Regulation of adenylate cyclase type VIII splice variants by acute and chronic $G_{i/o}$ -coupled receptor activation

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We previously reported that acute agonist activation of $G_{i/o}$ -coupled receptors inhibits adenylate cyclase (AC) type VIII activity, whereas agonist withdrawal following chronic activation of these receptors induces AC-VIII superactivation. Three splice variants of AC-VIII have been identified, which are called AC-VIII-A, -B and -C (with AC-VIII-B missing the glycosylation domain and AC-VIII-C lacking most of the C_{1b} area). We report here that AC-VIII-A and -B, but not -C, are inhibited by acute μ -opioid and dopaminergic type D_2 receptor activation, indicating that the C_{1b} area of AC-VIII has an important role in AC inhibition by $G_{i/o}$ -coupled receptor activation. On the other hand the glycosylation sites in AC-VIII did not play a role in AC-VIII regulation. Although AC-VIII-A and -C differed in their capacity to be inhibited by acute agonist exposure, agonist withdrawal

after prolonged treatment led to a similar superactivation of all three splice variants, with no significant change in AC-VIII expression. AC-VIII superactivation was not affected by pre-incubation with a cell permeable cAMP analogue, indicating that the superactivation does not depend on the agonist-induced reduction in cAMP levels. The superactivated AC-VIII-A, -B and -C were similarly re-inhibited by re-application of agonist (morphine or quinpirole), returning the activity to control levels. These results demonstrate marked differences in the agonist inhibition of the AC-VIII splice variants before, but not after, superactivation.

Key words: adenylate cyclase type VIII, cAMP, dopamine receptor, G-protein-coupled receptor, opiate receptor, superactivation.

INTRODUCTION

Stimulation of seven-transmembrane domain inhibitory receptors activates $G_{i/o}$ proteins, as a result of which the $G\alpha_{i/o}$ subunit exchanges its GDP for GTP, and both $G\alpha_{i/o}$ and $G\beta\gamma$ dimers become able to interact with their effectors. For example, the released $G\alpha_{i/o}$ subunits and the $G\beta\gamma$ dimers were shown to interact with adenylate cyclase (AC), leading to its inhibition or stimulation, depending on the AC isoenzyme involved. In this regard, nine AC isoenzymes have been cloned to date and have been shown to differ in their characteristics, i.e. sensitivity to $G\alpha_{i/o}$, $G\beta\gamma$, Ca^{2+} and protein kinase C (for reviews, see [1–3]). Using cells transfected with individual AC isoenzymes, we and others have shown that acute exposure to agonists of $G_{i/o}$ -coupled receptors [e.g. μ -opioid, m_4 (muscarinic type 4), D_2 (dopaminergic type 2), CB_1 (cannabinoid type 1)] inhibits the activity of AC types I, V, VI and VIII, whereas it stimulates the activity of AC types II, IV and VII [2,4–8].

Withdrawal of the $G_{i/o}$ -coupled receptor agonist after chronic receptor activation has been shown to affect the activities of the various AC isoenzymes in an opposite manner as compared with the effect of acute activation. For example, such treatment has been shown to lead to an increase in the activities of AC types I, V, VI and VIII, a phenomenon referred to as AC superactivation or supersensitization [4,5,7,9–12]. On the other hand, chronic exposure to $G_{i/o}$ -coupled receptor agonists followed by their withdrawal led to inhibition of the activities of AC types II, IV and VII [5,7,10].

AC superactivation, originally observed in several cell lines and neuronal cells, has been suggested to represent a general means of cellular adaptation to the activation of inhibitory receptors, and to play an important role in the development of opiate addiction [9,13–16].

The first and best studied cell line was NG108-15 neuroblastoma $\times$ glioma hybrid cells, where chronic opiate, α_2 -adrenergic and muscarinic activation were shown to lead to AC superactivation [9,13,16,17]. The exact repertoire of AC isoenzymes in NG108-15 cells is not known. These cells were shown to contain a Ca^{2+} -stimulated AC activity [18] and AC-VI, but not AC-I or -II [19]. It is thus possible that these cells contain the other Ca^{2+} -stimulatable AC isoenzyme, namely AC-VIII. Moreover, in morphine-treated rats, AC superactivation was observed in the locus coeruleus [20]. This area contains AC-VIII, and it was suggested that increased expression of this AC isoenzyme is involved in opiate addiction [21,22]. Increased expression of AC-VIII was also described in several other brain areas upon repeated cocaine administration and in brains of alcoholics [23,24]. Thus AC-VIII seems to be an important target for regulation by addictive drugs.

Three splice variants of AC-VIII have been described: AC-VIII-A, -B and -C [25]. The original full-length AC-VIII [26] is referred to as the AC-VIII-A form. AC-VIII-B differs from AC-VIII-A by a deletion of 30 amino acids in the extracellular domain between the ninth and tenth putative transmembrane domains, resulting in the loss of two N-linked glycosylation sites and of the capacity of this molecule to be glycosylated, but no difference has been noted in its regulation by Ca^{2+} [25]. In this regard, the glycosylation of AC-VI was shown to play an important role in the $G_{i/o}$ -coupled receptor inhibition of this isoenzyme, whereas glycosylation of AC-IX did not show this effect [27,28]. AC-VIII-C differs from AC-VIII-A by a deletion of 66 amino acids in the non-conserved part of the middle intracellular domain (the C_{1b} domain of AC-VIII), resulting in a loss of most of this domain. It is interesting to note that the C_{1b} domain in AC-V was shown to contribute to the affinity of $G\alpha_i$ to the AC molecule [29]. However, the

Abbreviations used: AC, adenylate cyclase; CHO, Chinese-hamster ovary; 8-CPT-cAMP, 8-(4-chlorophenylthio)-3',5'-cAMP; DMEM, Dulbecco's modified Eagle's medium; D_2 receptor, dopaminergic type 2 receptor; D_2L receptor, long form of the D_2 receptor; FS, forskolin; IBMX, isobutylmethylxanthine; PTX, pertussis toxin.

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C_{1b} region is highly variable among the different AC isoforms and could have different functions in the different isoenzymes. Thus the two AC-VIII splice variants (B and C) offer natural deletion mutants of AC-VIII-A and allow for the analysis of the regulatory roles of these parts of the AC molecule in the acute inhibition and chronic-induced superactivation of the AC-VIII molecule.

In the present study, using African green monkey kidney (COS-7) cells transfected with μ -opioid or D₂ receptors (dopaminergic type 2 receptors) and either AC-VIII-A, -B or -C, we report that acute activation of G_{i/o}-coupled receptors leads to inhibition of AC-VIII-A and -B, but not of the AC-VIII-C splice variant. Chronic receptor activation leads to superactivation of all three splice variants, and the superactivated state of all AC-VIII isoenzymes can be re-inhibited by an additional acute agonist exposure.

EXPERIMENTAL

Materials

[2-³H]Adenine (30.0 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). Morphine and naloxone were obtained from the National Institute of Drug Abuse, Research Technology Branch (Rockville, MD, U.S.A.). Quinpirole and sulpiride were from Research Biochemicals (Natick, MA, U.S.A.). Ionomycin, 8-CPT-cAMP [8-(4-chlorophenylthio)-3',5'-cAMP] and the phosphodiesterase inhibitors IBMX (isobutylmethylxanthine), and RO-20-1724 were from Calbiochem (La Jolla, CA, U.S.A.). FS (forskolin) and cAMP were from Sigma (St. Louis, MO, U.S.A.). Tissue culture reagents were from Gibco BRL (Bethesda, MD, U.S.A.). PTX (pertussis toxin) was from List Biological Laboratories (Campbell, CA, U.S.A.).

Plasmids

The rat μ -opioid receptor in pCMV-neo, rat D₂L (long form of the D₂) receptor cDNA in pcDNAI-Amp, the human lymphocyte CD8 receptor in pcDNAI-Amp have been described previously [4,7]. AC-VIII-A, -B and -C [25,26] cDNAs in the mammalian expression vector pCMV5-neo were kindly provided by Professor J. Krupinski (Geisinger Clinic, Danville, PA, U.S.A.).

Transient cell transfections

Before transfection (24 h), a confluent 10-cm plate of COS-7 cells in DMEM (Dulbecco's modified Eagle's medium), supplemented with 5 % fetal calf serum, 100 units/ml penicillin and 100 μ g/ml streptomycin, in a humidified atmosphere (5 % CO₂/95 % air) at 37 °C, was trypsinized and divided between four 10-cm plates. The cells were transfected, using the DEAE-dextran chloroquine method as described previously [5], using 1 μ g/plate of rat μ -opioid or D₂L receptor cDNA and 4 μ g/plate of the appropriate AC isoenzyme cDNAs, or with CD8 in pcDNAI-Amp (for mock cDNA transfection), such that each transfection contained an equivalent amount of plasmid DNA. After 24 h, the cells were trypsinized and re-cultured in 24-well plates, and after an additional 48 h, the cells were assayed for AC activity as described below. Transfection efficiencies were normally in the range of 60–80 %.

cAMP accumulation assay

The assay was performed in triplicate as described previously [5], and the values obtained in mock (CD8)-transfected cells were subtracted in all the experiments (except Figure 1). In brief, cells cultured in 24-well plates were incubated for 2 h with 0.25 ml/well of fresh growth medium containing 5 μ Ci/ml of [³H]adenine, and then washed with 0.5 ml/well DMEM containing 20 mM Hepes

(pH 7.4) and 0.1 mg/ml BSA. This medium was replaced with 0.5 ml/well DMEM containing 20 mM Hepes (pH 7.4), 0.1 mg/ml BSA, the phosphodiesterase inhibitors IBMX (0.5 mM) and RO-20-1724 (0.5 mM), the μ -opioid ligand morphine or the D₂ ligand quinpirole (at the indicated concentrations), and 1 μ M ionomycin (and/or 1 μ M FS where indicated) to stimulate AC-VIII activity. After 10 min at room temperature (25 °C), the medium was removed and the reaction was terminated by the addition of perchloric acid containing 0.1 mM unlabelled cAMP, followed by neutralization with KOH, and the amount of [³H]cAMP was determined by a two-step column separation procedure as described previously [4,30]. Chronic agonist treatment was achieved by incubating the cells for 18 h with 1 μ M morphine or 0.2 μ M quinpirole (unless otherwise indicated). This was usually followed by agonist withdrawal which was obtained by either, (i) three rapid washes with DMEM containing 20 mM Hepes and 0.1 mg/ml BSA, or by (ii) one wash and the addition of the respective antagonists (5 μ M naloxone or 10 μ M sulpiride) just prior to the 10 min assay. The second method was used in most experiments, whereas the first method was used when the effect of the re-addition of agonist was studied (to avoid interference with excess of antagonist). Both withdrawal methods were shown to provide similar results [31]. We found that the uptake of [³H]adenine into the cells was not affected by the chronic opiate or dopaminergic treatment [4,7]. The time- and dose-response data were analysed, and the curves were plotted using the Graphpad Prism 3.0 software package.

SDS/PAGE and Western blotting

Transfected COS cells from 10-cm plates were washed with cold PBS without Ca²⁺ or Mg²⁺, scraped and spun down at 2000 g (4 °C for 5 min). Cell pellets were mixed with 100 μ l of Laemmli sample buffer per plate, sonicated and frozen. Prior to application on the gel, dithiothreitol (0.1 M final concentration) was added and the samples incubated for 1 h at 37 °C. Proteins were separated on 8 % polyacrylamide gels and transferred on to nitrocellulose. The nitrocellulose was blocked in PBS containing 5 % fat-free milk and 0.5 % Tween 20 for 1 h, followed by 1.5 h incubation with a 1:1000 dilution of BBC-4 monoclonal antibody against AC [32]. This antibody reacts well with all variants of AC-VIII. Blots were washed 3 times with PBS containing 0.3 % Tween 20 and secondary antibody (horseradish-peroxidase-coupled rat anti-mouse antibody; Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.) diluted 1:20000 in 5 % fat-free milk plus 0.5 % Tween 20, incubated with the blot for 1 h and the blot extensively washed (> 2 h) with PBS containing 0.3 % Tween 20. Peroxidase activity was evaluated by the ECL[®] chemiluminescence technique (Amersham, Little Chalfont, Bucks., U.K.).

Data analysis

Data are expressed as the means $\pm$ S.E.M. of three independent experiments, each performed in triplicate. In each experiment the data (in c.p.m.) was transformed to the percentage of control activity and the percentages of the various experiments averaged. The number of c.p.m. in different experiments varied due to changes in transfection efficiency. Results were analysed using the Student's *t* test.

RESULTS

Activation of AC-VIII-A, -B and -C by ionomycin and FS

COS-7 cells were transfected with the cDNAs for AC-VIII-A, -B or -C. AC-VIII is known to be stimulated by Ca²⁺ ions [25,26], and

Figure 1 FS, ionomycin, and FS plus ionomycin stimulation of AC activity in COS-7 cells expressing the various AC-VIII splice variants

COS-7 cells were co-transfected with μ -opioid receptor and with either CD8 or the indicated AC-VIII splice variant cDNAs. AC activity endogenously present in COS-7 cells (AC-COS) or the activity in COS cells transfected with AC-VIII-A, -B or -C are shown in unstimulated cells or in cells stimulated with 1 μ M FS, 1 μ M ionomycin or with FS plus ionomycin. The data represent the means $\pm$ S.E.M. of triplicate determinations of a representative experiment.

its activity is therefore stimulated by exposing the cells during the 10-min assay to the Ca^{2+} ionophore ionomycin. Indeed, as seen in Figure 1, cells transfected with AC-VIII-A, -B or -C displayed 12-, 4- and 5-fold increases in ionomycin (1 μ M)-stimulated cAMP levels respectively, whereas no such increase was observed for the endogenous AC activity in COS cells. Cells transfected with AC-VIII-A, -B or -C displayed 5-, 2.5- and 8-fold increases in FS (1 μ M)-stimulated cAMP levels respectively, whereas a 2.5-fold stimulation was observed upon the addition of FS to control COS cells not transfected with AC. Moreover, the effects of FS and ionomycin on the activities of the three splice variants of AC-VIII were found to be synergistic (yielding 42-, 30- and 29-fold increases versus the unstimulated activity), whereas no such synergism was observed for the endogenous AC activity in COS-7 cells (the activity was even reduced by 10%, compared with the activity with FS alone). These results indicate that AC-VIII-A, -B and -C are expressed and active in the transfected COS-7 cells, and that their activity can be distinguished from that of the AC endogenously found in COS-7 cells. Interestingly, AC-VIII-C was more sensitive to 1 μ M FS than to 1 μ M ionomycin, whereas AC-VIII-A and -B were more sensitive to ionomycin than to FS, indicating different sensitivities of these AC-VIII isoforms to FS versus Ca^{2+} .

Modulation of AC-VIII-A, -B and -C activity by acute and chronic receptor activation

The effect of acute and chronic morphine treatments (in μ -opioid-receptor-transfected cells) or of quinpirole treatments (in D_2L -receptor-transfected cells) on the ionomycin-stimulated activity of the three AC-VIII variants is shown in Figure 2. Acute (10-min) activation of the μ -opioid receptor by morphine (1 μ M) or of the D_2L receptor by quinpirole (0.2 μ M), added together with the 1 μ M ionomycin, led to a reduction in the ionomycin-stimulated activity of AC-VIII-A ($29 \pm 5\%$ and $31 \pm 3\%$ for morphine and quinpirole respectively; $n = 3$) and AC-VIII-B ($27 \pm 4\%$ and $54 \pm 6\%$ respectively; $n = 3$). On the other hand, the activity of AC-VIII-C was not significantly inhibited by either morphine or quinpirole. These results indicate a difference in the capacity of these inhibitory receptors to inhibit the activity of the various splice variants. On the other hand, chronic activation (18 h) of the μ -opioid receptor or the D_2L receptor followed by agonist withdrawal (obtained by the addition of the antagonist) led to a large increase in the ionomycin-stimulated AC activity

Figure 2 Acute and chronic activation of μ -opioid or D_2 -dopaminergic receptors and their effect on the activity of AC-VIII-A, -B and -C

The effect of morphine (a) or quinpirole (b) treatments on ionomycin (1 μ M)-stimulated cAMP accumulation in COS-7 cells transfected with μ -opioid (a) or D_2L (b) receptors and AC-VIII-A, -B or -C cDNAs. Control, cells to which no agonists were added; acute agonist, 1 μ M morphine or 200 nM quinpirole are present during the 10-min AC assay; withdrawal, cells are incubated with 1 μ M morphine or 200 nM quinpirole for 18 h followed by three rapid washes and immediate AC assay; withdrawal + agonist, 1 μ M morphine or 200 nM quinpirole (18 h) followed by three rapid washes and re-addition of 1 μ M morphine or 200 nM quinpirole respectively at the start of the AC assay. The data represent the means $\pm$ S.E.M. of three experiments. 100% represents the activity under control conditions. The 100% values in the three different experiments ranged between 3380–5217 c.p.m. for AC-VIII-A; 1185–2378 c.p.m. for AC-VIII-B and 1544–3402 c.p.m. for AC-VIII-C. * $P < 0.01$ compared with control; # $P < 0.01$ compared with withdrawal.

of all three AC-VIII isoforms, indicating that they were super-activated during the chronic agonist exposure. The values for the AC superactivation observed following chronic morphine exposure were: AC-VIII-A, 1.99 ± 0.08 -fold; AC-VIII-B, 1.75 ± 0.14 -fold; AC-VIII-C, 2.73 ± 0.43 -fold over non-treated cells, $n = 3$. The values for the superactivation observed following chronic quinpirole were: AC-VIII-A, 2.15 ± 0.49 -fold; AC-VIII-B, 4.29 ± 0.91 -fold; AC-VIII-C, 5.93 ± 0.11 -fold over non-treated cells, $n = 3$.

To examine the ability of the opiate and dopaminergic agonists to inhibit the superactivated states of AC-VIII-A, -B or -C, these agonists were re-applied to the cells immediately following withdrawal from the chronic treatment and AC activity immediately tested. In these experiments the withdrawal was achieved by extensive wash of the agonist. Under these conditions, the percentage of inhibition of ionomycin-stimulated AC-VIII-A activity by morphine and quinpirole in comparison with the superactivated state were $41 \pm 1\%$ and $55 \pm 3\%$ for morphine and quinpirole respectively ($n = 3$), and for AC-VIII-B, the values were $40 \pm 10\%$ and $68 \pm 5\%$ respectively ($n = 3$). These values are much higher than the levels observed for acute morphine or quinpirole inhibition in control cells prior to the chronic treatment. Moreover, re-applying morphine or quinpirole to the superactivated AC-VIII-C also resulted in a strong inhibition of this isoenzyme ($66 \pm 13\%$ and $78 \pm 6\%$ respectively, $n = 3$), a result which is very different from the relative insensitivity of this isoenzyme to morphine or quinpirole under the initial acute conditions. However, when the data are examined carefully, it seems that in all

Figure 3 Effect of various concentrations of morphine on the acute inhibition, superactivation and re-inhibition of AC-VIII-A and -C

COS cells were co-transfected with the cDNAs of the μ -opioid receptor and either AC-VIII-A or -C. (a) Inhibition of ionomycin (1 μ M)-stimulated AC-VIII-A and -C activities by various concentrations of morphine applied acutely during the 10-min assay. (b) Ionomycin-stimulated AC activity following rapid withdrawal (1 rapid wash and addition of 5 μ M naloxone) from chronic treatment (18 h) performed with the indicated concentrations of morphine. (c) Effect of re-added morphine (after 18 h pre-treatment with 0.1 μ M morphine and morphine withdrawal) on ionomycin-stimulated cAMP accumulation. In this experiment, withdrawal was achieved by 3 washes, and morphine was re-added at the indicated concentrations. The data represent the means $\pm$ S.E.M. of three experiments. 100% represents the activity under control conditions with AC-VIII-A values ranging between 969–1671 c.p.m. and AC-VIII-C between 869–1574 c.p.m. for the different experiments.

AC-VIII splice variants, the re-addition of the agonists in essence abolishes the increased AC activity obtained during the withdrawal following the chronic treatment. These results indicate that agonist withdrawal following chronic treatment induces a type of AC activity which is more sensitive to agonist inhibition than is the control AC-VIII activity.

Dose and time dependency of morphine treatment on AC regulation

The dose–response curves of acute morphine inhibition, chronic-induced superactivation and re-inhibition by morphine following withdrawal from chronic exposure for AC-VIII-A and -C are shown in Figure 3. The inhibition of AC-VIII-A activity by acute morphine (Figure 3a) is dose-dependent, with an EC_{50} of 2.2 ± 2.8 nM and maximal inhibition of $41 \pm 4\%$ (obtained with 1 μ M morphine). However, only minute inhibition of ionomycin-stimulated AC-VIII-C activity was observed in cells acutely treated with 0.1–100 nM morphine. Moreover, in the presence of 1 μ M morphine, there was no inhibition and sometimes even a slight stimulation could be observed. This could be due to the

Figure 4 Time course of chronic morphine pre-treatment on the activity of AC-VIII-A and -C

COS-7 cells were co-transfected with the cDNAs of either AC-VIII-A or -C, and with the μ -opioid receptor cDNA. Cells were incubated with 1 μ M morphine for the times indicated, followed by withdrawal (achieved by a rapid wash and the addition of 5 μ M naloxone) and the immediate assay of ionomycin (1 μ M)-stimulated AC activity. The data represent the means $\pm$ S.E.M. of three experiments. 100% represents the activity under control conditions with AC-VIII-A of 1930 ± 89 c.p.m. and AC-VIII-C of 1147 ± 97 c.p.m..

superactivation already taking place following 10 min with this concentration of morphine.

In the experiment shown in Figure 3(b), the transfected COS-7 cells were pre-treated (for 18 h) with various concentrations of morphine, and the agonist was rapidly withdrawn immediately prior to the AC assay. Significant superactivation of AC-VIII-A and -C were observed in cells pre-treated with morphine, as compared with control cells (not treated with the agonist). The increases in AC-VIII-A and -C activities were dose-dependent (reaching approx. 4.0- and 4.7-fold following 18 h with 1 μ M morphine), with an EC_{50} of 63 ± 11 nM and 50 ± 2 nM respectively, demonstrating a similar or equivalent dose–response for both AC-VIII-A and C.

To characterize the ability of re-added morphine to inhibit the activity of the superactivated AC-VIII isoenzymes, the cells were pre-treated with 100 nM morphine for 18 h, the agonist was rapidly withdrawn (by three quick washes), and morphine (0.1–1000 nM) was re-added immediately prior to the AC assay. We found that under these conditions (see Figure 3c), morphine inhibited AC-VIII-A and -C in a dose-dependent manner to a similar extent ($53 \pm 8\%$ and $62 \pm 4\%$ maximal inhibition respectively) and with similar EC_{50} values (9 ± 6 nM and 12 ± 4 nM respectively). These results also indicate that chronic activation of the μ -opioid receptor with morphine leads to only mild desensitization of the receptor signalling, as the EC_{50} for AC-VIII-A inhibition increased by only 4-fold following the chronic morphine treatment. In addition, it is clear from these results that AC-VIII superactivation requires stronger receptor activation (EC_{50} of approx. 56 nM) than do acute inhibition (EC_{50} of approx. 2.2 nM) or inhibition following chronic treatment and withdrawal (EC_{50} of approx. 10 nM). A similar observation was made with μ -receptor-transfected CHO (Chinese-hamster ovary) cells stimulated with morphine [33].

The development of AC superactivation for both AC-VIII-A and -C is time-dependent (Figure 4). The levels of superactivation reached approx. 3- and 4.5-fold over the activity of control non-treated cells after 30 h of treatment with 1 μ M morphine. Careful examination of the time dependency suggests that this process involves at least two time constants. With this assumption, the time constants were calculated to be (i) approx. 10–20 min and (ii) between 10 and 20 h for both AC-VIII-A and -C. The

Figure 5 Effects of various times with morphine on the acute inhibition, superactivation and re-inhibition of AC-VIII-A and -C

COS-7 cells were co-transfected with the cDNAs of either AC-VIII-A or C, and with the μ -opioid receptor cDNA. Cells were incubated with 1 μ M morphine for the times indicated (1 or 18 h), followed by withdrawal (achieved by a rapid wash and the addition of 5 μ M naloxone) or by extensive wash followed by the re-addition of 1 μ M morphine. AC activity was assayed in the presence of 1 μ M ionomycin (a, b) or 1 μ M FS (c, d). The data represent the means $\pm$ S.E.M. of three experiments after subtraction of values obtained in mock (CD8)-transfected cells. 100% represents control activity without pre-treatment with morphine. Control, cells to which no morphine was added; acute morphine, 1 μ M morphine present during AC assay; withdrawal, 1 μ M morphine for 1 or 18 h followed by withdrawal by one rapid wash and the addition of 5 μ M naloxone; withdrawal + morphine, 1 μ M morphine pre-treatment for 1 or 18 h followed by extensive washes (withdrawal) and re-addition of 1 μ M morphine at the start of the assay. 100% represents the activity under control conditions with AC-VIII-A, of 1017 ± 184 and 1185 ± 108 c.p.m. and AC-VIII-C of 887 ± 38 and 1711 ± 231 c.p.m. for ionomycin and FS respectively. * $P < 0.005$ compared with control; # $P < 0.005$ compared with withdrawal.

contribution of the slower process is larger than that of the fast process, and amounts to 70–80% of the total superactivation. Interestingly, these kinetics of the superactivation of AC-VIII-A and -C are very different from those observed for AC-V [4] and are much more similar to those found for AC-I following chronic activation of the D_2 receptor [7]. Similarly, in NG108-15 cells, the noradrenaline-dependent increase in cAMP content was found to be biphasic, where approx. 20% of the elevation was observed in the first hour, and the second larger increase took place between 5–10 h [17].

These findings raise the question of whether the increase in the agonist's capacity to acutely inhibit the superactivated AC is associated with one or both of the time-dependent components of the superactivation. In order to answer this, we examined the ability of morphine to re-inhibit AC-VIII-A and -C activity, after 1 or 18 h of chronic treatment followed by withdrawal (Figure 5). We found that after both short and long chronic treatments, the increased AC-VIII-A and -C activities could be inhibited by the re-addition of morphine. Similar results were obtained upon stimulation of AC activity with either ionomycin or FS. These results suggest that both the faster and slower components of the AC superactivation behave similarly with respect to the capacity of being re-inhibited by the re-addition of morphine.

PTX abolishes the regulation of AC-VIII by morphine

To study the role of G-proteins in the opiate regulation of AC-VIII-A and -C, we pre-treated the cells with PTX. PTX was found to

Figure 6 PTX pre-treatment blocks μ -opioid receptor regulation of AC-VIII-A and -C

COS-7 cells were co-transfected with μ -opioid receptor cDNA together with the cDNAs of either AC-VIII-A or -C. The cells were pre-incubated with 100 ng/ml PTX added to the cultures before (2 h) and during (18 h) the chronic treatment. For further details, see Figure 5. The PTX pre-treatment prevented the morphine-induced inhibition and superactivation of ionomycin (1 μ M)-stimulated AC-VIII-A (a) and -C (b) activity. The data represent the means $\pm$ S.E.M. of three experiments. 100% represents the activity under control conditions of cells transfected with AC-VIII-A or AC-VIII-C.

Figure 7 Effect of a cell permeable cAMP analogue on μ -opioid receptor regulation of AC-VIII-A and -C

COS-7 cells co-transfected with μ -opioid receptor and with AC-VIII-A (a) or AC-VIII-C (b) were exposed to 0.1 mM 8-CPT-cAMP before (2 h) and during (18 h) the chronic treatment. For further details, see Figure 5. The data represent the means $\pm$ S.E.M. of three experiments performed in triplicate. 100% represents the activity under control conditions with AC-VIII-A of 1575 ± 21 c.p.m. and AC-VIII-C of 1352 ± 85 c.p.m. Pre-treatment with 8-CPT-cAMP was found to reduce the [3 H]adenine uptake by approx. 15%.

abolish both the inhibition and superactivation of AC-VIII-A and -C by the acute and chronic morphine treatments (Figure 6a and b), whereas it had only a negligible effect on the level of stimulation of these ACs by ionomycin. It therefore follows that both the inhibition of AC-VIII-A and the superactivation of AC-VIII-A and -C (like that of AC-V) [4] are mediated via PTX-sensitive $G_{i/o}$ proteins.

Effect of modulation of cAMP concentration on AC regulation

It could be postulated that the prolonged reduction in cellular cAMP levels induced by inhibitory receptor activation could somehow induce the compensatory increase in AC activity observed after the chronic activation. To investigate the possible role of cAMP itself in the regulation of the activity of AC-VIII, we have exposed the transfected COS-7 cells to 0.1 mM of the cell permeable cAMP-derivative 8-CPT-cAMP before (2 h) and during (18 h) the chronic treatment (Figure 7). We found that this treatment did not affect the level of inhibition of ionomycin-stimulated AC-VIII-A activity by acute morphine, nor did it affect the superactivation or the re-inhibition of AC-VIII-A or -C activity after chronic morphine treatment. Thus the reduction in cAMP by

Figure 8 Effect of chronic μ -opioid receptor activation on the expression of AC-VIII-A, -B and -C

COS-7 cells were co-transfected with μ -opioid receptor cDNA and either AC-VIII types A, B or C, or with CD8 cDNA (for mock-transfection). Where indicated, cells were treated chronically (for 18 h) with 1 μ M morphine, homogenized and aliquots of crude homogenates containing equivalent amounts of total cellular proteins (100 μ g) were separated by SDS/PAGE (8% acrylamide) and analysed by Western blotting using BBC-4 monoclonal antibody against AC, as described in the Experimental section. The Figure depicts a representative experiment out of three performed with similar results.

the acute treatment does not seem to be required for the induction of AC superactivation.

Effect of chronic morphine on the expression of AC-VIII splice variants

The expression of AC-VIII-A, -B or -C in transfected COS cells was confirmed using the monoclonal antibody BBC-4, an antibody known to interact with different AC isoenzymes [32]. This antibody did not show high-molecular-mass immunoreactive bands in lysates from mock-transfected COS cells (Figure 8). However, high-molecular-mass immunoreactive bands were observed in lysates from COS cells transfected with all of the AC-VIII splice variant cDNAs. All the three splice variants showed an immunoreactive band which migrated with the mobility appropriate for proteins of approx. 125–140 kDa, whereas AC-VIII-A, and to a lesser extent AC-VIII-C, also showed an immunoreactive band of higher molecular mass (approx. 185 kDa). The calculated molecular masses of AC-VIII-A, -B and -C, according to their amino acid composition, are 139.8, 136.4 and 132.3 kDa respectively. According to these values, the low-molecular-mass band should represent the non-glycosylated forms of the three splice variants, whereas the approx. 185 kDa bands should represent the glycosylated forms. In agreement with this, the AC-VIII-B splice variant, which lacks the two glycosylation sites, did not show the high-molecular-mass band. Moreover, the lower band of AC-VIII-C showed a lower molecular mass than that of AC-VIII-A, in accordance with its smaller number of amino acids. Similar observations were made for these AC-VIII splice variants when transfected into HEK-293 cells, where the glycosylated molecules migrated at 165 kDa bands and the unglycosylated molecules as 125 kDa bands [25].

To investigate whether chronic morphine exposure affects the expression of the AC-VIII splice variants, the cells were treated with or without 1 μ M morphine for 18 h, and the cell homogenates analysed by SDS/PAGE and blotting with the antibody BBC-4. The results of these experiments (see Figure 8 for a representative result) demonstrate that chronic morphine treatment did not lead to significant changes in the expression of any of the three AC-VIII isoenzymes, suggesting that the superactivation of AC-VIII-A, -B or -C is not due to a change in the protein expression of these isoenzymes.

DISCUSSION

In this study, we found that the inhibition of AC-VIII by acute activation of $G_{i/o}$ -coupled receptors is splice-variant specific, as

AC-VIII-A and -B show significant acute inhibition, whereas AC-VIII-C does not. On the other hand, the capacity to undergo superactivation following chronic receptor activation is shared by all three splice variants. In addition, we show that the superactivated state of all three AC-VIII splice variants share the capacity to undergo stronger reduction in activity upon re-addition of receptor agonist, as opposed to control AC-VIII activities. These results were obtained with both μ -opioid and D_2L receptors, suggesting that it could be a general property of AC-VIII when activated via a variety of $G_{i/o}$ -coupled receptors.

As described above, we observed that AC-VIII-C differs from AC-VIII-A and -B in its inability to undergo significant inhibition by acute inhibitory receptor activation. This suggests that the part of the C_{1b} region which is missing in AC-VIII-C is important for acute AC-VIII inhibition. In this regard, the area of C_{1b} in AC-V has been shown to contribute to the potency of inhibition of this AC isoenzyme by $G\alpha_i$ [29]. However, after withdrawal from chronic treatment, all AC-VIII splice variants exhibit inhibition, suggesting that regions other than the C_{1b} area may also be involved in AC-VIII inhibition. This also suggests that the inhibition (or 're-reduction' in activity) of superactivated AC-VIII could have a different mechanism than that of the acute inhibition or, in other words, that the 'surplus' AC-VIII activity induced by withdrawal of the agonist following chronic treatment is more sensitive to inhibition by agonist application than the AC activity in control non-treated cells. This possibility is supported by the fact that the inhibition of the superactivated AC never attains a level lower than the 100% control level of AC activity. Thus, the superactivated AC molecules differ in their regulation characteristics from the 'regular' ionomycin-activated AC molecules. Altogether, it seems that the 66 amino acids of the C_{1b} region deleted to form AC-VIII-C, although needed for acute inhibition, are not essential for the induction of AC-VIII superactivation and its reversal.

It was reported that AC-VIII-C is at least as sensitive to Ca^{2+} /calmodulin as AC-VIII-A [25]. In agreement with this result, we found that the Ca^{2+} -dependent stimulation of AC-VIII is not dependent upon the 66 amino acids deleted from the C_{1b} area in AC-VIII-C, as the activity of AC-VIII-C is stimulated by ionomycin. On the other hand, C_{1b} was found to be critical for Ca^{2+} /calmodulin activation of AC-I [34] and for the Ca^{2+} -mediated inhibition of AC-V [35,36]. This apparent discrepancy can be explained by the finding that the calmodulin-binding sites on AC-VIII are located primarily in the C_{2b} area and on an ancillary site in the N-terminus and not in the C_{1b} area [37]. Moreover, the C_{1b} area of AC-VIII does not share extensive sequence similarity with the respective areas in AC-V and AC-I.

Interestingly, the fact that AC-VIII-B lacks 30 amino acids, leading to the loss of N-linked glycosylation (see Figure 8 and [25]), did not affect its regulation pattern by acute and chronic agonist exposure (in comparison with AC-VIII-A). This would seem to indicate that this part of the molecule, as well as the glycosylation itself, are not critical for either inhibition or superactivation of AC-VIII. Similarly, the inhibition of AC-IX by $G_{i/o}$ -coupled receptors activation was not affected by the glycosylation state of this isoenzyme [27]. However, it should be noted that unglycosylated AC-VI was found to be less sensitive to D_2 receptor inhibition [28]. Thus glycosylation may play a different role in different AC isoenzymes.

In the present paper and in previous publications, we show that ionomycin-stimulated AC-VIII-A activity is inhibited by acute stimulation of various $G_{i/o}$ -coupled receptors, including the D_2L , m_4 (muscarinic type 4), CB_1 and CB_2 (cannabinoid types 1 and 2) receptors, transfected into COS-7 cells [5,7,8]. In all these cases, the inhibition was much weaker than that

observed with AC-V or AC-VI. On the other hand, others have reported that A23187-stimulated AC-VIII (most likely AC-VIII-A) activity in HEK-293 cells was not, or only slightly, inhibited by the activation of endogenous somatostatin and transfected D₂L receptors respectively [38]. This difference in the capacity to be inhibited by different G_{i/o}-coupled receptors could be due to changes in composition of G_{i/o} proteins in the two cell systems and in the regulatory pattern of the various G_{i/o}-coupled receptors by the various G_i proteins [39].

We have reported that in COS-7 cells, AC isoforms I, V, VI and VIII-A are inhibited and superactivated by acute and chronic inhibitory receptor activation respectively, whereas AC isoforms II, IV and VII are stimulated by acute and inhibited by chronic inhibitory receptor activation [5,7]. From this pattern of inhibition and stimulation, it appears that the type of compensatory response (increase or decrease in activity) depends on the type of acute response (inhibition or stimulation). This raised the question of whether inhibition of AC and the subsequent reduction in cellular cAMP have a role in eliciting the superactivation process. In this regard, we found that the superactivation of AC-VIII and its reversal are not affected by the cell permeable cAMP analogue 8-CPT-cAMP, suggesting that these phenomena are not elicited by the reduction in cAMP levels. These results are in agreement with our previous data, showing that superactivation in CHO cells stably transfected with μ -opioid receptors was not affected by cholera toxin or by dibutyryl cAMP [33], and that the superactivation of AC-V and AC-VI in COS cells was not prevented by co-transfection with constitutively active G α_s (Gln²²⁷ → Leu) [5]. The unique property of AC-VIII-C of showing superactivation following chronic exposure, even though it was not inhibited by activation of G_{i/o}-coupled receptors, also supports the idea that AC superactivation is not directly dependent on acute inhibition.

Both the superactivation and the increase in susceptibility of AC-VIII to be inhibited arise as a result of the chronic treatment. It is therefore possible that the same mechanism that leads to superactivation may also cause the enhanced inhibition. The mechanism leading to these phenomena is not yet known. However, G $\alpha_{i/o}$ proteins are involved, as PTX prevented the superactivation. We have preliminary results suggesting that G $\beta\gamma$ dimers are involved in the superactivation of AC-VIII splice variants, as molecules that interfere with G $\beta\gamma$ activity (G $\beta\gamma$ scavengers) abolish AC-VIII superactivation and attenuate AC-VIII acute inhibition (D. Steiner, T. Avidor-Reiss, E. Schallmach, D. Saya and Z. Vogel, unpublished work). In this regard, our previous results and those of others show that G $\beta\gamma$ dimers have a role in the superactivation of AC-V and AC-VI following chronic opiate exposure [4,11].

Membrane preparations of neurons from the locus coeruleus (the major noradrenergic nucleus in the brain, which has an essential role in opiate addiction) [40] of rats chronically treated with morphine exhibit an increase in AC activity [20]. Using AC isoenzyme-specific antibodies, it was found that the locus coeruleus expresses AC-I and AC-VIII, as well as AC types III, IV and V [21]. Rats chronically treated with morphine exhibited a 43 % increase in mRNA of AC-VIII [22], and 24 % and 26 % increases in the levels of AC-I and AC-VIII immunoreactivities respectively, in the locus coeruleus [21]. It was therefore suggested that adaptation at the expression level of AC-VIII and possibly also of AC-I may be responsible, at least partially, for the increase in AC activity during withdrawal [21,22]. We, on the other hand, found no significant increase in the immunoreactivity of AC-VIII-A, -B or -C following chronic morphine treatment. Moreover, we [33] and others [41,42] have shown that AC superactivation observed in CHO and HT29 cells transfected with

inhibitory receptors is not prevented by pre-treatment with the protein synthesis inhibitor cycloheximide, suggesting that protein synthesis is not essential for the superactivation phenomenon. It is thus conceivable that *in vivo*, AC superactivation could lead (by virtue of the increase in cAMP) to a cascade of events which affect the expression of several proteins (including AC-VIII) which could have a role in the mechanism of addiction to opiate drugs.

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