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Chronic Opioid Treatment Induces Adenylyl Cyclase V Superactivation

INVOLVEMENT OF $G_{\beta\gamma}$*

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Tomer Avidor-Reiss‡, Igal Nevo‡, Rivka Levy‡, Thomas Pfeuffer§, and Zvi Vogel‡¶

From the ‡Department of Neurobiology, The Weizmann Institute of Science, 76100 Rehovot, Israel and the §Department of Physiological Chemistry II, University of Düsseldorf, Düsseldorf, D-40225 Germany

It has been known for some time that chronic treatment of neuronal cells and tissues with opioids, contrary to their acute effect, leads to an increase in cAMP accumulation. This phenomenon, defined as adenylyl cyclase superactivation, has been implicated in opiate addiction, yet the mechanism by which it is induced remains unclear. Here, we show that this phenomenon can be reproduced and studied in COS-7 cells cotransfected with adenylyl cyclase type V and μ-opioid receptor cDNAs. These cells display acute opioid inhibition of adenylyl cyclase activity, whereas prolonged exposure to the μ-agonist morphine or [D-Ala^2, *N*-methyl-Phe^4, Gly-ol^5]enkephalin leads to a time-dependent superactivation of adenylyl cyclase. This superactivated state is reversible, because it is gradually lost following agonist withdrawal. Adenylyl cyclase superactivation can be prevented by pertussis toxin pretreatment, indicating the involvement of $G_{i/o}$ proteins, or by cotransfection with the carboxyl terminus of β-adrenergic receptor kinase or with α-transducin (scavengers of $G_{\beta\gamma}$ dimers), indicating a role for the G protein $\beta\gamma$ dimers in adenylyl cyclase superactivation. However, contrary to several other $G_{\beta\gamma}$-dependent signal transduction mechanisms (*e.g.* the extracellular signal-regulated kinase 2/MAP kinase pathway), adenylyl cyclase superactivation is not affected by the Ras dominant negative mutant N17-Ras.

Opioid receptors are members of the seven-transmembrane domain GTP-binding protein (G protein)[1]-coupled receptor superfamily (Reisine and Bell, 1993; Uhl *et al.*, 1994). Stimulation of seven-transmembrane domain inhibitory receptors (such as the opioid receptors) activates the $G_{i/o}$ GTP-binding proteins, as a result of which these G proteins dissociate into $G_{i/o\alpha}$ and $G_{\beta\gamma}$ dimers (Birnbaumer *et al.*, 1990; Childers, 1991; Tang and Gilman, 1992). The G_{α} interacts with adenylyl cyclase (AC), leading to its acute inhibition and subsequently to a reduction in cAMP levels in the cell (Birnbaumer *et al.*, 1990; Taussig *et al.*, 1993a). However, chronic activation of several inhibitory receptors (such as α_2-adrenergic, m_2/m_4-muscarinic, or opioid) has been shown to lead to an increase in cAMP accumulation (Ammer and Schulz, 1993; Avidor-Reiss *et al.*, 1995a; Hamprecht, 1977; Law *et al.*, 1994; Sharma *et al.*, 1975, 1977; Thomas and Hoffman, 1987; Wang *et al.*, 1994). This phenomenon, which is particularly manifest upon withdrawal of the inhibitory agonist, is referred to as AC superactivation or AC "overshoot." Although the mechanism by which this effect is achieved is not understood, AC superactivation is believed to play an important role in the development of opiate tolerance and dependence, commonly observed upon prolonged exposure to opiate drugs (Avidor-Reiss *et al.*, 1995a; Childers, 1991; Nestler *et al.*, 1993; Sharma *et al.*, 1975).

Recently, we have shown that stable transfection of the μ- or κ-opioid receptor is sufficient to confer to Chinese hamster ovary cells the ability to display AC superactivation upon chronic treatment with opioids (Avidor-Reiss *et al.*, 1995a, 1995b), similarly to what was reported for the δ-opioid receptor by Law *et al.* (1994). We further showed that AC superactivation is due to an increase in the activity of endogenous AC and that it is mediated via a pertussis toxin (PTX)-sensitive $G_{i/o}$ protein. However, the nature of the AC isozyme and of the G protein subunits involved in this process are not currently known. In this regard, eight types of AC isozymes have recently been described (Choi *et al.*, 1993; Cooper *et al.*, 1995; Mons and Cooper, 1995; Pieroni *et al.*, 1993; Taussig and Gilman, 1995) that differ in their response to Ca^{2+}/calmodulin, α_s, protein kinase C, etc. It is of interest to determine which of them participates in the chronic opioid-induced superactivation. In the present work, we have reconstituted AC superactivation in African green monkey kidney COS-7 cells by transfecting the cells with μ-opioid receptor together with AC type V (AC-V) cDNAs and demonstrated the involvement of $G_{\beta\gamma}$ subunits in this process.

EXPERIMENTAL PROCEDURES

Materials—[2-^{3}H]Adenine (10.3 Ci/mmol) was purchased from Rotem Industries (Be'er Sheba, Israel). Opioid ligands were obtained from Research Biochemical International (Natick, MA) and from the National Institute of Drug Abuse, Research Technology Branch (Rockville, MD). The phosphodiesterase inhibitors 1-methyl-3-isobutylxanthine and RO-20-1724 were from Calbiochem (La Jolla, CA). 12-*O*-Tetradecanoylphorbol-13-acetate (TPA), forskolin (FS), and cAMP were from Sigma. PTX was from List Biological Laboratories (Campbell, CA). 4-[3-Chlorophenylcarbamoyloxy]-2-butynyltrimethyl ammonium chloride (McN-A-343) was obtained from Research Biochemical International (Natick, MA).

Cell Transfection—COS-7 cells were cultured in Dulbecco's modified Eagle's medium 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. 24 h before transfection, a confluent 10-cm plate was trypsinized and split into four 10-cm plates. The cells were transfected with 1 μg/plate of rat μ-opioid receptor cDNA

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¶ To whom correspondence should be addressed. Tel.: 972-8-934-2402; Fax: 972-8-934-4131.

[1] The abbreviations used are: G proteins, GTP-binding proteins; AC, adenylyl cyclase; βARK, β-adrenergic receptor kinase; DAMGE, [D-Ala^2, *N*-methyl-Phe^4, gly-ol^5]enkephalin; DPDPE, [D-penicillamine^2, D-penicillamine^5]enkephalin; FS, forskolin; $G_{\alpha t}$, α-transducin; HA, hemagglutinin; MAPK, mitogen-activated protein kinase; PTX, pertussis toxin; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; McN-A-343, 4-[3-chlorophenylcarbamoyloxy]-2-butynyltrimethyl ammonium chloride; AC-V, AC type V.

in pCMV-neo expression vector (Thompson *et al.*, 1993) and 2 μg/plate of either pXMD1-AC-V (Wallach *et al.*, 1994) or pXMD1-gal (Kluxen and Lübbert, 1993) (for mock DNA transfection) using the DEAE-dextran chloroquine method (Keown *et al.*, 1990). In the experiment described in Fig. 9, cells were transfected with the mouse δ-opioid receptor (DOR-1, in pcDM8) (Evans *et al.*, 1992) or the human m_2-muscarinic receptor (Hm2, in pcD) (Bonner *et al.*, 1987). 24 h later, the cells were trypsinized and re-cultured in 24-well plates. 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 (Lim and Chae, 1989).

AC Activity—The assay was performed in triplicate essentially as described (Avidor-Reiss *et al.*, 1995a; Salomon, 1991). 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 Dulbecco's modified Eagle's medium 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 Dulbecco's modified Eagle's medium 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 the absence of opioid ligands by the addition of either FS or TPA (final concentration, 1 μM). After 10 min at 37 °C, the medium was removed and the reaction was terminated with 1 ml of 2.5% perchloric acid containing 0.1 mM of unlabeled cAMP. After 15 min with perchloric acid at 4 °C, volumes of 0.9 ml were neutralized with 100 μl of a mixture of 3.8 M KOH and 0.16 M K_2CO_3. Aliquots of 0.9 ml of the supernatants were applied to a two-step column separation procedure as described previously. Aliquots of 50 μl were counted for determination of the total acid-soluble pool of [^{3}H]adenine and products (Salomon, 1991). We found (see also Avidor-Reiss *et al.*, (1995a)) that the uptake of [^{3}H]adenine into this pool is not affected by the various ligand exposures or the cDNA transfections.

Unless otherwise indicated, chronic opioid treatment was achieved by incubating the cells for 18 h with 1 μM of [D-Ala^2, *N*-methyl-Phe^4, Gly-ol^5]enkephalin (DAMGE), followed by opioid withdrawal (by three rapid washes with Dulbecco's modified Eagle's medium containing Hepes and bovine serum albumin) and the addition of FS or TPA to assay AC activity.

Extracellular Signal-regulated Kinase 2/MAP Kinase (MAPK) Transfection and Assay—COS-7 cells were cotransfected with the cDNA for μ-opioid receptor and an expression plasmid containing an amino-terminal hemagglutinin-tagged murine extracellular signal-regulated kinase 2/MAPK cDNA (HA-MAPK) (Crespo *et al.*, 1994; Her *et al.*, 1993). The determination of MAPK activation by opioid treatment was performed 48 h after transfection. Overnight serum-starved cells were exposed to opioids for the times indicated, the cells lysed, and HA-MAPK immunoprecipitated using a specific monoclonal antibody (12CA5 Babco) against the HA moiety, followed by application of protein G-Sepharose beads. The beads were washed three times and incubated for 30 min at 30 °C with 45 μg of myelin basic protein and 1 μCi of [γ-^{32}P]ATP in a total volume of 30 μl of kinase reaction buffer (Crespo *et al.*, 1995). The reaction was terminated by the addition of 5 × Laemli buffer, boiled, and separated by polyacrylamide gel electrophoresis. Phosphorylated myelin basic protein was visualized by autoradiography and quantitated by phosphoimaging (Molecular Dynamics PhosphorImager 445 SI) (for details, see Crespo *et al.* (1994, 1995)).

RESULTS

COS-7 cells were transfected with μ receptor cDNA either together with or without the cDNA for AC type V. Fig. 1 (*a* and *b*) shows that cells transfected with AC-V displayed a 2–3-fold increase in unstimulated and a 20–30-fold increase in FS-stimulated cAMP levels, as compared with control cells not transfected with AC-V. Moreover, TPA, in contrast to its lack of effect on the endogenous ACs of COS-7 cells, markedly stimulated (3–6-fold over the unstimulated level) the activity of the transfected AC-V. These results indicate that the transfected AC-V is expressed and active in COS-7 cells and that it can be distinguished from the AC endogenously found in COS-7 cells.

Acute exposure to the μ-opioid agonist DAMGE led to a marked inhibition of both the unstimulated and FS- or TPA-stimulated AC-V activity (Fig. 1*b*). Neither the κ-selective opioid agonist, U69593, nor the δ-selective opioid agonist, [D-penicillamine^2, D-penicillamine^5]enkephalin (DPDPE), had any effect on cAMP levels in the μ-opioid receptor-transfected cells (Fig. 1*c*). No inhibition by opioid agonists was observed with cells transfected with AC-V alone (data not shown). The inhibition of AC-V activity by the μ-selective opioid agonist DAMGE is dose-dependent with an EC_{50} of ~30 nM (Fig. 2*a*) and can be blocked by the opioid antagonist naloxone, which by itself has no effect on AC activity (Fig. 1*c*). Taken together, these results indicate that AC-V is inhibited by acute activation of μ-opioid receptors.

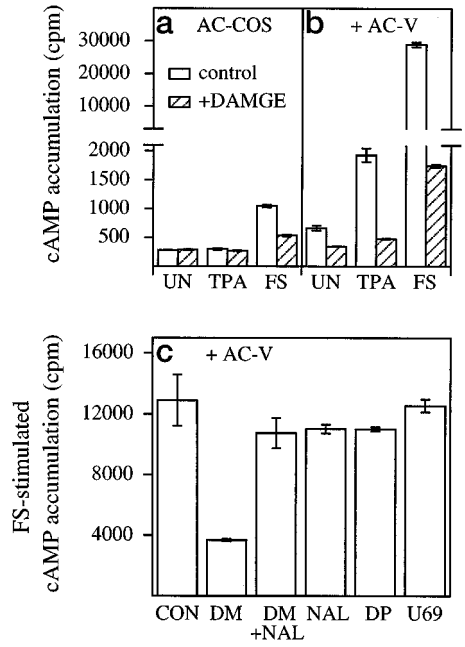

FIG. 1. **Activation of the μ-opioid receptor leads to inhibition of AC-V activity.** *a*, endogenous AC activity in COS-7 cells transfected with rat μ-opioid receptor is stimulated by 1 μM FS but not by 1 μM TPA. The FS stimulation is inhibited by the acute addition of 1 μM DAMGE. UN, unstimulated. *b*, in cells transfected with both rat μ-opioid receptor and rabbit myocardial AC-V, the cAMP levels are increased and stimulated by both FS and TPA. Acute addition of 1 μM DAMGE attenuates the increases in cAMP levels. *c*, FS-stimulated AC-V activity in cells transfected with μ-opioid receptor and AC-V cDNAs is inhibited by μ-agonists (1 μM DAMGE (*DM*)), and this inhibition is blocked by 1 μM naloxone (*NAL*). The δ- (1 μM DPDPE (*DP*)) and κ- (1 μM U69593 (*U69*)) agonists have no effect on cAMP accumulation. Data represent the means ± S.E. of triplicate determinations of a representative experiment out of three experiments that gave similar results. *CON*, control.

To examine the effect of chronic opioid exposure on AC-V activity, COS-7 cells transfected with AC-V and μ-opioid receptor were pretreated (18 h) with opioid agonist, and the agonist was rapidly withdrawn prior to the AC assay (by three quick washes). A significant increase in AC-V activity (superactivation) was observed in cells pretreated with 10–1000 nM DAMGE (Fig. 2*b*) as compared with control cells (not treated with the agonist). The increase in AC-V activity was dose-dependent (reaching 2.5-fold following 18 h with 1 μM DAMGE). Concentrations of 1 nM DAMGE and lower did not induce superactivation of AC. As shown in Fig. 3, AC superactivation could also be induced by chronic pretreatment with the μ-opioid receptor agonist morphine or with the μ/δ agonist [D-Ala-D-Leu]enkephalin and could be blocked by coincubation with the antagonist naloxone. Neither chronic treatment with the antagonist alone nor with the κ- or δ-selective opioid agonists U69593 or DPDPE had any effect on AC-V activity in these cells, indicating that the AC superactivation is mediated by the transfected μ-opioid receptor.

Fig. 4*a* shows that the development of AC-V superactivation in the transfected cells is time-dependent, requiring ~4 h of

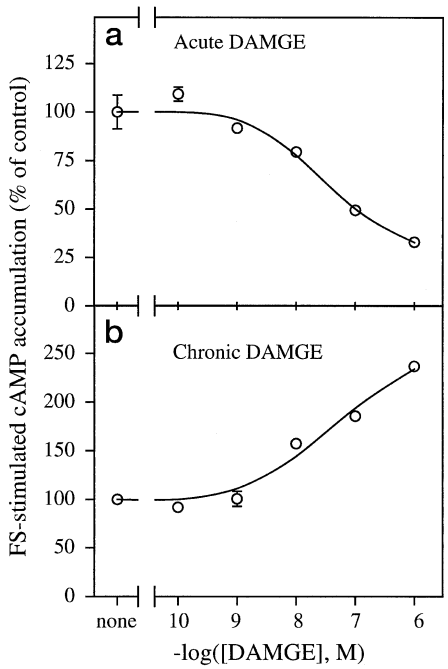

FIG. 2. **Dose-response of AC-V inhibition by acute DAMGE and superactivation by chronic DAMGE.** Cells were cotransfected with the cDNAs of AC-V and the μ-opioid receptor. *a* shows the inhibition of FS-stimulated AC-V by various concentrations of DAMGE applied acutely during the assay. *b* shows the FS-stimulated AC-V activity following rapid withdrawal (three washes) from chronic treatment (18 h) with the indicated concentrations of DAMGE. AC inhibition/superactivation curves were determined using the equation: $y = (a - d)/[1 + (x/c)^b] + d$, where a is the asymptotic maximum, b is the value of the slope, c is the inflexion point and is equivalent to the EC_{50} value, and d is the asymptotic minimum. The data represent the means ± S.E. of triplicate determinations of a representative experiment out of two to three experiments that gave similar results. 100% represents control activity without opioids and is equivalent to 10279 ± 530 cpm of [^{3}H]cAMP.

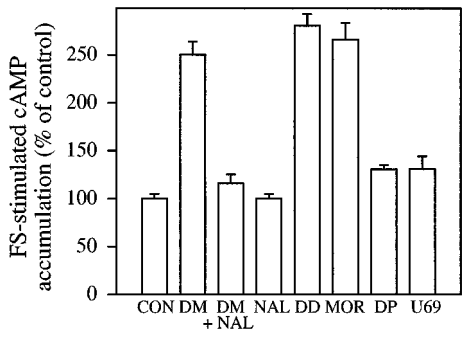

FIG. 3. **AC-V superactivation in AC-V/μ-transfected cells is specific to μ-agonists and is blocked by naloxone.** AC-V/μ-transfected cells were incubated for 18 h with 1 μM of the indicated opioids ([D-Ala-D-Leu]enkephalin (*DD*) and morphine (*MOR*)) and FS-stimulated AC activity determined following withdrawal by three quick washes. Data represent the means ± S.E. of triplicate determinations of a representative experiment out of two to three experiments that gave similar results. 100% represents control activity without opioids and is equivalent to 12889 ± 1686 cpm of [^{3}H]cAMP. *CON*, control; *DM*, DAMGE; *NAL*, naloxone; *DP*, DPDPE; *U69*, U69593.

pretreatment with 1 μM DAMGE to attain maximal level. Half the maximal level of AC superactivation was already observed after 1 h of incubation with the opioid agonist. Moreover, although chronic agonist treatment reveals the phenomenon of AC superactivation, prolonged exposure to the antagonist (1 μM naloxone, a concentration that effectively removes the agonist used; see Figs. 1*c* and 3) after the chronic treatment results in a gradual reduction (with a half-life of ~30 min) in AC-V

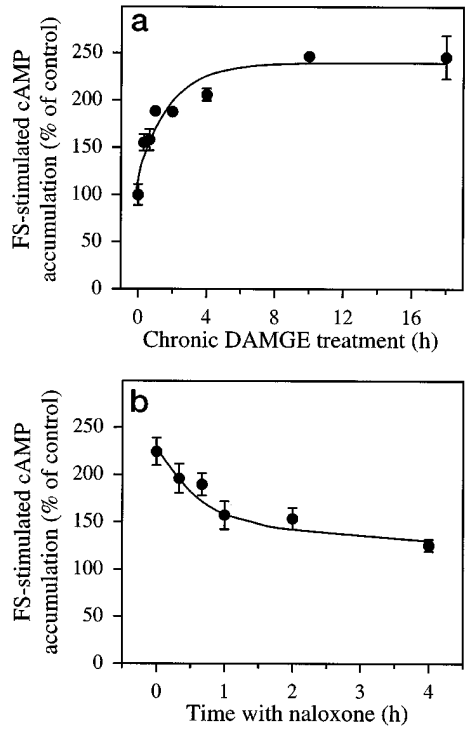

FIG. 4. **AC-V superactivation depends on sustained activation of the μ-opioid receptor.** *a*, time with 1 μM DAMGE and its effect on AC-V superactivation. *b*, reversibility of AC-V superactivation following long term treatment with naloxone; cells were incubated with 1 μM DAMGE for 18 h, followed by the addition of 1 μM naloxone for the times indicated prior to the AC assay. Data represent the means ± S.E. of triplicate determinations of a representative experiment out of two to three experiments that gave similar results. 100% represents control activity without opioids and is equivalent to 5640 ± 625 and 10957 ± 970 cpm of [^{3}H]cAMP for *a* and *b*, respectively.

superactivation, reaching nearly the original level after 4 h with the antagonist (Fig. 4*b*). It is therefore apparent that AC-V superactivation requires sustained activation of the receptor, whereas incubation with the antagonist (which blocks the activation of the receptor) slowly reverses and abolishes the superactivation state of AC-V.

AC-V superactivation upon chronic opioid treatment was observed not only when cells were stimulated with FS (Figs. 2–8) but also when the cells were stimulated with TPA (Figs. 5–7) or with the β_2-adrenergic agonist isoproterenol (data not shown). In addition, the opioid-induced superactivation could also be observed in cells not stimulated with either FS or TPA (data not shown), indicating that the phenomenon is independent of the method used to activate AC-V.

To examine whether the opioid receptor remains functional after the chronic opioid treatment, the agonist was re-applied to the cells following agonist withdrawal. Under these conditions, the percentage of inhibition of FS-stimulated AC-V (78.7 ± 4.7%, n = 3) was at the same level as that observed for acute inhibition in control cells (78.3 ± 3.7%, n = 3; see also Figs. 5 and 6, indicating no change in the functional receptor coupling to G protein and the capacity to inhibit AC-V. However, the level of cAMP in the agonist-withdrawn cells following re-addition of agonist was higher than that in control cells under conditions of acute opioid inhibition, probably due to the increased AC-V activity obtained during the chronic exposure.

PTX is known for its ability to ADP-ribosylate $G_{i/o}$ proteins at cysteine residues at the carboxyl terminus of the α subunit, thus preventing the activation of these G proteins (Birnbaumer *et al.*, 1990; Gilman, 1987). PTX was found to abolish both the inhibition and superactivation of AC-V activity by the opioid

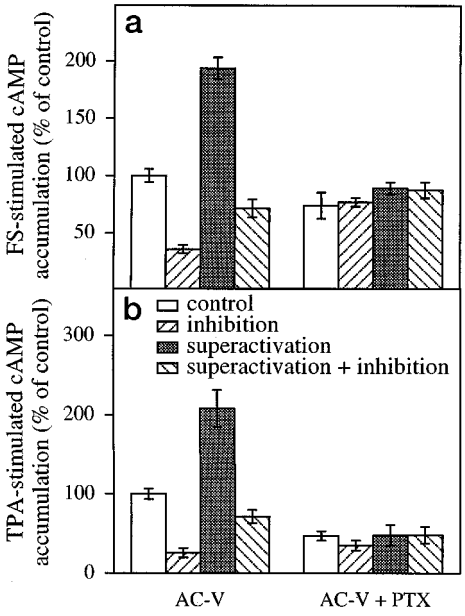

FIG. 5. **AC-V superactivation is blocked by PTX.** Pre-incubation of the cells with 100 ng/ml PTX (added to the cultures 18 h before the addition of [^{3}H]adenine and replenished upon the addition of [^{3}H]adenine) prevents the DAMGE-induced inhibition and superactivation of FS- (*a*) and TPA-stimulated (*b*) AC-V activity. *Control*, cells to which no opioids were added; *inhibition*, 1 μM DAMGE present during AC assay; *superactivation*, 1 μM DAMGE for 18 h followed by three rapid washes; *superactivation + inhibition*, 1 μM DAMGE (18 h) followed by washes and readdition of 1 μM DAMGE at the start of the assay. Data are from a representative experiment performed in triplicate. 100% represents 6084 ± 125 cpm (FS) and 1419 ± 21.1 cpm (TPA).

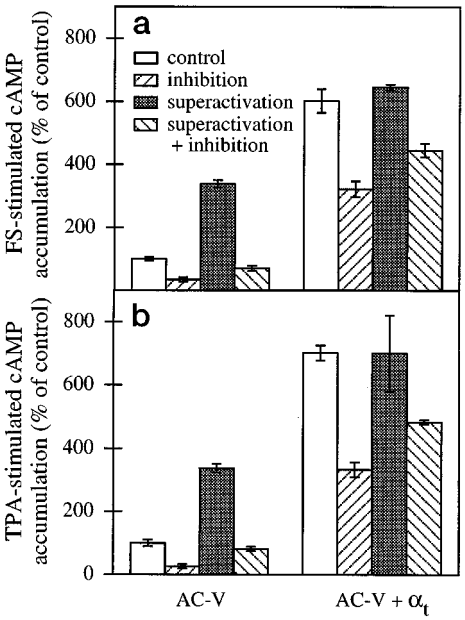

FIG. 6. **AC-V superactivation is blocked by α-transducin ($G_{\alpha t}$).** Cotransfection of the cells with $G_{\alpha t}$ cDNA (4 μg/plate, together with AC-V and the μ receptor cDNAs) prevents the chronic DAMGE-induced superactivation of FS- (*a*) and TPA-stimulated (*b*) AC-V activity. All other details are as described in the legend to Fig. 5. Data are from a representative experiment performed in triplicate. 100% represents 4613 ± 183 cpm (FS) and 1368 ± 149 cpm (TPA).

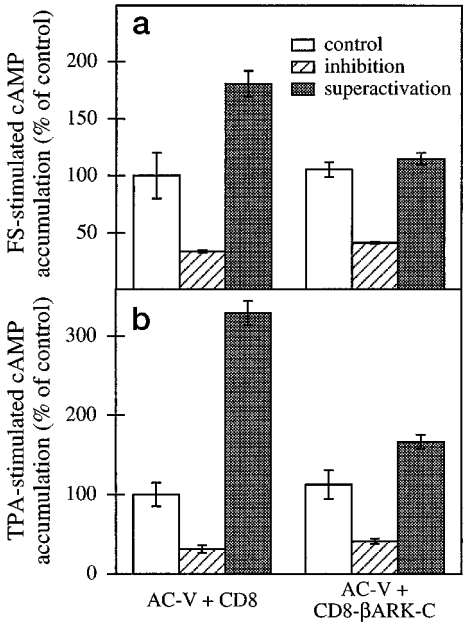

FIG. 7. **AC-V superactivation is blocked by the carboxyl terminus of βARK.** Cotransfection of the cells with CD8-βARK-C cDNA prevents the chronic DAMGE-induced superactivation but not the acute DAMGE-induced inhibition of FS- (*a*) and TPA-stimulated (*b*) AC-V activity. Cells were cotransfected with the cDNAs of either CD8 or CD8-βARK-C (4 μg/plate). All other details are as described in the legend to Fig. 5. Data are from a representative experiment performed in triplicate. 100% represents 12250 ± 2462 cpm (FS) and 2571 ± 147 cpm (TPA).

treatment (Fig. 5), whereas it had only a small inhibitory effect on the level of stimulation of AC-V by TPA or FS. It therefore follows that both the inhibition and superactivation of AC-V are mediated by PTX-sensitive $G_{i/o}$ proteins.

PTX, via the ADP-ribosylation of the α subunits, prevents the dissociation of the $G_{\beta\gamma}$ dimer from the α subunit of $G_{i/o}$. One of the useful tools for investigating the role of the $G_{\beta\gamma}$ dimer complex in signal transduction is cotransfection with the cDNA of α-transducin ($G_{\alpha t}$), which scavenges free $G_{\beta\gamma}$ (Crespo *et al.*, 1994; Faure *et al.*, 1994; Federman *et al.*, 1992; Lustig *et al.*, 1993). As seen in Fig. 6, cotransfection with $G_{\alpha t}$ abolished the ability to induce superactivation of AC following chronic opioid treatment of AC-V. However, in addition, $G_{\alpha t}$ led to an increase in FS- or TPA-stimulated cAMP accumulation and to some reduction in the percentage of inhibition of AC-V by the opioid agonist. The reason for the $G_{\alpha t}$-induced increase in AC-V activity is not clear, but one could argue that $G_{\alpha t}$ may alter the natural $G_{\alpha i}$ to $G_{\beta\gamma}$ ratio in a manner mimicking the chronic μ-receptor activation.

Another $G_{\beta\gamma}$ scavenger is the carboxyl terminus of β-adrenergic receptor kinase (βARK-C), which contains a $G_{\beta\gamma}$-binding domain (Crespo *et al.*, 1995; Koch *et al.*, 1994a, 1994b). In Fig. 7, it is shown that in cells cotransfected with a chimera of CD8 (which allows anchoring to the membrane) and βARK-C (CD8-βARK-C) (Crespo *et al.*, 1995), the ability to induce superactivation of AC following chronic opioid treatment was strongly attenuated. The same result was obtained when either FS or TPA were used to stimulate AC activity. The ability of TPA or FS to stimulate AC-V in control cells (not treated with opioids) was not affected by CD8-βARK-C. Moreover, the ability of opioids to inhibit AC-V activity following acute exposure was also not affected. Transfection with CD8 without the βARK-C moiety did not affect the superactivation of AC. Taken together, these results indicate that the $G_{\beta\gamma}$ dimer has an important role in AC-V superactivation, whereas the inhibition of AC-V by opioid agonists is not affected by $G_{\beta\gamma}$ scavengers and is mediated via the $G_{\alpha i/o}$ subunits and not via $G_{\beta\gamma}$.

$G_{\beta\gamma}$ dimers affect many signaling pathways. It has recently been shown that $G_{\beta\gamma}$ activates the c-*jun* amino-terminal kinase and extracellular signal-regulated kinase/MAPK cascades and

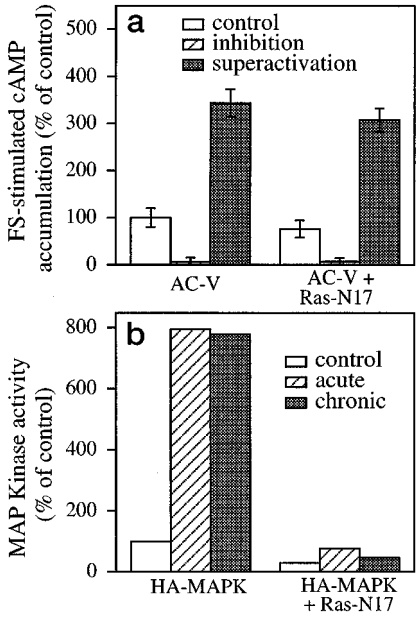

FIG. 8. **The Ras dominant negative mutant N17-Ras prevents extracellular signal-regulated kinase 2/MAPK activation by opioids but not AC-V inhibition or superactivation.** Cells were transfected with μ-opioid receptor and 2.5 μg/plate of either N17-Ras or CMV-gal cDNAs (for mock DNA transfection) and either 2 μg/plate AC-V or 1 μg/plate hemagglutinin-tagged MAPK (HA-MAPK). *a*, cotransfection with the cDNA of the Ras dominant negative mutant N17-Ras does not significantly affect AC-V inhibition or superactivation by acute (10 min) or chronic (18 h) DAMGE, respectively. The figure shows a representative experiment performed in triplicate. 100% represents 2198 ± 195 cpm. *b*, acute (5 min) or chronic (4 h) treatment with DAMGE stimulate extracellular signal-regulated kinase 2/MAPK activity. This stimulation is prevented by cotransfection with the Ras dominant negative N17-Ras. The figure shows a representative experiment (out of three that produced similar results). 100% is the activity of MAPK in the absence of DAMGE.

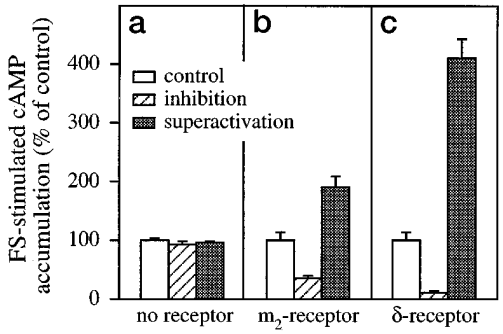

FIG. 9. **AC-V superactivation is induced by chronic activation of the δ-opioid or m_2-muscarinic receptors.** COS-7 cells were transfected with 2 μg/plate AC-V cDNA and 2 μg/plate of CMV-gal cDNA (for mock DNA transfection) (*a*), m_2-muscarinic receptor cDNA (*b*), or δ-opioid receptor cDNA (*c*). FS stimulation (1 μM) is inhibited by the acute (10 min) addition of 100 μM McN-A-343 (*b*) or 1 μM DPDPE (*c*). Superactivation of AC-V was measured after 18-h chronic exposure to the respective ligands followed by three quick washes. The figure shows representative experiments performed in triplicate. 100% represents 5169 ± 149 cpm (*a*), 3958 ± 533 cpm (*b*), and 7449 ± 687 cpm (*c*).

that Ras is involved in the activation of both pathways (Coso *et al.*, 1996; Crespo *et al.*, 1994; Hawes *et al.*, 1995; Inglese *et al.*, 1995; Koch *et al.*, 1994a). In Fig. 8, we show that cotransfection of the Ras dominant negative mutant N17-Ras did not affect the ability of chronic opioid treatment to induce AC-V superactivation to any significant extent. In a parallel experiment, we show that both acute (5 min) and chronic (4 h) morphine treatment stimulate the activity of HA-MAPK-transfected into the cells and that this activation is completely abolished by the Ras dominant negative mutant. As expected, and as found for other G protein-coupled receptors (Crespo *et al.*, 1994, 1995; Hawes *et al.*, 1995), this activity is also markedly reduced by scavengers of $G_{\beta\gamma}$ dimers (data not shown). Another effector system affected by $G_{\beta\gamma}$ dimers is phosphatidylinositol 3-kinase (Stoyanov *et al.*, 1995); we found, however, that wortmannin and LY294002, known to inhibit phosphatidylinositol 3-kinase (Nakanishi *et al.*, 1995), do not affect AC superactivation (data not shown). The nature of the signaling pathway downstream of $G_{\beta\gamma}$ affecting AC-V superactivation is currently under investigation.

The superactivation phenomenon has previously been shown in NG 108-15 cells when inhibitory δ-opioid and muscarinic receptors were exposed to chronic treatment with agonists of these receptors (Hamprecht, 1977; Sharma *et al.*, 1975; Westlind-Danielsson *et al.*, 1988). In Fig. 9, we demonstrate that in COS-7 cells transfected with AC-V together with either the m_2-muscarinic or the δ-opioid receptor, acute exposure to McN-A-343 or DPDPE (agonists of m_2-muscarinic and δ-opioid receptors, respectively) led to AC-V inhibition. Chronic treatment followed by agonist withdrawal, on the other hand, induced AC-V superactivation. No inhibition or superactivation were observed in cells transfected with AC-V, without m_2-muscarinic receptor, which were exposed to the muscarinic agonist McN-A-343. This result suggests that AC-V superactivation is common to the members of the G_i-coupled receptor superfamily.

DISCUSSION

In this study, we have used COS-7 cells expressing μ-opioid receptor and AC-V to gain information on AC superactivation. We have demonstrated that the μ- and AC-V-transfected cells are able to interact with μ-agonists and that this interaction leads to several processes of AC regulation, including inhibition of FS- or TPA-stimulated cAMP accumulation and the development of AC superactivation.

FS is presumed to activate AC directly (Tang and Gilman, 1995) and has been shown to stimulate the activity of AC-V (Ishikawa *et al.*, 1992; Jacobowitz *et al.*, 1993; Wallach *et al.*, 1994). In our work, TPA (1 μM) stimulates AC-V, presumably through the activation of PKC. This result is consistent with the finding of Kawabe *et al.* (1994), who showed that purified PKCα, in the presence of TPA, phosphorylates and activates purified recombinant AC-V. Moreover, these same authors showed that AC-V expressed in CMT cells is moderately stimulated by TPA. It is worthwhile noting that Jacobowitz *et al.* (1993) did not observe a clear activation of AC-V upon stimulation with TPA in lysed HEK-293 cells. These seemingly contradictory findings may be a result of differences in the assay conditions or in the cell lines used.

Superactivation of AC following chronic opioid exposure was reported in several neuronal cell lines (*e.g.* the neuroblastoma × glioma hybrid cells NG108-15 and the human neuroblastoma cells SH-SY5Y), in primary striatal neurons, and in several neuronal tissues (*e.g.* striatum, nucleus accumbens) (De Vries *et al.*, 1991; Sharma *et al.*, 1975; Terwilliger *et al.*, 1991; Van Vliet *et al.*, 1991; Wang *et al.*, 1994). AC superactivation was reconstituted here in COS cells using AC-V as the effector system. Eight types of AC isozymes that differ in their properties and tissue localization have recently been cloned. The brain contains predominantly types I, II, V, and VIII (Cali *et al.*, 1994; Furuyama *et al.*, 1993; Mons and Cooper, 1994; Pieroni *et al.*, 1993; Xia *et al.*, 1993). Interestingly, in the brain, AC-V has been shown to be predominantly and nearly exclusively localized to the striatum, nucleus accumbens, and olfactory tubercule (Glatt and Snyder, 1993; Mons and Cooper,

1994). The nucleus accumbens is considered to be one of the principal nuclei involved in the reinforcing effects of drugs of abuse and in the development of the withdrawal syndrome (Harris and Aston-Jones, 1994; Koob, 1992; Nestler *et al.*, 1993; Self and Nestler, 1995). These results suggest that AC-V in reward areas could play an important role in both acute and chronic agonist-induced AC regulation. The involvement of other isozymes in AC superactivation is currently under investigation; preliminary results indicate that this phenomenon seems to be isozyme-specific, with certain isozymes showing both opiate-induced inhibition and superactivation, whereas others only exhibit inhibition or do not show either inhibition or superactivation.

The characteristics of AC-V superactivation in transfected COS cells were found to be similar to those obtained in neuronal and Chinese hamster ovary cells in which the AC isozymes were not defined. AC superactivation is dose-dependent (Avidor-Reiss *et al.*, 1995a, 1995b) and can be blocked by an opioid receptor antagonist (Avidor-Reiss *et al.*, 1995a, 1995b; Sharma *et al.*, 1975). AC superactivation develops during chronic exposure and is accompanied by an apparent tolerance to the agonist (Avidor-Reiss *et al.*, 1995a; Sharma *et al.*, 1975). Upon withdrawal of the agonist and abolishment of the inhibition of the AC, a pronounced increase in AC activity is observed (Avidor-Reiss *et al.*, 1995a, 1995b; Sharma *et al.*, 1975; Wang *et al.*, 1994). AC superactivation depends on the constant stimulation of the receptor and is reversed upon long-lasting withdrawal (Avidor-Reiss *et al.*, 1995a; Wang *et al.*, 1994). Moreover, AC superactivation seems to be a property shared by other G_i-coupled receptors (Thomas and Hoffman, 1987). The signal transduction that is activated during the chronic treatment leading to AC superactivation was found to be PTX-sensitive (*i.e.* via $G_{i/o}$ proteins) (Avidor-Reiss *et al.*, 1995a, 1995b; Griffin *et al.*, 1985).

Here, we report that the $G_{\beta\gamma}$ dimer released from G_α upon receptor activation has an important role in AC-V superactivation, whereas the inhibition of AC-V by opioid agonists is not affected by $G_{\beta\gamma}$ and is mediated via the $G_{\alpha i/o}$ subunits. Using membrane preparations from baculovirus-transfected Sf9 cells expressing various AC isozymes, it was shown that AC-I is inhibited whereas AC-II and AC-IV are stimulated by addition of purified $G_{\beta\gamma}$ dimers, whereas the activity of AC-V did not seem to be affected (Choi *et al.*, 1993; Taussig *et al.*, 1993b). The fact that both the appearance of AC-V superactivation upon chronic exposure and its disappearance following withdrawal of the opioid drug have slow kinetics (half-life of ~1 h and 30 min, respectively) is compatible with the idea that $G_{\beta\gamma}$ does not affect AC-V directly but is affecting an as yet undefined slowly developing secondary signaling process.

$G_{\beta\gamma}$ subunits have been shown to regulate various additional effector systems, including K^+ channels, phospholipase C_β, G protein-dependent receptor kinase, phosphatidylinositol 3-kinase, MAPK, and c-*jun* amino-terminal kinase (Clapham and Neer, 1993; Coso *et al.*, 1996; Crespo *et al.*, 1994; Stoyanov *et al.*, 1995). Although the first four pathways seem to involve direct interaction of $G_{\beta\gamma}$ with the effector (Clapham and Neer, 1993; Stoyanov *et al.*, 1995), the activation of the last two by $G_{\beta\gamma}$ seems to be indirect. It has been suggested that the $G_{\beta\gamma}$ activates MAPK via a mechanism that involves tyrosing phosphorylation of Shc, leading to increased association of Shc, Grb2, and Sos, which then stimulate Ras (van Biesen *et al.*, 1995). Indeed, the activation of extracellular signal-regulated kinase/MAPK and c-*jun* amino-terminal kinase were found to be inhibited by the Ras dominant negative mutant N17-Ras and by $G_{\beta\gamma}$ scavengers (Coso *et al.*, 1996; Crespo *et al.*, 1994; Hawes *et al.*, 1995; Koch *et al.*, 1994a). Our results suggest that the signal transduction of AC-V superactivation does not involve either Ras or phosphatidylinositol 3-kinase. Moreover, as described above, it appears that there is no direct interaction between the $G_{\beta\gamma}$ dimers and AC-V and that the $G_{\beta\gamma}$ triggers a reversible secondary process, which participates in AC-V superactivation.

In summary, the phenomenon of AC superactivation following chronic opioid exposure, shown previously in various neuronal cells and tissues, was reconstituted using COS cells transfected with μ-opioid receptor and AC-V cDNAs. A similar superactivation was also observed in chronically treated COS cells transfected with other G_i-coupled receptors. The modulation of AC-V by opioids is affected via both G_α and $G_{\beta\gamma}$ subunits. The acute inhibition of AC-V results from a direct and rapid interaction between the enzyme and the $G_{\alpha i}$ subunit. Conversely, the released $G_{\beta\gamma}$ dimers, via a mechanism that remains to be determined, participate in the development of AC-V superactivation.

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