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Protein Kinase C-Mediated Down-Regulation of β_1-Adrenergic Receptor Gene Expression in Rat C6 Glioma Cells

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ABSTRACT

In the current study, we investigated the mechanism by which protein kinase C (PKC) regulates the expression of β_1-adrenergic receptor (β_1AR) mRNA in rat C6 glioma cells. Exposure of the cells to 4β-phorbol-12-myristate-13-acetate (PMA), an activator PKC, resulted in a down-regulation of both β_1AR binding sites and mRNA levels in a time- and concentration-dependent manner. This effect was not observed with phorbol esters that do not activate PKC and was blocked by bisindolylmaleimide, a specific PKC inhibitor. Activation of PKC did not reduce the half-life of β_1AR mRNA but significantly decreased the activity of the β_1AR promoter, as determined by reporter analysis. A putative response element, with partial homology to a consensus cAMP response element, was identified by mutation analysis of the promoter at positions −343 to −336, relative to the translational start site. Mutation of this putative regulatory element, referred to as a β_1AR-PKC response element, completely blocked the PKC-mediated down-regulation of β_1AR promoter activity. Gel mobility shift analysis detected two specific bands when C6 cell extracts were incubated with a labeled DNA probe containing the β_1AR-PKC response element sequence. Formation of one of these bands was inhibited by an oligonucleotide probe containing a consensus CRE and disrupted by an antibody for cAMP response element binding protein. Based on these studies, we propose that the PKC-induced down-regulation of β_1AR gene transcription in C6 cells is mediated in part by a cAMP response element binding protein-dependent mechanism acting on a novel response element.

Cross-regulation between two of the major signal transduction pathways, the receptor-coupled adenylyl cyclase and phospholipase C systems, is a well recognized phenomenon (Houslay, 1991). The second messengers generated by each of these systems activate protein kinase A and PKC, respectively. The effects of PKC on βAR-stimulated adenylyl cyclase have been investigated extensively and found to be complex. In some cells, desensitization has been observed (Garte and Belman, 1980; Kelleher *et al*., 1984; Sibley *et al*., 1984; Kassis *et al*., 1985). In other cells, potentiation of agonist-stimulated activity has been found (Bell *et al*., 1985; Sugden *et al*., 1985; Yoshimasa *et al*., 1987). The activation of PKC in a third class of cells leads to both potentiation and desensitization (Johnson *et al*., 1990; Bouvier *et al*., 1991; Zhou *et al*., 1994). The potentiation seems to be mediated by phosphorylation of the inhibitory G protein (Houslay, 1991) or the adenylyl cyclase catalyst (Yoshimasa *et al*., 1987; Simmoteit *et al*., 1991). The desensitization seems to be caused by PKC-catalyzed phosphorylation of βAR (Kelleher *et al*., 1984; Sibley *et al*., 1984; Bouvier *et al*., 1987, 1991). In this regard, a mutated β_2AR that lacks the consensus sites for PKC is no longer susceptible to phorbol ester-mediated phosphorylation and desensitization (Johnson *et al*., 1990; Bouvier *et al*., 1991).

Less is known about the effects of PKC activation on β_1AR expression. In rat C6 glioma cells, exposure of cells to phorbol esters that activate PKC leads to a loss of receptor binding activity (Kassis *et al*., 1985; Fishman *et al*., 1987). In addition to its role in the regulation of βAR, PKC may be involved in glial cell proliferation and differentiation (Kronfeld *et al*., 1995). C6 cells express both β_1AR and β_2AR subtypes (Fishman *et al*., 1994; Hosoda *et al*., 1994). We recently showed

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ABBREVIATIONS: PKC, protein kinase C; AR, adrenergic receptor; CRE, cAMP response element; CREB, cAMP response element binding protein; PMA, 4β-phorbol-12-myristate-13-acetate; CYP, cyanopindolol; SS, supershifted; DMSO, dimethylsulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AP-1, activator protein-1; PRE, protein kinase C response element; CREM, cAMP response modulator transcription factor.

that on exposure to agonist or other agents that activate protein kinase A, C6 cells coordinately down-regulate both receptor subtypes (Fishman *et al*., 1994; Hosoda *et al*., 1994). Receptor down-regulation seems to be mediated in part by down-regulation of receptor mRNA and to involve induction of a repressor protein that blocks gene transcription (Hosoda *et al*., 1994, 1995; Rydelek-Fitzgerald *et al*., 1996). We undertook the current study to determine whether similar mechanisms were involved in PKC-mediated down-regulation of βAR in C6 cells. Because β_1AR is the major subtype in C6 cells and less is known about its regulation, we concentrated on the effects of PMA on β_1AR expression.

Experimental Procedures

Materials. (−)-^{125}I-CYP (2200 Ci/mmol) and α-^{32}P-labeled nucleotides were obtained from Dupont-New England Nuclear (Boston, MA). Phorbol esters, bisindolylmaleimide I, HCl, and actinomycin D were purchased from Calbiochem (San Diego, CA). 8-(4-Chlorophenylthio)-cAMP was from Sigma Chemical (St. Louis, MO). *Pseudomonas* exotoxin A was from List Biological Laboratories (Campbell, CA). CGP 20712A was a generous gift from CIBA-GEIGY (Summit, NJ).

Cell culture. Rat C6 glioma cells were cultured as described previously (Hosoda *et al*., 1994). Cells (passages 42–70) were plated at 5–7 × 10^6 cells/175-cm^2 flask in 50 ml of medium. Four days later, the medium was changed to serum-free medium, and the cells were exposed to PMA (0.2 μM in 0.01% DMSO) or vehicle (DMSO) for the times indicated. In some experiments, cells were pretreated with exotoxin A at 0.3 μg/ml for ≥4 hr before adding the PMA. Cells (4–5 × 10^7/flask) were collected by removing the medium and adding serum-free medium buffered with 25 mM HEPES and containing 2 mM EGTA and 2 mM EDTA. The detached cells were centrifuged at 200 × *g* for 5 min, and the cell pellet was taken up in an ice-cold solution containing 4 M guanidine thiocyanate, 25 mM sodium acetate buffer, pH 6.2, and 0.5% 2-mercaptoethanol. The cell suspension was frozen at −80° for subsequent RNA isolation. In some experiments, cells were transfected with a plasmid expression vector containing the coding region for CREB according to the calcium-phosphate precipitation method. CREB was subcloned into pGEM vector and was under the control of the cytomegalovirus promoter.

Receptor binding assays. When β_1AR binding levels were determined, either a portion of the above detached cells was set aside or cells grown in separate flasks were used (Fishman *et al*., 1994). In each case, the cells were lysed in an ice-cold solution of 1 mM Tris·HCl and 2 mM EDTA, pH 7.4, and portions of the cell lysates were incubated with 100 pM ^{125}I-CYP in 0.5 ml of 50 mM HEPES, pH 7.5, 4 mM $MgC1_2$, and 0.04% bovine serum albumin. Nonspecific binding was determined in the presence of 1 μm (−)-propranolol, and 0.3 μM CGP 20712A was used to distinguish between β_1AR and β_2AR subtypes (Fishman *et al*., 1994). After the binding reactions were incubated at 30° for 75 min, they were terminated by filtration over glass-fiber filters (no. 32; Schleicher & Schuell, Keene, NH) using a Brandel (Montreal, Quebec, Canada) M-24R harvester. Competition analysis with a β_1AR antagonist CGP 20712A indicated the presence of two distinct sites (not shown). In agreement with previous studies (Fishman *et al*., 1994; Hosoda *et al*., 1994), the high affinity site represented β_1AR, and the low-affinity site represented β_2AR; the proportion of the two subtypes was ~2:1. Both βAR subtypes were down-regulated to a similar extent in PMA-treated cells (not shown).

RNA extraction. After homogenization of the cells in the buffered guanidine thiocyanate solution, total RNA was isolated by centrifugation at 150,000 × *g* at 20° for 21 hr through a 5.7 M cesium chloride step gradient (Davis *et al*., 1986). RNA then was suspended in 0.3 M sodium acetate, pH 5.2, and precipitated in ethanol, and the concentration was determined by spectrophotometry at 260 nm.

Riboprobe and cRNA preparation. A uniformly radiolabeled riboprobe corresponding to the antisense DNA strand of the rat β_1AR coding region (+266 to +398) (Machida *et al*., 1990) was synthesized as described previously (Hosoda *et al*., 1994). Briefly, the 133-base pair riboprobe fragment was isolated by a *Pst*I/*Sac*I digest and cloned into pBluescript II SK (Strategene, La Jolla, CA). The cDNA was linearized by *Eco*RI digestion 5′ to the insert, and ^{32}P-labeled riboprobes were synthesized with T3 RNA polymerase in a 25-μl reaction volume using [α-^{32}P]CTP (800 Ci/mmol). The specific activity of the typical riboprobe was ~1 × 10^9 dpm/μg. Unlabeled sense strand cRNA was prepared from the same plasmid and used as a hybridization standard. The plasmid was linearized 3′ to the DNA insert, and cRNA complementary to the riboprobe was synthesized using T7 RNA polymerase (Melton *et al*., 1984). The sense strand then was purified, quantified by absorbance at 260 nm, and frozen in aliquots at −70°.

RNase protection assay. RNase protection analysis was carried out as described previously (Hosoda *et al*., 1994). Briefly, 10–20 μg of total RNA were hybridized with ^{32}P-labeled riboprobe (10^5 cpm/sample) at 63° for 16–18 hr. The samples were digested with RNase at 37° for 45 min. For the filtration assay, 10% trichloroacetic acid was added, and the samples were filtered through Whatman GF/C glass fiber filters. The filters were washed extensively and quantified by liquid scintillation spectroscopy. For polyacrylamide gel analysis, samples were treated in a similar manner with modifications (Hosoda *et al*., 1994; Rydelek-Fitzgerald *et al*., 1996) and then analyzed on 6% polyacrylamide/8 M urea denaturing gels. The gels were dried, and labeled bands were detected by autoradiography.

mRNA stability analysis. To determine the half-life of β_1AR mRNA, the cells were incubated with actinomycin D to block transcription as described previously (Hosoda *et al*., 1994). Cells were incubated in the absence and presence of PMA as described above, actinomycin D (2 μg/ml) was added to the media, and the cells were harvested at different times (0–120 min). Total cellular RNA was extracted at each of the time points, and β_1AR mRNA levels were quantified by RNase protection assay as described above. This concentration of actinomycin D was shown to inhibit RNA synthesis by >98% (Hosoda *et al*., 1994).

β_1AR promoter-reporter constructs and analysis. Most of the β_1AR-luc constructs were provided by Dr. Curtis Machida and have been described previously (Searles *et al*., 1995). The [−331, −1]luc construct was made by removing the *Xma*I fragment from the [−1806, −1]luc construct, followed by recircularization of the plasmid. The [−263, −1]luc construct was made by removing the *Xma*I/*Bss*HII fragments from the [−1806,−1]luc construct, followed by filling in with Klenow and then recircularization of the plasmid. Mutations of the putative PMA response element and a consensus CRE were constructed by polymerase chain reaction-mediated mutagenesis. The cell transfection and reporter analysis were conducted as described previously (Searles *et al*., 1995; Rydelek-Fitzgerald *et al*., 1996). Briefly, ~4 × 10^6 C6 glioma cells were transfected with calcium-phosphate/DNA precipitates using 2 μg of the β_1AR-luc reporter DNA and 1 μg of the pCMVβgal (Clontech, Palo Alto, CA) for 6 hr. The cells were washed twice with phosphate-buffered saline and then incubated with fresh medium containing vehicle (DMSO) or PMA (0.2 μM). After 18 hr, the cells were lysed for 10 min at 25° in 250 μl of reporter lysis buffer, and the cell lysates were assayed for luciferase activity (Promega, Madison, WI) and β-galactosidase activity (Tropix Galacto-light Plus kit). Luminescence was measured for 5 sec on an Opticomp luminometer.

Gel shift analysis. Gel mobility shift analysis was conducted as described previously (Rydelek-Fitzgerald *et al*., 1996). Briefly, cells were homogenized in 20 mM HEPES, pH 7.9, 0.4 M NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM *p*-aminobenzamidine, 10 mg/ml leupeptin, 1 mg/ml pepstatin, 20% glycerol, and 1% Nonidet-P40 with a Dounce homogenizer (12 strokes). Homogenates were incubated on ice for 20 min and centrifuged at 15,000 × *g* for 20 min

at 4°. Supernatant was used for the gel shift analysis and protein was measured by Bradford analysis (BioRad). The sequence of the synthetic oligonucleotide containing the β_1AR-PRE was 5′-TCGAGCCTGACGCGCGGCC-3′ (−350, −332). The sequence of the mutated β_1AR-PRE was 5′-TCGAGCCTTCTGCGCGGCC-3′. A CRE probe derived from the somatostatin promoter, 5′-GGCTGACGTCAGAG-3′, and an AP-1 probe derived from the human metallothionein promoter, 5′- TCGACGTGACTCAGCGCG-3′, also were used for competition and supershift studies. Double-stranded oligonucleotide probes were labeled with [α-^{32}P]dTTP and [α-^{32}P]dGTP using Klenow DNA polymerase. Cell extracts (10–15 μg of protein) were incubated at room temperature for 20 min with 1 μg of poly(dI-dC), 40 μg of bovine serum albumin, 10 mM Tris·HCl, pH 7.9, 10 mM KCl, 1 mM EDTA, 4% glycerol, and 1 ng of ^{32}P-labeled probe. The samples then were electrophoresed, and the resulting gels were dried and exposed to X-ray film to visualize the labeled DNA/protein complexes by autoradiography. For competition and supershift studies, the cell extracts and assay components were incubated with either increasing amounts of unlabeled DNA (1–100 ng) for 20 min or varying amounts of antibody before adding the labeled DNA probe. The antibodies used were anti-c-Jun/AP-1 (D) (Santa Cruz Biotechnology, Santa Cruz, NM), anti-FRA (Fos-related antigen) antibody (provided by Dr. M. Iadorola, NIDR, National Institutes of Health, Bethesda, MD), and anti-CREB (provided by Dr. M. Greenburg, Harvard University, Boston, MA).

Other methods. Levels of intracellular cAMP were determined by radioimmunoassay (Zaremba and Fishman, 1984).

Results

Down-regulation of β_1AR mRNA in PMA-treated C6 cells. Incubation of C6 cells with 0.2 μM PMA for 24 hr down-regulated levels of β_1AR mRNA. Levels of β_1AR mRNA in C6 cells were quantified by RNase protection analysis and a riboprobe derived from a 133-base pair fragment of the cloned rat β_1AR gene (Machida *et al.*, 1990). We have shown previously that the amount of protected hybrid is proportional to the amount of total RNA or sense strand cRNA that is added and that the method is very quantitative for measuring β_1AR mRNA levels in rat brain or C6 cells (Hosoda *et al.*, 1994). As can be seen in Fig. 1, the amount of RNase-protected riboprobe was much less when total RNA from PMA-treated C6 cells was used compared with that from control cells. This down-regulation of β_1AR mRNA levels was relatively rapid (Fig. 1A) and was dependent on the concentration of PMA, with an EC_{50} value of ~20 nM (Fig. 1B). The down-regulation of β_1AR mRNA required an active phorbol ester because the inactive analog, 4α-phorbol-12, 13-didecanoate, was ineffective (Table 1). In addition, a PKC inhibitor, bisindolylmaleimide, blocked the down-regulation of β_1AR mRNA, indicating that these effects are mediated by PKC (Table 1). In contrast, the PKC inhibitor did not block down-regulation of β_1AR mRNA in response to activation of the cAMP pathway (Table 1), demonstrating the specificity of this inhibitor.

We have shown previously that inhibition of protein synthesis in C6 cells effectively blocks the cAMP-mediated down-regulation of β_1AR mRNA and binding (Hosoda *et al.*, 1994). Prior treatment of C6 cells with *Pseudomonas* exotoxin A (0.3 μg/ml) had minimal effects on the reduction in β_1AR mRNA levels (Fig. 2). This concentration of exotoxin A inhibits protein synthesis in C6 by 96% in 4 hr, the time of pretreatment (Hosoda *et al.*, 1994).

Down-regulation of β_1AR binding in PMA-treated C6 cells. Activation of PKC also resulted in down-regulation of β_1AR binding as reported previously (Kassis *et al.*, 1985; Fishman *et al.*, 1987). Down-regulation of β_1AR binding, determined with ^{125}I-CYP and the selective β_1AR antagonist CGP 20712A (see Experimental Procedures), occurred relatively slowly over a 24-hr period (Fig. 1A, *inset*), and there was no further loss by 48 hr (data not shown). The extent of β_1AR down-regulation was dependent on the concentration of PMA (Fig. 1B, *inset*), with an EC_{50} value of ~15 nM. This was similar to the concentration of PMA required for half-maximal binding to PKC in C6 cells (Kassis *et al.*, 1985; Fishman *et al.*, 1987). Down-regulation of β_1AR binding was not observed with the inactive phorbol ester and was blocked by the PKC inhibitor (Table 1). Incubation with the protein synthesis inhibitor slowed the rate of PMA-mediated down-regulation of β_1AR binding sites and slightly reduced the maximal loss (data not shown).

Effect of PMA on basal and agonist-stimulated cAMP levels in C6 cells. The effects of PMA on steady state levels of β_1AR binding and mRNA in C6 cells have some similarities to those of agents that elevate cAMP levels (Hosoda *et al.*, 1994). Thus, it was important to determine whether PMA was mediating its effects by elevating cAMP in the cells.

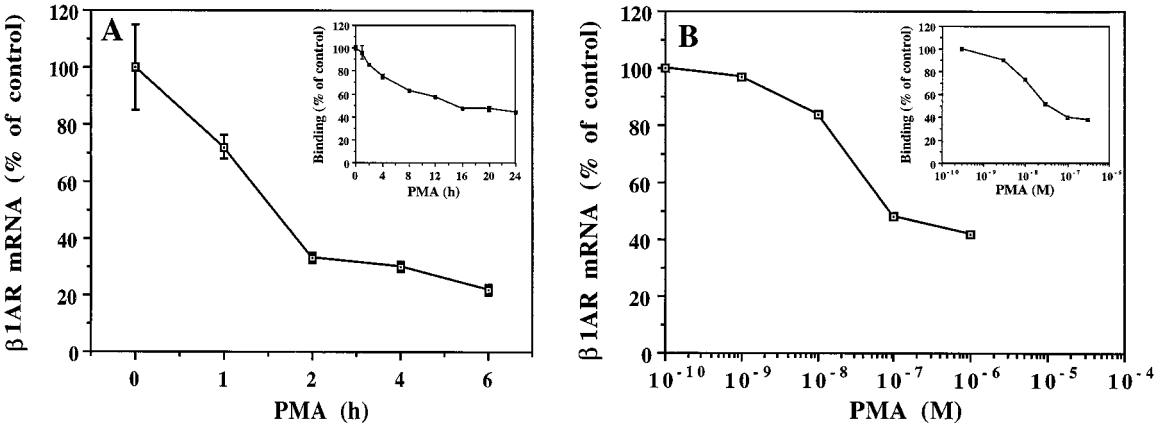

Fig. 1. Time and concentration dependence for PMA-mediated down-regulation of β_1AR binding activity and mRNA in rat C6 glioma cells. Cells were exposed to 0.2 μM PMA for the indicated times (A) or to increasing concentrations of PMA for 4 hr (B). Cells then were collected and assayed for β_1AR mRNA levels by solution hybridization analysis or specific binding of 100 pM ^{125}I-CYP or as described in Experimental Procedures. Data for mRNA values are the mean ± standard error of three separate experiments, each analyzed in duplicate. Binding data are mean ± standard deviation of triplicate values from one of five representative experiments.

Basal cAMP levels remained relatively constant in cells exposed to 0.2 μM PMA for up to 2 hr (from 14.7 ± 0.7 to 19.0 ± 1.8 pmol/mg protein, mean ± standard error). As expected, cAMP levels increased ~100-fold in cells stimulated with 1 μM isoproterenol for 20 min (1310 ± 7.4 pmol/mg protein, mean ± standard error), and this response was dramatically attenuated in cells treated with 0.2 μM PMA for 2 hr (94.6 ± 4.7 pmol/mg protein, mean ± standard error). The latter effect is consistent with the reported desensitization of βAR in C6 cells in response to activation of PKC (Kassis *et al*., 1985).

Effect of PMA treatment on β_1AR mRNA stability. To determine whether the PKC-mediated decrease in β_1AR mRNA levels might be due to a change in mRNA stability, we measured its half-life. Control and PMA-treated cells were exposed to actinomycin D to block further transcription, and β_1AR mRNA levels were assayed at different times. As shown in Fig. 3, PMA treatment did not increase receptor mRNA degradation. If anything, β_1AR mRNA was slightly more stable in PMA-treated cells; the respective half-lives were ~45 and ~75 min for control and PMA treated cells. We previously observed a similar, slight increase in β_1AR mRNA stability after the treatment of C6 cells with isoproterenol or forskolin (Hosoda *et al*., 1994). The half-life of 45 min obtained in the current study was similar to that observed in our earlier study (Hosoda *et al*., 1994) and somewhat faster than the 100 min reported by Kiely *et al*. (1994).

TABLE 1

Effect of phorbol esters and PKC inhibitor on β_1AR binding and mRNA in rat glioma C6 cells

Cells were exposed to the indicated phorbol ester (0.2 μM) or CPT-cAMP (200 μM) in the absence or presence of the PKC inhibitor BIS (1 μM) and analyzed for levels of β_1AR mRNA and binding as described in Experimental Procedures. The values are presented as percentage of control and are the mean ± standard error of three separate receptor binding experiments or two mRNA experiments conducted in duplicate.

Phorbol	β_1AR mRNA	β_1AR binding
None	100	100
PMA	38 ± 1	50 ± 3.5
4α-Phorbol-12,13-dideconoate	112 ± 4	105 ± 9.7
α-Phorbol	96 ± 1	106 ± 5.5
None	100	100
PMA	57 ± 4	51 ± 4
BIS	108 ± 6	98 ± 6
PMA + BIS	86 ± 2	94 ± 3
CPT-cAMP	50 ± 1	52 ± 1
CPT-cAMP + BIS	56 ± 1	50 ± 2

PMA-mediated repression of β_1AR promoter activity. Reporter studies were conducted to examine the influence of activation of PKC on β_1AR transcription rate. A large portion of the rat β_1AR promoter (−3354, −1) attached to the luciferase reporter, referred to as [−3354, −1]luc, was used for this study (Searles *et al*., 1995). C6 cells were transfected with [−3354, −1]luc and pCMV-βgal DNA, which served as a marker for transfection efficiency. The cells were routinely transfected for 6 hr and, after washing, were incubated with PMA or vehicle and assayed for luciferase and β-galactosidase activity after an additional 18 hr. As shown in Fig. 4A, PMA treatment significantly reduced the level of β_1AR[−3354, −1]luc activity. This reduction was observed after 6 hr of PMA treatment and lasted for up to 24 hr in PMA-treated cells (data not shown). To identify the promoter sequence or sequences mediating this effect of PMA, several truncated reporter constructs were analyzed (Fig. 4A). Like the longer β_1AR [−3354, −1]luc construct, the β_1AR truncated constructs [−1252, −1]luc and [−484, −1]luc were down-regulated in response to PMA treatment, whereas expression of a construct [−1252, −479]luc lacking the −478, −1 portion of the promoter was not affected by PMA treatment.

An additional series of truncation constructs were examined to define further the location of the PKC responsive element or elements in the β_1AR promoter (Fig. 4B). The reporter activity of constructs [−369, −1]luc and [−348, −1]luc also were significantly down-regulated by PMA treatment. In contrast, constructs containing smaller portions of the promoter, including those truncated at −331, −299, and −263, were not significantly influenced by PMA treatment. It was notable that PMA partially reduced the activity of the [−339, −1]luc construct. Based on these results, the PKC response element seemed to be located between −348 and −331, and it could include the sequence proximal to −339. The observation that the activity of [−484, −325]luc, but not [−484, −367]luc, also is decreased by PMA treatment is in agreement with this conclusion.

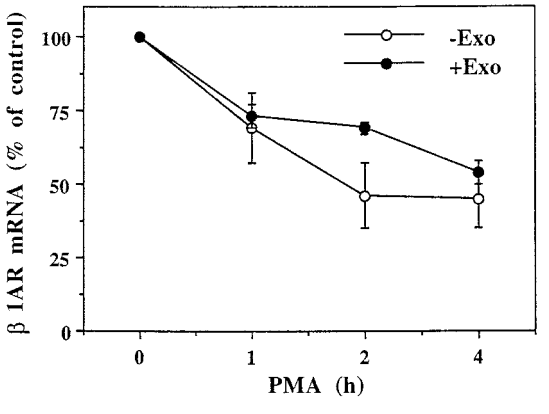

Fig. 2. Influence of inhibition of protein synthesis on PMA-mediated down-regulation of β_1AR mRNA in rat C6 glioma cells. Cells were treated without and with *Pseudomonas* exotoxin A (0.3 μg/ml) for 4 hr, exposed to 0.2 μM PMA for the indicated times, and then assayed for β_1AR mRNA levels by solution hybridization analysis as described in Experimental Procedures. Data are the mean ± standard error of three separate samples.

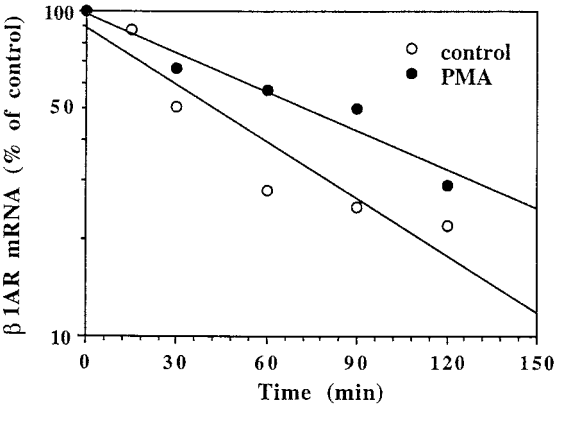

Fig. 3. Influence of PMA treatment on β_1AR mRNA stability in rat C6 glioma cells. Cells were exposed to vehicle or 0.2 μM PMA for 4 hr and then to actinomycin D (2 μg/ml). The cells were collected at the indicated times and analyzed for β_1AR mRNA levels by solution hybridization analysis as described in Experimental Procedures. The results are expressed as percent of control (0 time point, after the addition of actinomycin D) and are plotted on a log scale versus time. Data are the mean ± standard error of three separate experiments, each analyzed in duplicate.

A computer analysis of the sequence within this region indicated that there was only one potential motif with significant homology to known regulatory elements. The sequence −343, −336 (TGACGCGC) has partial sequence homology with consensus CRE (TGACGTCA) and AP-1 (TGACCTCA) response elements. This putative element was mutated to further investigate its role in mediating the PMA response. The sequence within the longest promoter construct (−3354, −1) was changed to TTCTGCGC (altered nucleotides are underlined). This mutation resulted in the complete loss of the PKC-induced down-regulation of the promoter activity (Fig. 5). In contrast, mutations of CRE (−1315 to −1308) or the inverted CCAAT (−354 to −358) sites located in the promoter did not influence the PKC response (Fig. 5). These results demonstrate that the partial CRE/AP-1 element mediates the PKC-induced repression of β_1AR promoter activity. We refer to this site as β_1AR-PRE.

Gel shift analysis of the PRE. Gel mobility shift analysis was used to further study the putative β_1AR-PRE site in the promoter. A double-stranded synthetic oligonucleotide containing this response element was used as a probe to detect DNA binding proteins. When C6 cell extracts were incubated with ^{32}P-labeled β_1AR-PRE oligonucleotide, two major retarded bands were observed (Fig. 6) (free radiolabeled probe runs near the bottom of the gel and is not shown). Treatment of the cells with PMA did not significantly influence the gel shift pattern either quantitatively or qualitatively. This is consistent with the finding that PMA-mediated down-regulation of β_1AR mRNA is not dependent on *de novo* protein synthesis (see Fig. 2). The putative DNA binding proteins

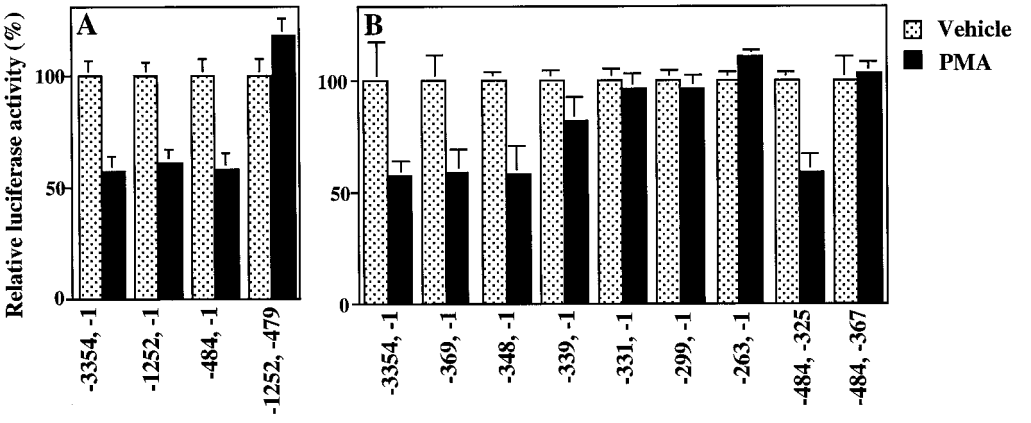

Fig. 4. Influence of PMA on β_1AR promoter activity in C6 cells with deletion analysis of the 5′ flanking region. C6 cells were cotransfected transiently with β_1AR promoter-luciferase reporter constructs containing the indicated portions of the promoter and with pCMVβgal. After 6 hr, the cells were incubated with either vehicle or 0.2 μM PMA for an additional 18 hr and assayed for luciferase and β-galactosidase activities as described in Experimental Procedures. The results, normalized for β-galactosidase activity, are expressed as percent of vehicle and are the mean ± standard error of three separate experiments, each analyzed in duplicate. A, Deletions of the −3354, −1 flanking region. B, Deletions of the −484, −1 flanking region.

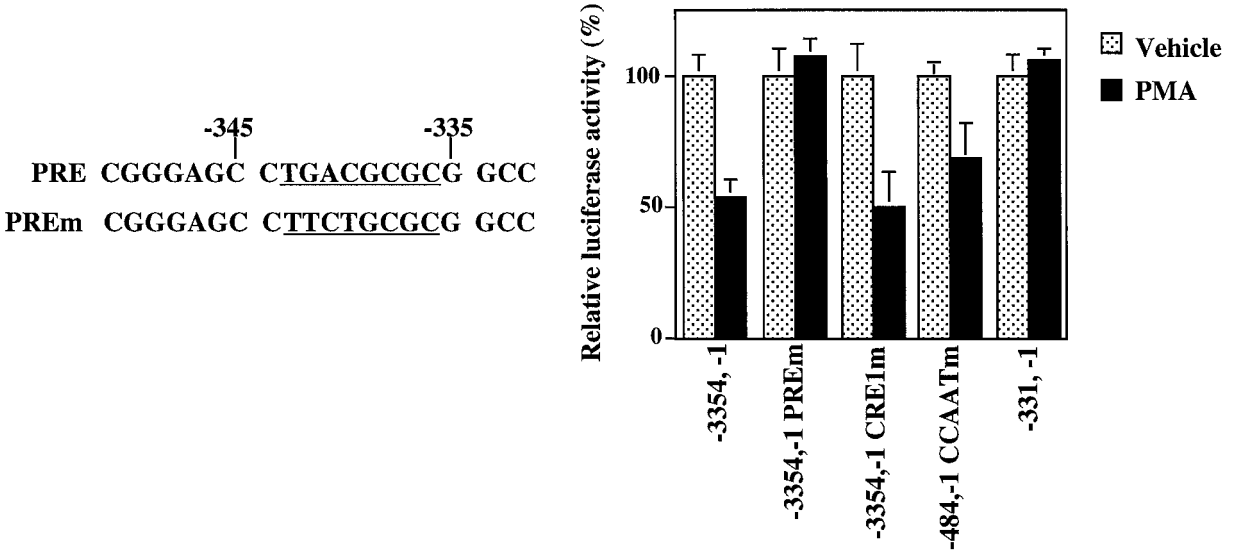

Fig. 5. Mutation analysis of the 5′ flanking region and regulation of β_1AR promoter activity in C6 cells. C6 cells were cotransfected transiently with β_1AR promoter-luciferase reporter constructs containing mutations of specific elements as described in the legend to Fig. 4 and Experimental Procedures. These included mutations of the putative PKC response element at −343 to −336 (−3354, −1PREm) as indicated (*right*), the CRE at −1315 to −1307 (−3354, −1CREm), and the inverted CCAAT at −354 to −358 (−484, −1 CCAATm). Data are the mean ± standard error of three separate experiments, each analyzed in triplicate.

were further characterized by competition and supershift experiments. Binding of the labeled probe to both bands was effectively blocked by unlabeled β_1AR-PRE, but to a much lesser extent by the mutated form of this oligonucleotide (TTCTGCGC) (Fig. 6), which indicates that both bands represent specific labeling. An unlabeled oligonucleotide containing a consensus CRE competed out the upper band but had much less of an effect on the lower band. In contrast, an oligonucleotide containing an AP-1 response element had very little effect on either band (Fig. 6).

The identity of the protein complex binding to the labeled PRE was examined further with transcription factor antibodies. Preincubation with a polyclonal CREB antibody disrupted the formation of the upper but not the lower complex (Fig. 7), whereas polyclonal antibodies to Jun or Fos (i.e., FRA) did not significantly influence binding to either band. When a labeled probe containing a consensus CRE was used, three major complexes were formed, and one corresponded in mobility to the upper PRE band. All three of these bands were disrupted by preincubation with CREB antibody, and an SS band was observed only with the SS CRE probe. To test further the hypothesis that the upper PRE binding complex contains CREB, the influence of recombinant CREB on PRE binding was examined. Transfection of C6 cells with recombinant CREB increased levels of the upper β_1AR-PRE binding complex, and this band was disrupted by preincubation with CREB antibody (Fig. 8). Based on these results, the upper β_1AR-PRE complex in the gel shift assay seems to contain CREB. The identity of the protein or proteins in the lower binding complex is currently unknown.

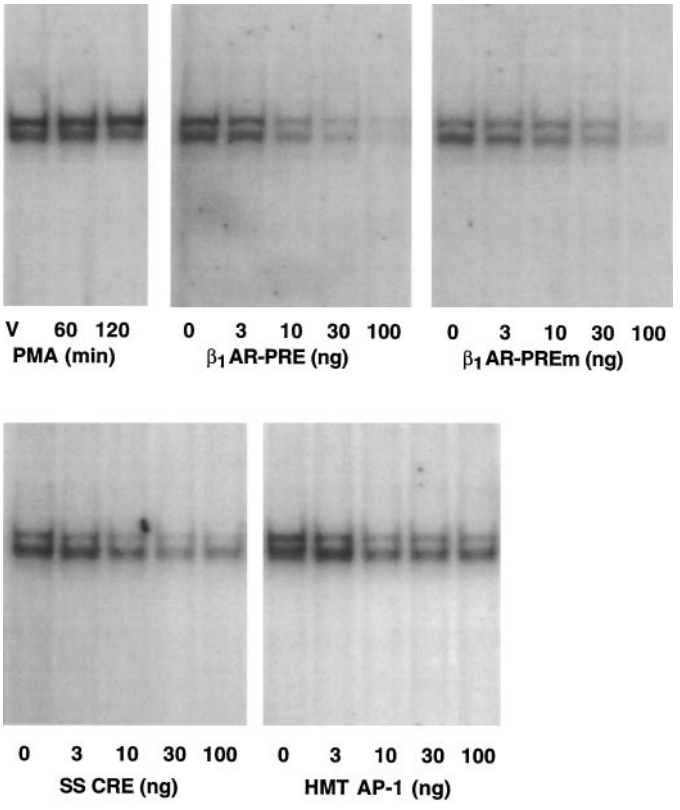

Fig. 6. Gel mobility shift analysis of complexes formed between β_1AR-PRE and DNA binding proteins in C6 cells. Extracts were incubated with a ^{32}P-labeled β_1AR-PRE oligonucleotide probe and subjected to gel mobility shift analysis as described in Experimental Procedures. *Top*, *first panel*, extracts are from control (*V*) and PMA-treated cells (0.2 μM for 1 and 2 hr). *Other panels*, results of competition experiments with 0–100 ng of unlabeled oligonucleotides containing the β_1AR-PRE, mutated β_1AR-PRE (*β_1AR-PREm*), the somatostatin CRE (*SS CRE*), and the human metallothionein AP-1 (*HMT AP-1*), respectively. Similar results were obtained in three separate experiments.

Discussion

In the current study, we observed that the treatment of C6 cells with PMA resulted in a down-regulation of both β_1AR steady state mRNA and binding levels. Because the reduction in mRNA temporally preceded the reduction in binding, it is likely that changes in β_1AR mRNA levels account for most of the loss of receptor binding activity. Both effects exhibited a similar dependence on PMA concentration with an EC_{50} value of 15–20 nM, required an active phorbol ester, and were blocked by a PKC inhibitor. Rat C6 glioma cells express at least four isoforms of PKC (α, δ, ϵ, and ζ) (Chen *et al.*, 1993). The first three are activated by PMA and inhibited by bisindolylmaleimide, and thus one or more of these may mediate the down-regulation of receptor expression observed in the present study. The down-regulation of β_1AR mRNA levels was not due to a decrease in receptor mRNA stability, as determined from mRNA half-life studies in actinomycin D-treated cells. Rather, using a β_1AR promoter-luciferase reporter construct, we found that the rate of β_1AR gene transcription was reduced ~50% by activation of PKC.

Prior exposure of C6 cells to exotoxin A, a potent inhibitor of protein synthesis, had little effect on the subsequent PKC-induced down-regulation of β_1AR mRNA levels. Based on these results, it is unlikely that PKC is mediating its effects on β_1AR gene transcription by inducing a repressor. This is in contrast to our recent findings that cAMP-mediated down-regulation of β_1AR gene transcription is blocked by exotoxin

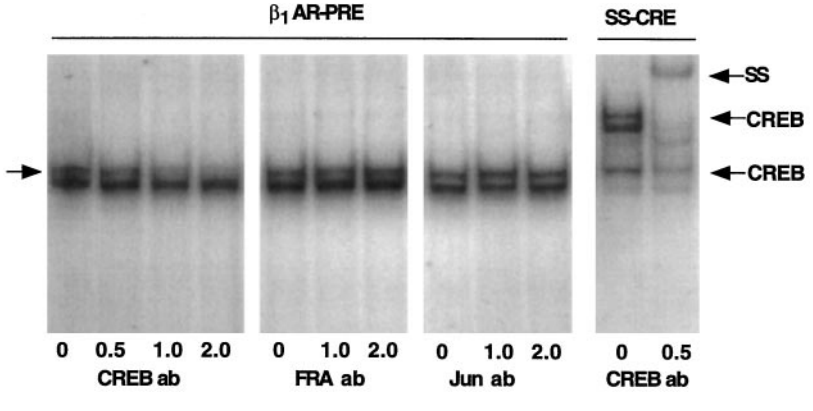

Fig. 7. Gel mobility supershift analysis of complexes formed between β_1AR-PRE and DNA binding proteins in C6 cells. Cell extracts were incubated with different amounts (in microliters) of antibodies raised against CREB, Fos-related proteins (FRA), or Jun as described in Experimental Procedures. The amount of antibody tested disrupts binding of complexes to consensus CRE (CREB) and AP-1 (FRA and Jun, not shown) elements. The reaction was started by the addition of ^{32}P-labeled oligonucleotide probes containing the β_1AR-PRE or SS-CRE sequence. Representative autoradiograms are shown for each condition. Similar results were obtained in three separate experiments.

A (Hosoda *et al.*, 1994) and results suggesting that this effect involves induction of a repressor known as the inducible cAMP early repressor (Rydelek-Fitzgerald *et al.*, 1996). More likely, activation of PKC results in phosphorylation and regulation of DNA-binding activity of an existing transcription factor or factors that repress β_1AR gene expression (see below). The results also indicate that down-regulation of β_1AR mRNA by PKC does not involve the cAMP system. First, activation of PKC does not result in a significant up-regulation of cAMP levels. Second, down-regulation of β_1AR mRNA by PKC is not blocked by inhibition of protein synthesis, which is in contrast to the cAMP-mediated down-regulation (Hosoda *et al.*, 1994).

We determined the location of the element that mediates the PKC response by deletion analysis of the β_1AR promoter. Deletions up to −348 did not influence the PKC-induced down-regulation of reporter activity, whereas additional deletions up to −339 (partially) or −331 (fully) blocked the response. Computer analysis of this region (−348, −331) revealed an element with partial homology to consensus CRE and AP-1 response elements. We then tested directly the involvement of this element in the PKC response by mutation analysis. The substitution of three of the four bases known to be critical for binding to the CRE and AP-1 sites completely blocked the PKC response. In contrast, mutation of a CRE site located further upstream (−1314, −1307) did not alter the ability of PKC activation to repress β_1AR promoter expression. Based on these results, there seems to be a CRE/AP-1-like site located at −343 to −336 (TGACGCGC) that mediates the negative effect of PKC on the rate of β_1AR gene transcription. We have referred to this site as β_1AR-PRE. The identical site is located in the mouse β_1AR gene (Cohen *et al.*, 1993). A putative PRE site also has been found at a similar location in the human β_1AR gene (TGACGCGA, −360 to −353) (Collins *et al.*, 1993) suggesting that PKC could decrease the transcriptional activity of the human gene through a similar mechanism. A highly homologous sequence (GGACGCGC, −140 to −133) is present in the rat β_2AR gene (Jiang and Kunos, 1995), and we have found that β_2AR binding also is down-regulated in PMA-treated C6 cells (not shown).

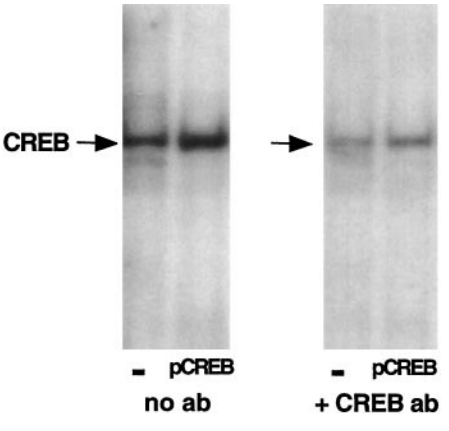

Fig. 8. Influence of recombinant CREB on β_1AR-PRE binding proteins in C6 cells. Cells were transfected with or without pCMV-CREB (*pCREB*), and cell extracts were incubated with a ^{32}P-labeled β_1AR-PRE oligonucleotide probe and subjected to gel mobility shift analysis. The influence of preincubation with CREB antibody (1 μl) on levels of the binding complex also was determined (*right*). Representative autoradiograms are shown for each condition. Similar results were obtained in two separate experiments.

We used gel mobility shift analysis to determine the presence of transcription factor or factors in C6 cells that bind to β_1AR-PRE. We found that a labeled oligonucleotide containing the β_1AR-PRE sequence formed two specific complexes that were not influenced by treatment of the cells with PMA. This is consistent with PKC regulation of these factors by phosphorylation, not by regulation of their expression. Binding to the labeled β_1AR-PRE probe was specific in that the unlabeled probe competed effectively compared with unlabeled oligonucleotides containing either a mutated β_1AR-PRE or an AP-1 sequence. Interestingly, an unlabeled CRE-containing oligonucleotide competed out the upper but not the lower complex. Furthermore, an antibody to CREB, but not antibodies to Fos or Jun, disrupted formation of the upper but not lower complex. Finally, expression of recombinant CREB increased levels of the upper, but not lower, complex. Based on these results, we believe that the upper, more retarded complex observed in the gel shift assay contains CREB, whereas the identity of the lower, less retarded complex is not known. However, it also is possible that the upper band contains a protein with considerable structural and immunochemical homology to CREB.

There are several CREB-like proteins that are known to inhibit the function of CREB. One family of CRE repressors are the CREMs (Foulkes *et al.*, 1991). There are several forms of CREM that act as transcriptional repressors and are regulated by phosphorylation, including CREMα and CREMβ. Inducible cAMP early repressor acts as CRE repressor but is regulated by its level of expression, not phosphorylation. CREM transcription factors can homodimerize or heterodimerize with CREB to form a nonactivating dimer that binds to CRE elements. It is possible that phosphorylation of a CREM repressor is responsible for the down-regulation of β_1AR in response to activation of PKC. However, preliminary supershift studies indicate that anti-CREM antibody does not disrupt the formation of either the upper or lower complexes with β_1AR-PRE (data not shown). Further gel shift studies will be required to determine the identity of the proteins binding to the β_1AR-PRE and to determine whether these proteins are substrates of PKC-mediated phosphorylation.

Although we are unaware of other reports on the regulation of β_1AR mRNA levels via activation of PKC, Feve *et al.* (1995) recently reported that activation of PKC induces the down-regulation of β_3AR binding activity and mRNA in 3T3-F442A adipocytes. Interestingly, PKC activation does not alter β_1AR mRNA in these cells. Because they found that the stability of β_3AR transcripts remains unchanged in PMA-treated adipocytes, they propose that activation of PKC leads to inhibition of β_3AR gene transcription. There are studies on other G protein-coupled receptors, including muscarinic cholinergic, α-adrenergic, and serotonergic receptors. All of these studies indicate that there are multiple mechanisms for such regulation. Activation of PKC in hamster smooth muscle DDT1 MF-2 cells causes an increase in α_{1B}AR gene transcription (Hu *et al.*, 1993), whereas in rabbit aortic smooth muscle cells, PKC activation induces a down-regulation by destabilizing the mRNA for this receptor (Izzo *et al.*, 1994). Destabilization of the rat m_1 muscarinic receptor mRNA also was observed in PMA-treated Chinese hamster

ovary cells stably transfected with this receptor gene (Earle-Hughes and Fraser, 1994). Rousell *et al*. (1995) found that m_2 muscarinic receptor gene transcription is down-regulated in human embryonic lung 299 cells by activation of PKC and that this effect is blocked by cycloheximide, suggesting that induction of a transcriptional repressor protein may be involved. In P11 cells derived from a rat pituitary tumor, PKC activation leads to a transient increase in serotonin 5-HT_{2A} receptor mRNA that is due not to increased transcription but rather to increased stability of the mRNA (Ferry *et al*., 1994).

Based on our current results and those of previous studies, there seems to be several mechanisms to regulate β_1AR gene expression in rat C6 glioma cells and other cells. Agonist stimulation of C6 cells leads to a transient increase, followed by a decrease, in gene transcription (Hosoda *et al*., 1994). Glucocorticoid treatment of C6 cells causes a decrease in gene transcription (Kiely *et al*., 1994), which may explain the steroid-mediated reduction in β_1AR mRNA observed previously in murine 3T3 adipocyte cell lines (Feve *et al*., 1990; Guest *et al*., 1990). In contrast, thyroid hormones transcriptionally up-regulate the β_1AR gene in cultured rat ventricular myocytes (Bahouth, 1991). Here, we show that activation of PKC causes a transcriptional down-regulation of the β_1AR gene expression in C6 cells. Thus, cross-regulation of β_1AR by PKC can occur at both post-transcriptional (by phosphorylation of the receptor protein) and transcriptional (by repression of gene expression) levels.

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