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Opiate-dependent modulation of adenylate cyclase

(neuroblastoma × glioma hybrid cells/enzyme regulation/morphine receptors/prostaglandin E_1 receptors/supersensitivity)

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ABSTRACT **Reactions mediated by the opiate receptors that inhibit adenylate cyclase (EC 4.6.1.1) are closely coupled to subsequent reactions that gradually increase adenylate cyclase activity of neuroblastoma × glioma NG108-15 hybrid cells. Opiate-treated cells have higher basal-, prostaglandin E_1-, and 2-chloroadenosine-stimulated activities than do control cells. However, NaF or guanosine 5′-(β,γ-imido)triphosphate abolishes most of the difference in adenylate cyclase activity observed with homogenates from control and opiate-treated cells. Cycloheximide blocked some, but not all, of the opiate-dependent increase in adenylate cyclase activity. These results suggest that the opiate-dependent increase in adenylate cyclase is due to conversion of adenylate cyclase to a form with altered activity. Protein synthesis also is required for part of the opiate effect. We propose that activity of adenylate cyclase determines the rate of conversion of the enzyme from one form to the other and that opiates, by inhibiting adenylate cyclase, alter the relative abundance of low- and high-activity forms of the enzyme.**

Inhibition of adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] by opiates is rapidly reversed upon removal of opiate (1–3). However, exposure of cells to an opiate for 12 or more hours results in a compensatory increase in adenylate cyclase activity that is long-lived and not readily reversed by withdrawal of the opiate (4–6). Similar dual regulations of adenylate cyclase activity are mediated by α-adrenergic receptors (7) and excitatory muscarinic acetylcholine receptors (S. K. Sharma and M. Nirenberg, unpublished data; ref. 8). Thus, dual regulation may be a general regulatory mechanism which alters the responsiveness of adenylate cyclase to activations mediated by other species of receptors. Because the mechanism of coupling inhibition of adenylate cyclase with a subsequent increase in enzyme activity is unknown, we examined the properties of adenylate cyclase from control cells and cells treated with opiates.

MATERIALS AND METHODS

The source of each chemical, growth conditions for culturing neuroblastoma × glioma NG108-15 hybrid cells, and preparation of homogenates have been described (2). Adenylate cyclase activity was measured by the procedure of Salomon *et al.* (9), modified slightly (2). Each tube contained 30 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$/160 mM sucrose/20 mM creatine phosphate/10 units of creatine kinase (65 μg of protein)/1 mM adenosine 3′:5′-cyclic monophosphate (cAMP)/0.5 mM 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724)/0.5% ethanol/1 mM [α-^{32}P]ATP (3 to 5 × 10^6 cpm) and 50–200 μg of homogenate protein in a final volume of 100 μl. Incubations were for 5 min at 37°.

RESULTS

Effect of Activators of Adenylate Cyclase. If the increase in adenylate cyclase activity of opiate-treated NG108-15 cells were due primarily to an increase in the number of molecules of adenylate cyclase, the relatively high enzyme activity of opiate-treated cells should be maintained under other assay conditions. For this reason, we studied the effects of activators and inhibitors on adenylate cyclase of cells grown in the presence or absence of morphine or etorphine for 3–5 days. Sodium fluoride is known to activate basal adenylate cyclase and inhibit activation of the enzyme by hormones, presumably by uncoupling the enzyme from the hormone-receptor complex (10). The effects of NaF upon basal and prostaglandin E_1 (PGE_1)-stimulated adenylate cyclase activity from control or etorphine-treated NG108-15 cells as a function of time are shown in Fig. 1. In the presence of 8 mM NaF, the basal activities of adenylate cyclase from control and opiate-treated cells were increased 4.3- and 2.6-fold, respectively, and the specific activities of the two enzyme preparations then were similar. Conversely, the activities of PGE_1-stimulated adenylate cyclase from control and opiate-treated cells were inhibited 44% and 60%, respectively, by NaF and the final specific activities were similar. These results show that adenylate cyclase from control cells is more responsive to activation by NaF than adenylate cyclase of opiate-treated cells and raise the possibility that the relatively high activity of adenylate cyclase of opiate-treated cells may be due to enzyme activation rather than to an increase in the number of enzyme molecules.

In other systems, guanosine 5′-(β,γ-imido)triphosphate [Gpp(NH)p] has been shown to activate basal adenylate cyclase and to activate or inhibit adenylate cyclase synergistically with hormones initially but ultimately to uncouple adenylate cyclase from the hormone-receptor complex (11, 12). The effects of Gpp(NH)p on basal or PGE_1-stimulated adenylate cyclase from control and etorphine-treated cells as a function of time are shown in Fig. 2. Incubation with Gpp(NH)p for 16 min increased basal adenylate cyclase activity of control cells 460% and that of opiate-treated cells, 280%. Conversely, Gpp(NH)p inhibited PGE_1-stimulated adenylate cyclase from control and opiate-treated cells 25% and 41%, respectively. In the presence of Gpp(NH)p, the specific activities of adenylate cyclase from control and etorphine-treated cells were almost the same. These results provide additional evidence for two forms of adenylate cyclase that differ in extent of response to Gpp(NH)p or NaF and suggest that the relatively high enzyme activity of opiate-treated cells may be due to enzyme activation.

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Abbreviations: cAMP, adenosine 3′:5′-cyclic monophosphate; Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; PGE_1, prostaglandin E_1; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate.

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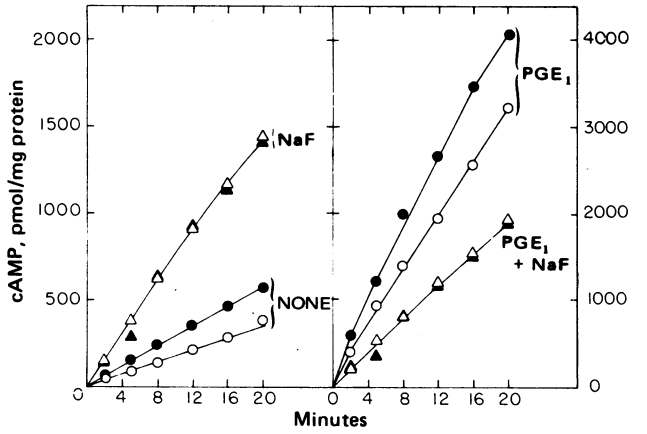

FIG. 1. Effect of NaF on basal (*Left*) and PGE_1-stimulated (*Right*) adenylate cyclase activities of homogenates prepared from NG108-15 cells (subculture 15) cultured for 3 days with or without 1 μM etorphine. Each point represents a 50-μl aliquot of a 400-μl reaction mixture that contained the components described in *Materials and Methods* and the following additions or exceptions: 15 mM magnesium acetate; 10 μM naloxone; and either 57 μg of homogenate protein from control cells cultured without etorphine or 59 μg of homogenate protein from cells cultured with etorphine. When present (*Right* panel), PGE_1 was 10 μM. Symbols: ○, no addition, control homogenate; ●, no addition, homogenate from etorphine-treated cells; △, 8 mM NaF, control homogenate; ▲, 8 mM NaF, homogenate from cells treated with etorphine.

The effects of different concentrations of NaF or Gpp(NH)p on the activities of adenylate cyclase from control or opiate-treated NG108-15 cells are shown in Fig. 3. At relatively low concentrations of activator [2 mM NaF or 5 μM Gpp(NH)p], basal adenylate cyclase of control cells was activated but not the enzyme of opiate-treated cells. Higher concentrations of NaF or Gpp(NH)p increased the enzyme activity from both control and opiate-treated cells to the same extent, but the specific activities of the two enzyme preparations were almost the same. Conversely, PGE_1-stimulated adenylate cyclase of control cells was inhibited less by NaF or Gpp(NH)p than the enzyme from opiate-treated cells. Both the PGE_1-stimulated

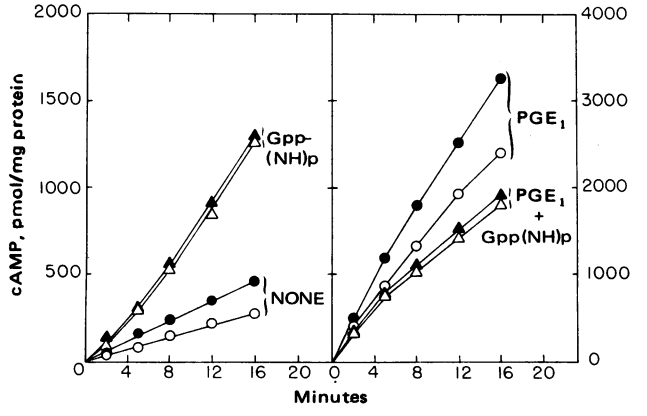

FIG. 2. Effect of Gpp(NH)p on the activity of basal (*Left*) and PGE_1-stimulated (*Right*) adenylate cyclase from NG108-15 cells (subculture 15) treated 3 days with or without 1 μM etorphine. The conditions were as described in the legend to Fig. 1 except that reaction mixtures contained, where specified: 100 μM Gpp(NH)p; 59 μg of homogenate protein from control cells; or 55 μg of homogenate protein from cells treated with etorphine. Symbols: ○, no addition, control homogenate; ●, no addition, homogenate from cells treated with etorphine; △, 100 μM Gpp(NH)p, control homogenate; ▲, 100 μM Gpp(NH)p, homogenate from cells treated with etorphine. (*Right*) Each reaction mixture contained 10 μM PGE_1.

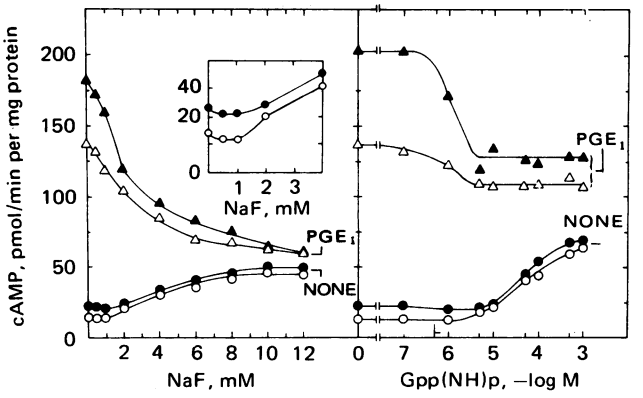

FIG. 3. Relationship between NaF (*Left*) or Gpp(NH)p (*Right*) concentration and adenylate cyclase activity in homogenates of NG108-15 cells treated for 5 days with or without 1 μM etorphine. Each 100-μl reaction mixture contained the components described in *Materials and Methods* with the following exceptions: 15 mM magnesium acetate; 10 μM naloxone; 10 μM PGE_1 where specified; NaF or Gpp(NH)p concentrations as specified. (*Left*) Each reaction mixture contained either (open symbols) 86 μg of homogenate protein from cells cultured 5 days without etorphine (control homogenate); or (solid symbols) 111 μg of homogenate protein from cells cultured 5 days with 1 μM etorphine (treated). The initial portions of the curves labeled "NONE" are enlarged in the *Inset* to show additional detail. (*Right*) Reaction mixture components were as in *Left* except that the reaction mixtures contained Gpp(NH)p instead of NaF, and each reaction mixture contained either 138 μg of homogenate protein from NG108-15 cells cultured 5 days without etorphine (control) or 170 μg of homogenate protein from NG108-15 cells treated 5 days with 1 μM etorphine (treated). Symbols: ○, no addition, control homogenate; ●, no addition, homogenate from etorphine-treated cells; △, 10 μM PGE_1, control homogenate; ▲, 10 μM PGE_1, homogenate from etorphine-treated cells.

and basal activities of adenylate cyclase from control cells almost equaled those of opiate-treated cells in the presence of 2 mM NaF or 5 μM Gpp(NH)p. The concentrations of Gpp(NH)p or NaF required for half-maximal inhibition of PGE_1-stimulated adenylate cyclase were similar to the concentrations required for half-maximal reversal of the difference between control and opiate-treated adenylate cyclase activity (3 μM Gpp(NH)p or 1.5 mM NaF). Higher concentrations of Gpp(NH)p (50 μM) or NaF (4 mM) were required for half-maximal activation of basal adenylate cyclase. Thus, reversal of the difference between control and opiate-treated adenylate cyclase activities resembles the uncoupling of a receptor from adenylate cyclase.

The hybrid cells were incubated with or without cycloheximide in the presence or absence of etorphine to determine whether inhibition of protein synthesis affected the opiate-dependent increase in adenylate cyclase activity (Fig. 4). Cycloheximide blocked the etorphine-dependent increase in adenylate cyclase at 10 and 20 hr of incubation but not at 4 hr. Similar results were obtained in other experiments not shown here. Although the toxic effects of cycloheximide on cells limited the duration of cell incubation and decreased the magnitude of the etorphine-dependent increase in adenylate cyclase activity, the results show that adenylate cyclase is a relatively stable enzyme that does not turn over rapidly under the conditions tested and suggest that most, but not all, of the increase in adenylate cyclase activity evoked by etorphine is dependent on protein synthesis. The results should be interpreted with caution because cycloheximide probably affects many reactions, directly or indirectly, during the long incubation period.

The effects of varying PGE_1 or 2-chloroadenosine (13, 14)

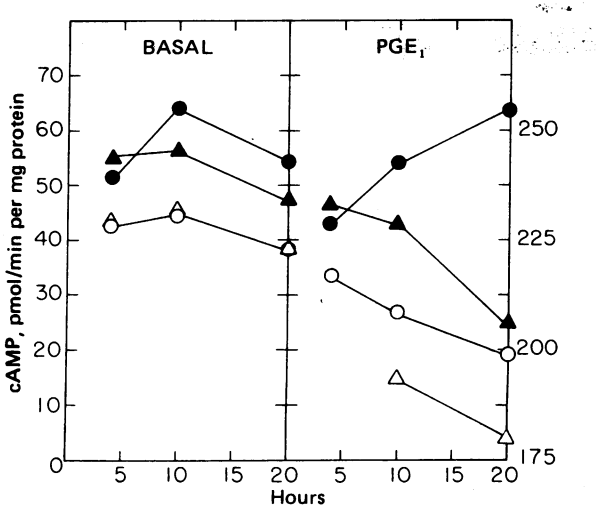

FIG. 4. Effect of cycloheximide on adenylate cyclase activity of control and etorphine-treated hybrid cells. (*Left*) Basal activity. (*Right*) Activity stimulated by 10 μM PGE_1. Triplicate cultures of NG108-15 cells (subculture 17) were treated as follows: ○, no addition; △, 70 μM cycloheximide; ●, 1 μM etorphine; ▲, 1 μM etorphine and 70 μM cycloheximide. In each 30-mm diameter dish, 770,000 cells were plated in 2 ml of 90% Dulbecco–Vogt modification of Eagle's medium supplemented with 10% fetal bovine serum, 100 μM hypoxanthine, 1 μM aminopterin, and 12 μM thymidine. Cycloheximide blocked incorporation of [^{3}H]proline into protein almost completely. After 20 hr under these conditions, cells tended to detach from the dish. Cell protein was harvested quantitatively after 4, 10, or 20 hr of incubation at 37° in 10% CO_2/90% air. Cells were removed and recovered by centrifugation at 400 × *g* for 5 min and washed three times with buffered saline adjusted to 340 mosmol/liter, pH 7.4. Each washed pellet was suspended in 1 ml of 320 mM sucrose/10 mM Tris·HCl, pH 7.5, and stored at −80° prior to assay for adenylate cyclase activity and for protein. Mean protein values per dish at 4, 10, and 20 hr were: control, 0.58, 0.66, and 0.80 mg; cycloheximide, 0.46, 0.51, and 0.52 mg; etorphine, 0.53, 0.65, and 0.75 mg; etorphine and cycloheximide, 0.46, 0.45, and 0.46 mg. Protein in the adenylate cyclase assay varied between 20 and 47 μg/100 μl of reaction mixture. Each point is the mean of triplicate values obtained with three homogenates, each from a separate dish, assayed in duplicate. Each adenylate cyclase reaction mixture contained 10 μM naloxone.

concentrations of adenylate cyclase activity from control and etorphine-treated cells are shown in Fig. 5. Half-maximal activations of adenylate cyclase from control and opiate-treated cells were obtained with approximately 0.15 μM PGE_1 or 1.8 μM 2-chloroadenosine. Concentrations of 2-chloroadenosine >50 μM were inhibitory. The results show that the increase in adenylate cyclase activity due to treatment of cells with opiates and stimulation of adenylate cyclase by PGE_1 or 2-chloroadenosine are expressed simultaneously and that treatment of cells with opiates does not alter the concentrations of PGE_1 or 2-chloroadenosine required for half-maximal activation of adenylate cyclase by PGE_1 or 2-chloroadenosine. Therefore, the supersensitivity of opiate-treated cells to PGE_1 or 2-chloroadenosine results from an increase in adenylate cyclase activity.

Interaction between Receptors Coupled to the Inhibition or Activation of Adenylate Cyclase. The relationship between morphine concentration and inhibition of adenylate cyclase from control and opiate-treated cells is shown in Fig. 6 *A* and *B*, and the effects of different concentrations of naloxone in reversing the inhibition of adenylate cyclase by 10 μM morphine are shown in Fig. 6 *C* and *D*. Half-maximal inhibition of basal adenylate cyclase from control and morphine-treated cells was obtained with 1 μM morphine and maximal inhibition with 5–10 μM morphine (Fig. 6*B*). However, in the presence

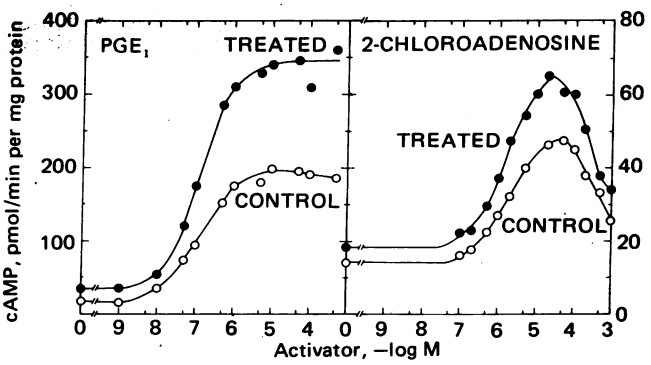

FIG. 5. Effect of PGE_1 (*Left*) or 2-chloroadenosine (*Right*) concentration on activity of adenylate cyclase from NG108-15 cells with or without 1 μM etorphine. (*Left*) Each reaction mixture contained the components described under *Materials and Methods* and 10 μM naloxone, the PGE_1 concentration indicated, and (○) 71 μg of homogenate protein from control cells; or (●) 87 μg of homogenate protein from cells treated 4 days with etorphine. (*Right*) Each reaction mixture contained the components described under *Materials and Methods*, 2-chloroadenosine as indicated, and (○) 87 μg of homogenate protein from control cells; or (●) 94 μg of homogenate protein from etorphine cells treated 5 days with etorphine.

of PGE_1, a 10- to 20-fold higher concentration of morphine (100 μM or more) was required for maximal inhibition of adenylate cyclase from control or opiate-treated cells (Fig. 6*A*). Conversely, a 10-fold lower concentration of naloxone (5 μM) was required for complete reversal of inhibition of adenylate cyclase by morphine in the presence of PGE_1 than in the absence of PGE_1 (50 μM naloxone). These results show that inhibitory opiate receptors and stimulatory PGE_1 receptors interact at the level of adenylate cyclase. Similarly, PGE_1 increased the effectiveness of naloxone in reversing the inhibition of adenylate cyclase by morphine. Perhaps both phenomena are a conse-

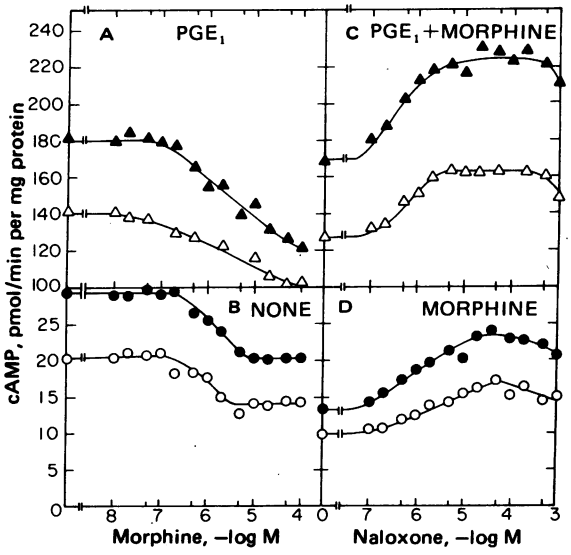

FIG. 6. Adenylate cyclase activity from homogenates of control NG108-15 cells (open symbols) or morphine-treated cells (solid symbols), cultured for 5 days in the absence or presence of 10 μM morphine, respectively. (*A*) and (*B*) Effect of morphine concentration on inhibition of adenylate cyclase in the presence of 10 μM PGE_1 or in the absence of PGE_1, respectively. (*C*) and (*D*) Effects of different concentrations of naloxone in reversing inhibition of adenylate cyclase due to 10 μM morphine in the presence and absence of 10 μM PGE_1, respectively. Each adenylate cyclase reaction mixture contained 87 μg of homogenate protein from control cells or 94 μg of homogenate protein from morphine-treated cells.

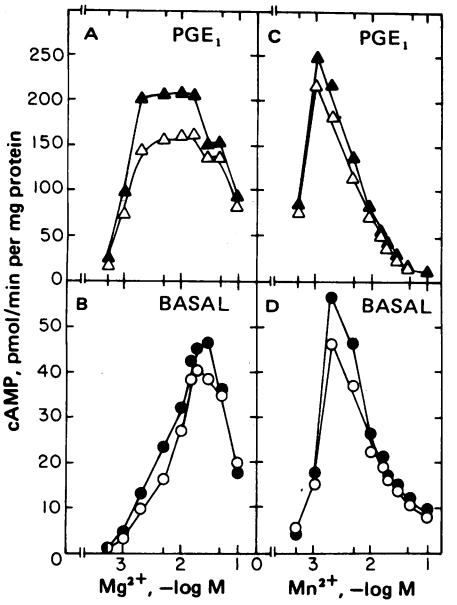

FIG. 7. The effects of Mg^{2+} (*A* and *B*) or Mn^{2+} (*C* and *D*) concentrations upon adenylate cyclase activity from homogenates of control (open symbols) or morphine-treated (solid symbols) NG108-15 hybrid cells. Homogenates are described in the legend to Fig. 6. In *A* and *C*, reaction mixtures contained 10 μM PGE_1; in *B* and *D*, basal adenylate cyclase activity was determined.

quence of the loss of positive cooperativity in the coupling of the morphine-opiate receptor complex with adenylate cyclase (Hill coefficient = 2). Naloxone and PGE_1 would be expected to act by different mechanisms because naloxone and morphine compete for a site on the opiate receptor, whereas the morphine-opiate receptor and the PGE_1-PGE_1 receptor either might compete for sites coupling receptors to adenylate cyclase, or both species of receptor might be coupled simultaneously to adenylate cyclase at separate sites and interact by an allosteric mechanism.

The data also show that the long-lived activation of adenylate cyclase that results from prolonged exposure of NG108-15 cells to an opiate and the transient inhibition of adenylate cyclase by morphine are expressed independently and simultaneously. Tolerance and dependence produced in animals by chronic exposure to opiates can be understood at the cellular level in terms of an increase in adenylate cyclase activity (4–6). Opiate-treated cells are tolerant to opiates because the relatively high adenylate cyclase activity of such cells must be inhibited to a greater extent by the opiate before levels of enzyme activity and of cAMP fall below the levels of control cells. They are dependent upon opiates because withdrawal of the opiate reverses the inhibition of adenylate cyclase and thereby increases the cAMP level well above that of control cells. Finally, opiate-treated cells are supersensitive to the opiate antagonist naloxone because opiate-treated cells have more adenylate cyclase activity and more opiate-inhibited enzyme activity than control cells. Consequently, at any concentration of naloxone, more adenylate cyclase activity is rescued with homogenates from opiate-treated cells than with those from control cells when both are assayed in the presence of morphine.

In experiments not shown here, the pH optimum of adenylate cyclase from control or morphine-treated cells was pH 8.5 for basal activity and pH 8.0 for PGE_1-stimulated activity. The apparent Michaelis constant (K_m) for ATP was 0.16 mM with adenylate cyclase from control cells and opiate-treated cells with or without PGE_1.

Table 1. Properties of adenylate cyclase from control and opiate-treated NG108-15 hybrid cells

	Control cells		Opiate-treated cells	
Addition	Basal	PGE_1	Basal	PGE_1
		μM		
ATP	160	160	160	160
Mg^{2+}	7000	1000	5000	1000
Mn^{2+}	1000	600	1000	500
PGE_1	—	0.14	—	0.16
2-Chloroadenosine	1.8	—	1.8	—
Gpp(NH)p	50	1.5	50	1.2
NaF	4000	2000	3600	1800
Morphine	1.0	2.0	1.0	2.0
Naloxone	1.4	0.5	0.9	0.4
pH optimum	8.5	8.0	8.5	8.0

The numbers shown are μM and are K_m values for ATP, K_a values for PGE_1 and 2-chloroadenosine, or concentrations required for half-maximal stimulation or inhibition of adenylate cyclase by $MgCl_2$, Gpp(NH)p, NaF, morphine, or naloxone (the last in the presence of 10 μM morphine). The PGE_1 concentration was 10 μM except where indicated.

The effects of Mg^{2+} and Mn^{2+} concentrations on basal and PGE_1-stimulated adenylate cyclase activities from control on morphine-treated cells are shown in Fig. 7. The concentrations of Mg^{2+} required for maximal activation of basal and PGE_1-stimulated adenylate cyclase were approximately 30 and 2 mM, respectively, with both enzyme preparations. Thus, Mg^{2+} and PGE_1 activate adenylate cyclase synergistically. Higher concentrations of Mg^{2+} inhibited adenylate cyclase, and the enzyme from morphine-treated cells was more sensitive to inhibition than that from control cells. The concentrations of Mn^{2+} required for maximal stimulation of basal and PGE_1-stimulated adenylate cyclase were 2 and 1 mM, respectively. Higher concentrations of Mn^{2+} were inhibitory.

In Table 1, K_m values for ATP, K_a values for PGE_1 or 2-chloroadenosine, or concentrations of Mg^{2+}, Mn^{2+}, Gpp(NH)p, or NaF required for 50% of the maximal effect are summarized and are shown to be the same for adenylate cyclase from control and opiate-treated cells. Similarly, no difference was found in the concentration of morphine required for half-maximal inhibition of adenylate cyclase or in the concentration of naloxone required for reversal of morphine inhibition of adenylate cyclase.

DISCUSSION

Opiates regulate adenylate cyclase in two ways, both mediated by the opiate receptor. Opiates inhibit adenylate cyclase rapidly and reversibly, and the inhibition is coupled to a gradual, long-lived increase in adenylate cyclase activity that is expressed even in the absence of the opiate. In this report, the properties of adenylate cyclase from neuroblastoma × glioma NG108-15 hybrid cells cultured with or without opiates for several days are compared. Adenylate cyclase preparations from control and opiate-treated cells do not differ appreciably with respect to the concentrations of PGE_1, 2-chloroadenosine, morphine, naloxone, ATP, Mg^{2+}, or Mn^{2+} required for half-maximal effects. However, Gpp(NH)p or NaF abolishes most, but not all, of the opiate-dependent increase in adenylate cyclase activity. Basal adenylate cyclase of opiate-treated cells is activated less by Gpp(NH)p or NaF, and the PGE_1-stimulated enzyme is inhibited more by these compounds, than the enzyme from control cells.

Because the mechanisms of activation and inhibition of adenylate cyclase by Gpp(NH)p or NaF are not understood and

the extent of activation of adenylate cyclase by Gpp(NH)p or NaF is low compared to that found with PGE_1, the effects of Gpp(NH)p or NaF on adenylate cyclase should be interpreted with caution. However, the results show that the opiate-dependent increase in adenylate cyclase is reversed by agents that are thought to uncouple adenylate cyclase from receptors and suggest that NG108-15 cells contain two or more forms of adenylate cyclase that differ in basal activity and in responsiveness to activators. Inhibition of protein synthesis with cycloheximide blocked most, but not all, of the opiate-dependent increase in adenylate cyclase. These results show that adenylate cyclase is a relatively stable enzyme that does not turn over rapidly and suggest that protein synthesis is required for the full expression of the opiate-evoked increase in adenylate cyclase activity. Further work is needed to identify the protein that is required for opiate-modulation of adenylate cyclase activity and the function of the protein.

Changes in cell membrane lipids have persistent effects on adenylate cyclase activity that resemble the opiate-dependent increase in adenylate cyclase activity. Increases were reported in basal activity and in hormone-, Gpp(NH)p-, or NaF-stimulated activity; however, NaF-stimulated activity was least affected (15–17). Cholera toxin also activates basal- and hormone-stimulated adenylate cyclase but not the NaF-activated enzyme (18).

A simple hypothesis that provides a mechanism for coupling inhibition of adenylate cyclase by opiates with a subsequent long-lived increase in enzyme activity is that activation of adenylate cyclase desensitizes the enzyme (19), perhaps via a cAMP-dependent reaction. Alternatively, inhibition of adenylate cyclase by opiates results in the conversion of the enzyme to an activated form. In either case, inhibition of adenylate cyclase by opiates would lead to an increase in enzyme activity, and stimulation would decrease enzyme activity by altering the relative proportions of low- and high-activity forms of the enzyme.

Other species of receptor coupled to the inhibition of adenylate cyclase have also been shown to regulate the amount of adenylate cyclase activity. Exposure of NG108-15 cells to carbamylcholine, which activates muscarinic acetylcholine receptors of the cells, or to norepinephrine, which activates α-receptors, also inhibits adenylate cyclase, and slowly increases adenylate cyclase activity (refs. 7 and 8; S. K. Sharma and M. Nirenberg, unpublished data). Such reactions that modulate the amount of adenylate cyclase activity may provide mechanisms for relatively long-lived (hours) regulation of the sensitivity and extent of neural responses to various neurotransmitters and thus may play a role in memory.

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