

Regulation of Adenylyl Cyclase Isozymes on Acute and Chronic Activation of Inhibitory Receptors

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Received December 29, 1997; Accepted May 11, 1998

This paper is available online at <http://www.molpharm.org>

ABSTRACT

Adenylyl cyclase superactivation, a phenomenon by which chronic activation of inhibitory $G_{i/o}$ -coupled receptors leads to an increase in cAMP accumulation, is believed to play an important role as a compensatory response of the cAMP signaling system in the cell. However, to date, the mechanism by which adenylyl cyclase activity is regulated by chronic exposure to inhibitory agonists and the nature of the adenylyl cyclase isozymes participating in this process remain largely unknown. Here we show, using COS-7 cells transfected with the various AC isozymes, that acute activation of the D_2 dopaminergic and m_4 muscarinic receptors inhibited the activity of adenylyl cyclase isozymes I, V, VI, and VIII, whereas types II, IV, and VII were stimulated and type III was not affected. Conversely,

chronic receptor activation led to superactivation of adenylyl cyclase types I, V, VI, and VIII and to a reduction in the activities of types II, IV, and VII. The activity of AC-III also was reduced. This pattern of inhibition/stimulation of the various adenylyl cyclase isozymes is similar to that we recently observed on acute and chronic activation of the μ -opioid receptor, suggesting that isozyme-specific adenylyl cyclase superactivation may represent a general means of cellular adaptation to the activation of inhibitory receptors and that the presence/absence and intensity of the adenylyl cyclase response in different brain areas (or cell types) could be explained by the expression of different adenylyl cyclase isozyme types in these areas.

The stimulation of seven-transmembrane domain G protein-coupled inhibitory receptors leads to inhibition of AC and a consequent reduction in cellular cAMP levels (Birnbaumer *et al.*, 1990). We and others have shown, however, that when certain of these inhibitory receptors (e.g., μ -, δ -, and κ -opioid; α_2 -adrenergic; somatostatin) are chronically activated, there is an increase in cAMP accumulation, which is particularly apparent on withdrawal of the inhibitory agonist (Sharma *et al.*, 1975, 1977; Parsons and Stiles, 1987; Thomas and Hoffman, 1987, 1992; Avidor-Reiss *et al.*, 1995a, 1995b, 1996, 1997; McDermott and Sharp, 1995). This phenomenon, referred to as AC superactivation (also termed AC overshoot, supersensitivity, or sensitization), is believed to represent a possible biochemical substratum for the development of opiate tolerance and dependence, commonly observed on prolonged exposure to opiate drugs (Sharma *et al.*, 1975; Nestler *et al.*, 1993). Moreover, it has been suggested that such regulation of AC could be a general means of cellular adaptation to the activation of inhibitory $G_{i/o}$ -coupled receptors (Thomas and Hoffman, 1987).

This work was supported by the National Institute of Drug Abuse (Grant DA06265), German-Israeli Foundation for Scientific Research and Development, Forscherheimer Center for Molecular Genetics, and Israeli Ministries of Science and Arts and of Absorption (fellowship to I.N.).

Despite the fact that this phenomenon has been studied for a long time, the mechanism by which it is evoked remains largely unknown. AC superactivation has been described in many different cell types (Sharma *et al.*, 1975; Parsons and Stiles, 1987; Thomas and Hoffman, 1992; Avidor-Reiss *et al.*, 1995a, 1997; McDermott and Sharp, 1995) and, as mentioned above, on chronic treatment with agonists of several different inhibitory receptors, yet under similar conditions, other cell types do not display AC superactivation (McDermott and Sharp, 1995; Puttfarcken and Cox, 1989) or do so only under certain stimulation conditions (Sharma *et al.*, 1977; Jones and Bylund, 1988; Ammer and Schulz, 1993). An explanation for this may reside in the fact that there are several isozymes of AC that differ in their properties and that different cell types vary in their AC isozyme populations. To date, mRNAs encoding nine distinct isozymes of AC have been identified, and it has been shown that they differ in their capacity to be inhibited or stimulated by G protein α_i , α_s , and $\beta\gamma$ subunits; protein kinase C; and Ca^{2+} (Mons and Cooper, 1995; Sunahara *et al.*, 1996).

Using COS cells cotransfected with μ -opioid receptor and various AC isozymes, we observed superactivation of AC-I, -V, -VI, and -VIII (Avidor-Reiss *et al.*, 1996, 1997). In addition, we have shown that AC-V transfected into COS-7 cells

ABBREVIATIONS: AC, adenylyl cyclase; DMEM, Dulbecco's modified Eagle's medium; HEK, human embryonic kidney; FS, forskolin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TSH, thyroid-stimulating hormone.

is susceptible to superactivation after chronic activation of the δ -opioid or m2 muscarinic receptors (Avidor-Reiss *et al.*, 1996). In partial agreement with those results, Thomas and Hoffman (1996) showed that AC-VI transfected into HEK 293 cells is susceptible to superactivation after chronic activation of the inhibitory m2 muscarinic or D_2 dopaminergic receptors. However, contrary to our results with the μ -opioid receptor, they did not observe superactivation of AC-I after chronic m2 activation. It therefore became of interest to determine the pattern of differential stimulation/inhibition of the various AC isozymes on acute and chronic activation by various inhibitory receptors. In the present study, we examined the effect of acute and chronic activation of the D_2 dopaminergic and m4 muscarinic receptors on the activities of AC isozyme types I–VIII.

Experimental Procedures

Materials. 2-[3H]adenine (18.0 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Ionomycin and the phosphodiesterase inhibitors 1-methyl-3-isobutylxanthine and RO-20-1724 were from Calbiochem (La Jolla, CA). FS, cAMP, TSH, and atropine were from Sigma Chemical (St. Louis, MO). (–)-Quinpirole, (–)-sulpiride, and McN-A-343 were from Research Biochemicals (Natick, MA). Tissue culture reagents were from Life Technologies (Gaithersburg, MD).

Plasmids. AC-containing plasmids (pXMD1-AC-I, pXMD1-AC-II, pXMD1-AC-III, pXMD1-AC-IV, pXMD1-AC-V, pCMV5-neo-AC-VI, pXMD1-AC-VII, pCMV5-neo-AC-VIII), pXMD1-gal, and pSG5-TSH have been described previously (Avidor-Reiss *et al.*, 1997). Other plasmids used were the rat D_{2L} dopamine receptor (referred to here as the D_2 receptor) cDNA in pcDNA1 Amp (obtained from Dr. S. Fuchs, Weizmann Institute, Rehovot, Israel) and human m4 muscarinic receptor cDNA in PCD (provided by Dr. T. Bonner, National Institute of Mental Health, National Institutes of Health, Bethesda, MD).

Transient cell transfection. Twenty-four hours before transfection, a confluent 10-cm plate of COS-7 cells in DMEM supplemented with 5% fetal calf serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin in a humidified atmosphere consisting of 5% CO_2 /95% air at 37° was trypsinized and split into four 10-cm plates. Using the DEAE-dextran chloroquine method as described previously (Avidor-Reiss *et al.*, 1997), the cells were transfected, unless otherwise indicated, with 2 μ g/plate of either one of the AC isozyme cDNAs or pXMD1-gal (for mock DNA transfection), and, where indicated, with 1 μ g/plate of D_2 dopamine receptor, m4 muscarinic receptor, or TSH receptor cDNA. Twenty-four hours later, the cells were trypsinized and recultured in 24-well plates, and after an additional 24 hr, the cells were assayed for cAMP content (as a measure of 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).

cAMP accumulation. The assay was performed in triplicate as described previously (Avidor-Reiss *et al.*, 1996, 1997). In brief, cells cultured in 24-well plates were incubated for 2 hr with 0.25 ml/well of fresh growth medium containing 5 μ Ci/ml [3H]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 the examined m4 or D_2 ligands by the addition of either FS, TSH, or ionomycin. After a 10-min (FS or TSH) or 20-min (ionomycin) incubation 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 [3H]cAMP was determined by a two-step column separation procedure as described previously (Avidor-Reiss *et al.*, 1996). Unless otherwise indicated, chronic agonist treatment was achieved by incubating the cells for 18 hr with 200 nM quinpirole (for the D_2 receptor) or 100 μ M McN-A-343 (for the m4 receptor), followed by agonist withdrawal (achieved by quick removal of medium and the addition of new medium containing antagonist: 10 μ M (–)-sulpiride for the D_2 receptor or 10 μ M atropine for the m4 receptor, concentrations that effectively remove the agonist used) and the addition of the appropriate AC stimulator (see above) to assay cAMP accumulation. The incubation with [3H]adenine took place during the last 2 hr of the chronic exposure. Uptake of [3H]adenine into the cells was not affected by the chronic agonist treatments.

Results

Effect of D_2 agonist on AC activity endogenously expressed in COS-7 cells. In COS-7 cells transfected with D_2 dopaminergic receptor cDNA, cAMP accumulation could be stimulated (by ~3-fold) on activation with 1 μ M FS (Fig. 1), and this stimulated cAMP accumulation could be inhibited (by ~35%) by the application of 200 nM of the D_2 agonist quinpirole. Although a high concentration (10 μ M) of the nonselective dopaminergic receptor antagonist (–)-sulpiride did not in itself affect FS-stimulated cAMP accumulation, application of quinpirole in the presence of the antagonist completely prevented the inhibitory effect of the agonist. Chronic pretreatment of the cells with quinpirole followed by withdrawal of the agonist led to an increase (of ~50%) in FS-stimulated cAMP accumulation, in agreement with the phenomenon of AC superactivation (Sharma *et al.*, 1975; Thomas and Hoffman, 1987; Avidor-Reiss *et al.*, 1995a, 1995b, 1996). Readdition of the agonist after chronic pretreatment with quinpirole reduced cAMP accumulation (by ~30%) to a level similar to that observed on acute application of the agonist, thus demonstrating that the D_2 receptor re-

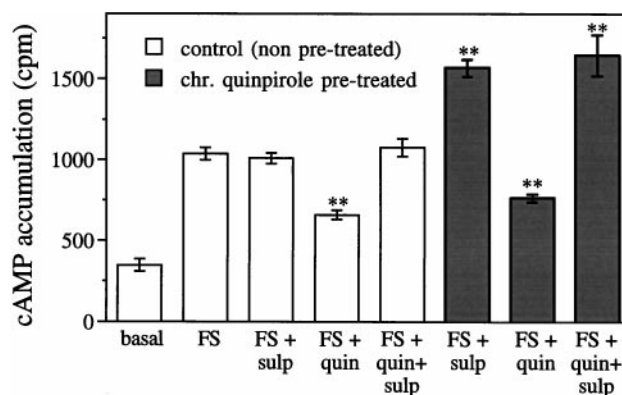


Fig. 1. Effect of D_2 dopamine receptor agonist on FS-stimulated AC activity endogenously expressed in COS-7 cells. COS-7 cells were transfected with the D_2 dopamine receptor, as described in Experimental Procedures. The endogenous AC activity in COS-7 cells is expressed in cpm of [3H]cAMP formed after acute (10-min) stimulation (□) with 1 μ M FS (basal, unstimulated), in the presence or absence of 200 nM of the agonist quinpirole (quin) and/or 10 μ M of the antagonist (–)-sulpiride (sulp), and after chronic (18-hr) treatment (■) with 200 nM quinpirole followed by a rapid wash and readdition (at the beginning of the cAMP accumulation assay) of either 10 μ M sulpiride (sulp; withdrawal conditions) or 200 nM quinpirole (quin) or by the readdition of 200 nM quinpirole together with 10 μ M sulpiride (quin + sulp). Data represent the mean \pm standard error of triplicate determinations of a representative experiment of three experiments that gave similar results. **, $p < 0.01$ versus FS-stimulated cAMP accumulation.

mains functional after this long period of chronic exposure. The addition of (–)-sulpiride together with the agonist after the chronic quinpirole treatment led to an equivalent level of AC superactivation (~50%) as that observed when the antagonist alone was added.

COS-7 cells cotransfected with the D₂ dopaminergic receptor and the TSH receptor displayed an elevated level of cAMP accumulation (5–7-fold) when stimulated with 0.1 μM TSH, with respect to unstimulated cells (Fig. 2a). In these cells, both acute activation of the D₂ receptor and chronic activation followed by agonist withdrawal produced a small reduction in cAMP accumulation, but no superactivation was observed. The Ca²⁺ ionophore ionomycin, which by increasing Ca²⁺ concentration is known to activate AC-I and AC-VIII (Mons and Cooper, 1995; Sunahara *et al.*, 1996), only very slightly stimulated the endogenous AC activity in these cells (Fig. 2b), suggesting that COS cells contain little if any of these AC isozymes.

The above results indicate that in COS-7 cells, D₂ receptor regulation of endogenous cAMP levels depends on the way in which AC is stimulated. The composition of AC isozymes present in COS cells is not well known, although this cell line has been shown to contain at least the AC-VII isozyme (Premont, 1994). To determine the nature of the regulation of the various AC isozymes by acute and chronic exposure to D₂ receptor agonists, we transfected AC types I–VIII into COS-7 cells and monitored the effect of acute and chronic agonist treatment on AC activity. All of the exogenous ACs were found to be functionally active, as determined by the increase in cAMP accumulation in the cells after stimulation with the appropriate stimulant (ionomycin for AC-I and -VIII or TSH for the other isozymes). Moreover, Western blotting performed for AC isozymes I, II, IV, V, VI, and VIII showed that these isozymes are expressed in the transfected cells (data not shown).

Regulation of AC-V by D₂ receptor activation. We have previously shown that AC-V expressed in COS-7 cells after cotransfection with μ-opioid receptor is acutely inhibited

by activation of μ receptors and undergoes superactivation on withdrawal from chronic treatment with μ receptor agonists (Avidor-Reiss *et al.*, 1996). Fig. 3 shows that similar results were obtained with the D₂ dopaminergic receptor. Acute application of quinpirole strongly inhibited TSH-stimulated cAMP accumulation (Fig. 3a), but this could be completely prevented by inclusion of (–)-sulpiride during the acute agonist exposure. Withdrawal from chronic quinpirole treatment led to AC superactivation (Fig. 3b). The continued presence of the antagonist prevented the development of AC superactivation by chronic agonist treatment, whereas chronic treatment with the antagonist alone had no effect on cAMP accumulation. Pertussis toxin, which is known for its ability to catalyze the ADP-ribosylation of G_{i/o} proteins at cysteine residues at the carboxyl terminus of the G_α subunit, thus preventing the activation of these G proteins (Birnbaumer *et al.*, 1990), had a small inhibitory effect on the level of stimulation of AC-V by TSH (Fig. 3c) but was found to abolish both the inhibition and superactivation of this isozyme by acute and chronic quinpirole treatment, respectively. It therefore follows that both the inhibition and superactivation of AC-V after short and prolonged activation of the D₂ receptor are mediated by the G_{i/o} family of G proteins.

Differential regulation of AC isozymes by D₂ receptor activation. In cells transfected with AC-I or AC-VIII, stimulation with ionomycin resulted in a large increase in cAMP accumulation compared with the unstimulated basal level (Fig. 4a). The finding that AC-I- and AC-VIII-transfected cells show strong activation by ionomycin is in agreement with previous reports demonstrating that Ca²⁺/calmodulin has a strong stimulatory effect on these isozymes (Mons and Cooper, 1995; Sunahara *et al.*, 1996) and indicates that the transfected AC-I and AC-VIII are expressed and functionally active because, as described earlier, the endogenous AC of COS is only very weakly stimulated by ionomycin (Fig. 2b). Acute activation of the D₂ receptor by quinpirole led to inhibition of the ionomycin-stimulated activity of these two isozymes, whereas chronic D₂ receptor activation fol-

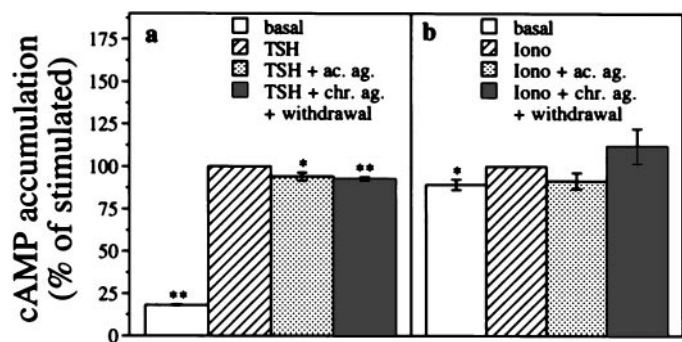


Fig. 2. Effect of D₂ dopamine receptor agonist on TSH- and ionomycin (Iono)-stimulated AC activity endogenously expressed in COS-7 cells. COS-7 cells were transfected with the D₂ dopamine receptor and with either the TSH receptor or β-gal cDNAs. The endogenous AC activity in COS-7 cells was measured during (a) a 10-min stimulation with 0.1 μM TSH (basal, unstimulated) or (b) a 20-min stimulation with 1 μM ionomycin. Where indicated, the assay was performed in the presence of 200 nM of the agonist quinpirole (ac. ag.) or after chronic (18 hr) agonist treatment and withdrawal [by rapid wash and the addition of 10 μM of the antagonist (–)-sulpiride; chr. ag. + withdrawal]. 100%, AC activity observed in cells stimulated with TSH (4359 ± 378 cpm of [³H]cAMP) or ionomycin (400 ± 31 cpm of [³H]cAMP). Data represent the mean ± standard error of four experiments. *, *p* < 0.05, **, *p* < 0.001 versus FS-stimulated cAMP accumulation.

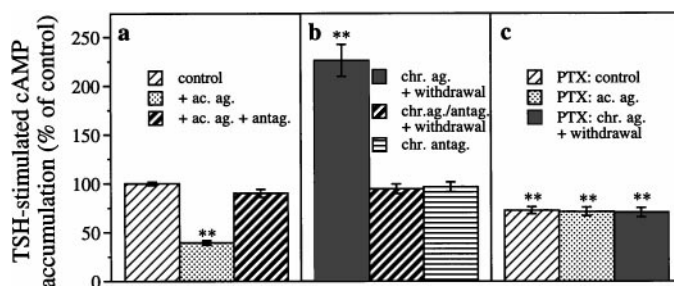


Fig. 3. Quinpirole regulation of AC-V activity in COS-7 cells transfected with AC-V and D₂ dopamine receptor. COS-7 cells were cotransfected with AC-V, the TSH receptor, and the D₂ dopamine receptor cDNAs. a, Acute effect of 200 nM quinpirole (ac. ag.) in the presence or absence of the antagonist (antag.) (–)-sulpiride (10 μM) on 0.1 μM TSH-stimulated AC-V activity. b, Effect of withdrawal after chronic (18-hr) treatment with the agonist (chr. ag. + withdrawal) compared with withdrawal after chronic treatment with a mixture of the agonist, 200 nM quinpirole, and the antagonist, 10 μM (–)-sulpiride (chr. ag./antag. + withdrawal) or chronic treatment with the antagonist alone (chr. antag.). c, Effect of pretreatment with pertussis toxin (PTX, 18 hr, 100 ng/ml) on TSH-stimulated cAMP accumulation after acute agonist exposure and after chronic agonist exposure and withdrawal. 100%, Control AC activity observed in cells stimulated with TSH (9833 ± 203 cpm of [³H]cAMP). Data represent the mean ± standard error of three experiments. **, *p* < 0.001 versus control.

lowed by withdrawal of the agonist led to superactivation (Fig. 4a).

Although none of the other AC isozymes are activated by Ca^{2+} in intact cells, their activities are stimulated by α -activating receptors or by a constitutively active α s mutant (Mons and Cooper, 1995; Sunahara et al., 1996). Stimulation of these AC isozymes, cotransfected together with D_2 and TSH receptors, by $0.1 \mu\text{M}$ TSH, led to a large increase in cAMP accumulation compared with the unstimulated basal level (Fig. 4, b–d). In cells transfected with the AC-V or -VI isozymes, acute quinpirole significantly inhibited the TSH-stimulated cAMP accumulation, whereas withdrawal of the agonist after chronic treatment led to superactivation. Similar results were observed when AC-V or -VI were stimulated with $1 \mu\text{M}$ FS (data not shown).

Taken together, the above results demonstrate that AC-I, -V, -VI, and -VIII exhibit inhibition and superactivation by acute and chronic D_2 agonist exposure, respectively, and that the two functions are not dependent on the agent used to stimulate the particular AC activity [i.e., FS, Ca^{2+} (ionomycin), or α s (TSH)].

In contrast to the above results, acute exposure to quinpirole of cells transfected with AC-II, -IV, or -VII (which are known to be closely related according to their sequences and regulatory patterns; Cooper et al., 1995; Sunahara et al., 1996), induced an increase in TSH-stimulated cAMP accumulation (Fig. 4c). Moreover, after withdrawal from chronic agonist treatment, not only was no superactivation of these isozymes apparent, but a reduction in their activities actually was observed.

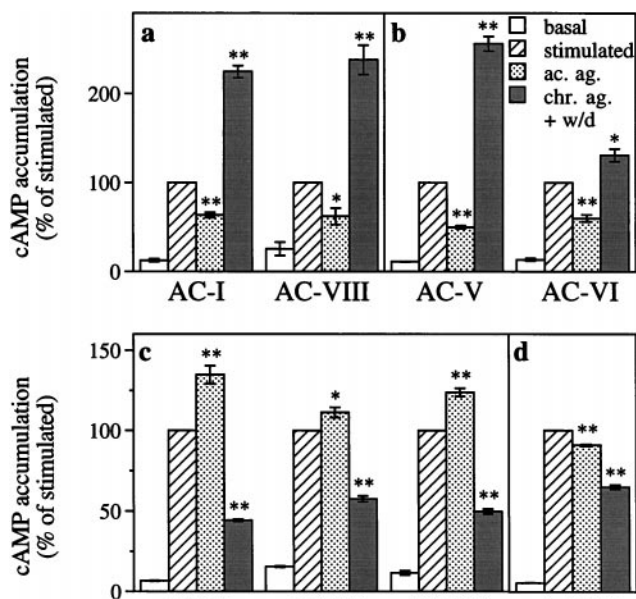


Fig. 4. Acute and chronic D_2 dopamine receptor activations differentially regulate the various AC isozymes. COS-7 cells were transfected with the cDNAs of the indicated AC isozymes together with cDNAs of the D_2 dopamine receptor and the TSH receptor (where necessary). AC was stimulated with $1 \mu\text{M}$ ionomycin (a) or $0.1 \mu\text{M}$ TSH (b–d), and the amount of cAMP was determined under basal (unstimulated) conditions (\square), after stimulation with ionomycin or TSH in the absence of quinpirole (\square), after stimulation in the presence of acute 200 nM quinpirole (\blacksquare), and after withdrawal (by rapid wash and addition of (–)-sulpiride, $10 \mu\text{M}$) after chronic quinpirole (18 hr, 200 nM) treatment (\blacksquare). 100%, AC activity observed in cells stimulated with either ionomycin (a) or TSH (b–d). Data represent the mean \pm standard error of three experiments. *, $p < 0.05$, **, $p < 0.01$ versus stimulated cAMP accumulation.

As depicted in Fig. 4d, the activity observed in AC-III-transfected cells was only slightly reduced by acute exposure to the D_2 agonist. However, when these cells were chronically treated with quinpirole and the agonist was withdrawn, a further reduction in AC-III activity was observed.

Taken together, the results show that based on criteria of agonist-induced inhibition and superactivation, the AC isozymes can be divided into three functional groups: (1) AC-I, -V, -VI, and -VIII exhibit inhibition by acute, and superactivation by chronic, D_2 agonist exposure; (2) AC-II, -IV, and -VII exhibit AC stimulation on acute D_2 agonist treatment and inhibition of AC after chronic exposure; and (3) AC-III is slightly inhibited by acute D_2 agonist treatment and further inhibited after chronic exposure.

Dose-response curves for the effects of acute and chronic agonist exposures for AC-I, -V, and -II are depicted in Fig. 5. Significant inhibition of AC-I (Fig. 5a) and V (Fig. 5c) activities were observed in cells acutely treated with quinpirole, with ED_{50} concentrations of 12 ± 3.6 and $8.7 \pm 2.9 \text{ nM}$, respectively. A dose-dependent superactivation was observed

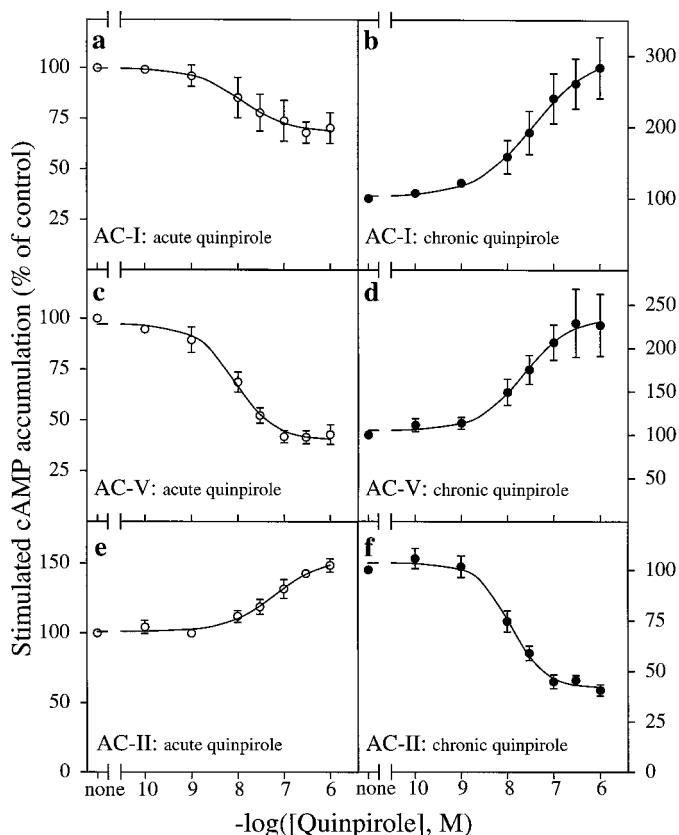


Fig. 5. Dose-response of acute and chronic quinpirole on AC-V, AC-I, and AC-II activities. COS-7 cells were cotransfected with the cDNAs of the D_2 dopamine receptor, the TSH receptor (where necessary), and the indicated AC isozymes. a, Inhibition of ionomycin-stimulated AC-I activity by various concentrations of quinpirole applied acutely during the assay. b, Ionomycin-stimulated AC-I activity after chronic treatment (18 hr) with the indicated concentrations of quinpirole and subsequent withdrawal of the agonist (rapid wash and the addition of $10 \mu\text{M}$ (–)-sulpiride). c and d, TSH-stimulated AC-V activity after acute and chronic quinpirole treatment. e and f, TSH-stimulated AC-II activity after acute and chronic quinpirole treatment. AC inhibition/superactivation curves were drawn 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 inflection point (which is equivalent to the EC_{50} value), and d is the asymptotic minimum. Data represent the mean \pm standard error of three experiments.

for both of these isozymes after chronic exposure (Fig. 5, b and d). Interestingly, and as shown previously for the μ - and κ -opioid receptors (Avidor-Reiss *et al.*, 1995a, 1995b), the ED_{50} concentrations obtained for the chronic superactivation (AC-I, 37 ± 7.7 nM; AC-V, 23 ± 5.9 nM) were higher than those observed for acute inhibition. Contrary to AC-I and -V, AC-II showed a dose-dependent increase in activity on acute exposure to quinpirole (Fig. 5e) and a dose-dependent inhibition of its activity after chronic treatment with the D_2 agonist (Fig. 5f), with ED_{50} values of 65 ± 24 and 12 ± 2.8 nM, respectively.

The development of superactivation (for AC-I, -V, -VI, and -VIII) and the decrease in AC activity (for AC-II, -IV, and -VII) on withdrawal after chronic quinpirole treatment are time dependent. The kinetics of these processes as a function of time of treatment with 40 nM quinpirole for AC types I, V, and II are depicted in Fig. 6, with all of the isozymes attaining a maximal level of superactivation/inhibition after ~8 hr

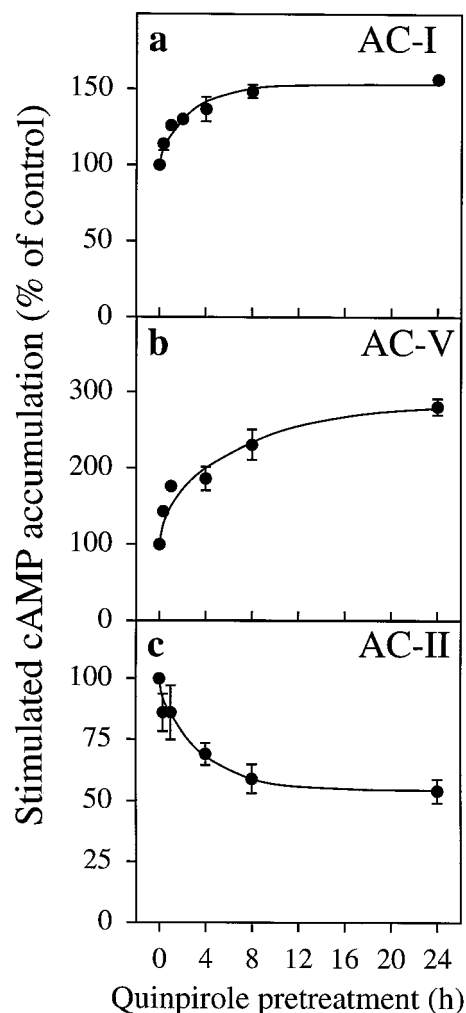


Fig. 6. Time-dependent development of AC-I and AC-V superactivation and decrease in AC-II activity on chronic quinpirole treatment. COS-7 cells were cotransfected with the cDNAs of the indicated AC isozymes, the D_2 dopamine receptor, and the TSH receptor (where necessary). The rate of development of superactivation of AC-I (stimulated with ionomycin) and AC-V (stimulated with TSH) (a and b) and the progressive inhibition of AC-II activity (stimulated with TSH) (c) after chronic treatment for the indicated times with 40 nM quinpirole and its withdrawal immediately before the cAMP accumulation assay. Data represent the mean \pm standard error of three experiments.

of pretreatment with quinpirole. As previously shown for the superactivation of AC-V after chronic activation of the μ -opioid receptor (Avidor-Reiss *et al.*, 1996), the chronic D_2 agonist-induced superactivation of this isozyme is reversible and could be abolished by prolonged exposure to the antagonist. Pretreatment for 18 hr with quinpirole followed by its withdrawal and incubation with (–)-sulpiride resulted in a nearly total abolishment of the superactivated state of AC-V and recovery of the original level of AC-V activity (data not shown).

Differential regulation of AC isozymes by m_4 muscarinic receptor activation. Cells transfected with the m_4 muscarinic receptor and the various AC isozymes were also tested on acute and chronic exposure to the muscarinic agonist McN-A-343 (Fig. 7). As seen previously (Fig. 4), AC isozymes I and VIII were stimulated by ionomycin (Fig. 7a), whereas activation of the transfected TSH receptor activated the other AC isozymes (Fig. 7, b–d).

As with the results obtained for stimulation of the D_2 dopaminergic receptor with quinpirole (Fig. 4), AC-I, -V, -VI, and -VIII exhibit inhibition and superactivation on acute and chronic exposure, respectively, of m_4 muscarinic receptors to McN-A-343 (Fig. 7, a and b).

The results obtained with the AC-II, -IV, and -VII isozymes (Fig. 7c) were similar to those observed with the D_2 receptor, albeit less marked. It should be noted that although the increases in AC activity observed on acute m_4 receptor activation are not evident (AC-VII) or small (AC-IV), one must take into account that the endogenous AC activity is in fact slightly inhibited by TSH stimulation (see Fig. 2a). Indeed, it can be seen from Fig. 7e, which depicts the ratios of the m_4 agonist-stimulated AC activity to the control TSH-stimulated activity, that although the increases in the TSH-stimulated activities of AC-II, -IV, and -VII are only slight to moderate, there is a decrease in endogenous TSH-stimulated AC activity on m_4 receptor activation. The result is that compared with endogenous AC activity, the activities of the AC-II, -IV, and -VII isozymes are significantly increased on m_4 receptor activation, as seen with the D_2 receptor. Taken together, these results demonstrate that AC-II, -IV, and -VII are similar in their patterns of regulation, exhibiting stimulation on acute m_4 receptor activation and inhibition after withdrawal from chronic agonist exposure.

Also similar to what was observed with the D_2 receptor, the activity in AC-III-transfected cells was slightly reduced by acute exposure to the m_4 agonist, and further so on chronic treatment with McN-A-343.

Discussion

We and others have reported that the chronic application of agonists of various inhibitory seven-transmembrane G protein-coupled receptors produces a time- and concentration-dependent increase (as opposed to the decrease observed on acute activation of these receptors) in the activity of AC (Sharma *et al.*, 1975; Parsons and Stiles, 1987; Thomas and Hoffman, 1992; Avidor-Reiss *et al.*, 1995a, 1995b). This phenomenon, originally described on chronic treatment of cells with opiates (Sharma *et al.*, 1975), is particularly evident on withdrawal of the agonist and has been referred to as AC superactivation, or “overshoot”. In the case of opiates, this phenomenon has been hypothesized to represent, at least in

part, a biochemical basis of opiate drug addiction (Sharma *et al.*, 1975; Nestler *et al.*, 1993).

However, the actual situation seems to be more complicated, as we now know that there are several subtypes of AC that differ in their activation and inhibition patterns (by α s, α i/o, $\beta\gamma$, and so on). To date, nine AC isozymes have been cloned (AC types I–IX); the activities of these AC isozymes seem to be stimulated by $G_{\alpha s}$, although to different extents. These ACs can be categorized according to sequence and functional similarities into five classes: (1) AC-I and -VIII are stimulated by Ca^{2+} /calmodulin; (2) AC-V and -VI are inhibited by low levels of Ca^{2+} ; (3) AC-II, -IV, and -VII are activated by $G_{\beta\gamma}$ subunits in the presence of activated $G_{\alpha s}$ and have been reported to be affected by activation of protein

kinase C; (4) AC-III has been reported to be either stimulated or inhibited by Ca^{2+} /calmodulin in the presence of $G_{\alpha s}$; and (5) AC-IX has thus far been found to be stimulated only by $G_{\alpha s}$ (Mons and Cooper, 1995; Sunahara *et al.*, 1996; Zimmermann and Taussig, 1996).

It was recently shown that acute activation of the m2 receptor inhibits the activity of AC types I and VI and stimulates that of type II, whereas chronic activation of this receptor results in superactivation of AC-VI, with no effect on the AC-I and -II isozymes (Thomas and Hoffman, 1996). D_2 receptor activation also was shown to lead to AC-VI superactivation. However, the effects of acute and chronic activation of inhibitory dopaminergic or muscarinic receptors on other AC isozymes have been for the most part unknown. Interestingly, as we show here for the D_2 and m4 receptors, and similar to what was observed by Avidor-Reiss *et al.* (1997) for the μ -opioid receptor, most of the AC isozymes are affected differentially by acute or chronic agonist activation. AC types I, V, VI, and VIII are inhibited by acute agonist application and exhibit superactivation on chronic treatment, whereas the activities of types II, IV, and VII are stimulated on acute exposure and inhibited on chronic agonist treatment; AC-III was only slightly inhibited by acute agonist application, whereas its activity was further reduced on chronic agonist treatment. Because the AC isozymes differ in the parameters that define their stimulation and inhibition characteristics, the fact that various tissues, brain areas, and cell types are known to vary in their repertoire of AC isozyme populations (Mons and Cooper, 1994, 1995) may offer an explanation to the different regulatory effects on AC activity of $G_{i/o}$ -coupled inhibitory receptor activation depending on the tissues examined. Indeed, whereas $G_{i/o}$ -coupled receptor activation normally inhibits AC activity, it has been shown that opioid (Olianas and Onali, 1995) or inhibitory muscarinic (Olianas and Onali, 1996) receptor activation can stimulate AC activity in the rat olfactory bulb and that cannabinoid receptor activation stimulates AC activity in the globus pallidus and in heart (Hillard *et al.*, 1990; Maneuf and Brothie, 1997). This also may explain the observation of different levels of AC superactivation in various cell types (Puttfarcken and Cox, 1989; McDermott and Sharp, 1995). For example, the COS-7 cells used in this study have been shown to contain at least the AC-VII isozyme (Premont, 1994). However, because AC-VII was shown here not to undergo superactivation, the fact that COS cells were found here to exhibit endogenous AC superactivation implies that at least one of the AC isozymes that does exhibit superactivation (AC-I, -V, -VI, or -VIII) must also be endogenously present in COS cells. The AC-I and VIII isozymes can be eliminated *a priori* because they are activated by Ca^{2+} , and ionomycin was not able to activate AC in nontransfected COS cells (Fig. 2b). It therefore follows that one or both of AC-V and AC-VI must be endogenously present in COS. It would be interesting to examine whether various brain areas also exhibit different patterns of AC superactivation and, if so, whether this can be correlated to localization patterns of the AC isozymes in the brain.

The data obtained here, taken together with previously published results (Avidor-Reiss *et al.*, 1997), indicate that the specific pattern of AC modulation observed here (with transfected COS cells) seems to be a general means of cellular adaptation to the activation of inhibitory receptors. It is

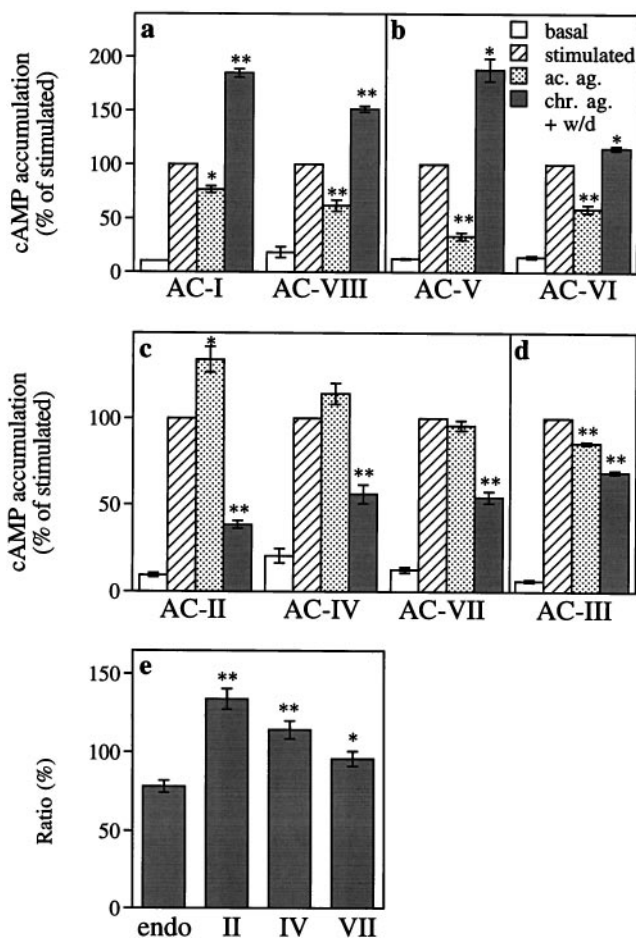


Fig. 7. Acute and chronic m4 muscarinic receptor activation differentially regulates the various AC isozymes. COS-7 cells were transfected with the cDNAs of the indicated AC isozymes together with cDNAs of the m4 muscarinic receptor and the TSH receptor (where necessary). Plasmid concentrations were as described in Experimental Procedures, except in the experiments with AC-II, -IV, and -VII, in which 2 μ g of m4 and 4 μ g of AC plasmids were used. AC was stimulated with 1 μ M ionomycin (a) or 0.1 μ M TSH (b–d), and the amount of cAMP was determined under basal (unstimulated) conditions (□), after stimulation with ionomycin or TSH in the absence of McN-A-343 (▨), after stimulation in the presence of acute 100 μ M McN-A-343 (▤), and after withdrawal (by rapid wash and the addition of 10 μ M atropine) after chronic McN-A-343 (18 hr, 100 μ M) treatment (■). 100% AC activity observed in cells stimulated with either ionomycin (a) or TSH (b–d). e, Ratio (in %) of m4 agonist-stimulated AC activity to control TSH-stimulated activity for endogenous AC (endo) and for AC-II, -IV, and -VII. Data represent the mean \pm standard error of three experiments. *, $p < 0.05$, **, $p < 0.01$ versus stimulated cAMP accumulation (a–d) or versus endogenous AC (e).

conceivable that other cell systems/brain areas (which may differ in their G protein subunits and various signal transduction components) also may exhibit AC isozyme-specific mechanisms for adaptation to inhibitory receptor activation, even though variations in the final result could be expected, depending on the AC isozyme pattern and G protein subunit composition.

In this regard, Thomas and Hoffman (1996) did not find superactivation of AC-I by chronic m2 muscarinic agonist application. Because both m2 and m4 inhibit AC via $G_{i/o}$ proteins, their chronic effects on AC-I could be expected to be similar. Indeed, in preliminary experiments, we observed AC-I superactivation on chronic m2 agonist application in COS cells transfected with the appropriate receptors, although it was much weaker than that produced in m4-transfected cells (data not shown). The difference between our observation of slight AC-I superactivation as opposed to that of Thomas and Hoffman that this isozyme is not superactivated by chronic m2 activation might be accounted for by the difference in the cell lines used in the two studies (HEK 293 cells as opposed to COS in the current study). For example, HEK 293 cells show a high level of background superactivation (in the nontransfected cells) on stimulation with FS (used as an AC stimulant by Thomas and Hoffman), making it more difficult to observe a weak increase in AC activity. Another possible explanation for the difference is the fact that these cell lines can differ in their G_i protein subunit populations. Indeed, $G_{\beta\gamma}$ dimers are known to have a role in AC superactivation (Avidor-Reiss *et al.*, 1996; Thomas and Hoffman, 1996), and we have recently shown that different β subunits display different profiles for activation of AC-II (Bayewitch *et al.*, 1998). Different α_i populations also can affect coupling to various receptor types in these cell lines, as shown, for example, regarding the differential coupling of m2 versus m4 (Migeon *et al.*, 1995).

It should be noted that the transfected m4 receptor is the only muscarinic receptor subtype that will be activated by application of the agonist McN-A-343, even though this ligand is not selective for the m4 receptor, because no muscarinic receptors are endogenously present in COS cells. This agonist was selected for these experiments due to the fact that it does not lead to significant down-regulation of muscarinic receptors (Heldman E and Vogel Z, unpublished observations). On the other hand, although carbachol (the more classically used nonspecific muscarinic agonist) also led to superactivation of AC-VI via activation of m2 (Thomas and Hoffman, 1996), this ligand has been shown to down-regulate all types of muscarinic receptors, including m4 (Maloteaux and Hermans, 1994).

The findings of the presence or absence of superactivation of the various AC isozymes on chronic D_2 receptor activation are of interest from several perspectives. First, in the brain, AC-V is known to be highly expressed in the nucleus accumbens (Glatt and Snyder, 1993; Mons and Cooper, 1994). This region, which is rich in D_2 receptors, is one of the key nuclei in the "dopamine reward pathway" in the brain (Koob, 1996). According to the dopamine reward theory, addiction phenomena would have as a common biochemical denominator a release of dopamine in the nucleus accumbens, which then would exert its effects predominantly via local D_2 receptors (White *et al.*, 1991; Rinaldi and Beninger, 1994). The positive reinforcement generated by the feeling of well-being pro-

duced by this release of dopamine would contribute to the development of dependence on the event that caused the initial release, thus initiating the vicious cycle of the development of addiction. If a drug that augments extracellular dopamine levels (e.g., opiates, which enhance its release, or cocaine, which inhibits its uptake) is present in the system for a long period of time, it is possible to envisage that the biochemical mechanism underlying the short and long term effects of this neurotransmitter could involve modulation of the activity of AC, particularly of the isozyme predominantly present in the nucleus accumbens: AC-V. This also could explain why certain drugs, which are addictive when taken over the long term, can in "naïve" users, who are using the drug for the first time, actually have effects that are diametrically opposed to those observed in chronic abusers (Gulati, 1995).

In this study, we found that chronic activation of the inhibitory m4 muscarinic receptor also led to a similar pattern of superactivation/inhibition of the various AC isozyme types. The finding that this is a characteristic common to several (e.g., m4, D_2 , μ -opioid), if not all, inhibitory G protein-coupled receptors, is of important physiological relevance. For instance, the basis of the action of many pharmaceutical drugs is the activation of inhibitory receptors. To illustrate this point, D_2 dopaminergic agonists such as bromocriptine or pergolide, alone or in conjunction with the dopamine precursor L-DOPA, are routinely used as antiparkinsonian agents (Lieberman and Goldstein, 1985; Gimenez-Roldan *et al.*, 1997). The abrupt discontinuation (withdrawal) of these antiparkinsonian drugs has been reported to lead to the emergence of the neuroleptic malignant syndrome, usually associated with neuroleptic (dopamine antagonist) medications and generally believed to be related to blockage of dopamine receptors in the brain (Olmsted, 1988; Ebadi *et al.*, 1990). It thus is conceivable that the chronic use of drugs that activate inhibitory receptors, even those that are not considered to have addictive properties, could lead to superactivation of AC or, more specifically, of particular AC isozymes. Indeed, one study reports that unilateral lesions of the nigrostriatal dopamine pathway in rats (a model of parkinsonism) enhanced the sensitivity of striatal AC to dopamine stimulation and that this AC hypersensitivity was further enhanced on prolonged L-DOPA administration (Groppetti *et al.*, 1986).

Furthermore, among the downstream effects of the phenomenon of AC superactivation, the augmented cAMP concentration resulting from the increased level of AC activity will affect protein phosphorylation, thereby increasing phosphorylation of, among others, cAMP-responsive element binding protein, which could in turn affect transcription factor regulation (Nestler *et al.*, 1993). It thus is evident that the implications of the chronic use of inhibitory receptor agonists as therapeutic treatments may be more far reaching than they would seem at first glance and that possible indirect effects of such drugs arising from AC regulation should be examined more closely.

Acknowledgments

We are grateful to the following scientists for the kind donation of the indicated plasmids: Dr. Sara Fuchs, Weizmann Institute, Rehovot, Israel (rat D_{2L} dopamine receptor); Dr. Shinji Kosugi, Kyoto University, Kyoto, Japan (rat TSH receptor); Dr. Tom Bonner, National Institute of Mental Health, National Institutes of Health,

Bethesda, MD (human m4 muscarinic receptor); Dr. Alfred Gilman, University of Texas Southwestern Medical Center, Dallas, TX (AC-IV); Dr. Franz-Werner Kluxen, University of Dusseldorf, Dusseldorf, Germany (pXMD1 plasmid and pXMD1-gal); Dr. Thomas Pfeuffer, Heinrich-Heine University, Dusseldorf, Germany (AC-I, AC-II, and AC-V in pXMD1); Dr. Randy Reed, Johns Hopkins School of Medicine, Baltimore, MD (AC-III); and Dr. John Krupinski and Dr. Peter A. Watson, Geisinger Clinic, Danville, PA (AC-VI, AC-VII, and AC-VIII).

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