# Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance

(neuroblastoma × glioma hybrid cells/cell culture/memory/synaptic transmission/opiate receptors)

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ABSTRACT    Narcotics affect adenylate cyclase [ATP py-rophosphate-lyase (cyclizing), EC 4.6.1.1] in two opposing ways, both mediated by the opiate receptor. The first process is the readily reversible inhibition of the enzyme by narcotics; the second is a compensatory increase in enzyme activity which is delayed in onset and relatively stable. Late positive regulation of the enzyme counteracts the inhibitory influence of morphine and is responsible for narcotic dependence and tolerance. The coupled inhibitory and positive regulatory mechanisms for adenylate cyclase provide a means of activating and deactivating neural circuits hours after the initial event and thus may play a role in a memory process.

Recent observations with neuroblastoma × glioma hybrid cells indicate that the binding of morphine and other opiates to narcotic receptors results in an inhibition of adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] activity (1–3) and a decrease in cAMP levels in intact cells (1, 2, 4, 5). Similar observations have been made with brain (6, 9) but the heterogeneity of cell types present and apparent lability of the brain enzyme may be responsible for conflicting observations (8–10). Both dependence upon opiates and tolerance to these compounds were hypothesized to result from either an increase in the number of molecules of adenylate cyclase or a long-lived factor which affects the rates of adenylate cyclase activity or turnover (1). In this report, we describe the results of experiments which were designed to test the hypothesis illustrated diagramatically in Fig. 1. The addition of morphine results in the rapid inhibition of adenylate cyclase activity and a resultant decrease in intracellular cAMP levels. Further incubation reveals a second regulatory process which involves a compensatory increase in adenylate cyclase activity termed late positive regulation. The increase in adenylate cyclase activity counteracts the inhibition of enzyme activity by morphine and cAMP levels are restored to the normal value. Cells now are tolerant to morphine and are also dependent upon the narcotic, since withdrawal of the drug or the addition of a specific narcotic antagonist will raise cAMP levels to abnormally high values and secondarily produce a gradual return to the normal level of adenylate cyclase activity.

In this communication we report data which demonstrate a rapid inhibition and a late positive regulation of adenylate cyclase which are dependent upon narcotics and account for the phenomena of narcotic dependence and tolerance.

## METHODS AND MATERIALS

The source of each chemical and the medium and growth conditions for neuroblastoma × glioma hybrid NG108-15 were described previously (1).

Assay of cAMP in Intact Cells and Medium. Growth medium was changed 12 hr before assay. Narcotic was added to the growth medium [Dulbecco's modification of Eagle's medium (DMEM), hypoxanthine-aminopterin-thymidine (HAT), and 10% fetal bovine serum] and plates were incubated in a humidified atmosphere of 90% air–10% $CO_2$. At appropriate times the phosphodiesterase inhibitor 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724), was added (0.5 mM, final concentration) and incubation was continued for 15 min. Reactions were initiated by the addition of narcotic and/or adenosine in $H_2O$ or prostaglandin $E_1$ (PGE₁) in ethanol. Ethanol (0.5% with Ro 20-1724 present, or 1.0% when both Ro 20-1724 and PGE₁ were present) had no effect upon cAMP formation. Reactions were terminated by the addition of 1.0 ml of trichloroacetic acid at 0° (final concentration, 5%). A solution containing 5 pmol of [¹⁴C]cAMP (3000 cpm) was added, and the suspension and two washes (each 1 ml) of 5% trichloroacetic acid were combined and centrifuged. Each supernatant fraction was applied to an 0.8 × 8 cm column of AG 50W-X4 resin, 200–400 mesh, H⁺ form (Bio-Rad) washed with $H_2O$. Each column was washed with 6 ml of $H_2O$ and cAMP was eluted with an additional 3 ml of $H_2O$, and applied to a 0.8 × 2.5 cm column of AG 1-X8 resin, 200–400
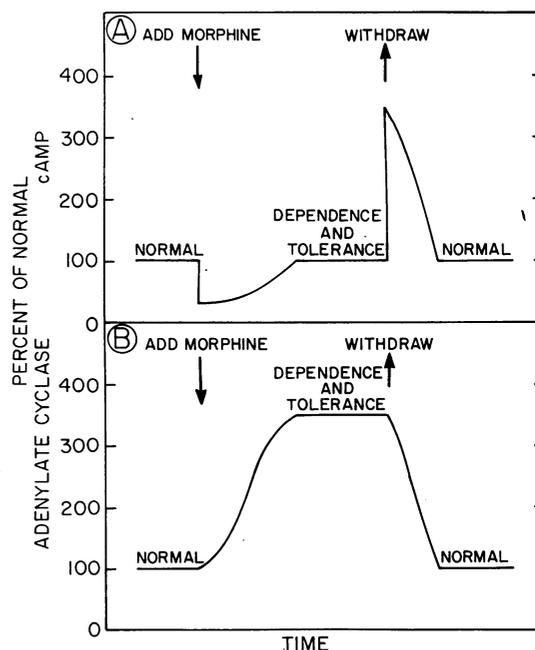


FIG. 1.    A model of the role of adenylate cyclase regulation in the development of morphine tolerance and dependence. Part A shows the effects of morphine upon cAMP levels, and part B, the effects of the opiate upon adenylate cyclase activity as a function of time.
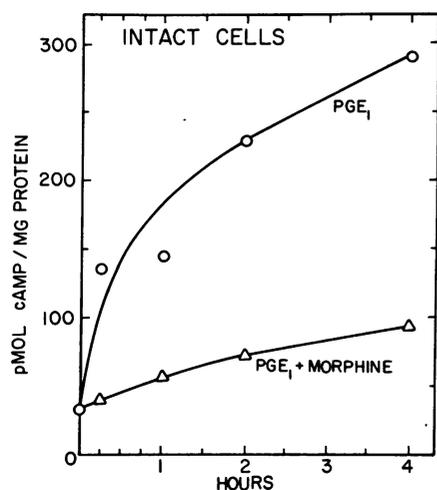
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FIG. 2. Levels of cAMP in intact NG108-15 hybrid cells and the medium measured as a function of time in the presence of 10 $\mu$M PGE$_1$ or 10 $\mu$M PGE$_1$ and 10 $\mu$M morphine sulfate. No phosphodiesterase inhibitor was present. The average 60 mm petri dish contained 3.5 mg of cell protein.
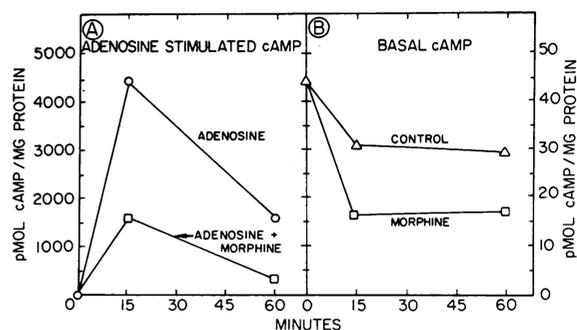


FIG. 3. Basal and adenosine-stimulated levels of cAMP in intact NG108-15 cells measured as a function of time in the presence or absence of morphine sulfate. The concentrations used were 100 $\mu$M adenosine and 10 $\mu$M morphine sulfate. Each 100 mm petri dish also contained 0.1 mM Ro 20-1724. The average dish contained 16.9 mg of protein.

mesh, formate form (Bio-Rad) equilibrated with water. The eluate and a subsequent 10 ml H$_2$O wash were discarded; cAMP was eluted with 4 N HCOOH and lyophilized. Each dried sample was taken up in 0.5 ml of H$_2$O and assayed for cAMP by the method of Gilman (11). Values reported are average values obtained with two to four replicate plates and are corrected to 100% recovery of cAMP.

At each time of incubation 6 to 8 dishes were washed free of serum (1) and the cells were transferred quantitatively to a tube. After centrifugation the pellets were dissolved in 1 N NaOH and protein was estimated by a modification of the method of Lowry *et al.* (12). The average value of protein at each time point was used to calculate the specific activity of cAMP.

The adenylate cyclase assay and preparation of homogenates have been described previously (1), except that Tris·HCl was 30 mM and pH 7.5 in the previous as well as this study.

**Opiate Receptor Assays.** Washed cells from two 100 mm culture dishes were homogenized in 10 ml of medium D1 (13), centrifuged for 10 min at 20,000 rpm (48,000 $\times$ g), and the pellet was resuspended in the original volume of D1 whose pH had been adjusted to 8.0 with 2 M Tris (free base). Specific binding of [$^3$H]naloxone (5 $\times$ 10$^{-9}$ M, 63,500 cpm/ml) was measured as described (13). Results are expressed in fmol of specifically bound naloxone per mg of protein in the whole homogenate.

## RESULTS

The effect of morphine on PGE$_1$-stimulated cAMP accumulation in neuroblastoma $\times$ glioma hybrid cells, (NG108-15), and cAMP excreted into the medium are shown in Fig. 2 as a function of time of incubation. The results show that morphine inhibited PGE$_1$-dependent accumulation of cAMP by more that 90% throughout the 4 hr period of incubation. Since a phosphodiesterase inhibitor was not present in this experiment, at least part of the PGE$_1$-dependent increase in cAMP specific activity may represent extracellular cAMP. No evidence for cellular tolerance to morphine was observed during the 4 hr incubation period.

The effect of morphine upon adenosine-dependent elevation of cAMP levels and basal cAMP levels is shown in Fig.

3A and B, respectively. Cells were preincubated with a phosphodiesterase inhibitor, Ro 20-1724, prior to the addition of adenosine and/or morphine. The addition of adenosine evoked a large increase in cAMP and morphine inhibited adenosine-dependent cAMP formation. Morphine also reduced the basal level of cAMP (Fig. 3B).

The effect of culturing cells in the presence of morphine for 0-4 days upon the specific activity of basal or PGE$_1$-stimulated adenylate cyclase activity is shown in Fig. 4A and B, respectively. Both basal and PGE$_1$-stimulated adenylate cyclase specific activities increased gradually during the period that cells were exposed to morphine. After 2-3 days of incubation, the specific activity of adenylate cyclase, assayed in the presence of morphine, equaled values found at zero time in the absence of morphine. Thus, culturing cells with morphine results in tolerance to the narcotic. However, a marked increase in adenylate cyclase specific activity was observed when enzyme activity was determined in the absence of morphine. Thus, morphine still inhibits adenylate cyclase activity but the inhibition is masked by a gradual, compensatory increase in the specific activity of the enzyme. Similar results were obtained in other experiments (not shown) with cells in the logarithmic phase of growth and with confluent cultures of cells, which multiply at a greatly reduced rate. One experiment of this type is shown in Fig. 5. Homogenates prepared from cells cultured with or
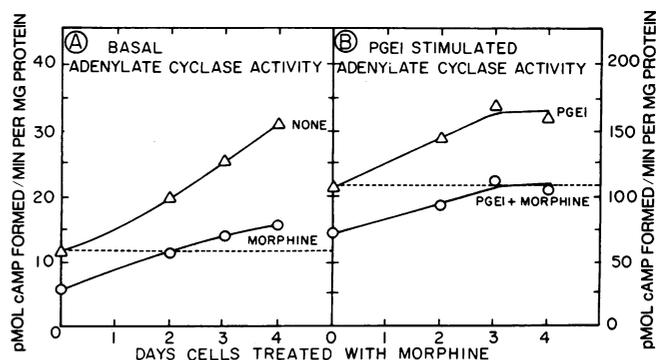


FIG. 4. Basal and PGE$_1$-stimulated adenylate cyclase activity of homogenates of NG108-15 cells cultured for the times shown in the presence of morphine. Confluent cultures (30-50%) in 100 mm petri dishes were divided into four groups; 10 $\mu$M morphine sulfate was first added to one group of cultures on day 0, to a second group on day 1, and to a third group on day 2. Media were changed and fresh morphine added daily. Cells were harvested, washed, homogenized, and assayed on day 4.
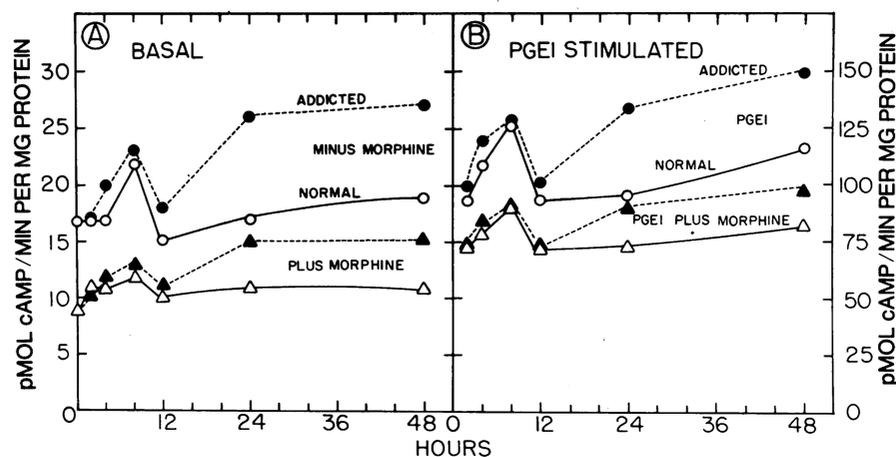
FIG. 5. Basal and PGE$_1$-stimulated adenylate cyclase activity of homogenates of NG108-15 cells cultured and assayed in the presence or absence of 10 $\mu$M morphine sulfate and/or PGE$_1$ as indicated. Cells 90% confluent in 100 mm petri dishes were cultured for the times indicated and were harvested, washed, and assayed immediately. Protein values ranged from 8.2 to 9.2 mg per dish. Filled symbols, addicted cells; empty, normal; triangles, plus morphine; circles, minus morphine.

without morphine for 0–48 hr were assayed in the presence or absence of morphine and/or PGE$_1$ as indicated in the figure. Some variation in the specific activity of adenylate cyclase was observed both with normal and addicted cells. The specific activities of adenylate cyclase of normal and addicted cells did not differ appreciably during the first 12 hr of incubation, whereas exposure of cells to morphine for 24–48 hr resulted in an increase in adenylate cyclase activity. Thus the delayed increase in adenylate cyclase specific activity requires more than 12 hr of exposure to the drug.

The effects of withdrawal, a narcotic antagonist, and stereoisomeric narcotics on positive regulation of adenylate cyclase are shown in Table 1. Cells were cultured with mor-

phine for 3 days and then for an additional day with or without morphine to test the effects of withdrawal. The results show that the morphine-dependent increase in adenylate cyclase activity was reversed, almost completely, 24 hr after withdrawal of the drug. In another experiment, not shown here, withdrawal for 30 min did not restore adenylate cyclase activity to normal levels. Thus the increase in adenylate cyclase activity due to narcotic-dependent positive regulation is stable > 30 min.

Table 1. Effects of withdrawal, a narcotic antagonist, and stereoisomeric narcotics on positive regulation of adenylate cyclase

| Additions during cell culture | pmol cAMP formed/ min per mg protein | |
|---|---|---|
| | H$_2$O | PGE$_1$ |
| *Cells cultured 4 days* | | |
| H$_2$O | 18 | 95 |
| Morphine | 27 | 115 |
| 3 Days Morphine | | |
| 1 Day Withdrawal | 21 | 98 |
| Morphine + Naloxone | 18 | 79 |
| *Cells cultured 3 days* | | |
| H$_2$O | 18 | 100 |
| Naloxone | 21 | 90 |
| Levorphanol | 30 | — |
| Dextrorphan | 21 | — |

NG108-15 hybrid cells were cultured in 100 mm petri dishes for 3 or 4 days in the presence or absence of 10 $\mu$M narcotic as indicated. Cultures were 50% confluent when narcotic was first added. The medium was replaced each day. For the withdrawal experiment cells were cultured 3 days with 10 $\mu$M morphine sulfate, then plates were washed three times with growth medium and then cultured for an additional 24 hr in growth medium devoid of morphine. The average protein per dish was 7.2 mg after 3 days and 8.2 mg after 4 days. Adenylate cyclase activity was assayed in the presence of 10 $\mu$M naloxone. The final concentration of PGE$_1$ was 10 $\mu$M. The activities found with morphine and levorphanol in the presence of naloxone were higher than those observed in the absence of naloxone; however, in all other cases little or no difference was observed in the absence of naloxone (data not shown).

Table 2. cAMP levels of hybrid cells grown in the presence or absence of morphine

| | | | pmol cAMP/mg protein | |
|---|---|---|---|---|
| Exp. no. | Hours cell culture | Additions to test cell responses | Control cells, no morphine | Addicted cells, morphine present |
| *1. Tested without phosphodiesterase inhibitor* | | | | |
| | 48 | H$_2$O | 21 | 23 |
| | 48 | Naloxone | 21 | 37 |
| | 48 | PGE$_1$ | 264 | 81 |
| | 48 | PGE$_1$ + Naloxone | 241 | 1183 |
| | 48 | Adenosine | 103 | 65 |
| | 48 | Adenosine + Naloxone | 72 | 217 |
| *2. Tested with phosphodiesterase inhibitor* | | | | |
| | 0 | H$_2$O | 89 | — |
| | 12 | H$_2$O | 54 | 40 |
| | 12 | Naloxone | 61 | 125 |
| | 24 | H$_2$O | 61 | 40 |
| | 24 | Naloxone | 60 | 285 |
| | 48 | H$_2$O | 63 | 38 |
| | 48 | Adenosine | 2000 | 859 |
| | 48 | Adenosine + Naloxone | 1430 | 3020 |

NG108-15 cells were cultured in 60 mm petri dishes until 50% confluent. Then 10 $\mu$M morphine sulfate or H$_2$O was added and incubation was continued as indicated in the table. In Exps. 1 and 2 at appropriate times cells were incubated for 15 min in the presence or absence of 0.5 mM Ro 20-1724 (final concentration). To test cell responses, 10 $\mu$M naloxone and/or PGE$_1$; 100 $\mu$M adenosine, or H$_2$O were added and incubation was continued for an additional 10 min. Reactions were terminated as described under *Methods and Materials*. Average mg of protein per dish at 0, 12, 24, and 48 hr were 2.6, 2.8, 3.5, and 4.4, respectively.

Table 3. Opiate receptors of hybrid cells cultured with or without morphine

| Cell culture conditions | fmol of [$^3$H]naloxone specifically bound/mg of protein |
|---|---|
| Control cells | 78 (67—92) |
| Cells cultured with morphine | 72 (63—80) |

Cells were cultured for 26 hr in the presence or absence of $10^{-5}$ M morphine. Homogenates of washed cells were assayed for opiate receptors as described in the *Methods and Materials* section. The values are based on duplicate determinations and are the average of four sets of duplicate dishes. Numbers in parentheses represent the range found.

Naloxone, a specific opiate antagonist with high affinity for the narcotic receptor, when present together with morphine reduced adenylate cyclase activity to levels below those of the control cells. Naloxone also reduced the PGE$_1$-dependent adenylate cyclase activity when cells were cultured with this compound. These results possibly suggest either that the cells synthesize or serum contains a morphine-like factor.

Levorphanol, a potent narcotic, increased adenylate cyclase activity of cells cultured in its presence for 3 days, whereas under the same conditions dextrorphan, the pharmacologically inactive stereoisomer, had little or no effect on basal adenylate cyclase activity.

In Table 2, cAMP levels of cells grown in the presence and absence of morphine and tested for 10 min with naloxone and/or activators of adenylate cyclase are shown. Cell response was tested in the absence of a phosphodiesterase inhibitor in Exp. 1. The data show that cells cultured in the presence of morphine have normal basal cAMP levels. In the presence of added naloxone, however, basal, PGE$_1$-, and adenosine-stimulated cAMP levels were considerably higher than those found with control cells. These data demonstrate morphine-dependent positive regulation of cAMP levels in intact cells, which agrees well with the increased adenylate cyclase activity found with homogenates. The data also show that cells grown in the presence of morphine are tolerant to morphine since their basal cAMP level is similar to that of control cells. Note that the cAMP assays were performed with cells still in the presence of growth medium and in the case of the addicted cells with morphine. The increase in cAMP levels found after short exposure of the cells to naloxone indicates that the cells have become dependent upon the presence of morphine for the preservation of normal cAMP levels. This phenomenon is, we believe, the biochemical counterpart of the abstinence syndrome seen with animals and man upon administration of antagonists to dependent individuals.

In Exp. 2, the phosphodiesterase inhibitor Ro 20-1724 was present when cell responses were tested. Cells grown in the presence of morphine for 12 hr had higher cAMP levels than control cells in the presence of naloxone. The difference was greater after 24 hr of growth with morphine. However, complete tolerance to morphine was not achieved under the conditions used even after 48 hr of culture with morphine. In the presence of naloxone, adenosine-stimulated cAMP levels of addicted cells were higher than those of control cells, similar to the observations made in the absence of Ro 20-1724.

In Fig. 6, morphine is shown to have no effect upon the rate of protein synthesis during 9 days of growth. The
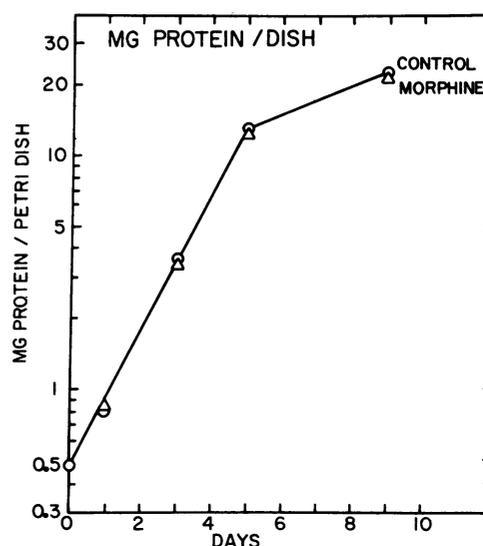


FIG. 6. Lack of effect of morphine on cell growth. Replicate cultures, each with 1.5 × 10$^5$ NG108-15 cells per 150 mm petri dish, were maintained in the presence or absence of 10 $\mu$M morphine sulfate. Cells were washed three times and the amount of protein per dish was determined.

amount of protein synthesized per dish is used as a measure of cell growth and replication. Thus, the effects of morphine upon adenylate cyclase activity of cells made tolerant to the narcotic are specific ones and are not due to an increase in total protein synthesis.

Another way in which the cells might have adapted to the presence of morphine is by changing the number or properties of opiate receptors. The data shown in Table 3 indicate that such changes have not taken place. The number of morphine receptors measured in homogenates prepared from well-washed hybrid cells grown in the presence of morphine is, within experimental error, unchanged from those of the control cells. The number of opiate receptors found in morphine-dependent rats also is similar to that of control animals (14).

## DISCUSSION

Narcotics affect adenylate cyclase in two ways, both mediated by the opiate receptor. The first process is a readily reversible inhibition of the enzyme by narcotics observed with homogenates and, indirectly, with intact cells. The second phenomenon, late positive regulation, results in an increase in adenylate cyclase specific activity which is dependent upon incubation of cells with narcotics for 12 or more hours. The effects of narcotics on both inhibition and positive regulation of adenylate cyclase are stereospecific and are reversed by naloxone. After culture with morphine for 1–2 days cells are tolerant to the narcotic, since the increase in adenylate cyclase activity due to positive regulation compensates for the inhibition of enzyme activity observed in the presence of the narcotic. Cells are also dependent upon morphine, since withdrawal, or displacement of morphine from the opiate receptor by the antagonist, naloxone, increases basal, PGE$_1$-, and adenosine-stimulated adenylate cyclase activities, which results in an overproduction of cAMP. This phenomenon can be likened to the abstinence syndrome in animals. Thus, NG108-15 cells become dependent upon morphine in 12–24 hr.

The mechanism of positive regulation of adenylate cyclase is not known. The long incubation in the presence of

narcotics required to increase adenylate cyclase activity, the fact that increases in basal, adenosine-, and $PGE_1$-stimulated adenylate cyclase activities are observed, and the relative stability of the elevated activity suggests that positive regulation represents an increase in the number of molecules of adenylate cyclase. The $PGE_1$- and adenosine-dependent elevations of cAMP levels in NG108-15 cells are not additive[§]; thus adenylate cyclase molecules rather than $PGE_1$ or adenosine receptors probably are limiting in these cells. We do not rule out the possibility that the enzyme activity is increased by the formation of a relatively long-lived modulator; however, no evidence for the formation of a diffusable activator or inhibitor of adenylate cyclase was detected when homogenates prepared from normal and narcotic-dependent cells were combined and assayed for adenylate cyclase activity. Goldstein and Goldstein (15) as well as Shuster (16) in 1961 proposed that enzyme induction might be a mechanism for drug tolerance and dependence.

Positive regulation of adenylate cyclase represents a new type of receptor-mediated control of adenylate cyclase activity. Inhibition of adenylate cyclase activity and late positive regulation are coupled, since both are initiated by interactions of narcotic with the opiate receptor. The inhibition of adenylate cyclase activity and concomitant reduction in cAMP levels may be required for positive regulation; that is, the concentration of cAMP or the activity of adenylate cyclase may regulate the number of adenylate cyclase molecules in a sequential feedback fashion. If so, each transmitter, hormone, or effector of adenylate cyclase is, in fact, a dual regulator, for transient stimulation or inhibition of adenylate cyclase may evoke a regulatory process in the opposite direction hours after the initial event.

The slowly expressed, relatively stable, positive regulatory process is a form of memory. Shifts in the number of molecules of adenylate cyclase or a long-lived modulator of enzyme activity would alter the sensitivity of cell responses to other activators or inhibitors of adenylate cyclase, and thus would profoundly affect the efficiency of trans-synaptic communication.

We think it likely that the endogenous morphine-like factor termed enkaphaline, a newly discovered peptide or family of peptides found in the nervous system which mimic the effects of opiates (17), will evoke both transient inhibition and late positive regulation of adenylate cyclase and, thus, may be involved in a memory process. Certainly any reaction affecting either the number of molecules needed for sending or receiving neural information or molecules that regulate their activities provides a potential for activating or deactivating neural circuits and thus may play a role in a memory process.

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