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Differential Superactivation of Adenylyl Cyclase Isozymes after Chronic Activation of the CB_1 Cannabinoid Receptor[1]

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ABSTRACT

Many types of cells exhibit increased adenylyl cyclase (AC) activity after chronic agonist treatment of $G_{i/o}$-coupled receptors. This phenomenon, defined as AC superactivation or sensitization, has mostly been studied for the opioid receptors and is implicated in opiate addiction. Here we show that this phenomenon is also observed on chronic activation of the CB_1 cannabinoid receptor. Moreover, using COS-7 cells cotransfected with CB_1 receptor and individual AC isozymes, we could show selective superactivation of AC types I, III, V, VI, and VIII. The level of superactivation was dependent on the concentration of agonist and time of agonist exposure and was not dependent on the AC stimulator used. No superactivation of AC types II, IV, or VII was observed in COS-7 cells cotransfected with CB_1. The superactivation of AC type V was abolished by pretreatment with pertussis toxin and by cotransfection with the carboxy terminus of β-adrenergic receptor kinase, which serves as a scavenger of $G_{\beta\gamma}$ dimers, implying a role for the $G_{i/o}$ proteins and especially $G_{\beta\gamma}$ dimers in the cannabinoid-induced superactivation of AC.

Two cannabinoid receptor subtypes, CB_1 and CB_2, have been cloned to date (Matsuda et al., 1990; Munro et al., 1993). Both receptors belong to the seven transmembrane domain GTP-binding protein (G protein)-coupled receptor superfamily. Whereas CB_2 is located in various immune cells and not in brain, the CB_1 receptors are found in many brain regions, including the hippocampus, cortex, caudate-putamen, globus pallidus, substantia nigra, and cerebellum (Gérard et al., 1991; Tsou et al., 1998). Activation of the CB_1 receptor with cannabinoid agonists was shown to lead to inhibition of adenylyl cyclase (AC) (Matsuda et al., 1990; Vogel et al., 1993; Howlett, 1995), inhibition of voltage-gated N- and Q-type calcium channels, and activation of voltage-sensitive potassium channels (Mackie et al., 1995). All these effects were shown to be mediated through pertussis toxin (PTX)-sensitive G proteins.

Chronic cannabinoid treatment results in the development of behavioral tolerance and dependence (Abood et al., 1993; de Fonseca et al., 1994). It was reported that chronic cannabinoid exposure in rats leads to down-regulation of cannabinoid receptors, which seems to parallel the tolerance phenomena observed at the neurobehavioral level (Oviedo et al., 1993; de Fonseca et al., 1994; Fan et al., 1996). In addition, chronic Δ^9-THC treatment time-dependently decreases WIN 55,212–2-induced GTPγS binding to various rat brain areas, demonstrating that such chronic treatment modulates cannabinoid receptor-G protein-effector signaling (Breivogel et al., 1999). Indeed, Dill and Howlett (1988) have shown that in N18TG2 neuroblastoma cells, chronic cannabinoid exposure leads to a reduction in cannabinoid-induced AC inhibition, suggesting that the observed cannabinoid tolerance may be due to alterations in the cannabinoid signal transduction pathways.

In this regard, we and others have shown that chronic activation of several $G_{i/o}$-coupled receptors (including the μ-, δ-, and κ-opioid; m_4-muscarinic; D_2-dopaminergic; and α_2-adrenergic) leads to an increase in cAMP accumulation, rather than the reduction in cAMP observed on acute activation of these receptors (Sharma et al., 1975; Thomas and Hoffman, 1987, 1996; Avidor-Reiss et al., 1995a,b; Nevo et al., 1998). This phenomenon has been referred to as AC superactivation, or AC sensitization, and in the case of the opioid receptors, is believed to play an important role in the development of drug tolerance and dependence (Sharma et al., 1975; Nestler and Aghajanian, 1997). However, until recently there was no knowledge regarding the induction of AC superactivation by chronic cannabinoid exposure. Moreover, nine AC isozymes have recently been cloned. These isozymes differ in their properties, including their capacity to be inhibited or stimulated by G protein α_i, α_s, and $\beta\gamma$ sub-

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ABBREVIATIONS: G protein, GTP-binding protein; AC, adenylyl cyclase; βARK-C, carboxy terminus of β-adrenergic receptor kinase; DMEM, Dulbecco's modified Eagle's medium; FAF-BSA, fatty acid-free bovine serum albumin; PK, protein kinase; PTX, pertussis toxin; TSH, thyroid-stimulating hormone; CHO, Chinese hamster ovary; CHO-CB_1, CHO cells expressing rat CB_1.

units, as well as by protein kinase (PK)C, and Ca^{2+}/calmodulin (Mons and Cooper, 1995; Sunahara et al., 1996; Simonds, 1999). We have previously shown that acute agonist activation of the cannabinoid receptors leads to a different response according to the AC isozyme studied, and whereas AC types I, V, VI, and VIII were inhibited, the activities of AC types II, IV, and VII were stimulated by acute cannabinoid receptor activation (Rhee et al., 1998). In this study we have studied the regulation of the various AC isozymes after chronic exposure of the CB_1 receptor to cannabinoid agonists.

Experimental Procedures

Materials. [^{3}H-2]adenine (18.0 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). The phosphodiesterase inhibitors, 1-methyl-3-isobutylxanthine and RO-20–1724 were purchased from Calbiochem (La Jolla, CA). Forskolin, cAMP, fatty acid-free bovine serum albumin (FAF-BSA), and thyroid-stimulating hormone (TSH) were obtained from Sigma (St. Louis, MO). PTX was purchased from List Biological Laboratories (Campbell, CA). The cannabinoid agonist, *R*(+)-WIN55,212–2 {*R*-(+)-(2,3-dihydro-5-methyl-3-[(4-morpholinyl) methyl] pyrolo-(1,2,3-de]-1,4-benzoxazin-6-yl)(1-naphtalenyl)methanone monomethanesulfonate}, was provided by Dr. R. Mechoulam (Jerusalem, Israel). The cannabinoid antagonist SR 141716A was provided by Research Triangle Institute (Research Triangle Park, NC). Tissue culture reagents were obtained from Life Technologies (Gaithersburg, MD).

Plasmids. β-gal cDNA in pXMD1 vector, the plasmid encoding a chimera of CD8 and the carboxy terminus of β-adrenergic receptor kinase (CD8-βARK-C), and the plasmids containing AC isozymes I to VIII and rat wild-type TSH receptor cDNAs were described previously (Avidor-Reiss et al., 1996, 1997; Rhee et al., 1998). The pSVL-CB_1 plasmid containing human CB_1 cDNA (Gérard et al., 1991) was provided by Dr. M. Parmentier (Bruxelles, Belgium).

Cell Cultures. COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere consisting of 5% CO_2 and 95% air, at 37°C. Chinese hamster ovary (CHO) cells expressing rat CB_1 (CHO-CB_1) were obtained from Dr. T. I. Bonner (National Institutes of Health, Bethesda, MD), and were cultured as described previously (Vogel et al., 1993) in DMEM supplemented with 8% fetal calf serum, nonessential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere consisting of 5% CO_2 and 95% air, at 37°C.

Transfection of COS Cells. Twenty-four hours before transfection, a confluent 10-cm plate of COS-7 cells was trypsinized and split into five 10-cm plates. The cells were transfected, using the DEAE-dextran chloroquine method (see Rhee et al., 1998), with 2 μg/plate of human CB_1 cDNA and 1 μg/plate either of one of the AC isozyme cDNAs or of pXMD1-gal (for mock DNA transfection), and, where indicated, with 1 μg/plate of TSH receptor cDNA. Twenty-four hours 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 to 80%, as determined by staining for β-galactosidase activity.

AC Activity. The assay was performed as described previously (Vogel et al., 1993; Rhee et al., 1998). In brief, cells cultured in 24-well plates were incubated for 2 h with 0.25 ml/well fresh growth medium containing 5 μCi/ml of [2-^{3}H]adenine. This medium was replaced with DMEM containing 20 mM HEPES (pH 7.4), 2 mg/ml FAF-BSA, and the phosphodiesterse inhibitors RO-20–1724 (0.5 mM) and 1-methyl-3-isobutylxanthine (0.5 mM). Cannabinoids diluted in 20 mg/ml FAF-BSA were then added. AC activity was stimulated in the presence or absence of cannabinoids by the addition of either forskolin or TSH (in the latter case, the assayed cells were cotransfected with the TSH receptor). After 10 min at 37°C, the medium was removed and the reaction terminated by adding to the cell layer 1 ml of 2.5% perchloric acid containing 0.1 mM unlabeled cAMP. Aliquots of 0.9 ml of the acidic extract were neutralized with 100 μl of 3.8 M KOH and 0.16 M K_2CO_3 and applied to a two-step column separation procedure, after which the [^{3}H]cAMP was eluted into scintillation vials and counted. Unless otherwise described, background levels (cAMP accumulation in the absence of stimulator) were subtracted from all values. Cannabinoids were added together with the forskolin or TSH for the 10-min assay period (acute treatment) or incubated with the cells for 18 h (or for the times indicated) before the 10-min assay (started by the addition of forskolin or TSH) (chronic treatment). Withdrawal of the chronically applied cannabinoid agonist was achieved by the addition of 1 μM of the selective CB_1 antagonist SR141716A to the assay medium. In experiments using PTX, it was added to the cultures, at 100 ng/ml, 20 h before the addition of [^{3}H]adenine, and was replenished on the addition of [^{3}H]adenine. All experiments were performed in triplicate.

Results

Effects of Acute and Chronic Cannabinoid Treatments on AC Activity in the CHO-CB_1 Cell Line. As shown in Fig. 1, the cannabinoid agonist WIN55,212–2, applied at 1 μM (for the duration of the 10-min AC assay), inhibited the forskolin-stimulated AC activity in CHO-CB_1 cells by 50%. The CB_1-selective antagonist, SR141716A (Rinaldi-Carmona et al., 1994), at 1 μM, completely reversed the inhibitory activity of WIN55,212–2. It should be noted that SR141716A by itself, at concentrations of 0.1 nM to 1 μM, did not affect the activity of AC in CHO-CB_1 cells (data not shown), neither did it affect the activity of AC-V in COS cells cotransfected with AC-V and human CB_1 cDNA (Fig. 3c). Moreover, control experiments showed that WIN55,212–2 had no effect on AC activity in parental CHO cells not transfected with CB_1 (data not shown), demonstrat-

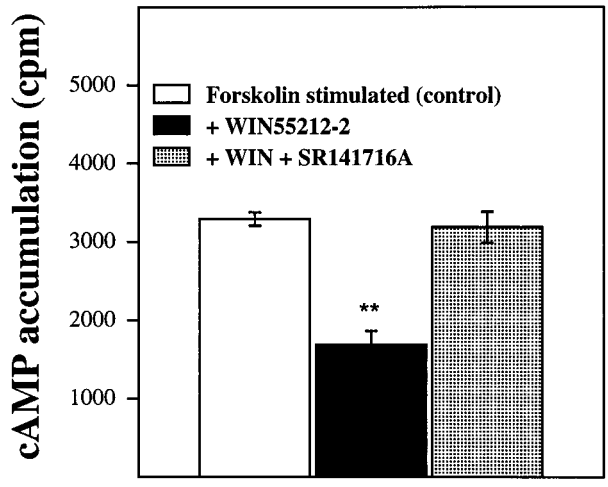

Fig. 1. Acute treatment with the agonist WIN55,212–2 inhibits forskolin-stimulated cAMP accumulation, and this is reversed by the antagonist, SR141716A. CHO-CB_1 cells were prelabeled for 3 h with [^{3}H]adenine. The endogenous AC activity in these CHO cells was activated with 1 μM forskolin for 10 min (control). Parallel cultures were activated with forskolin in the presence of 1 μM WIN55,212–2 (WIN) or 1 μM WIN55,212–2 together with 1 μM SR141716A. The data show the amount of [^{3}H]cAMP after forskolin stimulation and represent the means ± S.E. of triplicate determinations of a representative experiment of three experiments which gave similar results. ***$P < .001$, significant decrease compared with control forskolin-stimulated cAMP accumulation.

ing that the effect of WIN55,212–2 is mediated via the CB_1 receptor.

Contrary to the inhibition of AC activity observed on acute exposure to opiate agonists, an increase in PGE_1- or forskolin-stimulated AC activity has been reported after chronic opiate exposure in various cell lines containing opioid receptors (e.g., neuroblastoma × glioma hybrid NG108–15 cells and human neuroblastoma SH-SY5Y cells), as well as in cells (e.g., CHO or COS) transfected with opioid receptors. This increase in AC activity was particularly evident on removal of the inhibitory agonist either by wash or by the addition of antagonist (Sharma et al., 1975; Ammer and Schulz, 1993; Avidor-Reiss et al., 1995a,b, 1997). A similar phenomenon was also observed with several other receptor agonists, including the m_2- and m_4-muscarinic, α_2-adrenergic, and D_2-dopaminergic receptors (Avidor-Reiss et al., 1996; Thomas and Hoffman, 1996; Nevo et al., 1998). We examined whether a similar effect could be observed for the cannabinoids and the CB_1 receptor. For this purpose, either CHO cells transfected with CB_1, or COS-7 cells cotransfected with CB_1 and AC-V, were treated for 18 h with WIN55,212–2, and the agonist rapidly withdrawn just before the AC assay (by rapid wash and the addition of 1 μM of the antagonist SR141716A). Figure 2 shows the results of such an experiment in CHO-CB_1 cells. It shows that both acute (10-min) and chronic (18-h) exposure of the cells to 1 μM WIN55,212–2 lead to inhibition of the endogenous AC activity present in CHO cells. However, the removal of the agonist after the chronic exposure was found to lead to superactivation of AC activity in CHO-CB_1 by 65% compared with control untreated cells.

Effects of Acute and Chronic Cannabinoid Treatments in COS Cells Transfected with CB_1 and AC-V. A more detailed experiment is shown in Fig. 3 using COS cells transfected with CB_1 and AC type V. Figure 3a shows the inhibition of forskolin-stimulated AC-V activity by increasing concentrations of WIN55,212–2 applied acutely. The data shows that WIN55,212–2 inhibits AC-V activity (via CB_1 activation) with an EC_{50} of 18.6 ± 6.9 nM. A significant increase in AC-V activity (superactivation) was observed in cells treated for 18 h with 1 to 1000 nM WIN55,212–2 followed by withdrawal of the agonist before the assay (Fig. 3b). This increase in forskolin-stimulated AC-V activity was dependent on the concentration of WIN55,212–2 used during the chronic treatment. A 2-fold superactivation was obtained when the cells were pretreated with 0.1 to 1 μM WIN55,212–2, whereas almost no superactivation was observed when the cells were pretreated with 1 nM WIN55,212–2. As described above, efficient withdrawal of the inhibitory cannabinoid agonist was achieved by quickly washing the cells and adding 1 μM of the antagonist SR141716A. In the experiment shown in Fig. 3c, we have titrated the concentration of antagonist needed to obtain the maximal level of superactivation, and found it to be in the range of 0.1 to 1 μM. We have therefore used a concentration of 1 μM antagonist in all other experiments. In addition, this experiment shows that the presence of SR141716A during the AC assay without preincubation with WIN55,212–2 had no effect on AC activity. Thus, it is the chronic activation with the agonist and not the antagonist that leads to AC superactivation.

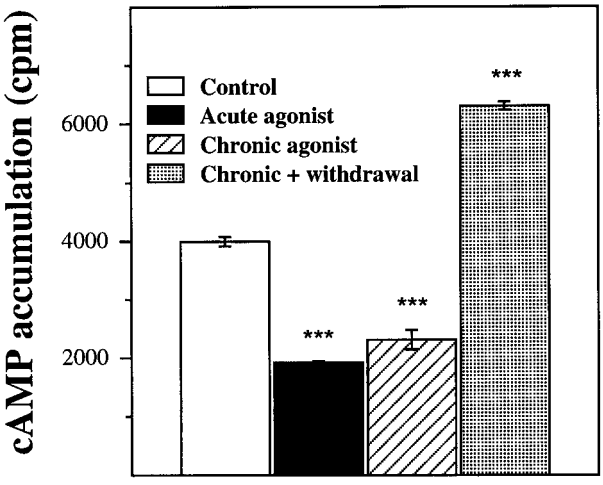

Fig. 2. Effects of acute and chronic cannabinoid receptor activation on forskolin-stimulated cAMP accumulation in CHO-CB_1 cells. Control, 10-min stimulation with 1 μM forskolin. Acute agonist, acute treatment with 1 μM WIN55,212–2 for the 10-min AC assay. Chronic agonist, exposure to 1 μM WIN55,212–2 for 18 h followed by AC assay in the presence of this agonist. Chronic + withdrawal, cells were incubated for 18 h with 1 μM WIN55,212–2, followed by a rapid wash and the addition of 1 μM the antagonist, SR141716A, just before the AC assay. The data represent the means ± S.E. of a representative experiment of three experiments that gave similar results. ***$P < .001$, significantly different from control forskolin-stimulated cAMP accumulation.

Figure 4a shows that the development of AC-V superactivation in the transfected cells is time-dependent. It shows that the cells had to be exposed to 1 μM WIN55,212–2 for ca. 12 h to reach the maximal level of AC superactivation. This level is still maintained after 24-h exposure to the drug. Moreover, although chronic agonist treatment leads to AC superactivation, prolonged exposure to the antagonist after withdrawal of the chronic agonist results in a gradual reduction in AC superactivation (Fig. 4b). The half-life of the disappearance of AC superactivation was ca. 35 min, and after 2 to 4 h in the presence of antagonist, the levels of cAMP in the cells returned to a level very close to their original values (i.e., to that obtained under normal, untreated conditions). It is therefore apparent that AC superactivation depends on sustained activation of the receptor and that the cells maintain the ability to return to the original levels of AC activity and cAMP concentration as a function of time after the withdrawal of the chronically applied cannabinoid agonist.

Both the inhibition (by acute agonist exposure) and superactivation (by chronic agonist exposure followed by agonist withdrawal) could be observed not only when the AC was stimulated with forskolin, but also when the cells were stimulated with TSH (which increases AC activity via the cotransfected TSH receptor and activation of $G_{\alpha s}$), indicating that both effects of the cannabinoids are not dependent on the method used to stimulate AC activity (Fig. 5). In addition, this figure shows that a significant desensitization to the inhibitory effect of WIN55,212–2 could be observed after 18-h exposure to this drug.

Both inhibition and superactivation of AC activity by acute and chronic cannabinoid treatments were dependent on the presence of CB_1 receptor in the treated cells (data not shown), suggesting that these phenomena require receptor signaling. Moreover, pretreatment of the cells with PTX prevented cannabinoid inhibition of AC-V, and blocked the SR141716A-induced increase in cAMP accumulation in the

chronically WIN55,212–2-treated cells (Fig. 6b). This finding demonstrates that the superactivation is dependent on the chronic activation of PTX-sensitive G_i/G_o proteins. PTX, via the ADP ribosylation of the $G_{\alpha i/o}$ subunits, interferes with the dissociation of $G_{\beta\gamma}$ subunits from the heterotrimeric G protein complex. To study the role of $G_{\beta\gamma}$ dimers in the superactivation of AC-V by chronic cannabinoid exposure, we have cotransfected the cells with cDNA encoding a chimera of CD8 (to allow anchoring to the membrane) and βARK-C (which contains a $G_{\beta\gamma}$-binding domain and serves as a $\beta\gamma$ scavenger; Crespo et al., 1994). We found that in cells transfected with CD8-βARK-C, the forskolin-stimulated activity of AC-V was

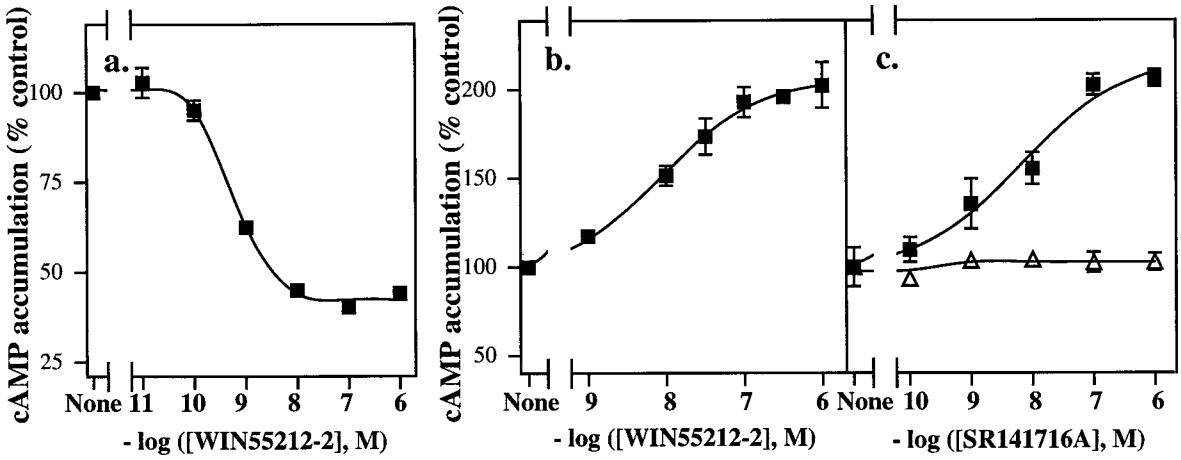

Fig. 3. Effects of acute and chronic exposure to WIN55,212–2 on cAMP accumulation in COS-7 cells transfected with human CB_1 and AC type V. a, inhibition of forskolin-stimulated cAMP accumulation by various concentrations of WIN55,212–2. b, induction of AC superactivation after chronic (18-h) exposure to various concentrations of WIN55,212–2 and withdrawal by rapid wash and the addition of 1 μM SR141716A at the start of the AC assay. c, effect of various concentrations of SR141716A on the induction of AC superactivation in 1 μM WIN55,212–2 (18 h)-pretreated cells (■) and in cells not pretreated with WIN55,212–2 (△). The latter curve shows that acute treatment with SR141716A alone does not affect cAMP accumulation. Data represent the means ± S.E. of three experiments.

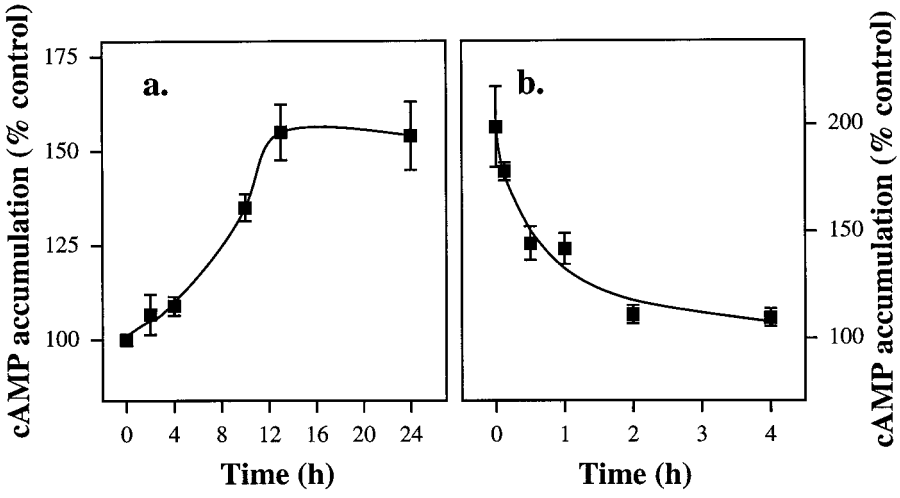

Fig. 4. Time course of AC-V superactivation by chronic WIN55,212–2 pretreatment and its reversal by prolonged incubation with SR141716A. a, COS cells transfected with CB_1 and AC-V were incubated with 1 μM WIN55,212–2 for the indicated periods, after which the WIN55,212–2 was quickly withdrawn by wash and addition of 1 μM SR141716A just before the AC assay. b, transfected COS cells were incubated with 1 μM WIN55,212–2 for 18 h followed by a rapid wash and incubation with 1 μM SR141716A for the times indicated just before the assay of forskolin-stimulated AC activity. The data represent the mean ± S.E. of three experiments.

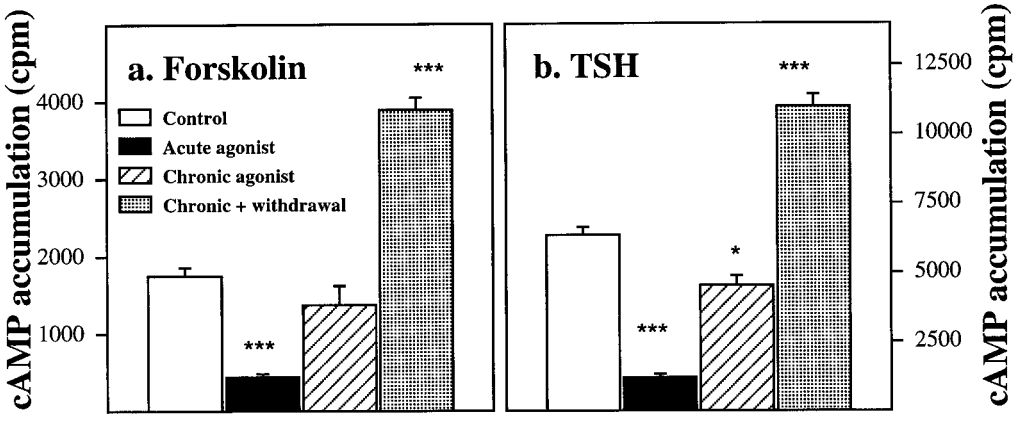

Fig. 5. Superactivation of AC-V by chronic activation of CB_1 is not dependent on the mechanism used to stimulate AC activity (forskolin or TSH). COS-7 cells transiently cotransfected with CB_1, TSH receptor, and AC-V were treated as described in Fig. 2, except that stimulation of AC was performed with either 1 μM forskolin (a) or 0.1 μM TSH (b). The data represent the mean ± S.E. of a representative experiment of three experiments that gave similar results. $^{**}P < .01$, $^{***}P < .001$, significantly different from control forskolin- or TSH-stimulated cAMP accumulation.

slightly elevated, whereas the ability of chronic WIN55,212–2 to induce AC superactivation was completely abolished. The inhibition of AC-V by the acute cannabinoid treatment was much less affected by the $G_{\beta\gamma}$ scavenger (Fig. 6c). These results suggest that $G_{\beta\gamma}$ dimers have a role in the regulation of AC-V activity and play an important role in the phenomenon of AC superactivation. A similar result was obtained regarding the superactivation of AC type V after chronic μ-opioid receptor activation and AC-VI after chronic D_2-dopaminergic receptor activation (Avidor-Reiss et al., 1996; Thomas and Hoffman, 1996), demonstrating the generality of the role of $G_{\beta\gamma}$ in the superactivation phenomenon.

As described in the introduction, nine distinct isozymes of AC have been identified to date (Sunahara et al., 1996; Simonds, 1999). We have previously shown that these isozymes differ in their response to acute cannabinoid activation (Rhee et al., 1998). Here, we show that they also differ in their response to chronic cannabinoid treatment (Fig. 7). We found that in addition to AC-V, acute activation of CB_1 leads to inhibition of AC-I, -III, -VI, and -VIII, whereas it produces stimulation of AC-II, -IV, and -VII. On the other hand, chronic activation followed by withdrawal of the inhibitory agonist leads to superactivation of AC-I, -III, -V, -VI, and -VIII, but to inhibition of AC-II, -IV, and -VII. In this experiment, cells transfected with AC-I, -III, -VI, and -VIII were stimulated with forskolin, whereas cells transfected with AC-II, -IV, and -VII were stimulated with TSH, because this second group of AC isozymes does not respond well to forskolin stimulation. However, as previously shown for the μ-opioid receptor (Avidor-Reiss et al., 1997) and for the acute activation of CB_1- and CB_2-cannabinoid receptors (Rhee et al., 1998), the results are not affected by the AC stimulant used (e.g., forskolin or TSH). Taken together, the results show that AC-I, -III, -V, -VI, and -VIII exhibit acute inhibition and chronic-induced superactivation, whereas AC-II, -IV, and -VII show acute activation and chronic-induced inhibition of AC activity.

Discussion

Relatively little is known about the effects of chronic cannabinoid exposure on cannabinoid signal transduction. On the other hand, there is much more information available regarding the in vivo and in vitro effects of chronic exposure to opiates. For example, chronic opiate exposure has been shown to lead to up-regulation of the cAMP pathway. This up-regulation involves superactivation of AC (a phenomenon first discovered in cultured NG108–15 cells by Sharma et al., 1975), increased immunoreactivity of AC types I and VIII, and increased activity of cAMP-dependent PKA (reviewed by Nestler and Aghajanian, 1997). This up-regulation of the cAMP pathway would oppose the continuous opiate agonist inhibition and thereby represent a form of physiological tolerance. On removal of the opiate agonist, the up-regulated cAMP pathway would become even more pronounced and contribute to the features of dependence and withdrawal (Nestler and Aghajanian, 1997).

In this study, we have used CHO and COS-7 cells expressing the rat and human CB_1 cannabinoid receptor to gain information on the regulation of AC by chronic cannabinoid

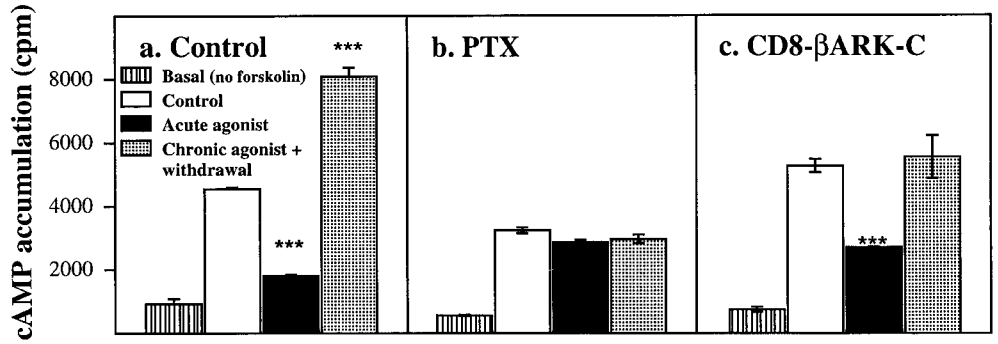

Fig. 6. Effects of PTX and the $G_{\beta\gamma}$ scavanger CD8-βARK-C on the inhibition and superactivation of forskolin-activated AC-V after acute and chronic cannabinoid treatments. a, COS-7 cells transfected with CB_1 and AC-V were treated as described in Fig. 2 and assayed for AC activity. b, cells were pretreated with 100 ng/ml of PTX for 18 h before the AC assay. c, COS-7 cells were cotransfected with the $\beta\gamma$ scavenger CD8-βARK-C. The data represent the mean ± S.E. of a representative experiment of three experiments that gave similar results. ***$P < .001$, significantly different from control forskolin-stimulated cAMP accumulation.

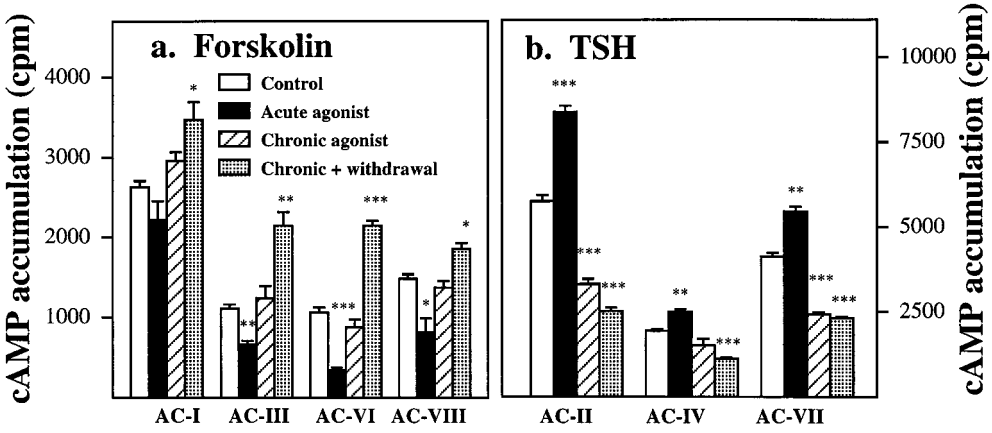

Fig. 7. Effects of various AC isozymes on forskolin- or TSH-stimulated cAMP accumulation. COS-7 cells were transfected with CB_1, with TSH receptor, and with the indicated AC isozymes. The transfected cells were treated with the cannabinoid agonist WIN55,212–2 as described in Fig. 2. AC activity was stimulated with either 1 μM forskolin (a) or 0.1 μM TSH (b). The data represent the mean ± S.E. of a representative experiment of three experiments that gave similar results. *$P < .05$, **$P < .01$, ***$P < .001$, significantly different from control forskolin- or TSH-stimulated cAMP accumulation.

agonist activation. We have demonstrated that the cannabinoid receptor-transfected cells are able to show different patterns of AC regulation, depending on the type of agonist treatment (acute versus chronic) and the type of AC isozyme involved. Most, if not all, of the cannabinoid agonists are very hydrophobic and cannot be easily washed away from the receptor. The availability of the CB_1 antagonist, SR141716A, which binds to the receptor selectively and with high affinity, has allowed us to study the regulation of AC after withdrawal of the cannabinoid agonist.

In this regard, it has been reported that SR141716A can act as an inverse agonist of CB_1 in several biological receptor assays, including GTPγS binding (Landsman et al., 1997), and mitogen-activated protein kinase and AC activity (Bouaboula et al., 1997). However, in the present study, we could not find any hints for inverse agonism of SR141716A on AC activity in either CHO or COS cells (see Figs. 1 and 3; see also Glass and Felder, 1997; Breivogel et al., 1998).

The fact that a different repertoire of AC isozymes could be expressed in a given cell line (Premont, 1994) and that AC isozymes are differentially distributed in various brain regions (Mons and Cooper, 1995), as well as the fact that these ACs are affected differently by forskolin, PKC, and Ca^{2+}, and by activation of hormone receptors (Sunahara et al., 1996; Simonds, 1999), may afford an explanation to the complex effect of cannabinoid receptor activation in the various cells studied, as well as in different areas of the central nervous system. It has been shown that contrary to the usual inhibition of AC, agonist activation of CB_1 in the absence of forskolin can lead to increased cAMP accumulation in globus pallidus slices (Maneuf and Brotchie, 1997). Similarly, Δ^9-THC at micromolar concentrations was found to increase the isoproterenol stimulation of AC in rat cardiac ventricular membranes (Hillard et al., 1990). Part of this AC activation could be via $G_{\alpha s}$, as suggested by Glass and Felder (1997) and Hillard et al. (1990). However, the composition of AC isozymes in the particular cells or tissues examined has been clearly shown to determine whether AC activity will be inhibited or activated by cannabinoids and other $G_{i/o}$-coupled receptor agonists (Mons and Cooper, 1995; Avidor-Reiss et al., 1997; Rhee et al., 1998).

All nine mammalian AC isozymes identified to date seem to be stimulated by $G_{\alpha s}$ as well as by forskolin, but to different degrees (Sunahara et al., 1996; Simonds, 1999). These isozymes can be categorized into six distinct classes based on their sequence and differential regulation by $G_{\alpha i/o}$ and $G_{\beta\gamma}$, as well as by PKs (PKA, PKC, and calmodulin kinase), and Ca^{2+} itself (Mons and Cooper, 1995; Bayewitch et al., 1998; Simonds, 1999): 1) AC-I is stimulated by Ca^{2+}/calmodulin, and is inhibited by $G_{\beta\gamma}$ subunits; 2) AC-VIII is also stimulated by Ca^{2+}/calmodulin, although its regulation by $\beta\gamma$ is not yet known; 3) AC-II, -IV, and -VII are activated by $G_{\beta\gamma}$; 4) AC-V and -VI are inhibited by $G_{\beta\gamma}$ as well as by low levels of Ca^{2+}; 5) AC-III is stimulated by a high concentration of Ca^{2+}/calmodulin in the presence of $G_{\alpha s}$, and has been reported to be unaffected by $G_{\beta\gamma}$ subunits (Tang and Gilman, 1991); and 6) AC-IX has thus far been found to be affected only by $G_{\alpha s}$. Exploring the nature of the AC isozymes that undergo superactivation versus those that do not, it seems that the superactivation correlates with the capacity of the isozyme to be inhibited by α_i, although there is still debate regarding the sensitivity of AC-VIII to α_i (see Nielsen et al., 1996). The other alternative is that the AC isozymes that undergo superactivation are those that can be inhibited by $G_{\beta\gamma}$, as this group now includes AC-I, -V, -VI, and probably AC-VIII (Mons and Cooper, 1995; Bayewitch et al., 1998). However, as described above, AC-III has not as yet been reported to be inhibited by $G_{\beta\gamma}$ (Tang and Gilman, 1991).

It is of interest to note that the desensitization observed after chronic exposure of COS cells transfected with either AC-I, -V, -VI, or -VIII (see Figs. 5 and 7) was much more evident than that observed with chronically treated CHO-CB_1 cells (see Fig. 2). This difference could possibly be attributed to the difference in the species of the CB_1 receptor (human versus rat), or to the fact that CHO cells contain both AC-VI and -VII (Varga et al., 1998), which, as can be seen from the results of Fig. 7, respond in opposite directions after chronic exposure to the cannabinoid agonist.

The superactivation of several AC isozymes on chronic cannabinoid exposure is of interest from several perspectives. First of all, in the brain, AC-V is known to be highly expressed in the nucleus accumbens (Glatt and Snyder, 1993; Mons and Cooper, 1995). This region also has high amounts of the CB_1 receptor (Tsou et al., 1998), and is one of the key nuclei in the "dopamine reward pathway" in the brain (Koob et al., 1998). Although the action of cannabinoids on dopaminergic transmission has been contradictory (Chen et al., 1990; Tanda et al., 1997; Diana et al., 1998), the reinforcing properties of cannabinoid agonists may be mediated by their action on the mesolimbic dopamine system (which projects from the ventral tegmental area to the nucleus accumbens). In this regard, it has recently been reported that the cannabinoids Δ^9-THC and WIN55,212–2 increase extracellular dopamine concentrations selectively in the shell of the nucleus accumbens, and that the antagonist, SR141716A, prevents these effects of the cannabinoid agonists (Tanda et al., 1997). In agreement with this, it has been shown that SR141716A precipitated an intense behavioral withdrawal syndrome in rats chronically treated with Δ^9-THC, and that this withdrawal from chronic cannabinoid administration reduced dopaminergic transmission in the limbic system (Diana et al., 1998).

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