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# μ-Opioid Receptors Inhibit Dopamine-Stimulated Activity of type V Adenylyl Cyclase but Enhance Dopamine-Stimulated Activity of Type VII Adenylyl Cyclase

MASAMI YOSHIMURA, HIROSHI IKEDA, and BORIS TABAKOFF

*Department of Pharmacology, University of Colorado School of Medicine, Denver, Colorado 80262*

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## SUMMARY

The introduction of $D_{1A}$ dopamine receptors and μ-opioid receptors into HEK 293 cells that were also transiently transfected with adenylyl cyclase cDNA imparted to dopamine and to μ-opioid receptor agonists the ability to modulate the activity of the expressed adenylyl cyclase. Dopamine added to cells expressing $D_{1A}$ receptors and type V adenylyl cyclase significantly stimulated type V enzyme activity. The concomitant addition of morphine produced a dose-dependent inhibition of dopamine-stimulated type V adenylyl cyclase activity. On the other hand, if the HEK 293 cells were transfected with cDNA for type VII adenylyl cyclase instead of the type V isoform, morphine stimulated this adenylyl cyclase activity beyond the stimulation produced by dopamine. Both the inhibitory and stimulatory effects of morphine were blocked by naloxone or pretreatment of the transfected HEK 293 cells with pertussis toxin. When expressed in the HEK 293 cells, the α subunit of transducin, which is considered to be the putative scavenger of the βγ subunits of G proteins, suppressed the stimulatory effect of morphine on type VII adenylyl cyclase. We also expressed the adenylyl cyclases in cells that were transfected with $D_{1A}$ receptor and $G_{\beta 1}$ and $G_{\gamma 2}$ cDNAs. Dopamine was more efficacious in stimulating type VII adenylyl cyclase activity in cells concomitantly transfected with the βγ subunit cDNAs than in cells not transfected with these G protein subunits. Transfection with βγ subunit cDNAs did not affect dopamine stimulation of type V adenylyl cyclase activity, and morphine-induced inhibition of type V adenylyl cyclase activity was still evident in cells cotransfected with the α subunit of transducin. These data support the contention that the effects on type VII adenylyl cyclase activity mediated through the $G_i/G_o$ proteins may depend on the actions of the βγ subunits. The same is not the case for type V adenylyl cyclase. Our data demonstrate that both qualitative and quantitative responses to μ-opioid receptor stimulation depend on the isoform of adenylyl cyclase expressed in neurons or other cells of the body.

Opioids produce a myriad of physiological and pharmacological effects through interactions with opioid receptors in the central nervous system and in the periphery. These effects range from analgesia and euphoria to the antitussive properties of the opioids to the inhibitory effects of opioids on colon motility (1). The receptors with which the opioids interact have been pharmacologically classified as μ, δ, and κ receptors (2). The recent cloning and structural analysis of μ and δ receptors (3–6) have substantiated prior work (for a review, see Ref. 7) in which these opioid receptors have been considered members of the family of G protein-coupled receptors. A number of studies with the cloned receptors expressed in model cell systems (8, 9) and with the endogenous μ and δ receptors of mammalian tissues (7) have demonstrated that agonists acting through μ and δ receptors exert their actions through pertussis toxin-sensitive G proteins (i.e., the $G_i/G_o$ family of proteins). Through their interactions with the $G_i/G_o$ proteins, the μ- and δ-opioid receptors have been shown to inhibit adenylyl cyclase activity, activate calcium-dependent potassium channels, and inhibit voltage-sensitive calcium channels (7, 9–11).

An increase in the knowledge regarding structural and functional features of the adenylyl cyclase has, however, demonstrated that several receptor systems previously thought to strictly mediate the inhibition of adenylyl cyclase activity can produce an activation of this enzyme activity, depending on which member of the growing family of adenylyl cyclases is expressed in a cell and the circumstance under which the activity of these catalytic units is being monitored (8, 12). Nine adenylyl cyclases have been cloned from mammalian sources and characterized with regard to regulation of their catalytic activity (13–17). These isoforms of adenylyl cyclase have been classified into subfamilies based on the structural similarities of these enzymes (18). Three adenylyl

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**ABBREVIATIONS:** DAMGO, [D-Ala$^2$,*N*-MePhe$^4$,Gly-ol$^5$]-enkephalin; DPDPE, [D-Pen$^2$,D-Pen$^5$]-enkephalin; PDBu, phorbol-12,13-dibutyrate; HEK, human embryonic kidney.

cyclase enzymes (types II, IV, and VII) constitute the type II subfamily of adenylyl cyclases (18). The type II enzyme is considered the prototype for this subfamily of enzymes, and the type II adenylyl cyclase has been shown to be particularly resistant to inhibition by $G_{i\alpha 1-3}$ (19) and to be regulated in its activity by $\beta\gamma$ subunits of the G proteins under circumstances in which there is concurrent activation by $G_{s\alpha}$ (12, 20). The $\beta\gamma$ subunits generate a further increase in type II adenylyl cyclase activity when this enzyme is activated by $G_{s\alpha}$. Chen *et al.* (21) recently proposed that an area of type II adenylyl cyclase structure, defined as amino acid residues 956–982, is the site of the interactions of the $G_{\beta\gamma}$ proteins with this enzyme. The amino acid sequence in this area is conserved in other effectors, such as type IV adenylyl cyclase, β-adrenergic receptor kinase, and the G protein-gated potassium channel GIRK-1, which are thought to be regulated by $G_{\beta\gamma}$ (21). This sequence is also evident in type VII adenylyl cyclase.

The presence of type VII adenylyl cyclase protein in mammals, including humans, and its structural similarity to type II adenylyl cyclase (16, 22) have raised interest in the regulatory properties of this adenylyl cyclase isoform. Although type VII adenylyl cyclase mRNA is expressed in a number of brain areas (23, 24), type VII enzyme is also abundantly expressed in various peripheral tissues that do not express type II adenylyl cyclase (16, 22, 23). We have previously examined some of the regulatory characteristics of type VII adenylyl cyclase of human origin, including the control of its activity by $G_{s\alpha}$ and by post-translational phosphorylation events (24). In the current study, we sought to determine the responsiveness of the enzyme to activation of the $G_i/G_o$ family of proteins.

In the central nervous system, the interactions between dopamine and opioid receptor systems have received much attention, particularly in brain areas (e.g., nucleus accumbens and striatum) related to the euphoria and the reinforcing and locomotor-activating effects of various pharmacological agents (25). Dopamine acting via the $D_1$ dopamine receptors stimulates adenylyl cyclase activity by promoting the activation of $G_s$ and through interactions of the GTP-activated $G_{s\alpha}$ ($G_{s\alpha*}$) with the adenylyl cyclase catalytic units (26). Although all currently known adenylyl cyclases are activated through interactions with $G_{s\alpha*}$, we considered whether activation of $G_i/G_o$ through the μ-opioid receptors would produce directionally different effects on dopamine-stimulated adenylyl cyclase activity depending on which adenylyl cyclase was expressed in an experimental system. For the purpose of our study, we chose to compare type V adenylyl cyclase, which is expressed at high levels in regions of brain receiving dopaminergic input (27), with type VII adenylyl cyclase, which has not yet been characterized with regard to its coordinate regulation by $G_{s\alpha}$ and the activation of the $G_i/G_o$ family of proteins.

## Materials and Methods

**Plasmids.** The mouse μ-opioid receptor cDNA (in the pRc/CMV vector) was a generous gift from Dr. L. Yu (Department of Medical and Molecular Genetics, Indiana University, Indianapolis, IN). The human $D_{1A}$ dopamine receptor (in the pCMV5 vector) was a generous gift from Dr. S. B. Liggett (Department of Medicine, University of Cincinnati, Cincinnati, OH). The construction of pcDNA-ACV (28) and pCMV-ACVII (24), which contain the coding sequences of type V and VII adenylyl cyclases, has been described previously. The cDNA clones for the β1 subunit ($G_{\beta 1}$) and the γ2 subunit ($G_{\gamma 2}$) of G protein (in the pCMV5) were generous gifts from Dr. G. L. Johnson (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO). The cDNA clone for the α1 subunit of transducin ($G_{t\alpha 1}$) was a generous gift from Dr. H. E. Hamm (Department of Physiology and Biophysics, University of Illinois, Chicago, IL). For experiments on the expression of $G_{t\alpha 1}$, pCMV-$G_{t\alpha 1}$ was constructed as follows. The coding sequence of $G_{t\alpha 1}$ was excised from the original construct (in the pML52 vector) through digestion with *Nco*I and *Kpn*I and inserted into a mammalian expression vector, pCMV-SK (24), which was digested with *Eco*RV and *Kpn*I. Before ligation, the *Nco*I end of the $G_{t\alpha 1}$ fragment was converted to a blunt end.

**Transient expression of receptors, adenylyl cyclases, and G protein subunits in HEK 293 cells.** Transfection of HEK 293 cells with DNA precipitated with calcium phosphate was carried out as described previously (29). Thirteen micrograms of plasmid DNA containing adenylyl cyclase cDNA were used for each flask (75 $cm^2$) (except for the experiments shown in Fig. 6A, for which 10 μg of DNA was used). The amount of plasmid DNA containing the receptor cDNA was 6 μg/flask, and the amount of plasmid DNA containing cDNA for $G_{t\alpha 1}$, $G_{\beta 1}$, or $G_{\gamma 2}$ was 3 μg/flask. For every transfection, the amount of DNA per flask was adjusted to 25 μg with pCMV-5. After transfection, the cells in a flask were replated onto 18 wells of 24-well culture plates. The cells were incubated for 2 days before we assayed adenylyl cyclase activity.

**Adenylyl cyclase activity assay.** Formation of cAMP in intact cells was measured by monitoring the conversion of [$^3$H]ATP into [$^3$H]cAMP after labeling the intracellular ATP pool with [$^3$H]adenine as described previously (29). Cells were treated with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (500 μM) for 10 min before the addition of a receptor agonist. The cells were incubated for an additional 5 min in the presence of agonist(s) and/or antagonists before termination of the reaction and quantification of the [$^3$H]cAMP. [$^3$H]ATP and [$^3$H]cAMP were separated through sequential chromatography with Dowex 50 and neutral alumina columns as described by Salomon *et al.* (30), and radioactive material was quantified with a Beckman LS 6000TA liquid scintillation spectrometer. cAMP production was calculated as a fraction of the available pool of [$^3$H]ATP converted to [$^3$H]cAMP as follows: cAMP accumulation over time (%) = $A/(A + B) \times 100$, where $A$ is $^3$H (in cpm) recovered in the cAMP fraction, and $B$ is $^3$H (in cpm) recovered in the ATP fraction. Details regarding the concentration of agonists added to assays and other assay variables are given in figure legends.

**Western blot analysis.** HEK 293 cells from three wells of a six-well plate were suspended in Dulbecco's phosphate-buffered saline containing 0.04% EDTA without $Ca^{2+}$ and $Mg^{2+}$ and then were harvested through centrifugation at 1000 × *g*. For detection of $G_{t\alpha 1}$, the cell pellet was suspended in 0.5 ml of lysis buffer (50 mM Tris·HCl, 2 mM $MgCl_2$, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamide, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml antipain, 20 μg/ml soybean trypsin inhibitor, and 2 μg/ml pepstatin A, pH 8.0), and disrupted with sonication, and stored at −80° until use. For detection of $G_\beta$, the cell pellet was suspended in 2 ml of the lysis buffer and homogenized with a Polytron homogenizer at a setting of 5–6 for 10 sec. Membranes were collected through centrifugation at 20,000 × *g* for 20 min at 4°. The pellet was suspended through sonication in 1 ml of the lysis buffer, and particulate material was collected through another centrifugation at 20,000 × *g* for 20 min at 4°. The final pellet was suspended through sonication in 0.5 ml of the lysis buffer and stored at −80° until use. Protein concentration was determined according to the BCA protein assay method (31). Sample preparations were reduced and denatured through boiling in Laemmli's sample buffer for 3 min before proteins (20 μg/lane) were separated on 10% polyacrylamide gels (32). After electrophoresis, the proteins were transferred to nitrocellulose membranes (33). The membranes were blocked with 5% nonfat dry milk and 0.2% Tween 20 in TBS (20 mM Tris·HCl, pH 7.5, and 500 mM NaCl) overnight, washed briefly with TBS containing 0.1%

Tween 20, and then incubated with the $G_{\alpha i1}$-specific antisera (Santa Cruz Biotechnology, Santa Cruz, CA) diluted at 1:1000 or the G protein β subunit-specific antisera, SW/1 (DuPont-New England Nuclear, Boston, MA), diluted at 1:5000 in TBS containing 1% fish gelatin and 0.05% Tween 20. After incubation with primary antiserum, the blots were incubated with horseradish peroxidase-conjugated IgG (BioRad, Hercules, CA) diluted at 1:25,000–100,000. Reactive bands were visualized through enhanced chemiluminescence (34). The immunoreactive bands were quantified with the use of image analysis using the National Institutes of Health IMAGE program (version 1.57) on a Macintosh II computer and a Sierra Scientific CCD video camera.

**Statistical analysis.** Results are reported as mean ± standard deviation values. Statistical significance of the mean values was determined with a Student's *t* test; $p < 0.01$ was taken to indicate significant differences in mean values. Data from concentration-response experiments were analyzed with a least-squares nonlinear regression computer program (SigmaStat, Jandel scientific software) according to the following equation: $f(x) = (a - d)/[1 + (x/c)^b] + d$, where $f(x)$ is cAMP accumulation, $x$ is morphine concentration, $a$ is asymptotic maximum, $b$ is slope parameter, $c$ is $EC_{50}$, and $d$ is asymptotic minimum.

## Results

We chose the $D_{1A}$ dopamine receptor and the μ-opioid receptor to study the effects of interaction of $G_s$ and $G_i/G_o$ on adenylyl cyclase activity because the couplings of these receptors to G proteins ($D_{1A}$ dopamine receptor to $G_s$ and μ-opioid receptor to $G_i/G_o$) have been well characterized (6, 7, 26, 35). cAMP accumulation in HEK 293 cells was not altered by the addition of dopamine (10 μM) or morphine (10 μM) without transfection of the cells with either the $D_{1A}$ or the μ-opioid receptor (data not shown). Dopamine stimulated cAMP accumulation in cells transfected with the $D_{1A}$ dopamine receptor (Fig. 1). cAMP accumulation stimulated by dopamine was inhibited in the presence of the $D_1$ dopamine receptor antagonist SCH-23390. Cells cotransfected with adenylyl cyclase (either type V or type VII isoform) and the $D_{1A}$ dopamine receptor showed 3–4-fold higher cAMP accumulation in response to dopamine than did cells transfected with the receptor alone (Fig. 1). The effects of SCH-23390 on cells transfected with both the $D_{1A}$ dopamine receptor and one of the adenylyl cyclases indicated that the effects of dopamine were mediated through $D_1$-type dopamine receptors (Fig. 1). A $D_2$ dopamine receptor antagonist, sulpiride, did not have any significant effect on dopamine-enhanced cAMP accumulation in cells expressing type VII adenylyl cyclase (data not shown).

To examine the effect of the μ-opioid receptor on cAMP accumulation in cells transfected with type V or type VII adenylyl cyclase, cells were cotransfected with the adenylyl cyclase, the $D_{1A}$ dopamine receptor, and the μ-opioid receptor. Morphine (10 μM), when added alone to cells transfected with the $D_{1A}$ and μ-opioid receptors and either one of the adenylyl cyclases, did not show any effects on cAMP accumulation without the addition of dopamine (data not shown). However, morphine and DAMGO, the μ-selective opioid receptor agonists (36), inhibited dopamine-stimulated cAMP accumulation in cells transfected with type V adenylyl cyclase (Fig. 2A). On the contrary, these opioid receptor agonists enhanced dopamine-stimulated cAMP accumulation in cells transfected with type VII adenylyl cyclase (Fig. 2B). The concentrations of morphine and DAMGO were selected to be

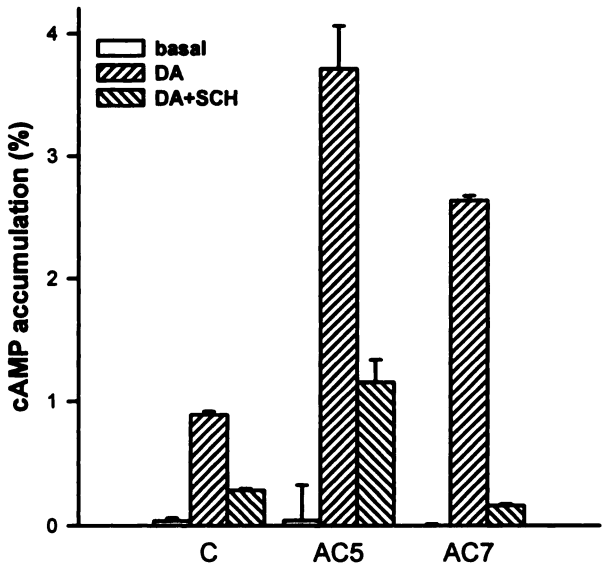


**Fig. 1.** Dopamine-mediated stimulation of cAMP accumulation in transfected HEK 293 cells. Cells transfected with $D_{1A}$ dopamine receptor (*C*), with this receptor and type V adenylyl cyclase (*AC5*), and with this receptor and type VII adenylyl cyclase (*AC7*) were incubated for 5 min in the presence of the dopamine and the dopamine antagonists: *basal*, no dopamine or antagonists; *DA*, 1 μM dopamine; *SCH*, 1 μM SCH-23390. cAMP accumulation during the 5-min incubation is reported as percent conversion of [$^3$H]ATP to [$^3$H]cAMP calculated as described in Materials and Methods. Data are mean ± standard deviation values derived from triplicate determination in one set of experiments; two additional experiments gave similar results. For all three samples, the values for dopamine were significantly greater than the values for basal conditions ($p < 0.0005$), and values for dopamine plus SCH-23390 were significantly less than the values for dopamine ($p < 0.0005$).

saturating at the μ-opioid receptor (based on their dissociation constants at the μ-opioid receptor) but to be below their dissociation constants at the δ-opioid receptor (36). Both inhibitory and stimulatory effects of morphine and DAMGO were reversed by the addition of naloxone, an opioid receptor antagonist. Although used at concentrations at least 16-fold higher than its dissociation constant at the δ-opioid receptor (36), DPDPE, a δ-selective opioid agonist (36), produced only a slight inhibition of cAMP accumulation in cells transfected with either type V or type VII adenylyl cyclase, and this effect was not reversed by naloxone (Fig. 2, A and B). Analysis of the concentration-response curves for morphine (Fig. 3) indicated that both the inhibitory and the stimulatory effects of morphine on cAMP accumulation were concentration dependent. The $EC_{50}$ value for morphine for inhibition of type V adenylyl cyclase (12.0 nM; 95% confidence limits, 6.7–17.4 nM) was significantly lower than the $EC_{50}$ value of morphine for stimulation of type VII adenylyl cyclase (54.7 nM; 95% confidence limits, 52.0–55.9 nM).

To examine the involvement of $G_i/G_o$ in the observed effects of the μ-selective opioid receptor agonists, cells transfected with the $D_{1A}$ and μ-opioid receptor and either type V or type VII adenylyl cyclase were treated with pertussis toxin. The toxin treatment did not affect cAMP accumulation in response to added dopamine, whereas the inhibitory effect of morphine on the activity of type V adenylyl cyclase and the stimulatory effect of morphine on type VII enzyme activity were completely abolished by pertussis toxin pretreatment (Fig. 4).

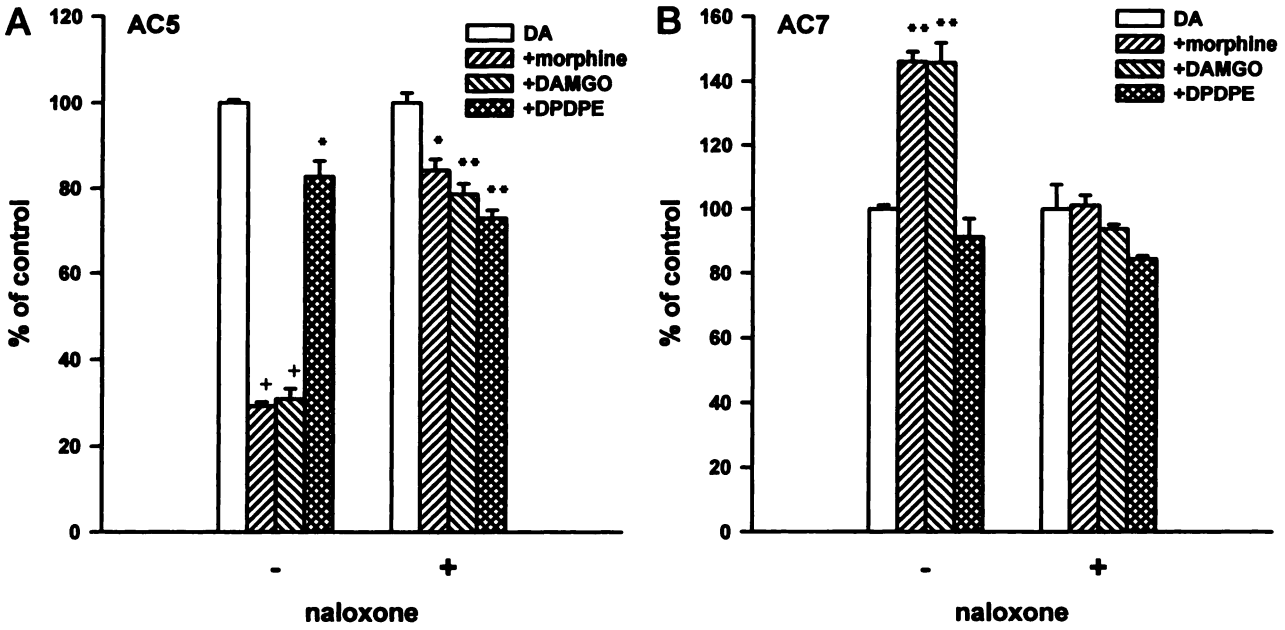


**Fig. 2.** Opioid-mediated modulation of cAMP accumulation in transfected HEK 293 cells. A, Cells transfected with the $D_{1A}$ dopamine receptor, the $\mu$-opioid receptor, and type V adenylyl cyclase (*AC5*) were incubated for 5 min in the presence of 1 $\mu$M dopamine plus opioid drugs: *DA*, dopamine alone; *+morphine*, 100 nM morphine; *+DAMGO*, 10 nM DAMGO; and *+DPDPE*, 20 nM DPDPE. In addition, 1 $\mu$M naloxone was added where indicated. cAMP accumulation during the 5-min incubation was measured as described in Materials and Methods. The results are expressed as a percentage of the cAMP accumulation measured in the samples incubated with dopamine alone (2.19 ± 0.02% conversion of [$^3$H]ATP to [$^3$H]cAMP) or with dopamine and naloxone (1.92 ± 0.05% conversion of [$^3$H]ATP to [$^3$H]cAMP). B, Cells transfected with the $D_{1A}$ dopamine receptor, the $\mu$-opioid receptor, and type VII adenylyl cyclase (*AC7*) were used for measurement of the effects of the opioid receptor agonists/antagonists on cAMP accumulation. The concentrations of the drugs were 1 $\mu$M dopamine, 100 nM morphine, 50 nM DAMGO, 100 nM DPDPE, and 1 $\mu$M naloxone. The results are expressed as a percentage of the cAMP accumulation measured in the samples incubated with dopamine alone (3.02 ± 0.04% conversion of [$^3$H]ATP to [$^3$H]cAMP) or with dopamine and naloxone (2.90 ± 0.22% conversion of [$^3$H]ATP to [$^3$H]cAMP). Data are mean ± standard deviation values derived from triplicate determination in one set of experiments; two additional experiments gave similar results. *, $p < 0.005$; **, $p < 0.0005$; and +, $p < 0.0001$, significantly different from the values for dopamine.

The activity of both type V and type VII adenylyl cyclase has been reported to be enhanced by activation of protein kinase C (16, 24, 37). Because opioids have been shown to stimulate the activity of phospholipase C (8, 38), which in turn can promote the activation of protein kinase C, we examined the effect of staurosporine, a potent serine/threonine protein kinase inhibitor (39), on modulation of cAMP accumulation by $\mu$-opioid receptor agonists. Treatment with staurosporine did not affect cAMP accumulation in cells transfected with type V adenylyl cyclase and the two receptors, regardless of the agonists used (dopamine alone or dopamine plus morphine) (Fig. 5A). Staurosporine reduced dopamine-stimulated cAMP accumulation in cells transfected with type VII adenylyl cyclase and the two receptors. However, the degree of stimulation by morphine (percent stimulation over that obtained with dopamine alone) was not altered by treatment with staurosporine (27% without staurosporine and 24% with staurosporine) (Fig. 5A). Simultaneous treatment with PDBu and dopamine of the HEK 293 cells that were transfected with the $D_{1A}$ dopamine and $\mu$-opioid receptors and type VII adenylyl cyclase generated a 62% greater response to dopamine compared with the response to dopamine with cells not treated with PDBu (Fig. 5B). The cAMP accumulation in response to dopamine plus morphine in the presence of PDBu was quantitatively greater than in the absence of PDBu. However, when the effect of morphine was considered as a percentage increase in the response to dopamine in the presence and absence of PDBu, morphine produced a 53% increase in dopamine-stimulated cAMP accumulation in the absence of PDBu and a similar 69% increase in the presence of PDBu (Fig. 5B).

To examine the involvement of $G_{\beta\gamma}$ in the enhancement of cAMP accumulation produced by $\mu$-opioid receptor agonists in cells transfected with type VII adenylyl cyclase, cells were transfected with $G_{t\alpha 1}$ in addition to the two receptors and type VII or type V adenylyl cyclase. In the cells expressing type VII adenylyl cyclase and $G_{t\alpha 1}$, morphine no longer enhanced dopamine-stimulated cAMP accumulation (Fig. 6A). Interestingly, the dopamine-stimulated cAMP accumulation in cells expressing type VII adenylyl cyclase was also consistently reduced by ~30% in the presence of $G_{t\alpha 1}$ compared with in the absence of $G_{t\alpha 1}$. In the cells expressing type V adenylyl cyclase, expression of $G_{t\alpha 1}$, however, produced an increase in dopamine-stimulated cAMP accumulation (see legend to Fig. 6). In these same cells (i.e., those expressing type V adenylyl cyclase), morphine produced a 77% inhibition of dopamine-stimulated cAMP accumulation when cells were not transfected with $G_{t\alpha 1}$ and a 61% inhibition of dopamine-stimulated cAMP accumulation when the cells were cotransfected with $G_{t\alpha 1}$ (Fig. 6A). Western blotting analysis demonstrated similar levels of $G_{t\alpha 1}$ in all cells transfected with $G_{t\alpha 1}$, the two receptors, and type VII or type V adenylyl cyclase. $G_{t\alpha 1}$ was not detected in HEK 293 cells without transfection with the $G_{t\alpha 1}$ cDNA (Fig. 7). To further examine the effect of $G_{\beta\gamma}$, cells were cotransfected with one of the adenylyl cyclases, the $D_{1A}$ dopamine receptor, and $G_{\beta\gamma}$ (Fig. 6B). Without the addition of dopamine, coexpression of $G_{\beta\gamma}$ did not affect cAMP accumulation in the cells transfected with either type

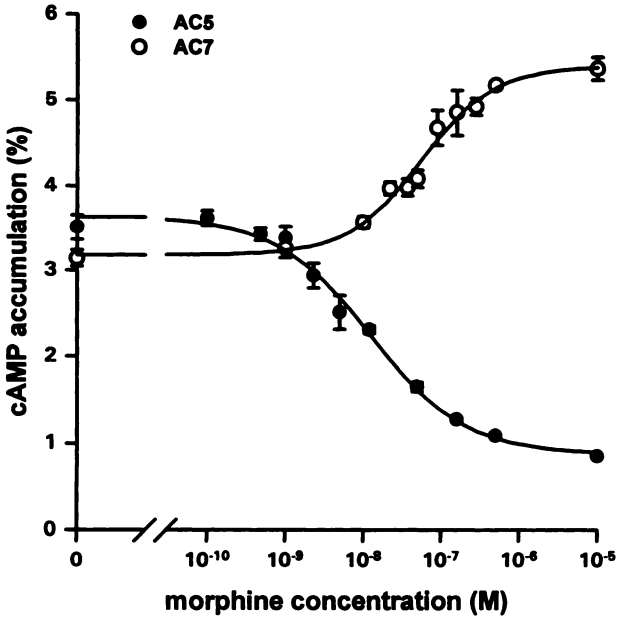


**Fig. 3.** Concentration-dependent effect of morphine on the activity of type V and type VII adenylyl cyclase. Cells transfected with the $D_{1A}$ dopamine receptor, μ-opioid receptor, and adenylyl cyclase [type V (*AC5*) or type VII (*AC7*)] were incubated with 1 μM dopamine for 5 min in the presence of various concentrations of morphine. cAMP accumulation during the 5-min incubation was measured as described in Materials and Methods. Data are mean ± standard deviation values derived from triplicate determination in one set of experiments; two additional experiments gave similar results. The curves were fitted by nonlinear regression as described in Materials and Methods.

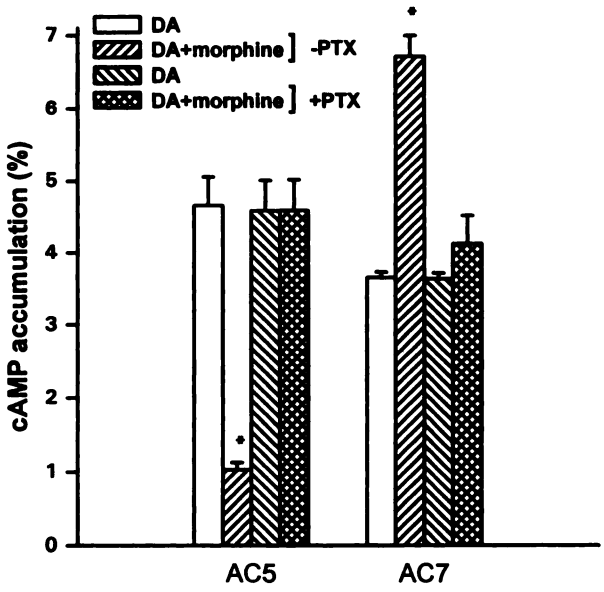


**Fig. 4.** Effects of pertussis toxin on μ-opioid receptor-mediated modulation of type V and type VII adenylyl cyclase activity. Cells transfected with the $D_{1A}$ dopamine receptor, the μ-opioid receptor, and adenylyl cyclase [type V (*AC5*) or type VII (*AC7*)] were treated with 100 ng/well pertussis toxin for 20 hr as indicated (±*PTX*) and then incubated for 5 min in the presence of 10 μM dopamine (*DA*) or 10 μM dopamine plus 10 μM morphine (*DA+morphine*). cAMP accumulation during the 5-min incubation was measured as described in Materials and Methods. Data are mean ± standard deviation values derived from triplicate determination in one set of experiments; two additional experiments gave similar results. *, Significantly different from the corresponding values for dopamine ($p < 0.0005$).

V or type VII adenylyl cyclase (data not shown). Cotransfection with $G_{\beta\gamma}$ also did not affect dopamine-stimulated cAMP accumulation in cells transfected with type V adenylyl cyclase (Fig. 6B). However, cAMP accumulation in response to dopamine in cells transfected with type VII adenylyl cyclase isoform was increased by cotransfection of $G_{\beta\gamma}$ (Fig. 6B). Western blot analysis demonstrated that transfection of HEK 293 cells with $G_{\beta 1}$ and $G_{\gamma 2}$ cDNAs produced a 40–140% increase in the amount of $G_{\beta}$ protein in the HEK 293 cell membranes regardless of the type of adenylyl cyclase coexpressed (data not shown).

## Discussion

The pharmacological characteristics of the expressed $D_{1A}$ and μ-opioid receptors fulfilled our expectations. The actions of dopamine on adenylyl cyclase activity were blocked by SCH-23390, a $D_1$ dopamine receptor antagonist, and were unaffected by sulpiride, a $D_2$ dopamine receptor antagonist (26). The μ-opioid receptor agonists morphine and DAMGO (36) had similar effects in HEK 293 cells expressing the μ-opioid receptors, and their effects were reversed by naloxone. DPDPE, a δ-opioid receptor selective drug (36), had little effect on adenylyl cyclase activity in cells expressing the μ receptors, and the modest effects of DPDPE (Fig. 2A) were not reversed by naloxone. The addition of dopamine to HEK 293 cells expressing the $D_{1A}$ receptor increased the formation of cAMP in all cases, and the formation of cAMP in response to dopamine was significantly greater in cells expressing type V or type VII adenylyl cyclase than in cells expressing the $D_{1A}$ receptor in the absence of transfection with one of these isoforms of adenylyl cyclase. Because the $D_{1A}$ dopamine receptor has been clearly demonstrated to be a $G_s$ protein-coupled receptor (26, 35), the activation by dopamine of cAMP formation by both type V and type VII adenylyl cyclase indicates that interactions of dopamine with the expressed $D_{1A}$ receptors generate activated $G_{s\alpha}$ ($G_{s\alpha*}$), which in turn stimulates the catalytic activity of both of the studied isoforms of adenylyl cyclase. Both type V and type VII adenylyl cyclase have been previously demonstrated to be activated by $G_{s\alpha*}$ (13, 14, 16, 24).

The structural characteristics of type V and type VII adenylyl cyclase have been used to classify these enzymes into different subfamilies of the adenylyl cyclases (18), and type V enzyme was recently shown to be particularly sensitive to the inhibitory actions of the $G_{i\alpha}$ proteins (19). On the other hand, little is known of the sensitivity of type VII enzyme to $G_{i\alpha}$ proteins. The type VII enzyme belongs to the same subfamily as type II adenylyl cyclase (18), which was found to be immune to the inhibitory actions of $G_{i\alpha 1,2}$ or $G_{i\alpha 3}$ (19). A motif has been identified in the sequence of type II adenylyl cyclase that has been proposed to constitute the binding site for $G_{\beta\gamma}$ (21). A similar motif is evident in the structure of type VII adenylyl cyclase (16, 22), and one might speculate that type VII adenylyl cyclase may respond to $G_{\beta\gamma}$ and the $G_{i\alpha}$ proteins as does type II enzyme. The type II adenylyl cyclase is coordinately regulated by $G_{s\alpha}$ and $G_{\beta\gamma}$, such that the $G_{\beta\gamma}$ proteins produce further stimulation of type II catalytic activity when the enzyme is concurrently activated by $G_{s\alpha*}$ (12, 20).

The interaction of μ-opioid receptors with the $G_i/G_o$ family of coupling protein promotes the dissociation of the G proteins into activated (GTP-bound) α subunits ($G_{i\alpha*}$ and $G_{o\alpha*}$)

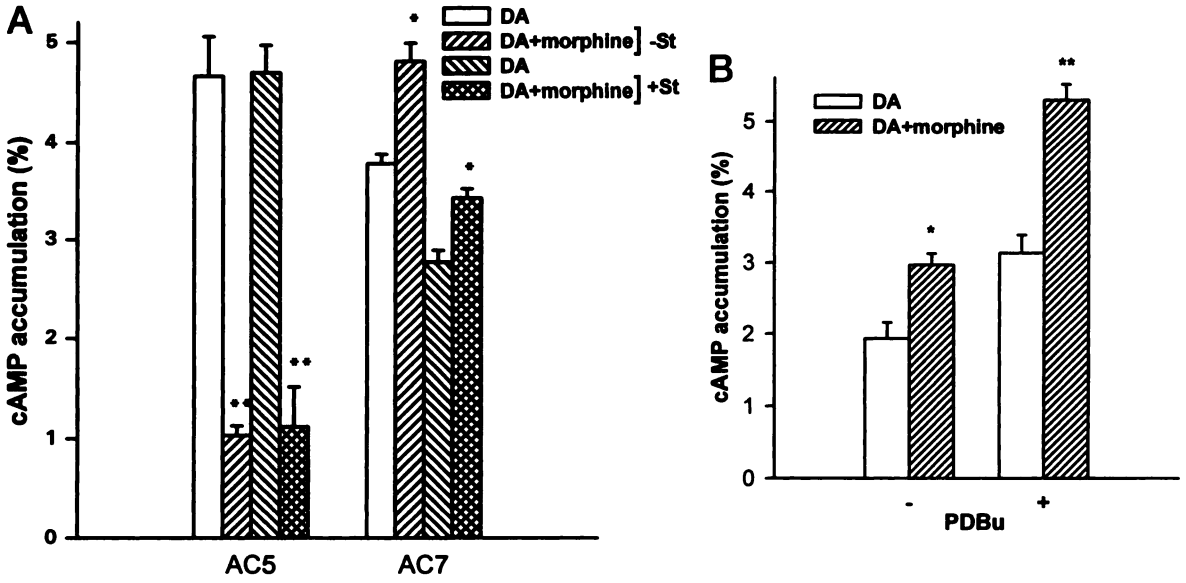


**Fig. 5.** Effect of staurosporine and PDBu on $\mu$-opioid receptor-mediated modulation of adenylyl cyclase activity. A, Cells transfected with the $D_{1A}$ dopamine receptor, the $\mu$-opioid receptor, and adenylyl cyclase [type V (*AC5*) or type VII (*AC7*)] were treated with 100 nM staurosporine for 20 min as indicated (±*St*) and then incubated for 5 min in the presence of 10 $\mu$M dopamine (*DA*) or 10 $\mu$M dopamine plus 10 $\mu$M morphine (*DA+morphine*). B, Cells transfected with the $D_{1A}$ dopamine receptor, the $\mu$-opioid receptor, and type VII adenylyl cyclase were treated with 10 $\mu$M dopamine (*DA*) or 10 $\mu$M dopamine plus 10 $\mu$M morphine (*DA+morphine*) for 5 min in the presence or absence of 100 nM PDBu as indicated. cAMP accumulation during the last 5-min incubation was measured as described in Materials and Methods. Data are mean ± standard deviation values derived from triplicate determination in one set of experiments; two additional experiments gave similar results. The values for dopamine plus morphine are significantly different from the corresponding values for dopamine: *, $p < 0.005$; **, $p < 0.0005$.

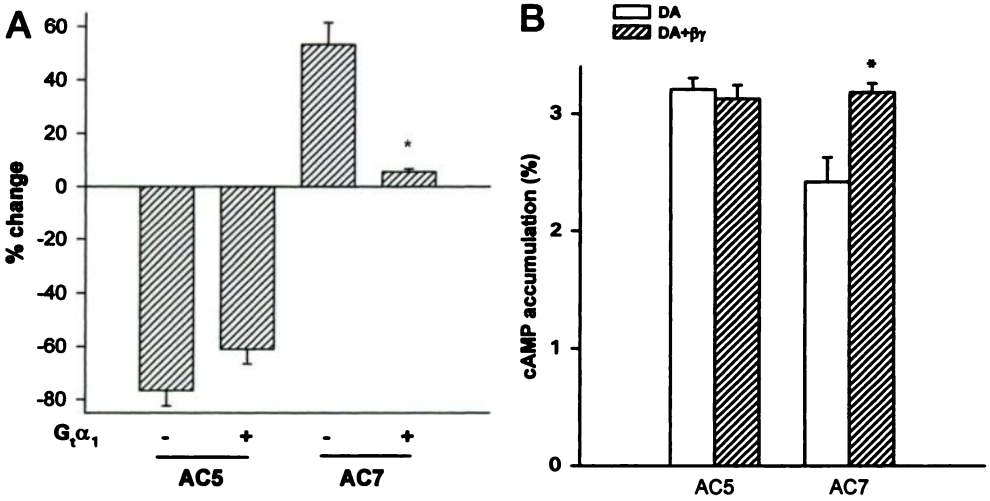


**Fig. 6.** Effects of $G_{t\alpha 1}$ and $G_{\beta\gamma}$ on the activity of type V and type VII adenylyl cyclase. A, Cells were transfected with $G_{t\alpha 1}$, as indicated (− or +) in addition to being transfected with the $D_{1A}$ dopamine receptor, the $\mu$-opioid receptor, and adenylyl cyclase [type V (*AC5*) or type VII (AC7)]. The cells were incubated for 5 min in the presence of 1 $\mu$M dopamine (*DA*) or 1 $\mu$M dopamine plus 10 $\mu$M morphine (*DA+morphine*). cAMP accumulation during the 5-min incubation was measured as described in Materials and Methods. Results are expressed as the percentage change in dopamine-stimulated cAMP accumulation by the addition of morphine. The dopamine-stimulated cAMP accumulation are: AC5 ($-G_{t\alpha 1}$), 1.17 ± 0.08%; AC5 ($+G_{t\alpha 1}$), 3.53 ± 0.24%; AC7 ($-G_{t\alpha 1}$), 1.93 ± 0.23%; and AC7 ($+G_{t\alpha 1}$), 1.34 ± 0.06%. Data are mean ± standard deviation values derived from triplicate determination in one set of experiments; two additional experiments gave similar results. *, Expression of $G_{t\alpha 1}$ significantly changed the effect of morphine ($p < 0.001$). B, Cells transfected with the $D_{1A}$ dopamine receptor, adenylyl cyclase [type V (*AC5*) or type VII (*AC7*)], and $G_{\beta\gamma}$ as indicated ($+\beta\gamma$), were incubated for 5 min in the presence of 10 $\mu$M dopamine (*DA*). cAMP accumulation during the 5-min incubation was measured as described in Materials and Methods. Data are mean ± standard deviation values derived from triplicate determination in one set of experiments; two additional experiments gave similar results. *, Significantly different from the corresponding values for DA ($p < 0.005$).

and $\beta\gamma$ subunits. Pertussis toxin blocks the interactions of the $\mu$-opioid receptor and $G_i/G_o$ by ADP-ribosylating the $\alpha$ subunits of $G_i/G_o$ and, through this ribosylation, preventing the exchange of GDP for GTP, thus locking the $\beta\gamma$ subunits into an inactive complex with the $G_{i\alpha}$ and $G_{o\alpha}$ subunits (40).

The transfection of HEK 293 cells with $D_{1A}$ and $\mu$-opioid receptors and type V adenylyl cyclase generated an experimental system in which the $\mu$-opioid receptor agonists efficaciously and potently inhibited dopamine-stimulated adenylyl cyclase activity. Pretreatment of the cells with pertussis toxin blocked the actions of the $\mu$-receptor agonists. The action of morphine and DAMGO (Fig. 2A) is reminiscent of

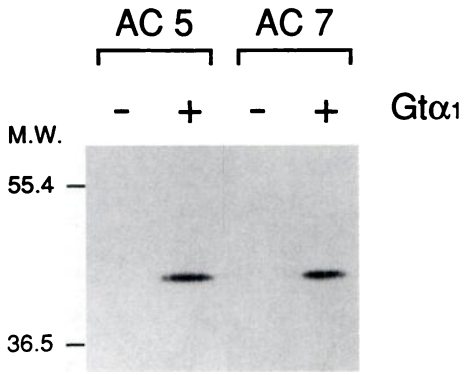


**Fig. 7.** Detection of $G_{t\alpha 1}$ in transfected HEK 293 cells. Proteins (20 μg/lane) from cells transfected with $G_{t\alpha 1}$, as indicated (− or +) in addition to the $D_{1A}$ dopamine receptor, the μ-opioid receptor, and adenylyl cyclase [type V (*AC5*) or type VII (*AC7*)] were separated on 10% acrylamide gels and transferred to a nitrocellulose membrane. The membrane was incubated with a specific antisera against $G_{t\alpha 1}$ and horseradish peroxidase-conjugated IgG as described in Materials and Methods. Proteins with which the antiserum reacted were detected through enhanced chemiluminescence. *Numbers at left*, position and size of molecular mass markers (in kDa).

prior studies (e.g., Ref. 41) with brain tissue, in which activation of μ receptors inhibited dopamine-stimulated adenylyl cyclase activity. The μ receptor-mediated inhibition of adenylyl cyclase activity in mammalian tissues has not, however, been a universal finding. Wang and Gintzler (42) demonstrated that sufentanil, a μ-opioid receptor agonist, enhanced cAMP accumulation in the myenteric plexus when applied concomitantly with electrical stimulation of this tissue, and stimulatory effects of opioid agonists on adenylyl cyclase activity have also been demonstrated in studies with olfactory bulb tissue (43).

In the HEK 293 cells transfected with type VII adenylyl cyclase and the $D_{1A}$ and μ-opioid receptors, the dopamine-stimulated adenylyl cyclase activity was further enhanced by the μ receptor-selective agonists (morphine and DAMGO). This action of the μ receptor agonists was also blocked by pertussis toxin. In the F-11 neuroblastoma sensory neuron hybrid cell line, opioid receptors (μ and δ) have been proposed to couple directly to $G_s$ (44). However, type VII adenylyl cyclase activity was not stimulated by morphine in our study in the absence of concomitant activation of $D_{1A}$ receptor by dopamine. Morphine was also ineffective in modulating the activity of either type V or type VII adenylyl cyclase without dopamine in cells treated with pertussis toxin. It is therefore unlikely that μ-opioid receptors couple to $G_s$ in the HEK 293 cells. Opioid agonists have also been shown to activate phospholipase C in a pertussis toxin-sensitive manner (8, 38). The release of diacylglycerol through the actions of phospholipase C would be expected to activate protein kinase C (39), and we have demonstrated that the basal activity and response of type VII adenylyl cyclase to $G_{s\alpha}$ are enhanced by protein kinase C-mediated phosphorylation events (24). Although staurosporine reduced the dopamine-stimulated activity of the expressed type VII adenylyl cyclase, the additional enhancement of the actions of dopamine by morphine was still evident in the presence of staurosporine. In cells transfected with the two receptors and type VII adenylyl cyclase, both dopamine-stimulated and dopamine-plus-morphine-stimulated cAMP accumulation were further enhanced by the addition of the phorbol ester PDBu. However, the proportional increase in cAMP accumulation produced by morphine was similar in the presence or absence of PDBu (Fig. 5B). One could therefore consider that the actions of morphine on adenylyl cyclase activity were not mediated by the activation by morphine of serine/threonine kinases such as protein kinase C. On the other hand, the absolute magnitude of the potentiation by morphine of dopamine-stimulated activity of type VII adenylyl cyclase could be expected to depend on the actions of protein kinase C.

The results we obtained in cells cotransfected with either $G_{t\alpha 1}$ or $G_{\beta\gamma}$ cDNA (Fig. 6) suggest that the presence of an excess of $G_{\beta\gamma}$ protein enhances dopamine-stimulated type VII adenylyl cyclase activity. If we assume that morphine or other μ receptor agonists are interacting with $G_i/G_o$ to release βγ subunits as well as the α subunits of these proteins, the results of our experiments indicate that type VII adenylyl cyclase, similar to the other members of the type II subfamily of adenylyl cyclases (20, 45), is an enzyme that can be further activated through interactions with $G_{\beta\gamma}$ when this enzyme is simultaneously activated by $G_{s\alpha *}$. Because type VII enzyme activity demonstrated only enhancement of the actions of dopamine through a wide range of morphine concentrations, type VII adenylyl cyclase appears to be insensitive to the released α subunits of the $G_i/G_o$ proteins. The studies of Federman *et al.* (12) demonstrated that $G_{t\alpha}$ can dampen the effects of $G_{\beta\gamma}$ on type II adenylyl cyclase. Our results demonstrate not only that coexpression of $G_{t\alpha 1}$ with $D_{1A}$ dopamine and μ-opioid receptors blocked the potentiating effects of morphine on dopamine-stimulated type VII adenylyl cyclase activity but also that an excess of $G_{t\alpha 1}$ could reduce the dopamine stimulation *per se* of type VII adenylyl cyclase activity. One of the several explanations of this effect may be that both the sα* and the βγ subunits generated by $D_{1A}$ dopamine receptor activation of $G_s$ can act in our cell system to enhance type VII adenylyl cyclase activity.

Our studies demonstrated that the potency of morphine for coordinate stimulation of type VII adenylyl cyclase activity by dopamine and morphine was lower than the potency of morphine for inhibition of dopamine-stimulated type V adenylyl cyclase activity. Because the same expressed receptors and endogenous G proteins are presumably involved in morphine-mediated stimulation and inhibition of adenylyl cyclase, the differences in the potency of morphine may be due to the type of adenylyl cyclase being expressed in the cells under examination and/or the characteristics of the interaction of G protein subunits with particular adenylyl cyclases. If the observed stimulatory or inhibitory effects are mediated solely through release of $G_{\beta\gamma}$ and $G_{i\alpha *}$, respectively, the potency of $G_{\beta\gamma}$ for generating stimulatory effects on type VII adenylyl cyclase would necessarily be lower than the potency of $G_{i\alpha *}$ for generating inhibition of type V adenylyl cyclase. Taussig and Gilman (46) discussed the evidence for differential effectiveness of $G_{s\alpha *}$ and $G_{\beta\gamma}$ on type II adenylyl cyclase. $G_{s\alpha *}$ is effective at picomolar concentrations, whereas $G_{\beta\gamma}$ effects on type II enzyme are noted at nanomolar concentrations. Studies with recombinant type V adenylyl cyclase and recombinant myristolated $G_{i\alpha *}$ proteins have indicated that the $EC_{50}$ for inhibition of type V enzyme by $G_{i\alpha *}$ is ~100 nM (47). Thus, the potency of $G_{\beta\gamma}$ actions on type II enzyme seems to be equal to or greater than the potency of $G_{i\alpha}$ action on type V adenylyl cyclase. The type VII isoform may, how-

ever, be distinguished by a lower affinity for $G_{\beta\gamma}$ than type II enzyme, and further studies will be necessary to evaluate such speculation.

Our current data with type VII adenylyl cyclase extend the principle of coordinate regulation of adenylyl cyclase activity by $G_{s\alpha*}$ and $G_{\beta\gamma}$ to another member of the type II subfamily of adenylyl cyclases. Because type VII adenylyl cyclase is expressed in dopaminergically innervated brain areas, such as the striatum (12), and because these areas of brain also express significant quantities of $\mu$-opioid receptors (48), the cAMP-mediated cellular responses to the simultaneous presence of dopamine and opioids in the extracellular milieu may be determined not only by the complement of dopamine and opioid receptors of a cell but also by the isoform of adenylyl cyclase expressed by the cell in question. The importance of the isoform of adenylyl cyclase in determining the quantitative characteristics, as well as the direction of the cAMP cellular response to opioids, is also relevant for peripheral tissues expressing the opioid receptors and type VII or other isoforms of adenylyl cyclase.

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**Send reprint requests to:** Boris Tabakoff, Ph.D., Department of Pharmacology, University of Colorado School of Medicine, Campus Box C236, 4200 East Ninth Avenue, Denver, CO 80262. E-mail: boris.tabakoff@uhsc.edu