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ACCELERATED COMMUNICATION

Naturally Occurring Opioid Receptor Agonists Stimulate Adenylate Cyclase Activity in Rat Olfactory Bulb

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SUMMARY

In homogenate of rat olfactory bulb, the opioid receptor agonists β-endorphin, Leu-enkephalin, and dynorphin A stimulated adenylate cyclase activity in a concentration-dependent manner, with half-maximal effects displayed at 22, 63, and 176 nM, respectively. The maximal stimulation of the enzyme activity corresponded to about a 40% increase of basal activity for all three peptides. Naloxone antagonized the stimulation of β-endorphin, Leu-enkephalin, and dynorphin A, with pA_2 values of 8.0, 7.7, and 8.1, respectively. Kinetic analysis performed with Leu-enkephalin showed that the opioid peptide increased the V_{max} of the enzyme, without changing the K_m for the substrate Mg-ATP. Moreover, the opioid stimulation was associated with a significant increase of the affinity of the enzyme for Mg^{2+} activation and occurred in membranes incubated in a Ca^{2+}-free medium. Addition of exogenous GTP at micromolar concentrations was absolutely necessary for the detection of the opioid effect. Treatment of olfactory bulbs with cholera toxin did not alter the stimulation of adenylate cyclase by Leu-enkephalin. However, the opioid stimulation disappeared in membranes obtained from bulbs injected with pertussis toxin. These results demonstrate the presence in the brain of a new functional class of opiate receptors coupled to stimulation of adenylate cyclase via a transduction mechanism that is Ca^{2+} independent and seems to involve a pertussis toxin-sensitive GTP-binding protein.

Among the different second messenger systems modulated by opioids, the inhibition of adenylate cyclase activity is considered to be a primary signal transduction mechanism in the action of opioid receptors (1). A number of studies have demonstrated that opioid agonists elicit a decrease of cyclic AMP formation in both brain and cultured neuronal cells (1) and that this inhibition involves the interaction of the opioid receptors with a G_i protein that is sensitive to pertussis toxin (2). Moreover, there is evidence indicating that changes in the opioid inhibition of adenylate cyclase may correlate with the development of tolerance to and dependence on opioid agonists (3), thus reinforcing the role of this biochemical event in opioid action.

In the present study, however, we show that inhibition of adenylate cyclase is not the sole response of adenylate cyclase to opioid agonists but that, in rat brain, activation of this enzyme system may also occur after stimulation of opioid receptors. In fact, in homogenates of rat olfactory bulb, naturally occurring opioid peptides increase adenylate cyclase activity, thus indicating that in this brain structure an increase rather than a decrease of intracellular cyclic AMP may mediate the action of opioids.

Materials and Methods

[α-^{32}P]ATP (30–40 Ci/mmol) and [2,8-^{3}H]cAMP (25 Ci/mmol) were purchased from Du Pont de Nemours (Bad Homburg, FRG). Opioid peptides were obtained from Peninsula Laboratories (Merseyside, UK). Naloxone was obtained from Salars (Como, Italy). Pertussis toxin (islet-activating protein) was purchased from List Biological Laboratory, Campbel, CA. Staurosporine was generously provided by Kyowa Hakko Kogyo Co. (Tokyo, Japan). Cholera toxin, PMA, and the other chemicals used for adenylate cyclase assay were obtained from Sigma Chemical Co. (St. Louis, MO).

Adenylate cyclase assay. Male Sprague-Dawley rats (200–300 g) were sacrificed by decapitation, and the olfactory bulbs were homogenized in 10 volumes (v/w) of ice-cold buffer containing 10 mM HEPES NaOH, 1 mM EGTA, 1 mM $MgCl_2$, and 1 mM dithiothreitol (pH 7.4), using a tight Teflon-glass tissue grinder (12 up-and-down strokes by hand). The homogenate was diluted 6-fold with homogenization buffer and centrifuged at 27,000 × *g* for 20 min at 4°. The pellet was resuspended in 10 volumes of homogenization buffer by aspiration of the tissue through a 19-gauge needle (six passages). The homogenate was diluted with the same buffer to give a protein concentration of 1.0–1.4 mg/ml and was used immediately for adenylate cyclase assay. Unless otherwise specified, the enzyme activity was routinely assayed in a 100-μl reaction mixture containing 50 mM HEPES-NaOH (pH 7.4), 2.3 mM $MgCl_2$, 1.3 mM dithiothreitol, 0.2 mM [α-^{32}P]ATP (45 cpm/pmol), 1

ABBREVIATIONS: G_i, inhibitory GTP-binding protein; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; G_s, stimulatory GTP-binding protein; G protein, GTP-binding protein; PMA, phorbol 12-myristate 13-acetate.

mM [^{3}H]cAMP (80 cpm/nmol), 0.3 mM EGTA, 1 mM 3-isobutyl-1-methylxanthine, 5 mM phosphocreatine, 50 units/ml creatine phosphokinase, 100 μM GTP, 50 μg of bovine serum albumin, 10 μg of bacitracin, and 10 kallikrein inhibitor units of aprotinin. The reaction was started by addition of the tissue preparation (30–40 μg of protein) and was carried out at 30° for 10 min. [^{32}P]cyclic AMP was isolated according to the method of Salomon *et al.* (4). The enzyme activity was linear with tissue protein concentration and with time (at least for up to 10 min), both with and without opioid agonists. Assays were performed in duplicate.

Intracerebral injections. Pertussis toxin was dissolved in a solution containing 50 mM sodium phosphate buffer and 250 mM NaCl (pH 7.0). Cholera toxin was dissolved in 25 mM Tris·HCl, 100 mM NaCl, 1.5 mM NaN_3, 0.5 mM EDTA (pH 7.5). The animals were anesthetized with chloral hydrate (400 mg/kg, intraperitoneally) and placed in a stereotaxic frame. The toxins (3.50 μg each) were injected in the right olfactory bulb at two different positions (1.75 μg in 3.5 μl over a 10-min period), A + 6.7, L − 1.5, V − 4.5, and A + 8.0, L − 1.3, V − 4.0, according to the atlas of Paxinos and Watson (5), with bregma as zero. Control animals were injected with an equal volume of vehicle containing 3.50 μg of bovine serum albumin. Sixty-eight hours after surgery, the animals were sacrificed and homogenates were prepared from vehicle- and toxin-treated olfactory bulbs. Five different tissue preparations were tested.

Protein content was determined by the method of Bradford (6), using bovine serum albumin as the standard.

The concentrations of free Ca^{2+}, free Mg^{2+}, and Mg-ATP were calculated with a computer program (EQCAL; Biosoft, UK), using the stability constants for metal-chelate and metal-nucleotide complexes reported by Bartfai (7).

Statistical analysis. Results are reported as mean ± standard error. The concentration of each stimulant that produced a half-maximal effect (EC_{50}) was calculated from log-probit plots of the stimulation of the enzyme activity, expressed as a percentage of the maximal effect, at different concentrations of the agent. The potency of naloxone in antagonizing the opioid-induced stimulation of adenylate cyclase was determined using Schild analysis (8). For each opioid agonist tested, the pA_2 value of naloxone was determined from the x-intercept of the Schild plot calculated by least-squares regression analysis. Statistical significance of the differences between concentration-response curves was determined by analysis of variance. In the other evaluations, Student's t test was used.

Results

As shown in Fig. 1, different opioid receptor agonists, such as β-endorphin, Leu-enkephalin, and dynorphin A, stimulated adenylate cyclase of rat olfactory bulb. For each peptide, the effect was concentration dependent and clearly saturable. The EC_{50} values were as follows: β-endorphin, 22.0 ± 2.6 nM; Leu-enkephalin, 63.0 ± 4.0 nM; dynorphin A, 176.0 ± 24.0 nM (four experiments). The maximal stimulation elicited by β-endorphin, Leu-enkephalin, and dynorphin A corresponded to 42.2 ± 1.9% ($p < 0.001$), 40.0 ± 1.3% ($p < 0.001$), and 42.5 ± 1.6% ($p < 0.001$) increases of basal enzyme activity, respectively. The metabolically stable opioid receptor agonist [D-Ala^2, D-Leu^5]-enkephalin maximally stimulated the enzyme activity by 39.5 ± 0.9% ($p < 0.005$; three experiments), with an EC_{50} value of 76 ± 2.0 nM (result not shown).

The addition of increasing concentrations of the opioid receptor antagonist naloxone progressively shifted to the right the concentration-response curves of the agonists (Fig. 1). Schild plots of naloxone competition experiments (Fig. 1, *insets*) yielded pA_2 values of 8.0 ± 0.07, 7.7 ± 0.08, and 8.1 ± 0.1 for β-endorphin, Leu-enkephalin, and dynorphin A, respectively. The corresponding inhibitory constant (K_i) values were

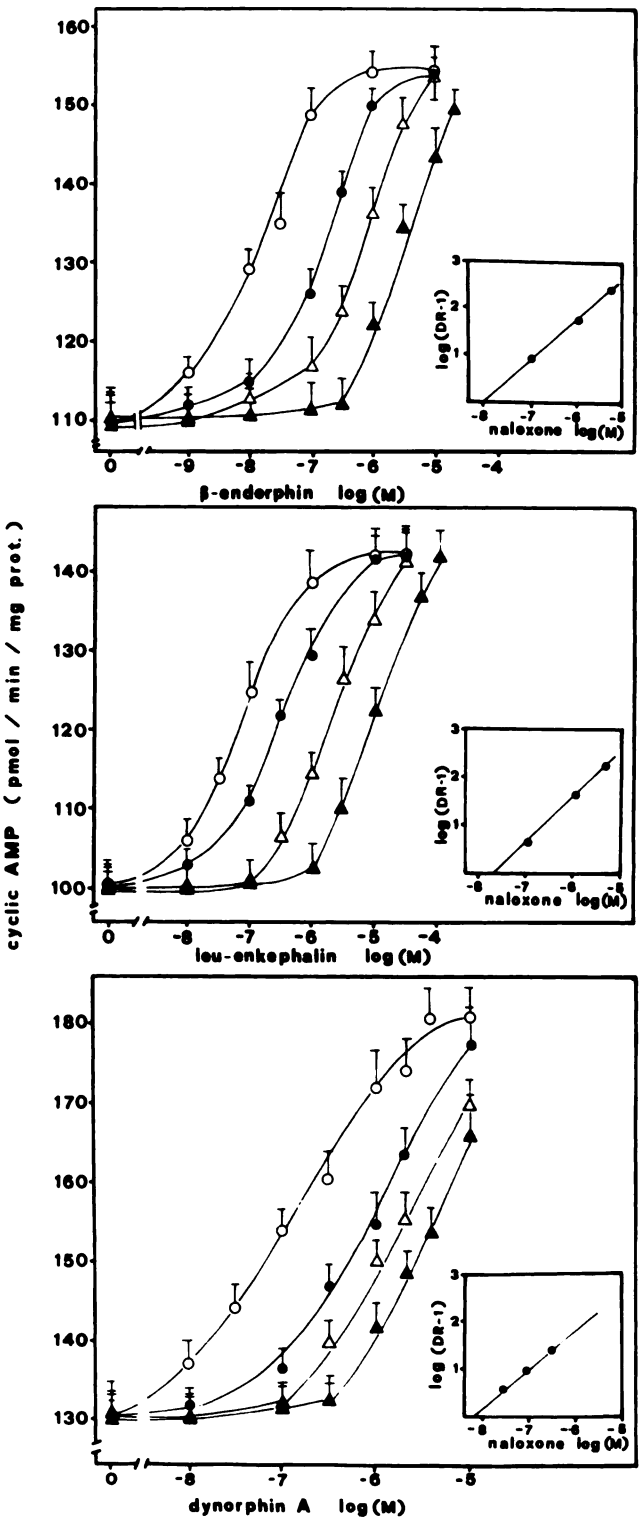

Fig. 1. Concentration-dependent stimulation of adenylate cyclase activity of rat olfactory bulb by β-endorphin, Leu-enkephalin, and dynorphin A and antagonism of each response by naloxone. Adenylate cyclase activity was assayed with the indicated concentrations of the opioid receptor agonists, in the absence and in the presence of increasing concentrations of naloxone. The reaction mixture contained 100 μM GTP. In the experiments using β-endorphin and Leu-enkephalin: ○, vehicle; ●, 0.1 μM naloxone; △, 1 μM naloxone; ▲, 5 μM naloxone. In the experiments using dynorphin A: ○, vehicle; ●, 0.05 μM naloxone; △, 0.1 μM naloxone; ▲, 0.5 μM naloxone. Values are the mean ± standard error of three experiments. *Insets*, Schild plots of the naloxone antagonism of each opioid agonist stimulation, where the log of the dose ratio (DR) − 1 is plotted as a function of naloxone concentration. pA2 values were obtained by linear regression analysis.

10.0, 20.0, and 8.0 nM, respectively. The slope values of the plots were 1.10 for β-endorphin, 0.95 for Leu-enkephalin, and 0.83 for dynorphin A. Kinetic analysis of the stimulation of adenylate cyclase activity by Leu-enkephalin as a function of the concentration of Mg-ATP indicated that the addition of the peptide increased the apparent V_{max} of the enzyme activity from 211.1 ± 15.3 to 313.8 ± 20.1 pmol of cyclic AMP/min/mg of protein ($p < 0.01$; three experiments) (Fig. 2A). The apparent K_m values for Mg-ATP were similar in the absence and in the presence of the opioid agonist, being 111.0 ± 9.8 μM and 129.2 ± 12.3 μM, respectively. Examination of the dependence of the enzyme stimulation on Mg^{2+} ion revealed that the opioid effect was associated with a significant decrease in the apparent activation constant (K_{act}) of the enzyme for Mg^{2+}, from 5.2 ± 0.2 mM to 3.6 ± 0.2 mM ($p < 0.01$; three experiments), whereas it did not significantly modify the V_{max} of Mg^{2+} stimulation (Fig. 2B). Maximal enzyme velocities (expressed as pmol of cyclic AMP/min/mg of protein, mean ± standard error) were 342 ± 10 for control and 360 ± 15 for Leu-enkephalin (three experiments). Under the same experimental conditions, the β-adrenergic agonist l-isoproterenol, which stimulated the enzyme activity by the same extent as Leu-enkephalin, shifted the k_{act} value for Mg^{2+} to 3.8 ± 0.1 mM, without significantly affecting the V_{max} value of the enzyme reaction (Fig. 2B).

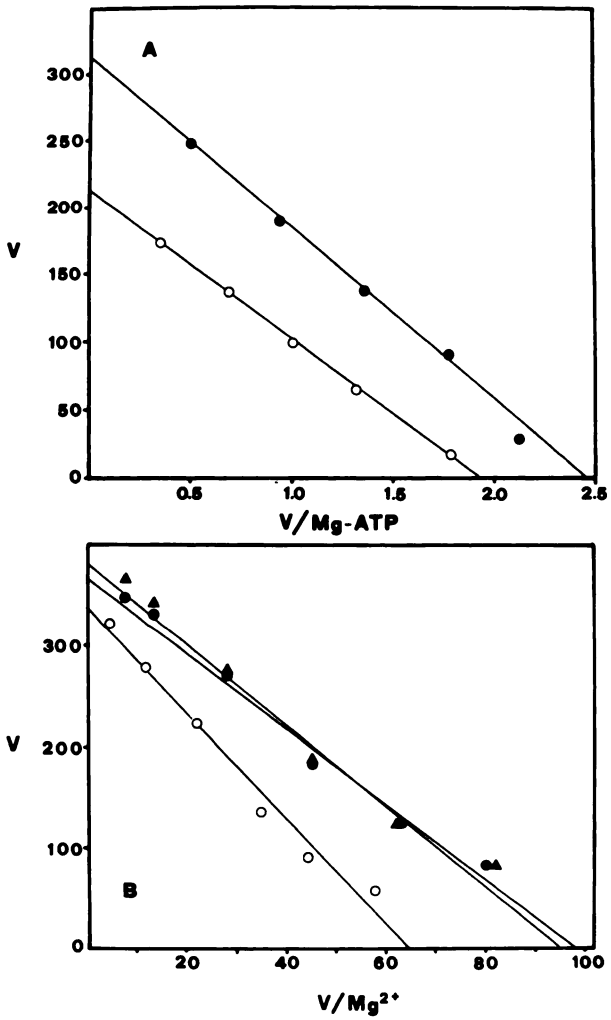

Fig. 2. A, Eadie-Hofstee plot of the adenylate cyclase activity of rat olfactory bulb as a function of the concentration of Mg-ATP, in the absence (○) and in the presence (●) of 10 μM Leu-enkephalin. The enzyme activity was assayed at concentrations of Mg-ATP ranging from 10 to 500 μM. The concentration of free Mg^{2+} was kept constant at 2.0 mM. Values are the mean of three experiments. B, Eadie-Hofstee plot of the adenylate activity as a function of the concentration of free Mg^{2+}. Enzyme activity was assayed in the absence (○) and in the presence of 10 μM Leu-enkephalin (●) or 100 μM l-isoproterenol (▲). The concentration of free Mg^{2+} varied from 1 to 50 mM. The calculated concentration of Mg-ATP ranged from 189.0 to 199.7 μM. Values are the mean of three experiments. In A and B, the concentration of GTP in the reaction mixture was 100 μM. Enzyme velocities are expressed as pmol of cyclic AMP/min/mg of protein. V_{max} and K_m values were obtained by linear regression analysis.

The stimulation of adenylate cyclase activity by Leu-enkephalin appeared to be independent of the concentration of free Ca^{2+}. In fact, the opioid effect was optimal in membranes prepared in the presence of 1 mM EGTA and incubated in an essentially Ca^{2+}-free medium, and it remained constant at concentrations of free Ca^{2+} ranging from 1 nM to 0.8 μM (Fig. 3).

The addition of the protein kinase inhibitor staurosporine (0.1 μM) (9) failed to affect the stimulation of adenylate cyclase by Leu-enkephalin (Table 1). In contrast, staurosporine completely antagonized the stimulatory effect elicited by PMA, a protein kinase C activator (10). The combined addition of Leu-enkephalin and PMA produced an increase of enzyme activity that was equal to the sum of the effects elicited by each agent alone (Table 1).

The addition of exogenous GTP was required for detection of the opioid stimulation of adenylate cyclase activity. As shown in Fig. 4, GTP maximally increased basal enzyme activity by about 350% with an EC_{50} value of 1.1 ± 0.2 μM. In the absence of added nucleotide, Leu-enkephalin failed to affect the enzyme activity, whereas a significant stimulation occurred at concentrations of GTP higher than 1 μM, with a maximum at 100 μM. The concentration of the nucleotide allowing half-maximal stimulation by Leu-enkephalin was 2.7 ± 0.3 μM. The stimu-

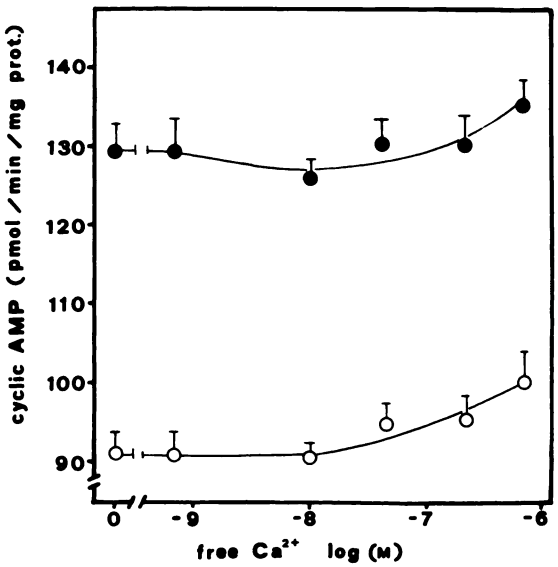

Fig. 3. Stimulation of adenylate cyclase activity by Leu-enkephalin at different concentrations of free Ca^{2+}. Tissue homogenate was prepared in a medium containing 1 mM EGTA. Enzyme activity was assayed in the presence of vehicle (○) or 10 μM Leu-enkephalin (●), at the indicated free Ca^{2+} concentrations. Zero Ca^{2+} was obtained by omitting Ca^{2+} and adding 1 mM EGTA. The reaction mixture contained 100 μM GTP. Values are the mean ± standard error of three experiments.

TABLE 1

Effect of the combined addition of Leu-enkephalin, staurosporine, and PMA on adenylate cyclase activity of rat olfactory bulb

Tissue homogenate was prepared as described in Materials and Methods, and the enzyme activity was assayed in the presence of 100 μM GTP, with the indicated agents alone and in combination. Values are the mean ± standard error of three experiments.

	Adenylate cyclase activity
	pmol of cAMP/min/mg of protein
Basal	119.1 ± 4.0
Leu-enkephalin (10 μM)	168.4 ± 7.0[a]
Staurosporine (0.1 μM)	128.1 ± 6.1
Staurosporine + Leu-enkephalin	176.5 ± 9.4[b]
PMA (5 μM)	151.6 ± 5.0[c]
Staurosporine + PMA	129.8 ± 3.6[d]
Leu-enkephalin + PMA	203.5 ± 10.1[e]

[a] $p < 0.005$ versus basal.
[b] $p < 0.01$ versus staurosporine alone.
[c] $p < 0.05$ versus basal.
[d] Not significantly different from staurosporine alone.
[e] $p < 0.05$ versus Leu-enkephalin alone.

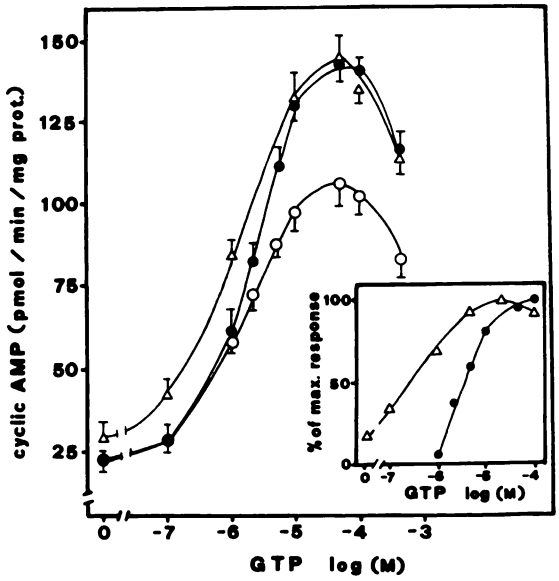

Fig. 4. GTP dependence of stimulations of adenylate cyclase activity by either Leu-enkephalin or *l*-isoproterenol in rat olfactory bulb. Adenylate cyclase activity was assayed at the indicated concentration of added GTP, in the absence (○) and in the presence of either 10 μM Leu-enkephalin (●) or 100 μM *l*-isoproterenol (△). Values are the mean ± standard error of four experiments. *Inset*, the percentage of maximal enzyme stimulation elicited by Leu-enkephalin (●) or *l*-isoproterenol (△) is plotted as a function of the concentration of added GTP.

lation of adenylate cyclase activity by *l*-isoproterenol was also GTP dependent, with the half-maximal effect occurring at 0.25 ± 0.05 μM GTP (Fig. 4, *inset*).

In vivo treatment of olfactory bulbs with cholera toxin increased basal adenylate cyclase activity by 151.5 ± 20.0% in the absence of added GTP ($p < 0.001$; five experiments) and by 55.8 ± 3.1% at 100 μM GTP ($p < 0.001$; five experiments). As shown in Fig. 5A, this toxin did not significantly change the enzyme stimulation elicited by either Leu-enkephalin or *l*-isoproterenol. In contrast, treatment of olfactory bulbs with pertussis toxin completely prevented the stimulation of adenylate cyclase activity by Leu-enkephalin (Fig. 5B). As compared with the response observed in vehicle-treated tissue, the maximal stimulation of the enzyme activity by *l*-isoproterenol was

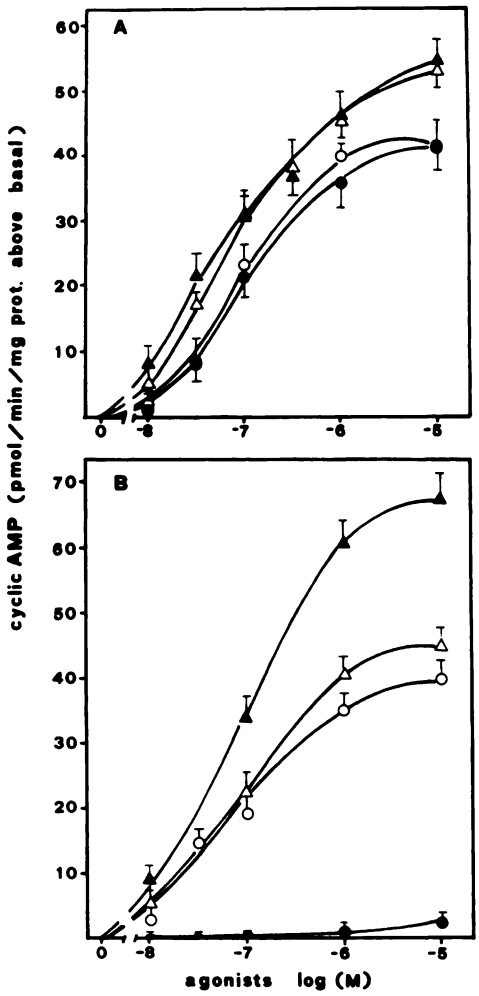

Fig. 5. Effect of treatment of rat olfactory bulb with cholera toxin (A) and pertussis toxin (B) on the stimulation of adenylate cyclase by either Leu-enkephalin or *l*-isoproterenol. Toxins or vehicles were injected *in vivo* into the olfactory bulbs, and tissue homogenates were prepared as described in Materials and Methods. Adenylate cyclase activity was assayed in vehicle- (*open symbols*) and toxin- (*closed symbols*) treated tissues, in the presence of the indicated concentrations of Leu-enkephalin (*circles*) or *l*-isoproterenol (*triangles*). The concentration of GTP added to the reaction mixture was 100 μM. Values represent the net increase of enzyme activity elicited by each agonist above basal activity and are the mean ± standard error of four (A) or five (B) experiments. Basal enzyme activities (expressed as pmol of cyclic AMP/min/mg of protein, mean ± standard error) were: in A, vehicle, 125.4 ± 11.9; cholera toxin, 195.8 ± 17.2 ($p < 0.05$; four experiments); in B, vehicle, 115.4 ± 8.5; pertussis toxin, 112.9 ± 7.4. Statistical analysis by two-way analysis of variance of the difference between *l*-isoproterenol concentration-response curves in vehicle- and pertussis toxin-treated tissue yields $p < 0.05$.

potentiated by 52.0 ± 7.1% in membranes obtained from pertussis toxin-treated olfactory bulbs.

Discussion

The present study shows that, in homogenates of rat olfactory bulb, stimulation of opioid receptors causes an increase of adenylate cyclase activity. As opioid receptor agonists, we have used the naturally occurring peptides β-endorphin, Leu-enkephalin, and dynorphin A, which display different affinities for the opioid receptor subtypes. In fact, β-endorphin is a potent agonist for both δ and μ receptors, with a slight preference for

δ, Leu-enkephalin is preferentially a δ agonist, and dynorphin A is considered a rather selective κ agonist (11). All three peptides elicit a stimulatory response of adenylate cyclase, which is concentration dependent and saturable and, in agreement with other studies on opioid receptor binding and function (12, 13), requires nanomolar concentrations of the agonists. β-Endorphin, Leu-enkephalin, and dynorphin A appear equally effective in stimulating adenylate cyclase activity but show different affinities, with a rank order of potency of β-endorphin > Leu-enkephalin > dynorphin A. The metabolically stable opioid receptor agonist [D-Ala^2, D-Leu^5]-enkephalin increases the enzyme activity with a potency and an efficacy similar to those of Leu-enkephalin, thus limiting the possibility that products of opioid peptide degradation may be responsible for the enzyme activation.

As demonstrated by Schild plot analysis, naloxone counteracts the opioid stimulation with slope values close to unity for all three peptides, indicating an antagonism of the competitive type. The calculated K_i values of naloxone range from 8.0 to 20 nM. These values are about 10–20 times higher than the reported affinity of naloxone for μ sites but are quite close to those for δ and κ receptors (14). Moreover, the finding that naloxone antagonizes the effects of the three peptides with similar potency fits well with the idea that the stimulation of adenylate cyclase activity is mediated by the activation of a single class of opioid receptors. Thus, the agonist rank order of potency and the sensitivity to naloxone would suggest that the opioid receptors mediating stimulation of adenylate cyclase belong to the δ subtype. This conclusion agrees with the autoradiographic distribution of δ and μ receptors in rat brain, demonstrating that δ receptors are highly concentrated in the external plexiform layers and glomerular layers of the rat olfactory bulb, whereas μ receptors show low density in this brain region (15). However, further studies employing various agonists and antagonists that are selective for the different opioid receptor subtypes are required to better define the pharmacological nature of this opioid response.

As shown for other receptor systems coupled to stimulation of adenylate cyclase (16, 17), the increase of enzyme activity elicited by opioid receptors is due to an increase of the apparent V_{max} of the enzyme, with no significant change in the K_m for the substrate Mg-ATP. Moreover, the adenylate cyclase stimulation elicited by Leu-enkephalin is associated with a significant increase in the apparent affinity of the enzyme for Mg^{2+}. This effect is analogous to that of other stimulatory hormones, which are known to decrease the Mg^{2+} requirement for the activation of G_s of adenylate cyclase (18). Indeed, a similar change in the Mg^{2+} requirement is elicited by the β-adrenergic agonist *l*-isoproterenol, which also stimulates adenylate cyclase activity of rat olfactory bulb.

Another property of the opioid stimulation that is shared with other receptor-mediated effects on adenylate cyclase is the GTP dependence. In fact, there is no effect in the absence of added GTP, whereas half-maximal opioid stimulation occurs at 2.7 μM GTP. This value is much higher than the GTP concentration generally required for stimulation of adenylate cyclase by other hormone receptor systems (19). This difference is not due to a unique responsiveness of adenylate cyclase to GTP in rat olfactory bulb, because *l*-isoproterenol requires 10-fold less GTP than Leu-enkephalin for stimulating the enzyme activity. A possible explanation of the discrepancy in the GTP requirement is that the opioid stimulation of adenylate cyclase results from receptor interaction with a G protein different from G_s, which is known to mediate the β-adrenergic stimulation of the enzyme (20).

This possibility is supported by the results obtained by treatment of the olfactory bulbs with cholera and pertussis toxins. Cholera toxin ADP-ribosylates the α subunit of G_s, which mediates hormonal activation of adenylate cyclase and activation of Ca^{2+} channels, and causes a stable activation of this protein (21). Cholera toxin can also ADP-ribosylate and activate G_{olf}, a G protein that is structurally homologous to G_s and that is considered to mediate the stimulation of adenylate cyclase by odorants in olfactory neurons (22). G_{olf} can interact with β-adrenergic receptors when expressed in S49 cyc$^-$ Kin$^-$ cells that are transfected with cDNA encoding the α subunit of G_{olf} (22). G_s is not a substrate for pertussis toxin, which instead ADP-ribosylates a variety of other G proteins, such as those mediating hormonal inhibition of adenylate cyclase (termed G_i), activation of K^+ channels (termed G_k), and stimulation of some phospholipase A_2 and C activities (termed G_p) (21). Pertussis toxin also ADP-ribosylates transducin and other G proteins whose function is not yet fully understood, such as G_o (21). As a result of exposure to pertussis toxin, the receptor regulation of the effector system is impaired, likely because of receptor-G protein uncoupling (21). The injection of cholera toxin into the olfactory bulb increases basal adenylate cyclase activity but fails to modify the stimulation of the enzyme activity by either Leu-enkephalin or *l*-isoproterenol. In contrast, treatment with pertussis toxin completely prevents the increase of enzyme activity elicited by Leu-enkephalin and potentiates the β-adrenergic stimulation. These results indicate that the opioid stimulation of adenylate cyclase is mediated by a G protein that is a pertussis but not cholera toxin substrate, and they provide further evidence that the G protein involved is different from G_s. The finding that pertussis toxin potentiates the *l*-isoproterenol response is in agreement with previous observations that the toxin enhances the stimulation of brain adenylate cyclase by other G_s-coupled receptors, such as dopamine D1 receptors, and may be explained by postulating that the toxin impairs G_i inhibition of G_s (23). At the present time, however, we do not know which of the G proteins that are reported to be sensitive to pertussis toxin may be involved in the opioid effect. Very recently, it has been reported that the α subunits of two molecular species of G_i, $G_{i\text{-}1}$ and $G_{i\text{-}2A}$, cause stimulation rather than inhibition of adenylate cyclase in a reconstituted system (24). Studies are in progress to ascertain whether these G proteins may be involved in the opioid stimulation of adenylate cyclase. It is also possible that opioid receptors cause adenylate cyclase stimulation indirectly, through a G protein-mediated coupling to another biochemical pathway. For instance, there is abundant evidence that, in intact cells, various neurotransmitters may increase cyclic AMP formation by stimulating other second messenger systems, such as those linked to phospholipases A_2 and C (25). As mentioned above, receptor-induced phospholipid breakdown may be mediated by G proteins that are sensitive to pertussis toxin. However, this second messenger-mediated stimulation of adenylate cyclase generally requires cell integrity, because no significant increase of the enzyme activity is observed in cell lysates, whereas opioid stimulation of adenylate cyclase is observed in tissue homogenate. Moreover, in a number of cases,

second messenger stimulation of adenylate cyclase by neurotransmitter requires Ca^{2+} (25), whereas the opioid stimulation of the enzyme is Ca^{2+} independent. The lack of Ca^{2+} requirement also lessens the possibility that opioid receptors stimulate adenylate cyclase activity by enhancing the binding of calmodulin to the enzyme.

The protein kinase inhibitor staurosporine fails to affect the stimulation of adenylate cyclase by Leu-enkephalin, whereas it completely antagonizes the increase of enzyme activity elicited by PMA. Furthermore, the stimulatory effects of Leu-enkephalin and PMA are additive. These results would suggest that activation of protein kinase C is unlikely to be involved in the opioid effect.

Recently, we have reported that, in rat olfactory bulb, activation of muscarinic receptors also stimulates adenylate cyclase activity (26). Like the opioid stimulation, the muscarinic effect requires relatively high concentrations of GTP, is Ca^{2+} independent, and is apparently not mediated by an increased phospholipid hydrolysis (27). These similarities suggest that the stimulatory response elicited by opioids in rat olfactory bulb may not be due to a peculiar property of the receptor molecules but rather to the ability of opioid receptors to activate a particular transduction mechanism, which can be utilized also by other receptor systems.

Previous studies have reported a stimulatory effect of morphine on adenylate cyclase activity of striatal homogenates (28, 29). This enzyme stimulation was observed with high concentrations (ranging from 1 μM to 100 μM in Ref. 29 and from 500 μM to 5 mM in Ref. 28) of morphine and has been interpreted as being due to the nonspecific ionic effect of the drug on adenylate cyclase activity, similar to that of monovalent cations (30). In homogenate of mouse spinal cord-ganglion explants, the opioid receptor agonist levorphanol (2 μM) was found to stimulate basal adenylate cyclase activity (31). However, in that study, naloxone (2 μM) elicited an even greater stimulation, thus making the interpretation of the results difficult. Conversely, the present study describes a stimulatory effect of opioids, which displays pharmacological and biochemical properties consistent with a receptor-mediated event and, therefore, can be considered as the first demonstration of the occurrence of a positive coupling of opioid receptors to adenylate cyclase.

In the olfactory bulb, the presence of opioid peptides has been demonstrated in perikarya and fibers/terminals (11). Enkephalin is also known to participate in the modulation of synaptic transmission in the olfactory bulb, by suppressing the dendrodendritic inhibition of mitral cells (32). It will be important to investigate the role of stimulation of adenylate cyclase in the cellular events regulated by opiate receptors in the olfactory bulb and to determine whether an increase in cyclic AMP formation mediates the opioid action in other brain areas.

References

1. Loh, H. H., and A. P. Smith. Molecular characterization of opioid receptors. *Annu. Rev. Pharmacol. Toxicol.* **30**:123–147 (1990).
2. Burns, D. L., E. L. Hewlett, J. Moss, and M. Vaughan. Pertussis toxin inhibits enkephalin stimulation of GTPase of NG 108-15 cells. *J. Biol. Chem.* **258**:1435–1438 (1983).
3. Sharma, S. K., W. A. Klee, and M. Niremberg. Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. *Proc. Natl. Acad. Sci. USA* **72**:3092–3096 (1975).
4. Salomon, Y., C. Londos, and M. Rodbell. A higly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541–548 (1974).
5. Paxinos, G., and C. Watson. *The Rat Brain In Stereotaxic Coordinates.* Academic Press, Sidney (1982).
6. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
7. Bartfai, T. Preparation of metal-chelate complexes and the design of steady-state kinetic experiments involving metal nucleotide complexes. *Adv. Cyclic Nucleotide Res.* **10**:219–242 (1979).
8. Schild, H. O. pA, a new scale for the measurement of drug antagonism. *Br. J. Pharmacol.* **2**:189–206 (1947).
9. Rüegg, U., and G. M. Burgess. Staurosporine, K-252 and UCN-01: potent but not specific inhibitors of protein kinases. *Trends Pharmacol. Sci.* **10**:218–220 (1989).
10. Nishizuka, Y. The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature (Lond.)* **308**:693–698 (1984).
11. Akil, H., S. J. Watson, E. Young, M. E. Lewis, H. Khachaturian, and J. M. Walker. Endogenous opioids: biology and function. *Annu. Rev. Neurosci.* **7**:223–255 (1984).
12. Pasternak, G. W. Opioid receptors, in *Psychopharmacology, The Third Generation of Progress* (H. Y. Meltzer, ed.). Raven Press, New York, 281–288 (1987).
13. Leslie, F. M. Methods used for the study of opioid receptors. *Pharmacol. Rev.* **39**:197–249 (1987).
14. North, R. A. Opioid receptor types and membrane ion channels. *Trends Neurosci.* **9**:114–117 (1986).
15. Quirion, R., J. M. Zajac, J. L. Morgat, and B. P. Roques. Autoradiographic distribution of *mu* and *delta* opiate receptors in rat brain using highly selective ligands. *Life Sci.* **33**:227–230 (1983).
16. Birnbaumer, L., and M. Rodbell. Adenylate cyclase in fat cells. I. Properties and effects of adrenocorticotropin and fluoride. *J. Biol. Chem.* **244**:3468–3476 (1969).
17. Steer, M. L., and A. Levitzki. The control of adenylate cyclase by calcium in turkey erythrocyte ghosts. *J. Biol. Chem.* **250**:2080–2084 (1975).
18. Iyengar, R., and L. Birnbaumer. Hormone receptor modulates the regulatory component of adenylyl cyclase by reducing its requirement for Mg^{2+} and enhancing its extent of activation by guanine nucleotides. *Proc. Natl. Acad. Sci. USA* **79**:5179–5183 (1982).
19. Cooper, D. M. F. Bimodal regulation of adenylate cyclase. *FEBS Lett.* **138**:157–163 (1982).
20. Caron, M. G., R. A. Cerione, J. L. Benovic, B. Strulovici, C. Staniszewski, R. J. Lefkowitz, J. Codina-Salada, and L. Birnbaumer. Biochemical characterization of the adrenergic receptors: affinity labelling, purification, and reconstitution studies. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **19**:1–12 (1985).
21. Birnbaumer, L., J. Abramowitz, and A. M. Brown. Receptor-effector coupling by G proteins. *Biochim. Biophys. Acta* **1031**:163–224 (1990).
22. Jones, D. T., S. B. Masters, H. R. Bourne, and R. R. Reed. Biochemical characterization of three stimulatory GTP-binding proteins: the large and small forms of G_s and the olfactory specific G-protein, G_{olf}. *J. Biol. Chem.* **265**:2671–2676 (1990).
23. Olianas, M. C., and P. Onali. Pertussis toxin attenuates D2 inhibition and enhances D1 stimulation of adenylate cyclase by dopamine in rat striatum. *J. Neurochem.* **48**:1443–1447 (1987).
24. Newton, D. L., and W. A. Klee. Guanine nucleotide dependent and independent reconstitution of G-proteins with adenylate cyclase: stimulation or attenuation of the enzyme by G_i α subunits. *FEBS Lett.* **271**:207–210 (1990).
25. Enna, S. J., and E. W. Karbon. Receptor regulation: evidence for a relationship between phospholipid metabolism and neurotransmitter receptor-mediated cAMP formation in brain. *Trends Pharmacol. Sci.* **8**:21–24 (1987).
26. Onali, P., and M. C. Olianas. Positive coupling of cholinergic muscarinic receptors to adenylate cyclase activity in membranes of rat olfactory bulb. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **342**:107–109 (1990).
27. Olianas, M. C., and P. Onali. Ca^{2+}-independent stimulation of adenylate cyclase activity by muscarinic receptors in rat olfactory bulb. *J. Neurochem.* **55**:1083–1086 (1990).
28. Tang, L. C., and G. C. Cotzias. Morphine sulfate stimulates the adenylate cyclase in mouse caudate nuclei. *Proc. Natl. Acad. Sci. USA* **75**:1546–1548 (1978).
29. Puri, S. K., J. Cochin, and L. Volicer. Effect of morphine sulfate on adenylate cyclase and phosphodiesterase activities in rat corpus striatum. *Life Sci.* **16**:759–768 (1975).
30. Law, P. Y., J. Wu, J. E. Koehler, and H. H. Loh. Demonstration and characterization of opiate inhibition of the striatal adenylate cyclase. *J. Neurochem.* **36**:1834–1846 (1981).
31. Makman, M. H., B. Dvorkin, and S. M. Crain. Modulation of adenylate cyclase activity of mouse spinal cord-ganglion explants by opioids, serotonin and pertussis toxin. *Brain Res.* **445**:303–313 (1988).
32. Nicoll, R. A., B. E. Alger, and C. E. Jahr. Enkephalin blocks inhibitory pathways in the vertebrate CNS. *Nature (Lond.)* **287**:22–25 (1980).

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