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Morphine Receptors as Regulators of Adenylate Cyclase Activity

(neuroblastoma × glioma hybrid/prostaglandin E_1/narcotic dependence)

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ABSTRACT **Morphine inhibits adenylate cyclase (EC 4.6.1.1) activity of neuroblastoma × glioma hybrid cells. The inhibition is stereospecific and is reversed by the antagonist, naloxone. The relative affinities of narcotics for the opiate receptor agree well with their effectiveness as inhibitors of adenylate cyclase. Morphine-sensitive and -insensitive cell lines were found, and the degree of sensitivity was shown to be dependent upon the abundance of narcotic receptors. Thus, morphine receptors are functionally coupled to adenylate cyclase. A molecular mechanism for narcotic addiction and tolerance is proposed.**

A neuroblastoma × glioma hybrid cell line with morphine receptors has been described (1). The receptors are stereospecific with respect to narcotic binding, and receptor affinities for narcotics closely resemble those of rat brain (2–4) and correlate well with their pharmacologic potencies.

Collier and Roy have reported that prostaglandin E dependent cAMP synthesis is inhibited by narcotics in rat brain homogenates (5, 6). In this communication the effects of morphine and other narcotics upon adenylate cyclase [EC 4.6.1.1; ATP pyrophosphate-lyase (cyclizing)] activity of neuroblastoma × glioma hybrids and parental cells are examined. We find that morphine and other narcotics inhibit adenylate cyclase activity and that the inhibition is dependent upon morphine receptors.

MATERIALS AND METHODS

Materials. [*G*-^{3}H]Naloxone (26.3 Ci/mmol) and [*G*-^{3}H]-cAMP (22.1 Ci/mmol) were from New England Nuclear; [α-^{32}P]ATP (6–10 Ci/mmol) was from ICN; and [*U*-^{14}C]-cAMP (0.295 Ci/mmol) was from Amersham/Searle. Morphine sulfate was from Merck; naloxone·HCl was a gift of Endo Labs; etorphine was from Dr. R. Willette, NIDA; other narcotics were from Dr. Everette May; PGE_1 was from Dr. John Pike, Upjohn Co.; Ro20-1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone was from Hoffman La-Roche; IBMX (3-isobutyl-1-methylxanthine) was from Aldrich; Gpp(NH)p (5′-guanylylimidodiphosphate) was from ICN; cAMP, creatine phosphate, and creatine kinase (155 U/mg of protein) were from Sigma.

Cell Lines. Neuroblastoma × glioma hybrid NG108-15 was obtained§ by fusion of mouse neuroblastoma clone N18TG-2 (7) and rat glioma clone C6BU-1 (8), derived from C6 (9). Cells were grown as described (1).

Assay of cAMP of Intact Cells. Confluent cells (3–4 mg of protein 60 min dish) were washed 3 times with 5 ml of medium A [Dulbecco's modified Eagle's medium with 25 mM Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) pH 7.4, instead of $NaHCO_3$, adjusted to 340 mosmol/liter with 1.1 g of NaCl/liter] and incubated with medium A plus 0.5 mM Ro20-1724 and 0.5 mM IBMX for 30 min at 37°. Reactions were initiated by the addition of 30 μl of narcotic in water, 30 μl of PGE_1 in ethanol, or solvent. Ethanol (0.5% with Ro20-1724 present or 1% when Ro20-1724 and PGE_1 were present) had no effect upon cAMP formation. After incubation the medium was discarded and 3 ml of 5% trichloroacetic acid with 5 pmol of [^{14}C]cAMP (3000 cpm) at 3° were added. The extract and 2 washes (each 1.5 ml of 5% trichloroacetic acid) were combined and centrifuged and the supernatant fluids applied to 0.8 × 8-cm columns of AG 50W-X4 resin, 200–400 mesh, H^+ form (BioRad). The 3 ml of eluate following a 6-ml water wash was assayed for cAMP by the method of Gilman (10). Values reported are for duplicate dishes and are corrected to 100% recovery of cAMP.

Adenylate Cyclase Assay. Cells that had been washed three times were homogenized in 0.32 M sucrose, 10 mM Tris·HCl, pH 7.4 (15 mg of protein per ml) with 10–15 strokes, by hand, of a ground-glass homogenizer. Enzyme activity was determined by a modification of method C of Salomon *et al.* (11). Each tube contained: 45 mM Tris·HCl, pH 7.4; 5 mM $MgCl_2$; 160 mM sucrose; 20 mM creatine phosphate; 10 U of creatine kinase; 1 mM cAMP; 0.5 mM Ro20-1724 (0.5% ethanol, final concentration); 1 mM [α-^{32}P]ATP (3 to 5 × 10^6 cpm); and 100–300 μg of homogenate protein in a final volume of 100 μl. The reaction was terminated by addition of 50 μl of 15% trichloroacetic acid. For product characterization, the ^{32}P product purified through the alumina column step (11) was subjected to Dowex-1 formate column chromatography. The 2 M formic acid eluate was lyophilized, and the radioactivity was characterized by paper or thin-layer chromatography in: (*A*) isopropanol–NH_4OH–0.1 M boric acid (7:1:2); (*B*) 1 M ammonium acetate–95% ethanol (3:7); (*C*) isobutyric acid–2 M NH_4OH (2:1); and (*D*) H_2O-washed polyethyleneimine-cellulose thin-layer plates developed sequentially with H_2O and 0.25 M LiCl. Greater than 90% of the radioactivity recovered from the alumina columns was cAMP.

Opiate Binding. The assay (1) was modified so that incubation mixtures contained the components of the adenylate cyclase assay and 875 μg of homogenate protein; 5 × 10^{-8} M [^{3}H]naloxone (4150 cpm); and unlabeled narcotic in a final volume of 200 μl. Under these conditions, narcotic affinities

Abbreviations: Ro20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; IBMX, 3-isobutyl-1-methylxanthine; Gpp(NH)p, 5′-guanylylimidodiphosphate; PGE_1, prostaglandin E_1.

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§ B. Hamprecht, T. Amano, and M. Nirenberg, in preparation.

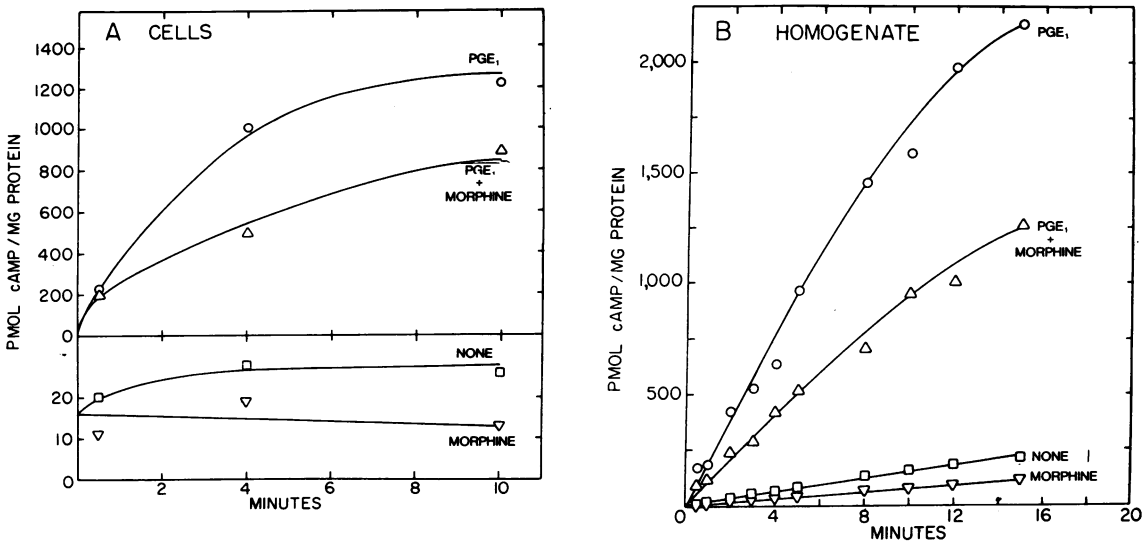

FIG. 1. Inhibition by morphine of the rate of cAMP accumulation in intact neuroblastoma × glioma hybrid NG108-15 cells and of adenylate cyclase activity in homogenates. The effect of morphine on basal and PGE_1-stimulated cAMP levels in intact cells is shown in part A. In B, the rate of adenylate cyclase activity in NG108-15 homogenates is shown. Concentrations of PGE_1 and morphine were 10 μM.

for the receptor are lower than those as measured (1) by the following factors: naloxone, 2; etorphine, 13; dextrorphan, 67; levorphanol, 667; morphine, 200; and 3-allylprodine, 100.

RESULTS

The effects of morphine upon basal and PGE_1-stimulated cAMP levels in intact NG108-15 hybrid cells and on adenylate cyclase activity in cell-free homogenates are shown in Fig. 1A and B, respectively. The addition of PGE_1 to intact cells results in a 40-fold increase in intracellular cAMP levels. The addition of morphine markedly reduces cAMP levels, both in the presence and absence of PGE_1. As shown in panel B, the addition of PGE_1 to homogenates similarly results in a 10-fold increase in adenylate cyclase activity (5- to 12-fold increase in other experiments). Morphine inhibits basal and PGE_1-dependent adenylate cyclase activity about 45% (30–60% inhibition in other experiments). The rate of cAMP formation was linear for about 8 min; therefore, in all subsequent experiments reaction mixtures were incubated for 5 min.

The effect of morphine upon adenylate cyclase activity was verified in the following ways: (*i*) the [^{32}P]product of the reaction was shown to have the chromatographic mobility of authentic cAMP in five systems (see *Materials and Methods*); (*ii*) the concentration of substrate, ATP, did not change appreciably during incubation by direct measurement with the luciferase assay (12); and (*iii*) the specific activity of ATP changed less than 5% during the incubation.

The effect of naloxone, an antagonist that displaces morphine from the opiate receptor but has no narcotic activity, is shown in Table 1. Naloxone reverses the inhibitory effect of morphine upon adenylate cyclase activity both in the presence and absence of PGE_1, but naloxone alone has little or no effect upon basal or PGE_1-stimulated adenylate cyclase activity.

The relation between molarity of PGE_1 and the specific activity of adenylate cyclase is shown in Fig. 2. Adenylate cyclase is stimulated by concentrations of PGE_1 greater than 10^{-8} M. A discontinuity in the concentration curve is observed at 5×10^{-7} M PGE_1.

The effects of different concentrations of naloxone, in the presence and absence of morphine, on adenylate cyclase are shown in Fig. 3. The concentration of morphine was 10^{-5} M, which inhibits adenylate cyclase activity maximally. Naloxone completely reverses the inhibitory effect of morphine on adenylate cyclase activity at 2×10^{-5} M; naloxone at higher concentrations stimulates adenylate cyclase activity 15–20%. Stimulation has not been observed in all experiments, and further experiments are needed to clarify this observation.

In Fig. 4 the decrease in binding of [^{3}H]naloxone to the narcotic receptor, as a function of narcotic concentration, is compared with the effectiveness of the narcotics as inhibitors of adenylate cyclase activity. Naloxone, a pure antagonist, binds with high affinity to the opiate receptor but does not inhibit adenylate cyclase activity (panel A). Etorphine, levorphanol, morphine, and 3-allylprodine, each a potent narcotic, inhibit adenylate cyclase at essentially the same

TABLE 1. *Reversal of morphine inhibition of adenylate cyclase by naloxone in cell-free preparations of NG108-15*

Addition*	Homogenate†	Membrane‡ fraction
	(pmol of cAMP formed/ min per mg of protein)	
None	17	24
Morphine	8	17
Naloxone	17	22
Morphine + naloxone	17	23
PGE_1	113	124
PGE_1 + morphine	68	101
PGE_1 + naloxone	110	
PGE_1 + morphine + naloxone	100	122

* 10 μM, each component.

† Unfractionated homogenate.

‡ The homogenate was centrifuged at 1500 × *g* for 10 min and the supernatant fraction at 17,000 × *g* for 30 min. The 17,000 × *g* pellet was washed and resuspended in the homogenizing medium (membrane fraction).

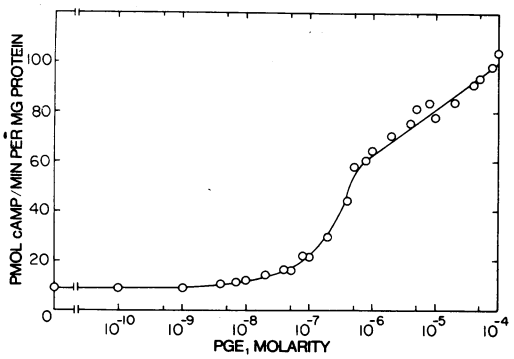

FIG. 2. Activation of adenylate cyclase in homogenates of NG108-15 by PGE_1 as a function of PGE_1 concentration.

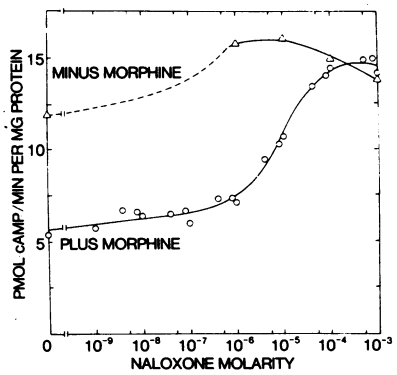

Fig. 3. The effect of naloxone upon adenylate cyclase activity of an NG108-15 homogenate in the presence and absence of 10 μM morphine.

concentrations as those needed to displace [^{3}H]naloxone from the opiate receptor (panels B, C, E, and F). In contrast to levorphanol, the analgesically inactive enantiomer dextrorphan is without effect on adenylate cyclase activity and has a much lower affinity for the opiate receptor (panel D).

Apparent dissociation constants for [narcotic·opiate receptor] complexes are shown in Table 2, along with apparent K_i values for adenylate cyclase calculated from the data of Fig. 4. A striking similarity is observed between the dissociation constants and the concentrations of narcotics required for 50% inhibition of adenylate cyclase activity.

Narcotic binding and enzyme inhibition curves shown in Fig. 4 are not superimposable, even though the narcotic concentrations for half-maximal inhibition of binding and enzyme activity agree well. With each of the four narcotics, enzyme activity changes over a relatively small range of narcotic concentration compared to narcotic binding. This relationship is seen more clearly in Fig. 5A and B, which shows Hill plots of narcotic binding and adenylate cyclase inhibition, respectively. Each slope for narcotic binding is approximately 1, whereas the maximum slopes for adenylate cyclase inhibition are 2.3. These results suggest that the binding of narcotics to receptors is not a cooperative process, whereas the effects of narcotics on adenylate cyclase activity exhibit a cooperative character.

The relation between the molarity of Gpp(NH)p, a relatively stable analog of GTP, and adenylate cyclase activity, in the presence and absence of morphine, is shown in Fig. 6.

TABLE 2. *Comparison of narcotic affinity for the opiate receptor and ability to inhibit adenylate cyclase*

Narcotic	K_d* Narcotic receptor nM	K_i† Adenylate cyclase nM
Etorphine	5	10
Levorphanol	200	200
Morphine	4,000	2,000
3-Allylprodine	10,000	50,000
Dextrorphan	10,000	—
Naloxone	20	—

* Apparent dissociation constant of the narcotic–receptor complex.

† Narcotic concentration required for 50% of maximal inhibition of adenylate cyclase.

—, no inhibition.

The GTP analog activates adenylate cyclase of NG108-15 homogenates, similar to the effects reported with other systems (13). Complete reversal of morphine inhibition of adenylate cyclase was observed in the presence of 1×10^{-5} M

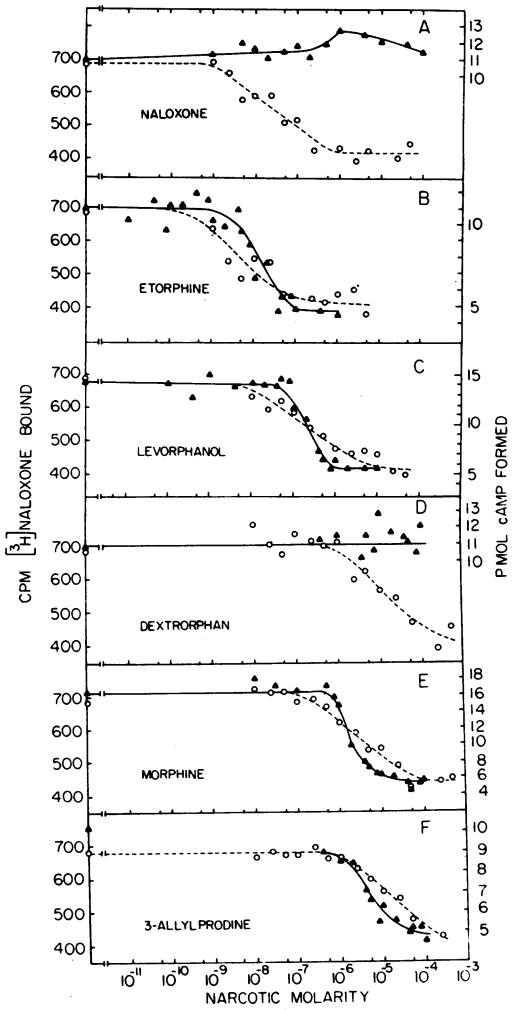

FIG. 4. The effectiveness of various narcotics as inhibitors of adenylate cyclase activity of NG108-15 homogenates is compared with their ability to displace [^{3}H]naloxone from the opiate receptor. Symbols represent the following: O, [^{3}H]naloxone bound to receptor; ▲, [^{32}P]cAMP formed per 5 min per tube (250 μg of protein per tube).

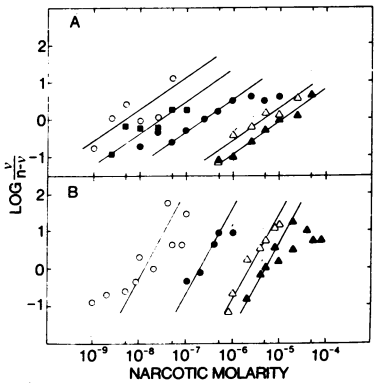

FIG. 5. (A) Hill plots of narcotic binding to receptor; and (B) inhibition of adenylate cyclase activity by narcotics. The symbols represent the following: O, etorphine; ■, naloxone; ●, levorphanol; △, morphine; and ▲, 3-allylprodine. In panel A the symbol n in the ordinate represents the maximum amount of [^{3}H]-naloxone displaced at saturating concentrations of unlabeled narcotic and ν is the amount of [^{3}H]naloxone displaced at nonsaturating concentrations of narcotic. In panel B, n represents the maximum inhibition of adenylate cyclase observed at saturating narcotic concentrations and ν is the inhibition observed at each nonsaturating concentration of narcotic.

Gpp(NH)p. In the absence of morphine, $<1 \times 10^{-5}$ M Gpp-(NH)p has little effect upon adenylate cyclase activity. Similar results were obtained with partially purified membrane preparations (not shown).

The effects of morphine upon adenylate cyclase activity of intact neuroblastoma N18TG-2 and glioma C6BU-1 cells, the parents of hybrid NG108-15, are shown in Fig. 7A and B, respectively. Morphine reduces cAMP levels of neuroblastoma N18TG-2 cells slightly but does not affect cAMP levels of glioma C6BU-1 cells.

The effects of morphine and naloxone upon adenylate cyclase activity of homogenates prepared from parent neuroblastoma N18TG-2 and glioma C6BU-1 cells are shown in Table 3. Morphine inhibits adenylate cyclase activity of N18TG-2 slightly, and the inhibition is reversed by naloxone; however, these compounds do not affect adenylate cyclase activity of C6BU-1. Thus, the neuroblastoma parent, which has few morphine receptors,¶ is slightly sensitive to morphine whereas the glioma parent, which lacks these receptors, is insensitive to morphine.

DISCUSSION

The results show that morphine reduces basal and PGE_1-stimulated cAMP levels of intact neuroblastoma × glioma hybrid cells and inhibits adenylate cyclase activity of homogenates. The effects of morphine are blocked by the narcotic antagonist, naloxone, which competes with narcotics for sites on the opiate receptor. The inhibition of adenylate cyclase activity by narcotics is specific for the levorotatory, pharmacologically active, stereoisomer. The relative affinities of narcotics for the opiate receptor agree well with their effectiveness as inhibitors of adenylate cyclase and with their pharmacologic potency. Morphine-sensitive and -insensitive cell lines

¶ Neuroblastoma clone N18TG-2 can be shown to have a relatively small number of morphine receptors when binding experiments are performed with [^{3}H]naloxone in place of the [^{3}H]-dihydromorphine used previously (1). Glioma clone C6BU-1 shows no specific binding of either naloxone or dihydromorphine.

TABLE 3. *Effect of morphine on adenylate cyclase activity of neuroblastoma and glioma parents*

Addition*	Homogenate: Neuroblastoma N18TG-2 (pmol of cAMP formed/min per mg of protein)	Homogenate: Glioma C6BU-1 (pmol of cAMP formed/min per mg of protein)
None	6	20
Morphine	4	19
Naloxone	5	20
Morphine + naloxone	5	20
PGE_1	75	24
PGE_1 + morphine	63	25
PGE_1 + naloxone	72	24
PGE_1 + morphine + naloxone	70	23

* 10 μM, each component.

were found, and the degree of sensitivity to morphine is dependent upon the abundance of narcotic receptors.

Thus, two kinds of adenylate cyclase complex were detected, one sensitive, the other insensitive, to narcotics. Sensitivity to narcotics is dependent upon functional interaction of the [morphine·receptor] complex with adenylate cyclase. The partial inhibition of adenylate cyclase observed in the presence of saturating concentrations of narcotics may be due, at least in part, to the presence of narcotic-sensitive and -insensitive adenylate cyclase molecules within cells and, possibly, heterogeneity in the cell population with respect to the number of narcotic receptors per cell.

Interactions that couple morphine receptors with adenylate cyclase exhibit positive cooperativity; however, the interaction of narcotic with receptor is not a cooperative process. The Hill coefficient of 2.3 suggests that multiple interactions between [narcotic·receptor] and the adenylate cyclase complex may be required for inhibition. Alternate mechanisms for apparent cooperativity may be envisaged involving threshold effects and spare receptors. The cooperative activation of adenylate cyclase has been reported for thyroid stimulating hormone (14), but glucagon (15) and oxytocin (16) do not exhibit cooperativity.

The available information suggests that one molecule of

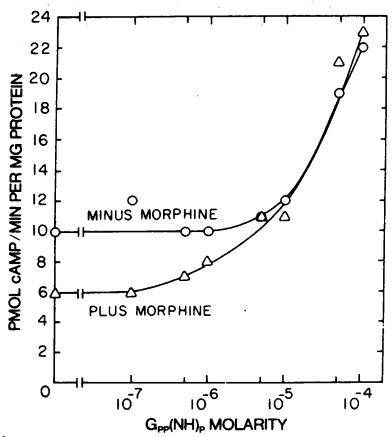

FIG. 6. Effect of Gpp(NH)p on adenylate cyclase activity of an NG108-15 homogenate, in the presence and absence of 10 μM morphine.

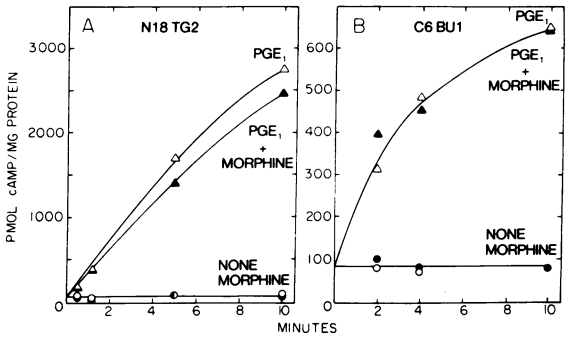

FIG. 7. Cyclic AMP accumulation, as a function of time, in intact parental cells. Cells used were neuroblastoma N18TG-2 in panel A and glioma C6BU-1 in panel B. Concentrations of morphine and PGE_1 were 10 μM.

adenylate cyclase can respond to different kinds of hormones, depending upon the species of receptors present, but that only one kind of hormone is translated at a time. Usually, hormones activate adenylate cyclase but enzyme inhibition mediated by some α- or prostaglandin receptors has been reported (17, 18). Morphine thus resembles an inhibitory hormone.

In essence, three kinds of mechanism have been proposed for the regulation of adenylate cyclase activity by hormones and other effector molecules: (*i*) Hormones and GTP are allosteric effectors that activate the enzyme by reducing its affinity for free ATP, a competitive inhibitor of the substrate [ATP·Mg] (19, 20). (*ii*) Mg^{++} or Ca^{++} bind to other sites and thereby regulate enzyme activity (21, 22). (*iii*) Adenylate cyclase is inactivated by phosphorylation, catalyzed by a protein kinase, and activated by dephosphorylation, catalyzed by a phosphatase (23).

Reactions that terminate reading of messages by adenylate cyclase regulate the duration of response of the enzyme to each message. GTP may be involved both in initiation and termination of reading of hormonal messages by adenylate cyclase in ways that may be analogous to the roles of GTP in aminoacyl-tRNA binding to ribosomes and translocation reactions in protein synthesis.

The GTP analog, Gpp(NH)p, activates adenylate cyclase, and morphine reduces the threshold for activation 10-fold. Thus morphine and Gpp(NH)p act in a concerted fashion, which suggests that both are physically coupled to the adenylate cyclase complex. The K_m for glucagon stimulation of adenylate cyclase is reduced 10-fold by GTP (24). The possibility that GTP is required for morphine action, as it is for hormone action, should be considered.

Collier and Roy have presented cogent arguments supporting the thesis that PGE-stimulated adenylate cyclase is the primary site of morphine action (5, 6). Our results support their view, although we find that morphine inhibits adenylate cyclase both in the presence and absence of added PGE_1. The inhibition of adenylate cyclase should have many secondary consequences which might account for the varied pharmacologic effects of morphine.

We wish to propose the following hypothesis concerning the molecular basis for narcotic addiction and tolerance. Inhibition of adenylate cyclase by morphine reduces intracellular levels of cAMP. This may lead to a compensatory shift in enzyme synthesis, degradation, or activity which restores the normal level of cAMP. The cell then is dependent upon narcotic because the level of cAMP is normal in the presence of the drug and abnormally high upon withdrawal. Our working hypothesis is that the number of adenylate cyclase molecules increases as cells become addicted to narcotics. This also leads to tolerance since, at a given narcotic concentration, the amount of uninhibited enzyme is greater in addicted than in normal cells.

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