025$ by χ^2 test.

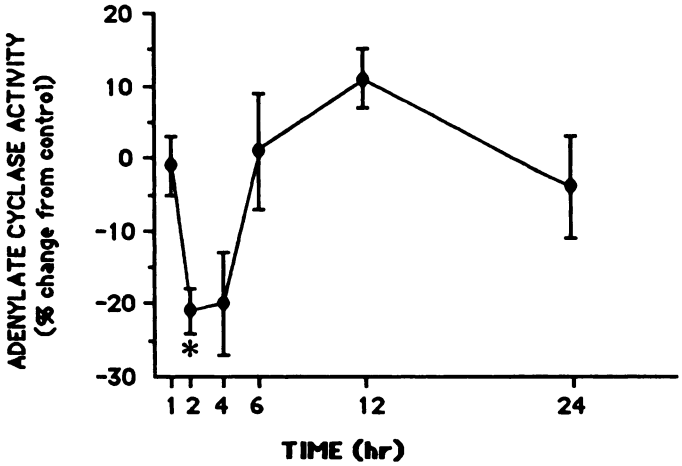

Fig. 3. Time course of acute *in vivo* morphine inhibition of forskolin-stimulated adenylate cyclase activity in rat LC. Single morphine pellets (75 mg) were implanted subcutaneously in rats. The animals were sacrificed at the times indicated, and isolated LC nuclei were assayed for adenylate cyclase activity. The data shown represent forskolin (5 μM)-stimulated adenylate cyclase activity, and data are expressed as per cent change from control ± standard error. The number of animals used ranged from 3 to 13. $p < 0.001$ by χ^2 test.

antagonist naloxone in the assay, and the opiate agonist DADLE produced the same degree of *in vitro* inhibition of adenylate cyclase in LC membranes from control and morphine-treated animals. Rather, it appears that acute *in vivo* morphine treatment imparts some persistent change to the adenylate cyclase system that is not dependent on the continuous presence of the opiate. This effect is clearly distinct from other previously reported actions of morphine on adenylate cyclase activity in the LC as well as in other tissues. Acute *in vitro* opiate exposure inhibits enzyme activity in isolated membranes of the LC and many other tissues (see Introduction), but this inhibition is readily and completely reversed by the inclusion of naloxone in the assay. On the other hand, chronic opiate treatment has been found to increase adenylate cyclase activity in the LC, dorsal root ganglion explants, and neuroblastoma cells (1, 7, 8).

The results of the present study indicate that there are two distinct mechanisms by which opiates can decrease adenylate cyclase activity in the LC, acute *in vitro* inhibition of the enzyme described previously and the acute *in vivo* decrease demonstrated here. The relationship between the two phenomena is unclear. It will be interesting in future studies to determine whether the persistent decrease in enzyme activity is triggered, specifically in the LC, by the initial, naloxone-reversible inhibition of adenylate cyclase activity, which is seen in the LC and other brain regions as well.

Interestingly, the effect of acute *in vivo* morphine on adenylate cyclase activity is short-lived. The opiate-induced decrease is maximal 2–4 hr after morphine administration, but adenylate cyclase activity returns to normal levels within 6–12 hr after pellet implantation, even though morphine is steadily being absorbed during this time (30) and LC neurons remain inhibited by the absorbed morphine (12). These results indicate that the effect is indeed a transient one or, alternatively, that it is being overcome by compensatory mechanisms, such as those that lead to increased adenylate cyclase activity with chronic morphine treatment in the LC (7).

A similar decrease in adenylate cyclase activity was observed with acute *in vivo* administration of clonidine, an α_2-adrenergic receptor agonist. Analogous to the decrease observed with acute *in vivo* morphine, the decrease produced by acute *in vivo* clonidine treatment was not reversed by the inclusion of yohimbine, an α_2-adrenergic antagonist, in the adenylate cyclase assay. Acute *in vivo* morphine and clonidine decreased adenylate cyclase in a receptor-specific manner. The morphine-induced decrease was reversed by the systemic co-administration of naltrexone, an opiate receptor antagonist, but not by yohimbine, whereas the clonidine-induced decrease was reversed by yohimbine, but not by naltrexone. Physiologically, it has been shown that, although they act through different receptors, α_2-adrenergic receptor and opiate receptor agonists produce similar effects through common post-receptor mechanisms in the LC (27). The effects of morphine and clonidine are mediated by pertussis toxin-sensitive G proteins (i.e., G_o and G_i) (14, 31), and partially mediated through decreases in a cAMP-dependent inward current, an action presumably achieved through the inhibition of adenylate cyclase (32). The fact that co-administration of maximal doses of morphine and clonidine in the present study did not decrease adenylate cyclase activity in an additive manner provides evidence that this action of the drugs is also achieved through a common post-receptor mechanism.

There are several possible mechanisms whereby acute *in vivo* morphine or clonidine treatment could decrease adenylate cyclase activity in the LC. The drug could alter either the enzyme itself, i.e., the intrinsic activity of its catalytic unit, the functional activity of various G protein subunits, or the interaction between the catalytic unit and the G proteins. One way to partially differentiate changes in the catalytic unit of the adenylate cyclase system from G protein-mediated ones is to assay the enzyme under conditions of "maximal activation." High levels of forskolin (50–100 μM) combined with 2 mM Mn^{2+} provide near maximal activation of adenylate cyclase and decrease the regulatory contributions made by G proteins[2] (25, 26). The inhibitory effects of acute *in vivo* morphine and clonidine treatment were not attenuated under such assay conditions. These results suggest that the persistent decrease of adenylate cyclase activity is due at least in part to changes in the catalytic activity of the enzyme itself. Such a persistent decrease in the adenylate cyclase catalytic unit could represent a covalent modification of the enzyme. For example, phosphorylation of the enzyme has been reported in a number of systems (33, 34) and represents one possible mechanism through which such an intrinsic change in the catalytic activity of the enzyme could be produced.

However, the above observations do not rule out a contribution of G proteins to this decrease in adenylate cyclase activity. In fact, our laboratory has shown that pertussis toxin-mediated ADP-ribosylation of $G_{i\alpha}$ and $G_{o\alpha}$ is decreased after acute *in vivo* morphine treatment (15). More recently, we have found that acute *in vivo* morphine treatment does not alter levels of immunoreactivity of $G_{i\alpha}$ or $G_{o\alpha}$, as well as $G_{s\alpha}$ and G_{β}, in the rat LC by immunoblot analysis.[3] It is possible that the decreased levels of ADP-ribosylation of $G_{i\alpha}$ and $G_{o\alpha}$, without a change in their total amount, reflect a functional change in the G proteins, which could also contribute to the changes observed in adenylate cyclase activity.

There is now a large body of evidence that implicates the LC as an important brain region with respect to opiate tolerance, dependence, and withdrawal. Electrophysiological studies have demonstrated the development of tolerance and dependence in LC neurons after chronic opiate treatment (12, 13), and these changes in LC neuronal excitability appear to play a role in mediating the behavioral effects of opiates in animals, including primates (see Ref. 11). In addition, morphine tolerance and dependence have been shown to be associated with changes in the cAMP system, at several levels, specifically in the LC. After chronic morphine treatment, adenylate cyclase activity (7), the levels of the G protein subunits $G_{i\alpha}$ and $G_{o\alpha}$ (15), and cAMP-dependent protein phosphorylation (16, 17) are all increased in the LC, but not in the other brain regions studied. The acute *in vivo* morphine decrease in adenylate cyclase activity observed in the present study also appeared to be specific to the LC, in that it was not observed in the dorsal raphe, neostriatum, or frontal cortex. The regional specificity of these various actions of morphine could be due to the heterogeneity of the other brain regions examined (in contrast to the homogeneity of the LC) and/or to specific properties of LC neurons (see Ref. 16). This regional specificity is in striking contrast to the acute *in*

[3] D. B. Beitner, R. S. Duman, and E. J. Nestler, unpublished observations.

vitro inhibition of adenylate cyclase by opiates, which appears to be widespread; it has been reported in a large number of different brain regions (2–8).

In addition to opiate addiction, LC neurons have also been shown electrophysiologically to exhibit tolerance to and dependence on clonidine (21), and we have found that chronic clonidine treatment increases adenylate cyclase activity and cAMP-dependent protein kinase activity in the LC (35). Interestingly, the only two drugs in the present study that decreased adenylate cyclase activity in the LC after acute *in vivo* administration are also the two that produce both addiction and an up-regulated cAMP system in the LC after chronic treatment. The other drugs tested, which included diazepam, dextromethorphan, and chloral hydrate, did not significantly influence adenylate cyclase under the conditions used.

Based on the regional and pharmacological specificity of this phenomenon, it can be hypothesized that this transient decrease in adenylate cyclase activity may be one of the initial molecular steps in the development of opiate (or clonidine) tolerance and dependence in the LC. Such a persistent change in adenylate cyclase activity could, possibly via a negative feedback pathway, serve to trigger the up-regulation of the cAMP system that is observed in opiate-addicted animals. Such an up-regulated cAMP system, which would be expected to increase the excitability of LC neurons (14, 32), could, in turn, contribute to opiate addiction. Clearly, more work needs to be done to characterize the mechanism by which acute *in vivo* morphine treatment decreases adenylate cyclase activity in the LC and to further investigate its role in the mechanisms underlying opiate addiction.

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