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Cycloheximide Induces the α_{1B} Adrenergic Receptor Gene by Activation of Transcription in DDT$_1$ MF-2 Smooth Muscle Cells

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SUMMARY

α_1-Adrenergic receptors play important roles in mediating a wide range of important cellular responses; regulation of expression of these receptors may have pathophysiological significance in diseases such as hypertension. In order to pursue understanding of mechanisms involved in the regulation of expression of α_1 receptors, the effects of protein synthesis inhibitor cycloheximide on α_{1B} receptor gene expression were examined in DDT$_1$ MF-2 smooth muscle cells. Cycloheximide markedly induced accumulation of the α_{1B} receptor mRNAs in a concentration- and time-dependent manner as detected by Northern blotting assays. The increased accumulation of α_{1B} receptor mRNA could be detected at 1 hr (1.7 ± 0.2-fold) and the maximal accumulation occurred at 6 hr (5.4 ± 0.3-fold, $p < 0.01$). Nuclear runoff assays reveal that cycloheximide markedly increased the transcriptional rate of the α_{1B} receptor gene. The stability of α_{1B} receptor mRNAs measured by RNase protection assays was essentially unchanged by cycloheximide. Incubation of DDT$_1$ MF-2 cells with two additional protein synthesis inhibitors, anisomycin and emetine, had similar effects to those of cycloheximide. However, a further inhibitor, puromycin, did not induce α_{1B} receptor mRNAs when protein synthesis was almost completely inhibited. Furthermore, puromycin did not inhibit the capacity of cycloheximide to induce transcription of the α_{1B} receptor gene. These observations suggest that cycloheximide induces α_{1B} receptor gene expression through direct activation of gene transcription rather than inhibition of protein synthesis.

α_1-Adrenergic receptors (ADR) are members of the class of G protein coupling membrane receptors and mediate many of the important physiological effects of catecholamines such as epinephrine (1–3). α_1-ADR play a particularly important role in control of cardiovascular responses such as regulation of blood pressure via activation of smooth muscle contraction (4). α_1-ADR also mediate cardiac and vascular smooth muscle growth and hypertrophy by catecholamines (5, 6). However, relatively little is known concerning the molecular mechanisms for regulation of α_1-ADR gene expression in either physiological or pathophysiological conditions.

We have demonstrated previously that glucocorticoids regulate expression of the α_{1B}-ADR gene in DDT$_1$ MF-2 smooth muscle cells which might be associated with the role of glucocorticoids in the increased sensitivity of vascular smooth muscle to catecholamines (7). Also, we have recently found that activation of protein kinase C by phorbol esters also leads to a several-fold induction of α_{1B}-ADR mRNA accumulation in these cells (8). Both glucocorticoids and phorbol esters enhance transcription of the α_{1B}-ADR gene. The effects of protein kinase C on expression of α_{1B}-ADRs are particularly interesting since it has recently been suggested that these receptors are potential oncogenes (9).

For some genes, exposure of cells to inducers of gene expression in the presence of inhibitors of protein synthesis leads to a greater induction of gene expression than the inducers alone; this phenomenon has been termed as "superinduction" (10, 11). According to this definition, inhibitors of protein synthesis function as cofactors to augment the effect of the inducer on gene expression. It has been demonstrated that some inhibitors of protein synthesis themselves activate gene expression in the absence of other inducers (12–14). Protein synthesis inhibitors may reveal mechanisms for regulation of gene expression at different levels (15). For example, it was thought that a prerequisite for induction of many eukaryotic genes by growth factors, hormones, and phorbol esters involved *de novo* synthesis of transcription factors (16, 17). However, it has been shown that induction of transcription of some genes, such as the proto-oncogenes c-*fos* and c-*jun*, does not require *de novo* protein synthesis since this activation is not blocked by inhibitors of protein synthesis (13, 14), suggesting that induction mechanisms may be related to the posttranslational modification of preexisting transcriptional factors (18). However, a number of studies have indicated that the effects of protein synthesis inhibitors on gene expression may result either from consequence of inhibition of protein synthesis or from direct effects of the compounds leading to activation of transcription (13–15).

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ABBREVIATIONS: ADR, adrenergic receptor; CHX, cycloheximide; PMA, 4β-phorbol 12-myristate 13-acetate; SDS, sodium dodecyl sulfate.

To determine possible role of inhibitors of protein synthesis in the induction of the α_{1B}-ADR gene expression in DDT_1 MF-2 smooth muscle cells, we incubated DDT_1 MF-2 cells with cycloheximide and found that it, as well as the protein synthesis inhibitors anisomycin and emetine, markedly induced α_{1B}-ADR mRNA expression in DDT_1 MF-2 smooth muscle cells. However, puromycin, which markedly attenuated protein synthesis in these cells, did not induce expression of the α_{1B}-ADR gene.

Experimental Procedures

Materials. Cycloheximide, emetine, anisomycin, and puromycin were purchased from Sigma (St. Louis, MO); [^{32}P]dCTP (2000 Ci/mmol), [^{32}P]UTP (3000 Ci/mmol), Hybond nylon filters, and random primer labeling system from Amersham Corp. (Arlinton, IL); nitrocellulose membrane from Schleicher and Schuell (Keene, NH); pGEM plasmid, T7 RNA polymerase from Promega Corp. (Madison, WI); RNase A and oligo(dT)-cellulose from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ); RNase T1, proteinase K, and actinomycin D from Boehringer Mannhein Biochemicals (Indianapolis, IN); RNase-free DNase I from United States Biochemical (Cleveland, OH); restriction enzymes, T4 DNA ligase from New England Biolabs (Beverly, MA); and [^{14}C]leucine (50 Ci/mmol) from New England Nuclear (Boston, MA). cDNA of α_{1B}-ADR was a gift of Dr. Lefkowitz and colleagues of Duke University; cell culture medium and newborn bovine serum were from Gibco/BRL (Grand Island, NY). All other chemicals were reagent or molecular biology grade and were obtained from standard commercial sources.

Cell culture and preincubation of cells with inhibitors of protein synthesis. In all experiments, DDT_1 MF-2 hamster smooth muscle cells were maintained in Dulbecco's modified Eagle's medium containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated newborn calf serum at 37° in air containing 5% CO_2 (19). The cells were seeded in 100-mm dishes at a low density, with confluence being reached about 6 days later. The medium was replaced every 2–3 days. The cells were treated with protein synthesis inhibitors or vehicle solution (as control) starting from the latest time point and the cells were harvested at the same time.

RNA preparation and Northern blot analysis. A single-step method of RNA isolation using acid guanidinium thiocyanate-phenol-chloroform extraction as described by Chomczynski and Sacchi (20) was used to isolate total RNA from the cultured smooth muscle cells. Briefly, the cultured DDT_1 MF-2 cells were rinsed with cold calcium-magnesium-free phosphate-buffered saline and then the cells were homogenized with a Polytron in 10 vol of denaturing buffer containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol. One volume of 2 M sodium acetate (pH 4.0), 10 vol of water-saturated phenol, and 2 vol of chloroform-isoamyl alcohol (49:1) were sequentially added to the homogenate with thorough mixing after addition of each reagent. The homogenates were incubated on ice for 20 min and centrifuged at 12,000 × g for 20 min. The aqueous phase was taken and RNA was precipitated from it with isopropanol (1:1 vol). The resulting RNA pellet was dissolved in the denaturing buffer and again precipitated with isopropanol by cooling and centrifugation. The RNA pellet was washed with 75% ethanol, sedimented, vacuum dried, and dissolved in TE buffer or autoclaved water to be used in experiments. The total RNA obtained was then purified by oligo(dT) cellulose column chromatography to obtain poly(A)$^+$ RNA (21). For Northern blot analysis, 2–5 μg of poly(A)$^+$ RNA were heated at 60° for 10 min, cooled rapidly on ice, and denatured with 6% formaldehyde. The mRNA was fractionated by 1% agarose gel electrophoresis, and transferred to a nylon filter by capillary blotting. The blot was prehybridized in 50% formamide, 5 × SSPE buffer, 5 × Denhardt's solution, and 0.5% sodium dodecyl sulfate (SDS), at 42° for 4 hr, and hybridized at 42° for 12–16 hr to the α_{1B}-ADR or the β-actin cDNA probes that were labeled by [^{32}P]dCTP using Amersham's random priming labeling kit. After hybridization, the filter was washed twice in 2 × SSPE and 0.1% SDS at 65° for 15 min, and once in 0.1 × SSPE and 0.1% SDS at 56° for 30 min. The filter was exposed to Kodak XAR-5 film at −70° with an intensifying screen for 16–24 hr. The autoradiograms were scanned using a laser densitometer. The amount of α_1-ADR mRNAs (both 2.1 and 2.3 kbp bands) was quantified relative to the amount of β-actin mRNA on the same filter.

Nuclear runoff transcription assays. To determine whether inhibition of protein synthesis changed the transcription rate of α_{1B}-ADR, nuclear run-on transcription assays were performed as described previously (22). The cells from two 100-mm dishes were used for isolation of the nuclei. The resulting pelleted nuclei were either stored at −70° or used immediately. The nuclei (2×10^7) were resuspended in 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.1 mM EDTA, and 20% glycerol at a concentration of 2×10^5 nuclei/μl. The prepared nuclei (200 μl) were added to 200 μl of reaction buffer composed of 10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.3 M KCl, 5 mM DL-dithiothreitol, 10 mM unlabeled GTP, ATP, CTP, and 10 μl of [α-^{32}P]UTP (3000 Ci/mmol), incubated for 30 min at 30°. After RNase-free DNase I and proteinase K treatments, the reaction products were extracted with phenol/chloroform and unincorporated [^{32}P]UTP was removed by trichloroacetic acid precipitation and filtration through a nitrocellulose filter. The radiolabeled RNA was treated again with DNase I and proteinase K, extracted with phenol/chloroform, and precipitated with 75% ethanol. This labeled RNA ($2–3 \times 10^7$ cpm) was dissolved in 1 ml of hybridization solution (same as described above) and hybridized at 42° for 36 hr with 20 μg of the pGEM-3Z plasmid immobilized to a nylon filter as control or with immobilized plasmid containing inserts of α_{1B}-ADR cDNA or β-actin cDNA (23). The filters were washed with 0.5 × SSPE and 0.1% SDS at 65° for 30 min and then treated with 2.5 μg/ml of RNase A and 5 units/ml of RNase T_1 at 37° for 30 min. After autoradiography, the film was scanned with laser densitometry and quantified by calculation of ratio of α_{1B}-ADR cDNA signal to β-actin cDNA signal.

Stability analysis of α_1-ADR mRNA by RNase protection assays. To determine whether cycloheximide produces variation in the rate of degradation of α_{1B} receptor mRNA, the stability of the α_1-ADR mRNA in cycloheximide-treated and control cells was measured by incubating the cells with 5 μg/ml actinomycin D to block RNA synthesis (24). After designated times of incubation with actinomycin D, total RNA was isolated from individual dishes of these actinomycin D-treated cells as described above and an RNase protection assay was performed. The RNA probe was produced by incubating a pGEM-3Z plasmid that contained a 0.3-kbp DNA fragment from the hamster α_{1B}-ADR cDNA with T7-RNA polymerase and [^{32}P]UTP (100 μCi) at 37° for 1 hr. The RNA probe (5×10^5 cpm) and 50 μg of total RNA were mixed and hybridized for 16 hr at 45° in 80% formamide, 0.4 M NaCl, 50 mM piperazine-N,N′-bis (2-ethanesulfonic acid), and 1 mM EDTA. RNase buffer containing RNase A (10 μg/ml) and RNase T1 (10 U/ml) was added to each assay tube and then incubated for 30 min at 30°. RNase-resistant hybrids were precipitated with ethanol and run on a 5% polyacrylamide, 8 M urea gel (25).

Measurement of inhibition of protein synthesis using [^{14}C] leucine incorporation. DDT_1 MF-2 cells were grown to near confluence in a series of 60-cm^2 dishes. Fresh Dulbecco's modified Eagle's medium was added and supplemented with the different concentrations of the indicated protein synthesis inhibitors. After 1 hr, [^{14}C]leucine (2 μCi/ml) was added, and incubation was continued for an additional 4 hr. Cells were washed four times with ice-cold phosphate-buffered saline and collected by scraping. Proteins were precipitated by addition of trichloroacetic acid to 10% (30 min, 4°), pelleted (12,000 × g, 15 min, 4°), washed, and pelleted again (26). The pellet was hydrolyzed in 1 N NaOH (20 min, 60°) and neutralized with 1 N HCl. An aliquot was assayed for protein by the method of Bradford (27) and for tritium by scintillation counting.

Data analysis. Data are presented as mean ± standard error of the mean, and treatment effects were compared by one-way analysis of

variance or Student's paired *t* test (two-tailed). A *p*-value of < 0.05 was taken as a significant level.

Results

To determine the effects of inhibitors of protein synthesis on expression of the α_{1B}-ADR gene, DDT_1 MF-2 cells were incubated with cycloheximide (5 μg/ml) for 15 min to 24 hr and then the cells were harvested to examine the accumulation of α_{1B}-ADR mRNA by Northern blot analysis. The yield of mRNA from cycloheximide-treated cells was only 40–50% of that from cells that had not been exposed to the protein synthesis inhibitor (data not shown); in addition, the proportion of the two signals was different in the cycloheximide-treated cells, as described previously (7). Cycloheximide caused a marked increase in accumulation of the α_{1B} receptor mRNAs and the smaller transcript (2.1 kb) was increased more greatly by cycloheximide (CHX) treatment than the larger transcript (2.3 kb; Fig. 1A). Preliminary experiments showed that throughout the experimental period there was no change in abundance of β-actin mRNA after CHX treatment (Fig. 1A); consequently, we used β-actin mRNA to confirm that equal amounts of poly(A)$^+$ RNA were applied to the gel. The time course of the induction of α_{1B}-ADR mRNA by CHX revealed that an increase in the α_{1B}-ADR mRNA could be detected as early as 1 hr (1.7 ± 0.2-fold of control) and the maximal expression occurred by 4–8 hr (5.4 ± 0.3-fold of control, $p < 0.01$) and was sustained for at least 24 hr (Fig. 1, A and B).

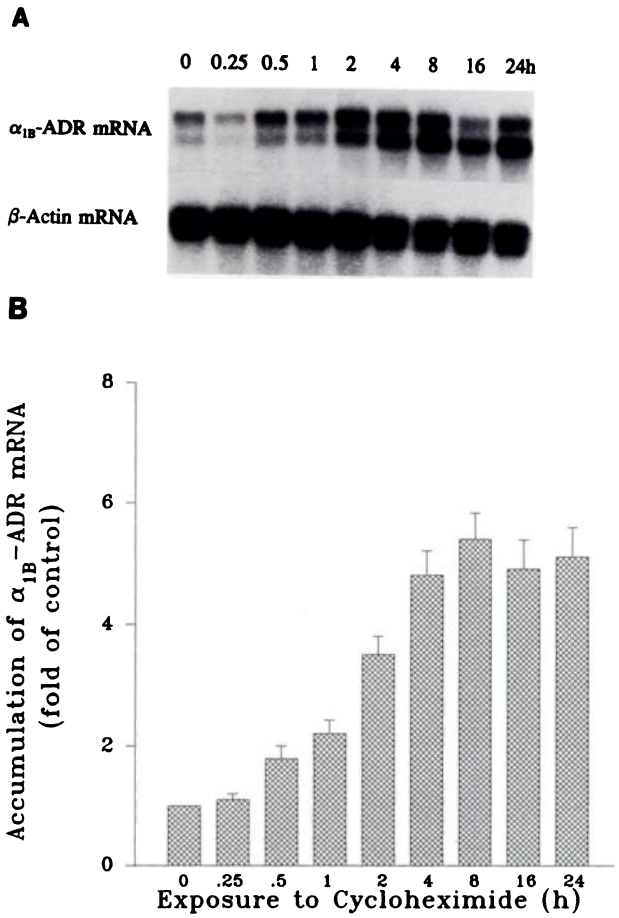

Fig. 1. Time course of CHX-induced increase in accumulation of α_{1B}-ADR mRNA. A, poly(A)$^+$ RNAs were isolated from the DDT_1 MF-2 cells treated in the absence or presence of 10 μg CHX for the indicated times. Two to 5 μg mRNA were subjected to Northern blot analysis and probed with ^{32}P-labeled hamster α_{1B}-ADR cDNA. The blots were hybridized and washed as outlined in Experimental Procedures. The transcript sizes were estimated from a positions of 18 and 28 S ribosomal RNA. β-actin mRNA was used as an internal control. B, the autoradiograms of α_{1B} mRNA and β-actin mRNA were scanned by laser densitometry; the ratio of α_{1B} mRNA relative to β-actin mRNA was calculated and is expressed as the fold of control values. The data shown are the average ± standard error of three experiments.

Increased expression of α_{1B}-ADR mRNA by CHX could be detected at a CHX concentration as low as 0.5 μg/ml; maximal induction of expression of α_{1B}-ADR mRNA occurred with a CHX concentration of 16 μg/ml (5.5 ± 0.2-fold; Fig. 2A). At higher concentrations of CHX (32 μg/ml), induction of α_{1B}-ADR mRNA declined compared with lower concentrations of CHX (data not shown), possibly related to toxic effects of CHX at high concentrations (28). To explore the relationship between induction of α_{1B}-ADR mRNAs by cycloheximide and inhibition of protein synthesis, the extent of protein synthesis measured with [^{14}C]leucine and induction of α_{1B}-ADR mRNAs were measured at various concentrations of CHX (Fig. 2, A and B). A concentration of CHX which caused a 55% inhibition of protein synthesis was associated with significant induction of the α_{1B}-ADR mRNA. CHX at a concentration of 2 μg/ml led to the maximal inhibition of protein synthesis (90%) that could be obtained with this compound. Interestingly, higher concentrations of CHX further augmented accumulation of the α_{1B} receptor mRNAs (Fig. 2B).

To evaluate the possibility that CHX was having effects in addition to inhibition of protein synthesis on the expression of α_{1B}-ADR mRNA, the cells were treated with several other compounds which have different mechanisms of inhibiting protein synthesis (Fig. 3, A and B). Emetine (10 μg/ml), a blocker of polypeptide chain elongation (as is CHX), anisomycin (10 μM), an inhibitor of association of ribosomal subunits, and puromycin (50 μg/ml), an aminoacyl-tRNA analog that promotes peptide chain release from the ribosomal complex, were used. Induction of α_{1B}-ADR mRNAs was found after treatment of the cells with emetine and anisomycin as had been seen with CHX. However, at a concentration that led to a 95% inhibition of protein synthesis, puromycin did not significantly induce α_{1B}-ADR mRNAs (Fig. 3, A and B). Because of the apparent dissociation between protein synthesis inhibition by puromycin and its failure to induce α_{1B}-ADR mRNA, we constructed a dose-response curve for puromycin (Fig. 4). The inhibition of protein synthesis produced by puromycin was similar to that caused by CHX; however, a very modest induction of α_{1B}-ADR mRNAs occurred only at the highest concentration of puromycin (50 μg). Preincubation of cells with puromycin (10 μg/ml) for 60 min did not block cycloheximide-induced accumulation of α_{1B} mRNA (data not shown). These results suggest that the induction of α_{1B}-ADR gene is not simply a consequence of inhibition of protein synthesis, but rather represents an effect of some specific protein synthesis inhibitors.

A change in abundance of the α_{1B}-ADR mRNA could result from either an alteration in the transcription rate or degradation rate of this mRNA. The stability of the α_{1B}-ADR mRNA in the presence of transcriptional inhibitor actinomycin D (5 μg/ml) was compared in control and in CHX-treated cells (Fig. 5, A and B). After preincubation of cells with actinomycin D, CHX did not increase accumulation of α_{1B}-ADR mRNA. CHX

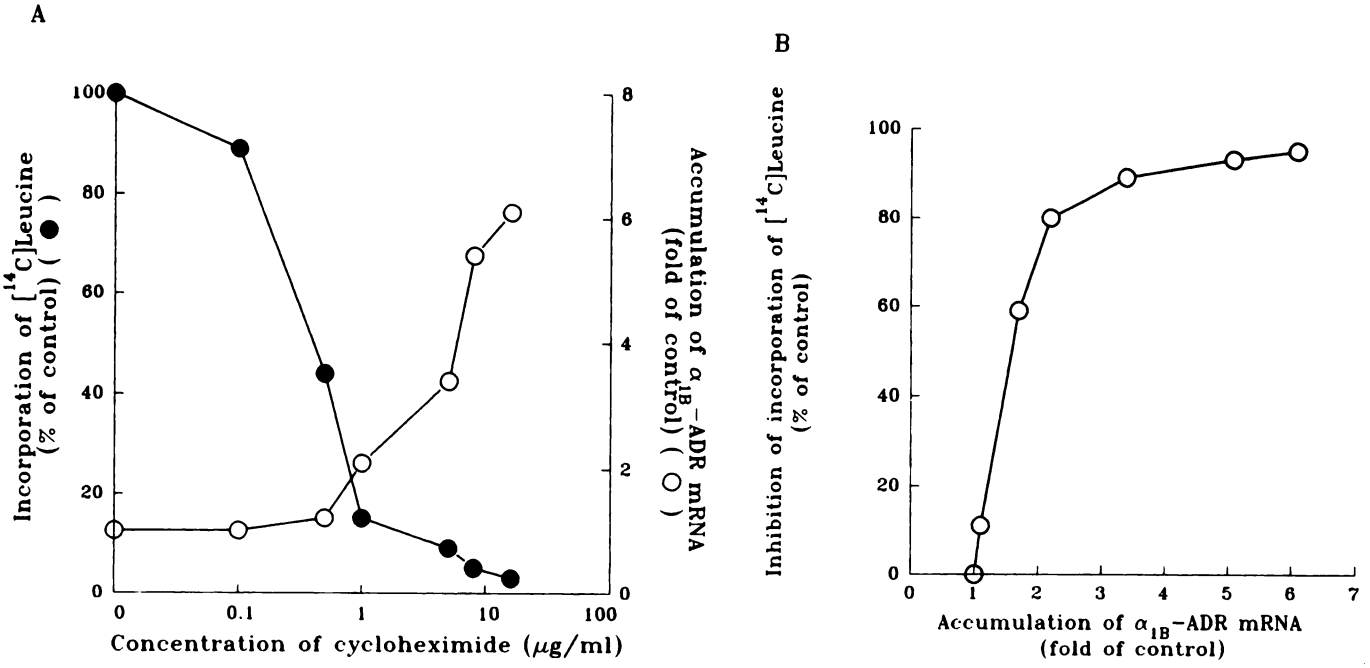

Fig. 2. Effects of CHX on protein synthesis and α_{1B}-ADR mRNA accumulation. A, DDT_1 MF-2 Cells were treated with an indicated concentration of CHX for 6 hr. Poly(A)$^+$ RNAs were isolated from control or CHX-treated cells and Northern blot analysis was performed as described in Experimental Procedures. For measurement of inhibition of protein synthesis, cells were grown to near confluence in a series of 60-cm^2 dishes. Fresh Dulbecco's modified Eagle's medium was added and supplemented with CHX at concentrations indicated in the figure. The inhibition of protein synthesis using incorporation of [^{14}C]leucine after 4 hr was determined as described in Experimental Procedures. B, Relationship between inhibition of protein synthesis and accumulation of α_{1B}-ADR mRNAs after exposure to CHX. The data are representative of three experiments.

treatment did not significantly change the degradation rate of the α_{1B}-ADR mRNA; the half-life of α_{1B}-ADR mRNA was approximately 6 hr in control and 6.5 hr in cells pretreated with CHX for 3 hr. Also, in separate experiments, cells were pretreated with CHX for 3 hr and then incubated with actinomycin D for 3 hr to block RNA synthesis. Under these conditions, the half-life of the α_{1B}-ADR mRNA was not significantly changed. These results suggest that the enhanced accumulation of α_{1B}-ADR mRNA in the presence of CHX is not due to increased stability of the α_{1B}-ADR mRNA. However, preincubation of cells with puromycin (10 μg/ml) did not inhibit CHX-induced activation of transcription of the α_{1B} gene (data not shown).

Using nuclear runoff assays, we measured the transcription rate of the α_{1B}-ADR gene in control and CHX-treated DDT_1 MF-2 cells. These experiments demonstrated that CHX (10 μg/ml) led to a marked elevation in transcription rate of the α_{1B}-ADR gene. The transcription rate of α_{1B}-ADR gene, calculated from the ratio of transcription rate of α_{1B}-ADR gene to that of the β-actin gene, was increased by 3.5 ± 0.3-fold and 6.1 ± 0.5-fold of control in nuclei from cells treated with CHX for 3 and 6 hr, respectively (Fig. 6).

We have demonstrated recently that phorbol esters increase the α_{1B}-ADR mRNA accumulation in DDT_1 MF-2 cells via activation of protein kinase C (8) and we wondered if CHX superinduces α_{1B}-ADR gene in the presence of a phorbol ester such as 4β-phorbol 12-myristate 13-acetate (PMA). DDT_1 MF-2 cells were incubated with PMA, CHX, or PMA with CHX for 3 hr. The results showed that CHX slightly superinduced α_{1B}-ADR mRNAs in the presence of PMA (Fig. 7).

Discussion

This study addresses the roles of protein synthesis inhibitors in the induction of the α_{1B}-ADR gene expression in DDT_1 MF-2 smooth muscle cells. The results demonstrate that CHX induces a time- and concentration-dependent increase in α_{1B}-ADR mRNA expression. Anisomycin and emetine had similar effects on inducing α_{1B}-ADR mRNA accumulation. However, puromycin, at the concentrations producing up to 95% inhibition of protein synthesis, had no effect on the induction of α_{1B}-ADR mRNA expression. Preincubation of cells with puromycin did not prevent CHX-induced accumulation of α_{1B} mRNA and activation of transcription of the α_{1B} gene. There was no measurable change in stability of α_{1B}-ADR mRNA induced by CHX. However, CHX induced transcription of the α_{1B}-ADR gene as detected by nuclear runoff experiments.

α_1-ADRs are expressed in many tissues including brain, heart, smooth muscle, and liver, where they function as important physiological regulators which mediate many biological effects of catecholamines, including regulation of blood pressure, glycogenolysis, and myocardial contraction (1–3). Knowledge of regulation of α_1-ADR gene expression in these tissues is relatively limited. A common mechanism to regulate gene expression in response of eukaryotic cells to extracellular signals is to modulate the activity of sequence-specific transcription factors (29–32), namely, an extracellular signal changes the activities of a protein kinase cascade that modulates transcription factor activity by protein phosphorylation (29, 31). Two transcription factors of particular importance for regulation of α_1-ADR genes are cAMP response element binding protein and activator protein-1. For example, catecholamines such as norepinephrine increase α_{1B}-ADR gene expression in DDT_1 MF-2 smooth muscle cells by activation of β-ADR,

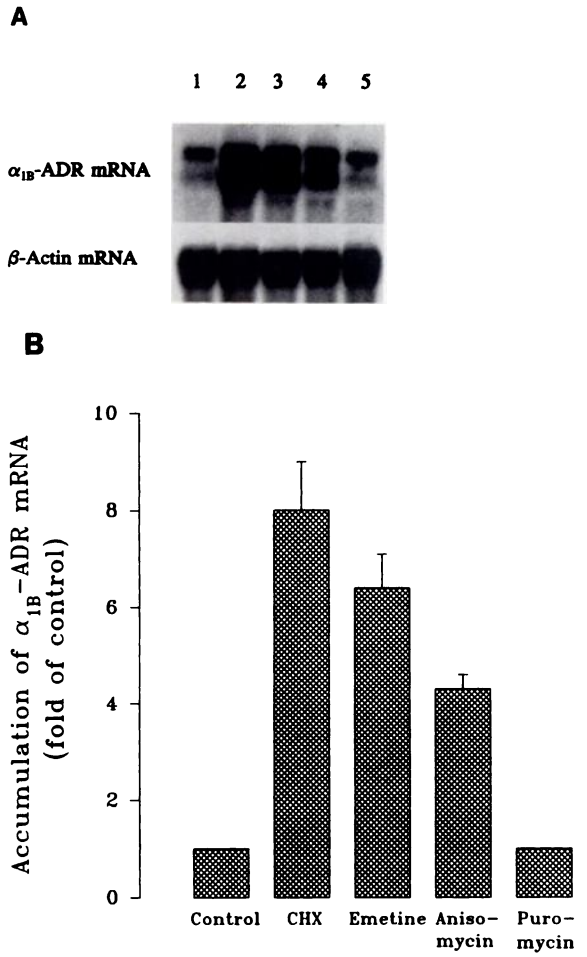

Fig. 3. Induction of α_{1B} receptor gene expression by different inhibitors of protein synthesis. A, poly(A)$^+$ RNAs were prepared from DDT$_1$ MF-2 cells incubated under control conditions (lane 1) or with 5 μg/ml of CHX (lane 2), 10 μg/ml of emetine (lane 3), 10 μM anisomycin (lane 4), or 50 μg/ml puromycin (lane 5) for 6 hr. Northern blot analysis of these poly(A)$^+$ RNAs was carried out using ^{32}P-labeled α_{1B}-ADR cDNA probes or β-actin cDNA probes. B, the average ± standard error of three experiments.

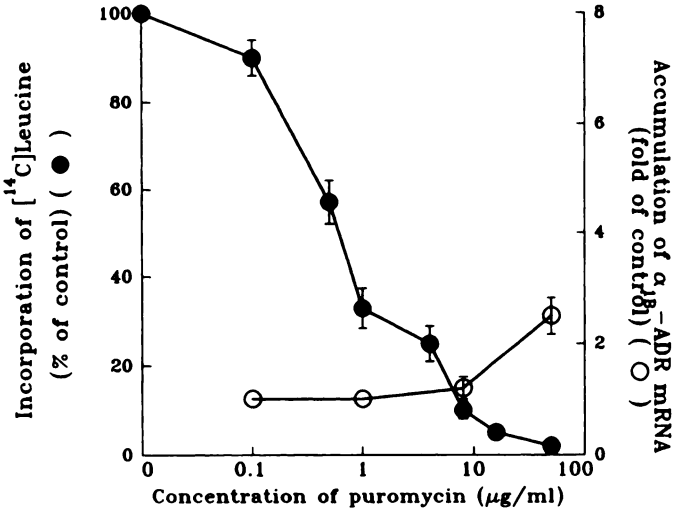

Fig. 4. Effects of puromycin on protein synthesis and accumulation of α_{1B}-ADR mRNAs. Cells were grown to near confluence and treated with puromycin at concentrations indicated in the figure. Measurement of inhibition of protein synthesis and accumulation of α_{1B}-ADR mRNAs were performed as described in the legend of Fig. 2. The data are the mean of three to five experiments.

probably by increasing the concentration of cAMP, likely leading to increased phosphorylation of cAMP response element binding protein (33, 34). We have recently found that activation of protein kinase C by phorbol esters induces α_{1B}-ADR gene expression via an increase in the rate of transcription (8), suggesting that the promoter region of the α_{1B}-ADR gene may contain a phorbol ester response element. A property of the genes containing a phorbol ester response element is that modulators such as growth factors, hormones, cytokines, interferons, and phorbol esters stimulate gene expression through phosphorylation of activator protein-1 (35, 36).

Although protein synthesis inhibitor-induced induction or superinduction of mammalian cell gene expression is a relatively common phenomena, the mechanisms for these actions of protein synthesis inhibitors are still not clear. Several mechanisms have been postulated to be involved in mediating these effects (13, 15). The existence of rapidly degraded nuclear repressors of transcription is frequently invoked as a possible mechanism of protein synthesis inhibitor-induced induction and superinduction (13). This hypothesis implies that inhibition of protein synthesis by any translational inhibitor is essential to the effects of protein synthesis inhibitors and should result in induction or superinduction of the gene expression (37, 38). For instance, it has been reported that unstimulated mouse fibroblasts contain repressors that can be titrated out by the addition of a high concentration of serum response element sequences (39). Also, a synthetic copy of the c-*fos* serum response element sequence is sufficient to confer CHX-dependent inducibility upon a heterologous promoter (13). However, although many genes have been suggested to be induced by inhibitors of protein synthesis via this mechanism, specific information about nuclear repressors is limited. We have found that four translational inhibitors with the different mechanisms of action did not all induce the α_{1B}-ADR mRNA accumulation, suggesting that inhibition of protein synthesis was not a sufficient explanation for the effects of these drugs on α_{1B}-ADR gene expression. Indeed, inhibition of protein synthesis without induction of gene expression suggests the lack of labile repressors controlling expression of the α_{1B}-ADR gene (14, 15, 17).

Although the inhibition of protein synthesis is not essential for the protein synthesis inhibitor-induced gene expression in some systems, translational arrest, the consequence of inhibition of protein synthesis, is another possible mechanism for the protein synthesis inhibitor-induced induction or superinduction (13, 15). Translational arrest may result in the suppression of synthesis of a repressing protein which functions as an autorepressing molecule to delay transcriptional shut-off (40), or suppression of synthesis of labile nucleases to extend the half-life of the mRNAs (41). The overall result of the translational arrest is an increase in stability of mRNAs. Generally, the genes whose mRNA turnover closely relates to a change in stability of the mRNAs have some common structural features, such as stem-loop structures or AU-rich sequences located in the 3′-untranslated regions of mRNAs (42, 43). However, these structures or sequences are not found in the α_{1B}-ADR mRNAs (34). Our own studies demonstrate that stabilization of mRNAs plays little role in the protein synthesis inhibitor-induced α_{1B}-ADR mRNA accumulation since the α_{1B}-ADR mRNA was not increased by CHX after inhibition of transcription by actino-

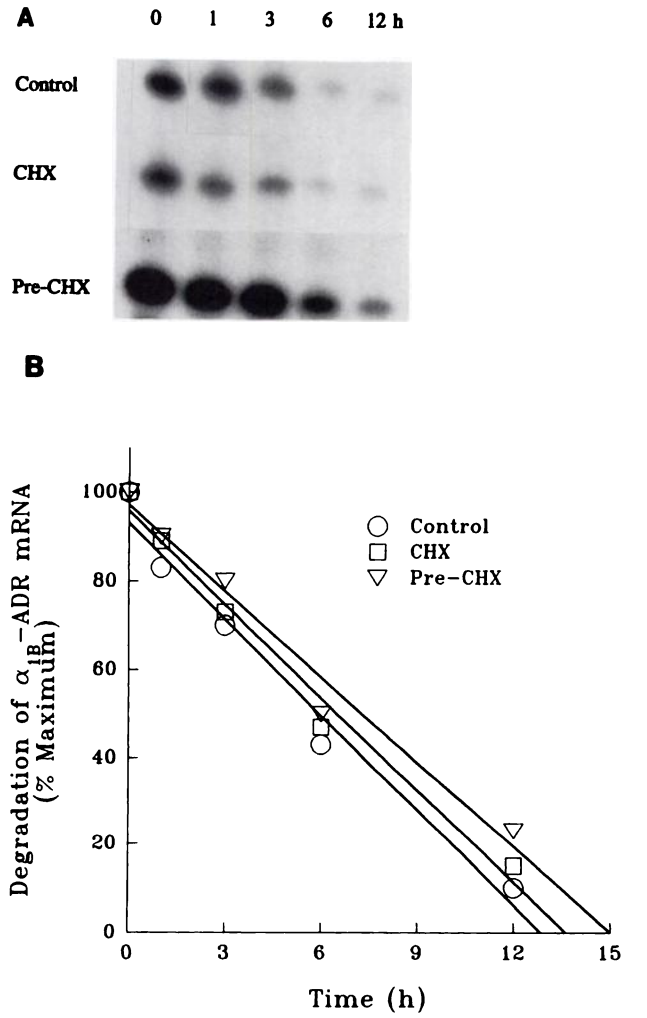

Fig. 5. CHX treatment does not change stability of the α_{1B} receptor mRNA. A, Cells were preincubated with 5 μg/ml actinomycin D for 3 hr to block transcription and then the cells were treated with vehicle (control) or 5 μg/ml of CHX for different times as indicated. Other cells were pretreated with 5 μg/ml CHX for 3 hr and then incubated with 5 μg/ml actinomycin D for 3 hr (pre-CHX). The total RNAs were isolated and RNase protection assays were performed as described in Experimental Procedures. B, The half-life of the α_{1B}-ADR mRNAs. The data are representative of three experiments.

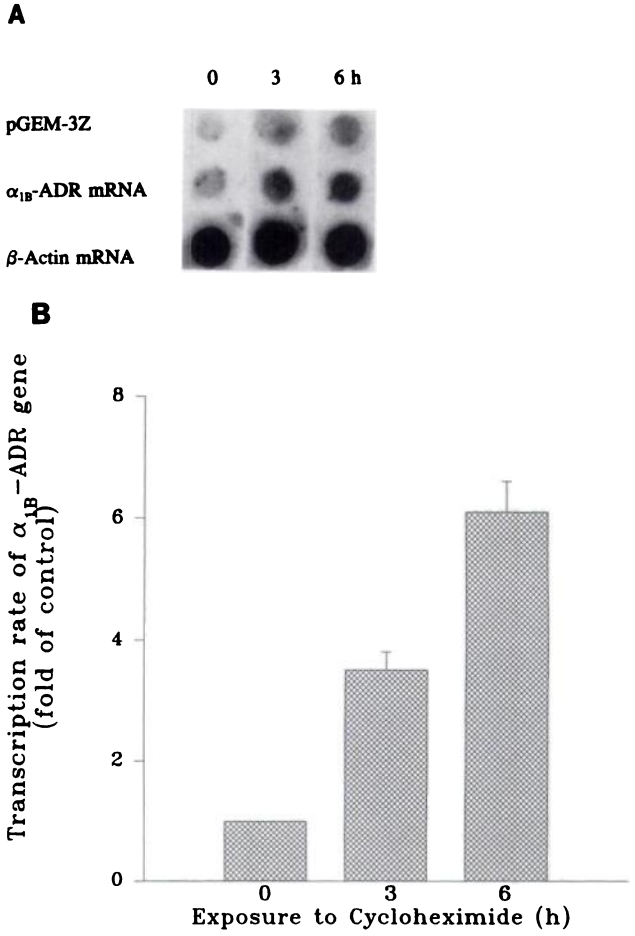

Fig. 6. Inhibition of protein synthesis by CHX activates transcription of the α_{1B}-ADR gene. A, DDT_1 MF-2 smooth muscle cells grown in Dulbecco's modified Eagle's medium were exposed to 10 μg CHX for 0, 3, and 6 hr. The cells were harvested and nuclei were prepared as outlined in Experimental Procedures and used immediately for the nuclear run-on assays. Transcript elongation was allowed to continue in the presence of [^{32}P]UTP and unlabeled nucleotides. After elongation, equal amounts of radiolabeled RNA were hybridized either to plasmid (20 μg/slot) harboring the full-length hamster α_{1B}-ADR cDNA, β-actin cDNA, or to the plasmid lacking the receptor cDNA insert (pGEM-3Z) as control. All of the plasmids were linearized by *Eco*RI before immobilization to the nylon membrane. B, Autoradiograms were analyzed by laser scanning densitometry to quantify the average increase in α_{1B}-ADR transcription rate. Data are average ± standard error of three experiments.

mycin D; also, the half-life of the α_{1B}-ADR mRNAs in control cells was similar to the that both in the CHX-treated cells or in the pre-CHX-treated cells as measured by RNase protection assays.

A third possible mechanism for protein synthesis inhibitor-induced induction or superinduction is that these inhibitors themselves function as intracellular nuclear signaling molecules triggering the transcriptional responses in some systems independently of protein synthesis inhibition (13–15). The inhibitors with nuclear signaling actions generally induce transcription of gene expression in absence of any inducers, while inhibitors without this effect may only superinduce gene expression, namely, induce gene expression in presence of inducers such as serum or growth factors. For example, incubation of C3H 10T1/2 cells with anisomycin or CHX results in the phosphorylation of cellular nuclear protein pp33/histone H3 (44) and subinhibitory concentrations of anisomycin and CHX increase the rate of c-*fos*/c-*jun* transcription (14, 15). However, protein synthesis inhibitors puromycin and emetine, which are without this action on intracellular nuclear signaling, do not cause nuclear protein phosphorylation and increase in the rate of c-*fos*/c-*jun* transcription in these cells (15). Our studies demonstrate that CHX induced α_{1B}-ADR mRNA accumulation via activation of transcription because nuclear run-off experiments revealed that CHX increased the rate of the α_{1B}-ADR gene transcription, and blocking RNA synthesis by actinomycin D completely prevented CHX-induced α_{1B}-ADR mRNA accumulation. In addition, puromycin, in DDT_1 MF-2 cells, at a concentration that caused 98% inhibition of protein synthesis, did not induce the α_{1B}-ADR mRNA accumulation. On the other hand, emetine, another protein synthesis inhibitor without demonstrated nuclear signaling in other cells (15), induced α_{1B}-ADR mRNA expression. A possible explanation for this could be that emetine may function as a nuclear signal

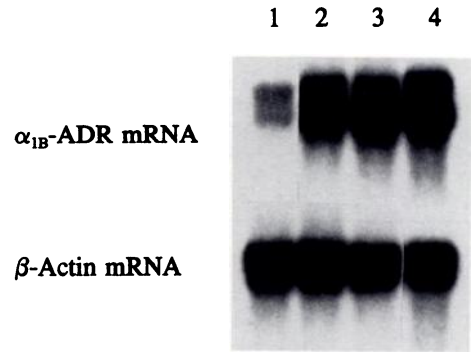

Fig. 7. Protein synthesis inhibitors superinduce the α_{1B}-ADr gene with PMA. Cells were grown as described in Experimental Procedures and treated by vehicle or agonists for 3 hr. Poly(A)$^+$ RNAs were prepared under control condition (lane 1), 100 nM PMA (lane 2), 5 μg/ml CHX (lane 3), and 100 nM PMA plus 5 μg/ml CHX (lane 4). Northern blot assays were performed as described in the legend of Fig. 1. Data are representative of three experiments.

agent in DDT_1 MF-2 cells, although we have not examined this possibility.

In summary, we have found that some protein synthesis inhibitors activate α_{1B}-ADR gene transcription; this activation of α_{1B}-ADR gene expression does not appear to be a direct consequence of inhibition of protein synthesis. The possible mechanisms for protein synthesis inhibitor-induced α_{1B}-ADR gene expression in DDT_1 MF-2 cells have been discussed and may involve phosphorylation of preexisting transcriptional factor(s) by protein synthesis inhibitors. The present results provide important information for understanding the complex mechanisms involved in the regulation of the α_1-ADR gene in smooth muscle cells.

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