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Opiate-induced Adenylyl Cyclase Superactivation Is Isozyme-specific*

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While acute activation of inhibitory $G_{i/o}$-coupled receptors leads to inhibition of adenylyl cyclase, chronic activation of such receptors leads to an increase in cAMP accumulation. This phenomenon, observed in many cell types, has been referred to as adenylyl cyclase superactivation. At this stage, the mechanism leading to adenylyl cyclase superactivation and the nature of the isozyme(s) responsible for this phenomenon are largely unknown. Here we show that transfection of adenylyl cyclase isozymes into COS-7 cells results in an isozyme-specific increase in AC activity upon stimulation (*e.g.* with forskolin, ionomycin, or stimulatory receptor ligands). However, independently of the method used to activate specific adenylyl cyclase isozymes, acute activation of the μ-opioid receptor inhibited the activity of adenylyl cyclases I, V, VI, and VIII, while types II, IV, and VII were stimulated and type III was not affected. Chronic μ-opioid receptor activation followed by removal of the agonist was previously shown, in transfected COS-7 cells, to induce superactivation of adenylyl cyclase type V. Here we show that it also leads to superactivation of adenylyl cyclase types I, VI, and VIII, but not of type II, III, IV, or VII, demonstrating that the superactivation is isozyme-specific. Not only were isozymes II, IV, and VII not superactivated, but a reduction in the activities of these isozymes was actually observed upon chronic opiate exposure. These results suggest that the phenomena of tolerance and withdrawal involve specific adenylyl cyclase isozymes.

The synthesis of cAMP by adenylyl cyclase (AC)[1] is modulated by hormones and neurotransmitters acting via receptors that activate GTP-binding proteins (G proteins). To date, mRNAs encoding nine distinct isozymes of AC have been identified (1–11). Sequence and functional similarities allow the categorization of these ACs into six classes: (*a*) AC type I (AC-I) is stimulated by Ca^{2+}/calmodulin, possibly independently of $G_{\alpha s}$ stimulation, and is inhibited by $G_{\beta\gamma}$ subunits; (*b*) AC-VIII is stimulated by Ca^{2+}/calmodulin; (*c*) AC-V and AC-VI are inhibited by low levels of Ca^{2+} but are unaffected by $G_{\beta\gamma}$ subunits; (*d*) AC-II, AC-IV, and AC-VII comprise a subfamily, where AC-II and AC-IV are highly activated by $G_{\beta\gamma}$ subunits in the presence of activated $G_{\alpha s}$, while AC-II and AC-VII are stimulated by activation of protein kinase C; (*e*) AC-III is stimulated by a high concentration of Ca^{2+}/calmodulin in the presence of $G_{\alpha s}$ but is unaffected by $G_{\beta\gamma}$ subunits; (*f*) AC-IX, which has only recently been cloned, has thus far only been found to be affected by $G_{\alpha s}$. The activities of all AC isozymes seem to be stimulated by $G_{\alpha s}$, but to different extents (5, 9, 10, 12–15).

Stimulation of seven-transmembrane domain inhibitory receptors (*e.g.* μ-, δ-, and κ-opioid receptors and m_2- and m_4-muscarinic receptors) activates $G_{i/o}$ proteins, as a result of which these G proteins dissociate into G_α and $G_{\beta\gamma}$ dimers (16–18). The $G_{\alpha i}$ subunit interacts with AC, leading to its acute inhibition and, subsequently, to a reduction in cAMP levels in the cell (11, 16, 19). However, chronic activation of inhibitory receptors has been shown to lead to an increase in cAMP accumulation. This phenomenon, which is particularly manifest upon withdrawal of the inhibitory agonist, is referred to as AC superactivation (20–28). While the inhibition of AC is considered to be one of the mechanisms underlying the acute effects of opiates, AC superactivation is believed to play a role in the development of tolerance and withdrawal upon prolonged opiate exposure (20, 23, 29). Tolerance and withdrawal are adaptive processes that are considered to underlie the development of drug dependence (30, 31).

AC superactivation was originally described in NG108–15 neuroblastoma × glioma hybrid cells that had been chronically treated with agonists of opioid, muscarinic, α_2-adrenergic, or somatostatin receptors (20, 32–34). This phenomenon was found not to be restricted to cells of neuronal origin, having been described in rat adipocytes treated with an A_1-adenosine receptor agonist (35) in somatostatin-treated S49 mouse lymphoma cells (34) and in opioid-treated Chinese hamster ovary cells transfected with μ-, δ-, or κ-opioid receptors (22–24). Nevertheless, it appears that there are cells that, under similar conditions, do not display AC superactivation, such as rat insulinoma RINm5F cells (36) and mouse 7315c cells (37). Moreover, there are cell types where AC superactivation is revealed only when AC is stimulated in a certain way (21, 38, 39).

As described above, several AC isozymes are known today that differ in their stimulation and inhibition characteristics, and the various cell types described above may vary in their AC isozyme populations. We have previously shown that AC-V transfected into COS-7 cells is susceptible to superactivation following chronic activation of μ- or δ-opioid or m_2-muscarinic receptors (40). Thomas and Hoffman (28) have shown that AC-VI transfected into HEK-293 cells is susceptible to superactivation following chronic activation of m_2-muscarinic or D_2-dopaminergic receptors. However, it is currently unknown whether other AC types could show superactivation following chronic treatment with inhibitory agonists. Here, we report that acute opiate treatment inhibits while chronic opiate treatment leads to superactivation of AC-I, AC-V, AC-VI, and AC-

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[1] The abbreviations used are: AC, adenylyl cyclase; DMEM, Dulbecco's modified Eagle's medium; FS, forskolin; G proteins, GTP-binding proteins; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TSH, thyroid-stimulating hormone; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate.

VIII. On the other hand, acute opiate treatment stimulates and chronic opiate treatment decreases the activity of AC-II, AC-IV, and AC-VII. The activity of AC-III is not affected by either acute or chronic opiate treatments.

EXPERIMENTAL PROCEDURES

Materials—[2-^{3}H]adenine (10.3 Ci/mmol) was purchased from Rotem Industries (Be'er Sheba, Israel). Morphine was obtained from the National Institute of Drug Abuse, Research Technology Branch (Rockville, MD). Ionomycin and the phosphodiesterase inhibitors 1-methyl-3-isobutylxanthine and RO-20–1724 were from Calbiochem. 12-*O*-tetradecanoylphorbol-13-acetate (TPA), forskolin (FS), cAMP, isoproterenol, thyroid-stimulating hormone (TSH), and carbachol were from Sigma. Tissue culture reagents were from Life Technologies, Inc. (Bethesda, MD).

Plasmids—AC-I cDNA was released from pSK-AC-I (1) using *Hin*dIII and *Xba*I, and ligated after "fill-in" into the *Sma*I site of the pXMD1 vector, which is under the control of the adenovirus-2 major late promoter (41). AC-II cDNA was released from pKS-AC-II (2) by *Eco*RI and ligated into the *Eco*RI site of pXMD1. These two plasmids, as well as pXMD1-AC-V (3), were provided by Prof. T. Pfeuffer. AC-III cDNA (4) was released from pBluescript KS (provided by Prof. R. Reed) by *Eco*RI and ligated into the *Eco*RI site of pXMD1. AC-IV cDNA (6) was released from pSK-AC-IV (provided by Prof. A. Gilman) by a partial cut with *Eco*RI and was ligated into the *Eco*RI site of pXMD1. AC-VII cDNA (8) was released from pBluescript (provided by Dr. P. Watson) by *Eco*RI and ligated into the *Eco*RI site of pXMD1. AC-VI (42), and AC-VIII (43) cDNAs in the mammalian expression vector pCMV5-neo were provided by Prof. J. Krupinski. β-galactosidase cDNA in pXMD1 (pXMD1-gal) was obtained from Dr. F.-W. Kluxen (41). Rat μ-opioid receptor cDNA in pCMV-neo was obtained from Prof. H. Akil (44). Rat wild-type TSH receptor cDNA inserted into the pSG5 vector was provided by Dr. S. Kosugi (45). Human m_1-muscarinic receptor cDNA in pCD vector was obtained from Dr. T. Bonner (46). α_s-Q227L in pcDNAId was obtained from Dr. H. Bourne (47).

Transient Cell Transfection—Twenty-four h before transfection, a confluent 10-cm plate of COS-7 cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere consisting of 5% CO_2 and 95% air at 37 °C, was trypsinized and split into four 10-cm plates. The cells were transfected, using the DEAE-dextran chloroquine method (48), with 1 μg/plate of rat μ-opioid receptor cDNA and 2 μg/plate of either one of the AC isozyme cDNAs or of pXMD1-gal (for mock DNA transfection) and, where indicated, with 1 μg/plate of m_1-muscarinic receptor, α_s-Q227L, or TSH receptor cDNA. Twenty-four h later, the cells were trypsinized and recultured in 24-well plates, and after an additional 24 h the cells were assayed for AC activity as described below. Transfection efficiencies were normally in the range of 40–80%, as determined by staining for β-galactosidase activity (49). The transfection of the various AC isozymes increased the amounts of cAMP in the cells compared with control (see "Results" for details). The expression of the μ-opioid receptor and of AC types I, II, IV, V, VI, and VIII was confirmed by the Western blotting technique, using selective antibodies (data not shown) kindly provided by Dr. G. Uhl (μ-opioid receptor) and by Dr. T. Pfeuffer (AC-I, -II, -IV, -V, and -VIII) or purchased from Santa Cruz Biotechnology (AC-VI). The expression of the μ-opioid receptor was not affected by the cotransfection with the various AC isozymes (data not shown).

AC Activity—The assay was performed in triplicate as described previously (23, 40, 50). In brief, cells cultured in 24-well plates were incubated for 2 h with 0.25 ml/well of fresh growth medium containing 5 μCi/ml of [^{3}H]adenine and then washed three times with 0.5 ml/well of DMEM containing 20 mM Hepes (pH 7.4) and 0.1 mg/ml bovine serum albumin. This medium was replaced with 0.5 ml/well of DMEM containing 20 mM Hepes (pH 7.4), 0.1 mg/ml bovine serum albumin and the phosphodiesterase inhibitors 1-methyl-3-isobutylxanthine (0.5 mM) and RO-20–1724 (0.5 mM). AC activity was stimulated in the presence or absence of opiate ligands by the addition of FS, TPA, ionomycin, carbachol, TSH, or isoproterenol. After 10 min (FS, TSH, or isoproterenol) or 20 min (ionomycin, TPA, or carbachol) at room temperature, the medium was removed, and the reaction was terminated by the addition of perchloric acid containing 0.1 mM unlabeled cAMP, followed by neutralization with KOH, and the amount of [^{3}H]cAMP was determined by a two-step column separation procedure as described previously (23, 51). Chronic opiate treatment was achieved by incubating the cells for 18 h with 1 μM of morphine followed by opiate withdrawal (by three rapid washes with DMEM containing 20 mM Hepes and 0.1 mg/ml bovine serum albumin) and the addition of stimulator (see above) to assay AC activity. We found that the uptake of [^{3}H]adenine into the cells was not affected by the chronic opiate treatment.

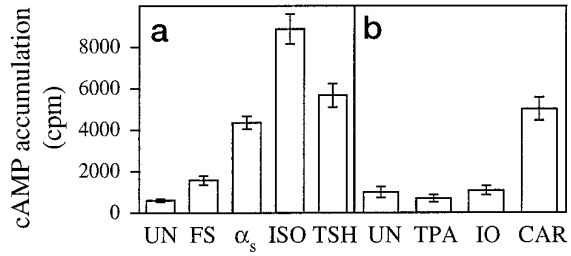

FIG. 1. **The endogenous AC activity in COS-7 cells is stimulated by FS, α_s-Q227L, isoproterenol, and carbachol.** COS-7 cells were transfected with the μ-opioid receptor and with either the TSH receptor, the m_1-muscarinic receptor, or α_s-Q227L cDNAs, as described under "Experimental Procedures." *a*, the endogenous AC activity in COS-7 cells is expressed in cpm of [^{3}H]cAMP formed following 10-min stimulation as follows. *UN*, unstimulated; *FS*, 1 μM FS; α_s, transfected α_s-Q227L; *ISO*, 10 μM isoproterenol (to stimulate endogenous β_2-adrenergic receptors); *TSH*, 0.1 μM TSH. *b*, the endogenous AC activity in COS-7 cells is expressed in cpm of [^{3}H]cAMP formed following 20-min stimulation as follows. *TPA*, 1 μM TPA; *IO*, 1 μM ionomycin; *CAR*, 100 μM carbachol. Data represent the means ± S.E. of at least three experiments performed in triplicate.

RESULTS

Effect of Opiates on AC Activity Endogenously Expressed in COS-7 Cells—In COS-7 cells transfected with μ-opioid receptor cDNA, cAMP accumulation could be stimulated upon activation with 1 μM FS (2–4-fold) or 10 μM isoproterenol (10–20-fold), which activates the endogenous β_2-adrenergic receptors, but not with 1 μM ionomycin or TPA (Fig. 1). Similarly, COS-7 cells transfected with the constitutively active $G_{\alpha s}$ mutant α_s-Q227L, displayed an elevated level of AC activity (5–10-fold). COS-7 cells transfected with the μ-opioid receptor and either the m_1-muscarinic or TSH receptor displayed an elevated level of cAMP accumulation when stimulated with 100 μM carbachol (4–6-fold) or 0.1 μM TSH (8–10-fold) with respect to unstimulated cells.

In a series of experiments (Fig. 2), we have utilized COS-7 cells transfected with μ-opioid receptor and investigated the effect of acute and chronic application of the μ agonist, morphine, on the activity of AC endogenously present in these cells. We found that application of 1 μM morphine, either acutely (10 min) or chronically (18 h), did not lead to any significant change in the level of cAMP in the cells ($n = 7$; Fig. 2*a*). However, acute application of morphine to cells stimulated with 1 μM FS led to marked inhibition (23.1 ± 3.4%, $n = 14$; $p < 0.0001$; see Fig. 2*b*) of cAMP accumulation, while not affecting AC activity in untransfected COS-7 cells (data not shown). Withdrawal from chronic opiate treatment (1 μM morphine, 18 h, followed by three rapid washes) led to an increase in FS-stimulated (18 ± 3%, $n = 13$; $p < 0.0001$) cAMP accumulation. This increase in cAMP accumulation, defined as AC superactivation (20, 22, 23, 25, 40), could be inhibited (28 ± 3%, $n = 13$) by readdition of morphine.

In cells transiently cotransfected with the μ-opioid receptor and either the constitutively active α_s mutant α_s-Q227L or TSH receptor, a small but significant inhibition in α_s- (15.3 ± 3.32%, $n = 4$; $p < 0.025$) and TSH-stimulated (14 ± 2.3%, $n = 11$; $p < 0.001$) AC activity could be observed (Fig. 2, *c* and *d*). There was no significant decrease ($n = 4$; Fig. 2*e*) in cellular cAMP levels when morphine was added to isoproterenol-stimulated μ-opioid receptor-transfected COS-7 cells. In all three conditions (TSH, α_s-Q227L, and isoproterenol), withdrawal from chronic opiate treatment did not lead to any significant increase in cAMP accumulation, and readdition of morphine following with-

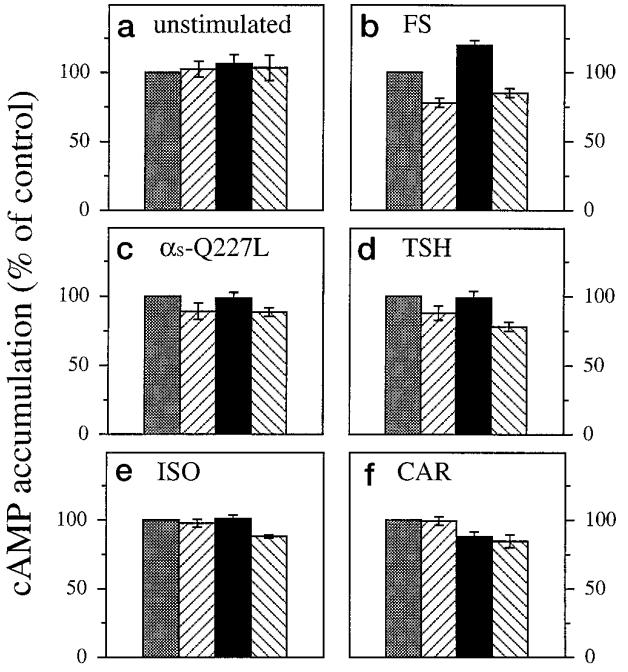

FIG. 2. **Morphine regulation of endogenous AC activity in COS-7 cells transfected with μ-opioid receptor is dependent on the stimulator used to activate AC.** COS-7 cells were transfected with the μ-opioid receptor and with either the TSH receptor, the m_1-muscarinic receptor, or α_s-Q227L cDNAs. The *figure* shows the effect of morphine on AC activity in unstimulated cells (*a*) and in cells stimulated with FS (*b*), α_s-Q227L (*c*), TSH (*d*), isoproterenol (*e*), or carbachol (*f*). *Control* (▨), cells to which no opiates were added; *Morphine* ( ), morphine present at 1 μM during AC assay; *Withdrawal* (■), 1 μM morphine for 18 h followed by three rapid washes; *Withdrawal* + *Morphine* ( ), 1 μM morphine (18 h) followed by three rapid washes and the readdition of 1 μM morphine at the start of the AC assay. Data represent the means ± S.E. of at least three experiments performed in triplicate. The level of stimulation by FS, α_s-Q227L, TSH, isoproterenol, and carbachol compared with unstimulated cells is shown in Fig. 1.

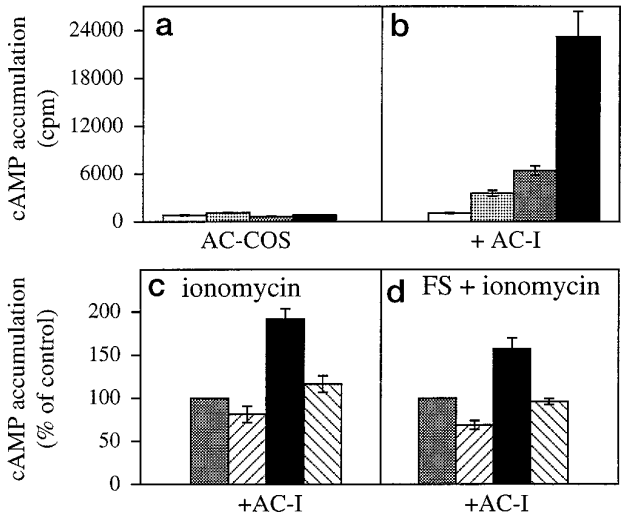

FIG. 3. **Acute μ-opioid receptor activation inhibits, and chronic activation superactivates, AC-I activity.** Effect of 1 μM FS (▨), ionomycin (▨), or FS + ionomycin (■), compared with unstimulated cells (□), on AC activity endogenously present in COS-7 cells (AC-COS) (*a*) or AC activity in AC-I-transfected COS-7 cells (*b*). The effect of morphine treatment on cAMP accumulation in COS-7 cells transfected with μ-opioid receptor and AC-I cDNAs was determined following stimulation by either ionomycin (*c*) or FS + ionomycin (*d*). *Control* (▨), cells to which no opiates were added; *Morphine* ( ), morphine present at 1 μM during AC assay; *Withdrawal* (■), 1 μM morphine for 18 h followed by three rapid washes; *Withdrawal* + *Morphine* ( ), 1 μM morphine (18 h) followed by three rapid washes and readdition of 1 μM morphine at the start of the AC assay. Data represent the means ± S.E. of at least three experiments performed in triplicate.

drawal led to inhibition of cAMP accumulation by 21 ± 2.4% ($n = 6$; $p < 0.0005$), 11.6 ± 2.5% ($n = 5$; $p < 0.05$), and 13.8 ± 3.5% ($n = 4$; $p < 0.05$), respectively.

On the other hand, in cells transiently transfected with the μ-opioid receptor and the m_1-muscarinic receptor cDNAs, no inhibition by morphine of the carbachol-stimulated AC activity could be observed ($n = 7$; see Fig. 2*f*). Withdrawal from chronic opiate treatment did not lead to any increase in cAMP accumulation. On the contrary, it led to a reduction of 12.6 ± 4.2% ($n = 7$; $p < 0.05$) in cAMP levels compared with control cells. Readdition of morphine under these conditions did not lead to a further inhibition of AC activity.

These results indicate that in COS-7 cells, opioid receptor regulation of endogenous cAMP levels depends on the way in which AC is stimulated. In order to evaluate the possibility that this phenomenon is due to activation of a particular AC isozyme or of a combination of isozymes present in the cells, we have transfected AC types I-VIII into COS-7 cells and monitored the effect of acute and chronic morphine on AC activity. All of the exogenous ACs were found to be expressed in the cells and to be functionally active, as determined by the increase in cAMP accumulation in the cells following stimulation with the appropriate stimulant (*e.g.* FS, ionomycin, TPA, or TSH; see below).

AC Isoforms I, V, VI, and VIII Are Inhibited and Superactivated by Acute and Chronic Opioid Receptor Activation, Respectively—In cells transfected with AC-I or AC-VIII, stimulation with FS or ionomycin resulted in a large increase in cAMP accumulation (3–5- and 3–10-fold, respectively), as compared with that obtained with the endogenous AC activity present in COS-7 cells (Figs. 3, *a* and *b*, Fig. 4*a*). The effects of FS and ionomycin on AC-I or AC-VIII activities were synergistic, whereas no such synergism was observed for the endogenous AC activity of COS-7 cells. The finding that AC-I- and AC-VIII-transfected cells show strong activation by ionomycin is in agreement with previous reports demonstrating that Ca^{2+}/calmodulin has a stimulatory effect on these isozymes (1, 43) and indicates that the transfected AC-I and AC-VIII are expressed and functionally active.

Activation of the μ-opioid receptor by morphine during the stimulation of AC-I led to inhibition of ionomycin- or ionomycin/FS-stimulated AC-I activity (20–30%; Fig. 3, *c* and *d*). On the other hand, activation of the opioid receptor for 18 h before the AC-I stimulation led, after washing of the agonist, to an increase in AC-I activity (by 1.6–1.9-fold over nontreated cells), indicating that AC-I is superactivated during the chronic opiate exposure. Readdition of morphine after chronic treatment and withdrawal led to a 30–40% inhibition of AC-I activity, indicating that the opioid receptor and its coupling to AC were still functional. However, cAMP levels in the cells were higher (by 30–45%) under these conditions as compared with acute inhibition, as expected for inhibition of up-regulated levels of AC activity. This suggests, as shown previously with AC endogenously found in Chinese hamster ovary cells (23), that the apparent tolerance due to chronic morphine exposure is a result of the superactivated state of the AC following the chronic exposure.

In Fig. 4*b*, we show that the opiate effect on AC-VIII is similar to that on AC-I. Acute activation of the opioid receptor led to inhibition of AC-VIII (by 21.3 ± 5.3%, $n = 6$), and chronic treatment followed by withdrawal led to superactivation (2.14 ± 0.15-fold, $n = 6$) of AC-VIII. Readdition of morphine after chronic treatment and withdrawal led to a 46 ± 5.4% ($n = 5$) inhibition of AC-VIII activity. As with AC-I, this level of

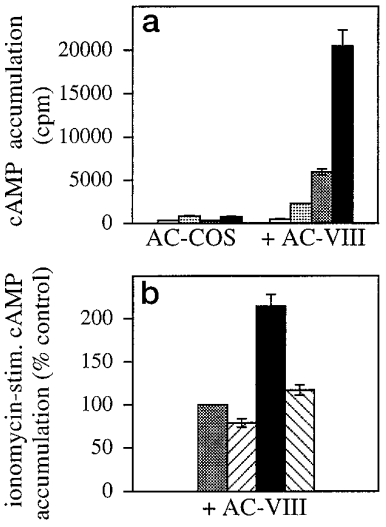

FIG. 4. **Acute μ-opioid receptor activation inhibits, and chronic activation superactivates, AC-VIII activity.** *a*, effect of 1 μM FS (▨), ionomycin (▤), or FS + ionomycin (■), compared with unstimulated cells (□), on AC-COS and on AC-VIII transfected into COS-7 cells. *b*, the effect of morphine treatments and ionomycin stimulation on cAMP accumulation in COS-7 cells transfected with μ-opioid receptor and AC-VIII cDNAs. *Control* (▤), cells to which no opiates were added; *Morphine* ( ), morphine present at 1 μM during AC assay; *Withdrawal* (■), 1 μM morphine for 18 h followed by three rapid washes; *Withdrawal + Morphine* ( ), 1 μM morphine (18 h) followed by three rapid washes and readdition of 1 μM morphine at the start of the AC assay. Data represent the means ± S.E. of at least three experiments performed in triplicate.

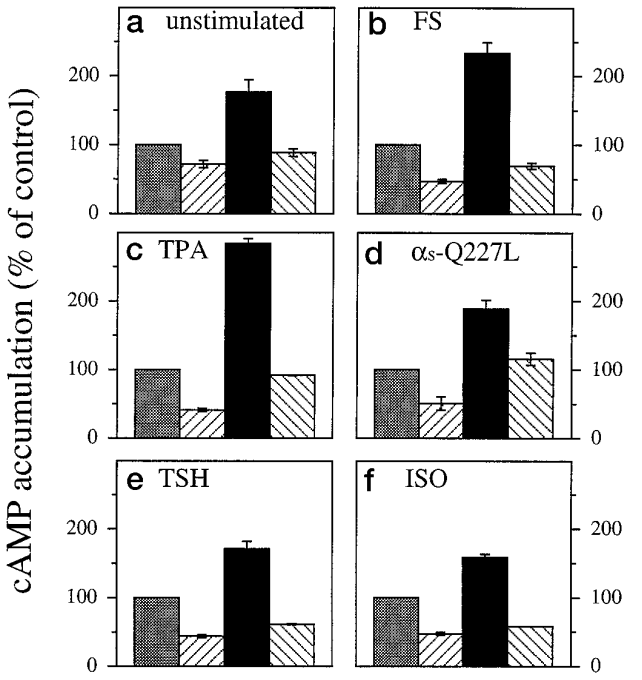

FIG. 5. **Acute μ-opioid receptor activation inhibits, and chronic activation superactivates, AC-V activity.** COS-7 cells transfected with μ-opioid receptor and AC-V cDNAs, and where appropriate with the cDNAs of α_s-Q227L or TSH receptors, were either unstimulated (*a*) or stimulated by FS (*b*), TPA (c), α_s-Q227L (*d*), TSH (*e*), or isoproterenol (*f*). All other details are as in the legend to Fig. 2.

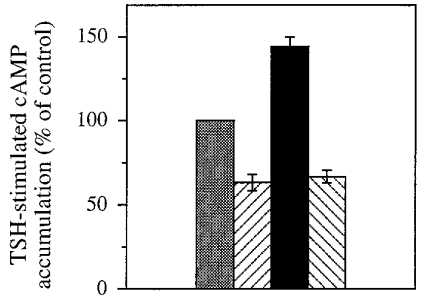

FIG. 6. **Acute μ-opioid receptor activation inhibits, and chronic activation superactivates, AC-VI activity.** COS-7 cells transfected with μ-opioid receptor, TSH receptor, and AC-VI cDNAs were stimulated by TSH, and cAMP accumulation was determined. All other details are as in the legend to Fig. 2.

cAMP is higher (49 ± 7.6%, $n = 5$) as compared with acute inhibition of nontreated cells.

Cells transfected with AC-V cDNA express an increase in unstimulated activity (158 ± 11%, $n = 10$) as well as in activity stimulated by FS (485 ± 63%, $n = 10$), TPA (473 ± 31%, $n = 4$), α_s-Q227L (361 ± 17%, $n = 3$), TSH (169 ± 11%, $n = 6$), and isoproterenol (162 ± 7%, $n = 3$), as compared with the activity endogenously present in COS-7 cells under the same conditions. We have recently shown that acute activation of the μ-opioid receptor leads to inhibition of FS- or TPA-stimulated AC-V, while chronic opiate exposure leads to its superactivation (40). In Fig. 5, we show that inhibition and superactivation can also be obtained when AC-V is stimulated by activation of the endogenous β_2-adrenergic or transfected TSH receptors as well as by the constitutively active α_s-Q227L. Acute morphine inhibited by about 25% the accumulation of cAMP in unstimulated cells. Inhibition of 50–60% was observed in the level of cAMP in cells stimulated with FS, TPA, α_s-Q227L, TSH, or isoproterenol. Removal of the agonist following chronic treatment led to superactivation (1.7–2.7-fold over control, nontreated cells) of AC-V activity in all of these cases. Reapplying the opiate agonist after withdrawal led to AC inhibition (40–70%).

In cells transfected with AC-VI, TSH-stimulated cAMP accumulation was higher (164 ± 6%, $n = 3$) than the activity endogenously present in COS-7 cells. AC-VI was recently shown to undergo inhibition by acute D_2- or m_2-agonist exposure, and superactivation following chronic exposure to these agonists (28). These observations are supported by results we obtained using COS-7 cells transfected with μ-opioid receptor and AC-VI. Fig. 6 shows, using TSH as a stimulant of AC, that acute μ-opioid receptor activation inhibited cAMP accumulation (by 36.3 ± 5.9%, $n = 3$) and that removal of the agonist following chronic treatment led to superactivation of AC-VI activity (1.44 ± 0.07-fold over control nontreated cells, $n = 3$). Reapplying the opiate agonist after withdrawal led to AC inhibition (54.1 ± 4.9%, $n = 3$).

Taken together, the above results demonstrate that AC-I, AC-V, AC-VI, and AC-VIII exhibit inhibition and superactivation by acute and chronic opiate exposure, respectively, and that the two functions are not dependent on the agent used to stimulate AC activity.

AC Isoforms II, IV, and VII Are Stimulated by Acute and Inhibited by Chronic μ-Opioid Receptor Activation—In AC-II-transfected cells (cotransfected with μ-opioid receptor), cAMP accumulation was higher than that obtained with the endogenous AC activity present in unstimulated COS-7 cells (177 ± 16%, $n = 8$) and in response to TPA (574 ± 91%, $n = 9$), α_s-Q227L (410 ± 40%, $n = 3$), TSH (342 ± 16%, $n = 4$), and carbachol (552 ± 27%, $n = 3$). Acute exposure to morphine induced a consistent increase (of 30–45%) in unstimulated as well as TPA-, α_s-Q227L-, or TSH-stimulated cAMP accumulation; its effect on carbachol-stimulated cAMP accumulation was lower, amounting to 15% (Fig. 7). However, no superactivation of AC-II was apparent following withdrawal from chronic morphine treatment using any of these stimulation

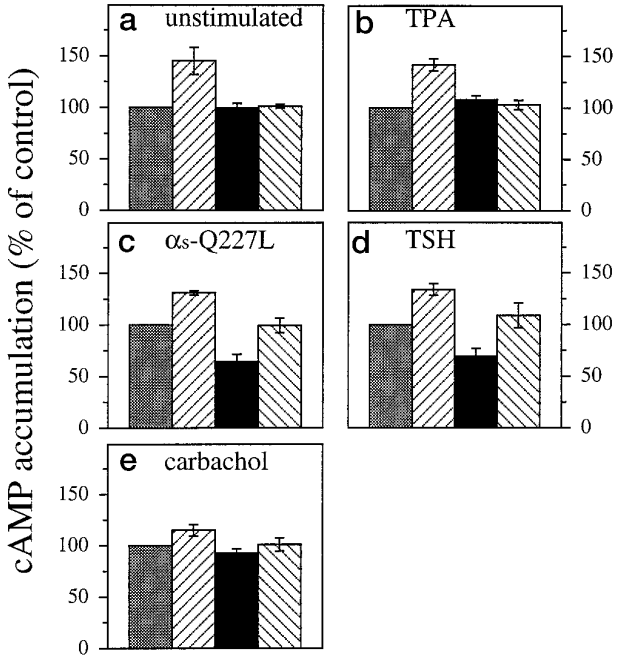

FIG. 7. **Acute μ-opioid receptor activation stimulates AC-II activity, but chronic activation does not lead to superactivation.** The effect of morphine treatments on cAMP accumulation in COS-7 cells cotransfected with μ-opioid receptor and AC-II cDNAs and other cDNAs is indicated following no stimulation of AC (*a*) or stimulation with TPA (*b*), α_s-Q227L (*c*), TSH (*d*), or carbachol (*e*). All other details are as in the legend to Fig. 2.

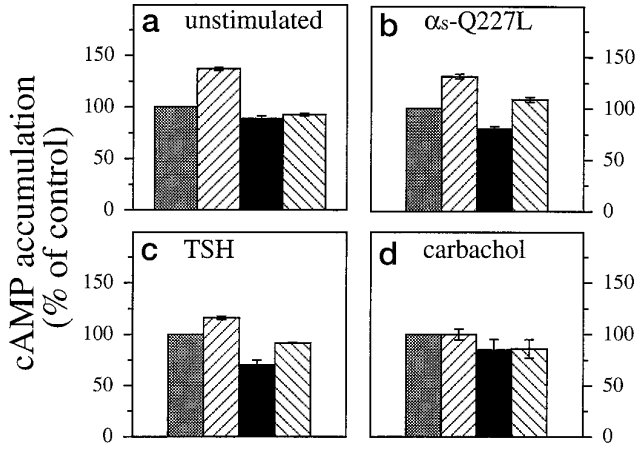

FIG. 8. **Acute μ-opioid receptor activation stimulates AC-IV activity, but chronic activation does not lead to superactivation.** The effect of morphine treatments on cAMP accumulation in COS-7 cells cotransfected with μ-opioid receptor and AC-IV cDNAs and other cDNAs is indicated following no stimulation of AC (*a*) or stimulation with α_s-Q227L (*b*), TSH (*c*), or carbachol (*d*). All other details are as in the legend to Fig. 2.

techniques. On the other hand, when AC-II-transfected cells were stimulated by α_s-Q227L or TSH, a reduction (of 30–40%, compared with control, nontreated cells) in AC activity was observed after removal of the agonist following chronic treatment (Fig. 7, *c* and *d*). Interestingly, the result of reexposure to morphine after chronic treatment and withdrawal was found to be dependent on the method used to activate AC. Such reexposure to morphine did not induce an increase in AC activity when the cells were unstimulated or stimulated by carbachol or TPA (Fig. 7, *a*, *b*, and *e*), while stimulation of AC-II by either α_s-Q227L or TSH (Fig. 7, *c* and *d*) led to an increase in AC activity, which was similar in magnitude to the increase in AC activity by acute morphine treatment. The reason for this difference is under investigation. In agreement with the results of others (52), we found that the increase in AC-II activity by acute opiate treatment is $G_{\beta\gamma}$-dependent and could be abolished by $G_{\beta\gamma}$ scavengers, *e.g.* the C′ terminus of β-adrenergic receptor kinase (data not shown).

AC-IV is known to be closely related to AC-II according to its sequence and regulatory patterns (11, 53). Transfection of AC-IV into COS-7 cells was found to increase AC activity of unstimulated cells (147 ± 5%, $n = 3$) or in cells stimulated by α_s-Q227L (198 ± 9%, $n = 5$), TSH (162 ± 16%, $n = 3$), or carbachol (156 ± 15%, $n = 3$). In Fig. 8, we show that the opioid receptor effect on AC-IV activity is similar to that on AC-II activity. Acute opioid receptor activation increases (by 15–35%) the activity of unstimulated AC, as well as α_s-Q227L- or TSH-stimulated AC-IV activity. Similarly to AC-II, which was only weakly stimulated by morphine, carbachol-activated AC-IV was not significantly stimulated by the opiate. No superactivation of AC-IV was apparent following chronic morphine treatment using any of these stimulation techniques. On the other hand, a reduction (of 10–30%) in AC-IV activity was observed after removal of the agonist following chronic treatment. Reexposure to morphine after chronic treatment and withdrawal led to an increase in AC-IV activity that was similar in magnitude to the increase in AC-IV provoked by acute morphine treatment. However, the final level of cAMP under these conditions was lower than upon acute exposure of morphine to cells not pretreated with the opiate.

AC activity in AC-VII-transfected COS-7 cells was found to be higher as compared with that obtained with the endogenous AC activity present in COS-7 cells in response to TSH (203 ± 33%, $n = 2$) and carbachol (274 ± 14%, $n = 6$). These results indicate that the transfected AC-VII is functionally active. In Fig. 9, we show that the opioid receptor effect on TSH-stimulated AC-VII activity is similar to that observed with AC-II and AC-IV activity. Acute opioid receptor activation increased (by ~25%) TSH-stimulated AC-VII activity. Furthermore, no superactivation of AC-VII was apparent following chronic morphine treatment. Conversely, a reduction (of 20–40%) in AC-VII activity was observed after removal of the agonist following chronic treatment. Reexposure to morphine after chronic treatment and withdrawal led to an increase in TSH-stimulated AC-VII activity that was similar in magnitude to the increase in AC-VII provoked by acute morphine treatment. As with AC-II and AC-IV, carbachol stimulation of cells transfected with AC-VII was only moderately affected by acute and chronic morphine, respectively.

Taken together, the above results demonstrate that AC-II, AC-IV, and AC-VII are similar in their patterns of regulation by opiates. These AC isoforms exhibit AC stimulation upon acute opiate agonist treatment and inhibition of AC following chronic exposure.

AC Type III Is Not Affected by μ-Opioid Receptor Activation—Transfection of AC-III into COS-7 cells was found to increase the AC activity of unstimulated cells (132 ± 7%, $n = 4$) or in cells stimulated by FS (205 ± 23%, $n = 3$), α_s-Q227L (294 ± 27%, $n = 4$), TSH (328 ± 30%, $n = 4$), or carbachol (201 ± 20%, $n = 4$).

In contrast to the other AC isozymes transfected into COS-7 cells, the activity of AC-III was not affected by opioid receptor activation (Fig. 10). The unstimulated activity, as well as that stimulated by α_s-Q227L, TSH, or carbachol, was not affected by acute opioid receptor activation, nor was it affected by removal of the agonist following chronic treatment, indicating that AC-III is insensitive to both acute and chronic effects of opioid receptors. FS-stimulated AC activity was found to be weakly inhibited by acute opioid receptor activation and weakly stimulated by removal of the opiate agonist following chronic treat-

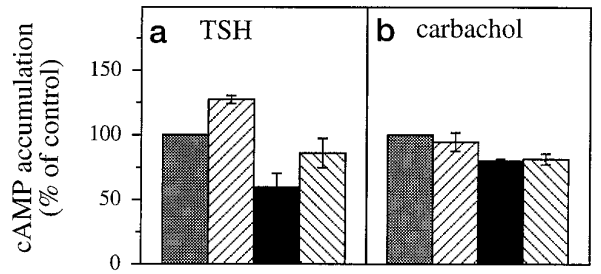

FIG. 9. **Acute μ-opioid receptor activation stimulates AC-VII activity, but chronic activation does not lead to superactivation.** Shown is the effect of morphine treatments on cAMP accumulation in COS-7 cells transfected with μ-opioid receptor and AC-VII cDNAs, stimulated with TSH (*a*) or carbachol (*b*). All other details are as in the legend to Fig. 2.

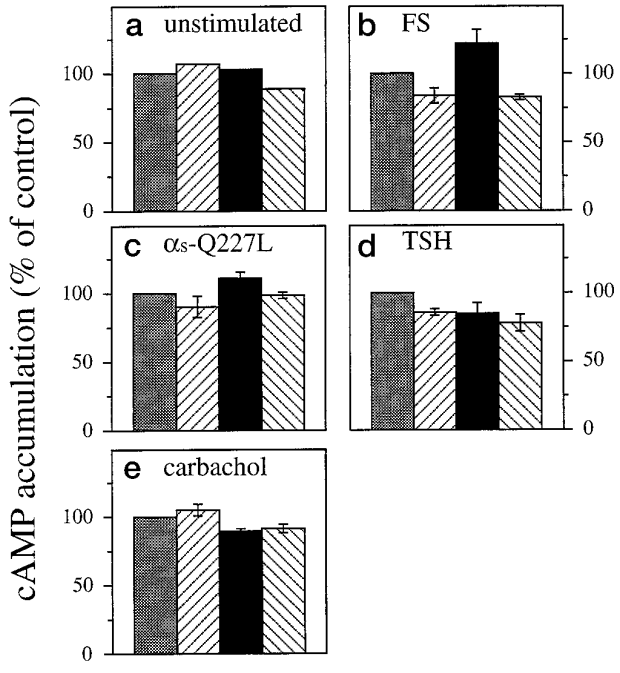

FIG. 10. **μ-Opioid receptor activation does not affect AC-III activity.** Shown is the effect of morphine treatments on cAMP accumulation in COS-7 cells transfected with μ-opioid receptor and AC-III cDNAs following no stimulation of AC (*a*) or stimulation with FS (*b*), α_s-Q227L (*c*), TSH (*d*), or carbachol (*e*). All other details are as in the legend to Fig. 2.

ment. However, these effects of FS are probably due to its effect on the endogenously expressed AC activity, which is FS-sensitive (see Fig. 2*b*).

Altogether, the results show that AC isozymes could be divided into three functional groups: (*a*) AC-I, AC-V, AC-VI, and AC-VIII exhibit inhibition by acute and superactivation by chronic opiate exposure; (*b*) AC-II, AC-IV, and AC-VII exhibit AC stimulation upon acute opiate agonist treatment and inhibition of AC following chronic exposure; and (*c*) AC-III is not affected by either acute or chronic opiate exposure.

DISCUSSION

In this study, we have used COS-7 cells transiently transfected with μ-opioid receptor together with various AC isozymes (I–VIII) to gain information on the mechanism by which inhibitory receptors, such as this opioid receptor, induce AC inhibition and superactivation. Three distinct parameters investigated in this study have demonstrated that transfection of the various AC isozyme cDNAs leads to the expression of functional AC: (*a*) a general increase in AC activity was observed in the transfected cells; (*b*) the transfected AC isozymes showed distinct, expected patterns of stimulation in response to FS, TPA, ionomycin, α_s-Q227L, and activation of the TSH or m_1-muscarinic receptors; (*c*) the transfected AC isozymes showed distinct effects upon acute opioid receptor activation and chronic treatment.

In agreement with the results of others, we have demonstrated that the activities of the various isozymes are differentially affected by various stimulants (5, 11, 53, 54). For example, while AC-I, AC-V, AC-VI, and AC-VIII are strongly and AC-III moderately stimulated by 1 μM FS, AC-II is only slightly stimulated, and AC-IV and AC-VII are not stimulated (data not shown). On the other hand, activation of the m_1-muscarinic receptor by carbachol strongly stimulates AC-II, AC-III, AC-IV, and AC-VII (see "Results"), whereas AC-I, AC-V, and AC-VI are not stimulated (data not shown). Only AC-I and AC-VIII were found to be stimulated by ionomycin.

Acute application of morphine has been found to inhibit AC-I, AC-V, AC-VI, and AC-VIII. These results are consistent with the known inhibitory effects of opiates on AC activity in many cell systems (for reviews see Refs. 56–58). These results are also in agreement with the finding that AC-I, AC-V, and AC-VI prepared from transfected Sf9 cells are susceptible to inhibition by purified $G_{\alpha i1-3}$ or $G_{\alpha o}$ (12, 13).

When opiate agonist was chronically applied to AC-V-, AC-VI-, AC-VIII-, or AC-I-transfected cells, an increase in cAMP levels was observed following withdrawal of the agonist, a phenomenon referred to as AC superactivation. This result is in agreement with previous reports by us (40) and by Thomas and Hoffman (28) showing that AC-V and AC-VI undergo such an inhibitory receptor-induced AC superactivation. In contrast to the endogenous AC activity in COS-7 cells (which showed superactivation only when induced with FS), the superactivation of AC-I and AC-V did not depend on the method of stimulation (hormone receptor activation, FS, TPA, or increased Ca^{2+} concentration) and was apparent under all conditions under which the particular AC isozymes could be activated.

Contrary to AC-I, AC-V, AC-VI, and AC-VIII, acute application of morphine increased AC-II, AC-IV, and AC-VII activities. These results are in line with the finding that in HEK-293 cells, α_2-adrenergic, D_2-dopaminergic, A1-adenosine, and δ-opioid receptor activation enhance the activated α_s (α_s-Q227L)-stimulated AC-II activity (52, 58, 59). Regarding the mechanism of such activation, it has been shown that AC-II in membrane preparations of transfected Sf9 cells is stimulated by purified bovine brain $G_{\beta\gamma}$ and is not inhibited by $G_{\alpha i}$ (13, 60, 61). Similarly, purified bovine brain $G_{\beta\gamma}$ was found to activate AC-IV activity (6). The ability of the $\beta\gamma$ dimer-sequestering fragment of β-adrenergic receptor kinase to prevent the stimulation of AC-II by opioid receptor activation indicates that the mechanism by which this stimulation occurs involves $G_{\beta\gamma}$ dimers (data not shown).

Unlike AC-I, AC-V, AC-VI, and AC-VIII, the AC-II, AC-IV, and AC-VII isozymes do not seem to undergo AC superactivation, since no increase in the activity of these isozymes was observed following chronic treatment and removal of the agonist. In fact, removal of morphine following chronic exposure yielded, in many cases, cAMP levels lower than those found in control, non-opiate-treated cells. Readdition of acute morphine led in these cases to a resumption of the stimulatory response.

As described in the Introduction, sequence and functional similarities allow the categorization of the nine ACs into six classes. Interestingly, three of these classes (AC-I, AC-VIII, and AC-V/VI) show inhibition and superactivation and were reported to have similar sequences (11). This may suggest that a common conserved structural element in this group of AC isozymes is responsible for the inhibition and superactivation.

AC-II, AC-IV, and AC-VII constitute a class of ACs that are stimulated by opioid receptor activation and whose activities are reduced upon chronic treatment. A region in AC-II that is critical for the interaction with $G_{\beta\gamma}$ dimers was identified, and this sequence of amino acids is conserved in the two other AC isozymes (IV and VII) of this class of ACs (62). On the other hand, sequence relationships indicate that AC-III is distinct from the other classes of ACs (11). We show here that this AC isozyme is also distinct in that it is not affected by acute or chronic stimulation of the opioid receptor.

According to some researchers (20, 26, 40, 55), the increase in cAMP induced by chronic opiate treatment could be a result of a secondary regulatory process that involves a compensatory increase in AC activity. Although the nature of this process is not known, it has been suggested to involve cAMP-dependent protein kinase, whose activity is reduced as a result of the decreased cAMP level during the initial agonist treatment (26). However, we and others (23, 63) have recently reported that increasing the levels of cAMP (*e.g.* by including a permeable cAMP analog) during the chronic treatment does not prevent the AC superactivation (23, 63).

A second possible mechanism involves activation of AC via the released $G_{\beta\gamma}$ of $G_{i/o}$ proteins. We have recently shown that superactivation of AC-V in transfected COS-7 cells is blocked by the $G_{\beta\gamma}$ scavengers α-transducin and the C′ terminus of β-adrenergic receptor kinase (40). Thomas and Hoffman (28) have recently shown that superactivation of AC-VI in transfected HEK-293 cells is blocked by the $G_{\beta\gamma}$ scavenger α-transducin. Since $G_{\beta\gamma}$ was not found to affect AC-V or AC-VI activity directly (5, 61), it has been speculated that $G_{\beta\gamma}$ activates another protein or enzyme that interacts with or modifies AC during the chronic treatment (see Refs. 28 and 40).

It has also been suggested that AC superactivation could result from the abolishment of a tonic inhibition of $G_{\alpha i}$ on AC (64). Our results, however, show that there is no apparent reduction in the ability of $G_{\alpha i}$ to inhibit AC-I, AC-V, AC-VI, or AC-VIII activity following chronic opiate treatment. Nevertheless, it is clear that the ability of opiates to inhibit AC and to induce AC superactivation are mediated by $G_{i/o}$, since treatment with pertussis toxin abolishes these phenomena (23, 40).

Ammer and Schulz (21) have suggested that in NG108–15 cells, AC superactivation may be due to enhanced coupling of the prostaglandin E_1 receptor to $G_{\alpha s}$. From our results, it appears that AC superactivation occurs with selective AC isozymes and could be observed even without receptor activation (*e.g.* with FS, ionomycin, TPA, or the use of a constitutively active mutant of $G_{\alpha s}$). It is therefore not likely that the superactivation phenomenon is due to a change in coupling between stimulatory receptors and $G_{\alpha s}$.

According to others (65, 66), AC superactivation could be a result of the ability of opioid receptors to couple to G_s and to stimulate AC. In this regard, we and others (23, 40, 64), have found that pertussis toxin pretreatment abolished AC superactivation in opioid receptor-transfected Chinese hamster ovary or COS-7 cells and in NG108–15 cells (which contain δ-opioid receptors), suggesting that it is mediated through $G_{i/o}$ proteins, and not through G_s. Moreover, prolonged activation of the inhibitory somatostatin receptor has been shown to increase AC activity in $G_{\alpha s}$-deficient S49 mouse lymphoma cells (67). Additional experiments are thus needed to determine the mechanism of induction of AC superactivation.

The fact that several ACs are usually expressed in a given cell line (68) and are differentially distributed in various brain regions (10, 11, 69) and that these ACs are affected differently by FS, protein kinase C, Ca^{2+}, and activation of hormone receptors may afford an explanation to the complex effect of opioid receptor activation on the activity of ACs endogenously expressed in the various cells studied as well as in different areas of the central nervous system. The composition of AC in the particular cells or tissues will determine whether it will be inhibited by $G_{\alpha i}$-coupled receptor agonists and whether it will show the superactivation phenomenon after chronic agonist treatment. For example, in NG108–15 cells, AC superactivation was detected under basal conditions and upon stimulation with FS, prostaglandin E_1, or adenosine, but not when the AC in cell membranes was stimulated with GTPγS or NaF (21, 38, 64). In HT29 human colonic adenocarcinoma cells, AC superactivation of 10–20-fold was observed when FS was used, 1.9-fold superactivation was observed with vasoactive intestinal peptide, and no superactivation was observed with isoproterenol (39). On the other hand, in rat adipocytes, a similar (2-fold) AC superactivation was detected for basal, and for isoproterenol, NaF, and FS stimulated AC activity (35).

We found that AC-I, AC-V, AC-VI, and AC-VIII are susceptible to superactivation following chronic opiate treatment, while AC-II, AC-III, AC-IV, and AC-VII are not. In this regard, it is worthwhile noting that AC-I and AC-VIII are expressed only in neurons, while AC-V and AC-VI are distributed among a number of tissues, including brain, heart, kidney, and liver (11). The mRNAs of the μ-opioid receptor as well as AC-VIII and AC-V, two of the AC isozymes that we found to be involved in AC superactivation, are highly expressed in the locus coeruleus and nucleus accumbens (69–73). This is interesting in view of the fact that these two brain nuclei have been found to exhibit AC superactivation in response to chronic morphine treatment and are known to play a central role in opiate addiction (29, 55, 74). This suggests that the AC superactivation observed in these brain nuclei, which presumably contributes to the physiological phenomenon of addiction, may in fact represent a superactivated state of the AC-V and/or AC-VIII isozymes in these areas.

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