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The Vascular Smooth Muscle Type I Angiotensin II Receptor mRNA Is Destabilized by Cyclic AMP-Elevating Agents

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SUMMARY

Although processes involved in mRNA degradation play a significant role in dictating steady state mRNA levels, the influence of cell surface signaling on mRNA stability control is understood incompletely. In this study, the effects of cAMP-elevating agents on type I angiotensin II receptor (AT_1-R) mRNA levels were assessed in cultured rat aortic vascular smooth muscle cells (VSMCs). AT_1-R mRNA levels are rapidly reduced by forskolin treatment, in which the maximal effect yields an 80% reduction in AT_1-R mRNA levels after 6 hr of treatment. The rate of AT_1-R mRNA decay in response to forskolin is greater than its apparent intrinsic decay, as assessed in the presence of the transcriptional inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, suggesting forskolin treatment destabilizes the AT_1-R mRNA. Nuclear run-on analysis indicates forskolin treatment does not affect transcription of the AT_1-R gene in VSMCs, implying induced AT_1-R mRNA destabilization accounts for the entire effect of forskolin in decreasing AT_1-R mRNA levels. Dose-effect studies that assessed AT_1-R mRNA levels and cAMP production were conducted using forskolin and the β-adrenergic receptor agonist isoproterenol as agonists. Isoproterenol is almost 3 orders of magnitude more potent at eliciting the reduction in AT_1-receptor mRNA levels than it is at stimulating cAMP production. Similarly, forskolin elicits reductions in AT_1-R mRNA, which occur at concentrations that fail to elicit a detectable production of cAMP. However, protein kinase A activity is stimulated maximally by isoproterenol and forskolin concentrations that do not stimulate detectable cAMP production. These data provide evidence that the mechanism for down-regulation of AT_1-R mRNA levels by cAMP-elevating agents in VSMCs occurs via a PKA-regulated mRNA destabilization pathway.

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The balance between RNA synthesis and degradation pathways dictates steady state levels of gene expression in cells (1). In eukaryotic cells, mRNA half-lives can range from seconds to many hours (for a review, see Ref. 2). For example, the expression of several short-lived cytokine and proto-oncogene mRNAs can be induced transiently by gene transcription, but rapid mRNA degradation prevents their accumulation inside the cell (3). A general picture has emerged in which these unstable mRNAs are transcribed into an environment in which the destabilizing machinery preexists in a fully active form.

The mechanisms responsible for controlling the activities of iron-response element binding proteins and their interactions with mRNA provide what is now the most well understood class of gene expression control in which mRNA stability is controlled by changing extracellular conditions (4). Here, the intracellular concentration of iron, and perhaps oxidative metabolites, regulates the ability of responsive *trans*-acting mRNA binding proteins to control mRNA translation and degradation (5, 6). There are relatively few examples in which stabilization and destabilization of specific mRNAs have been documented in response to receptor-mediated activation of intracellular signaling pathways (for a recent review, see Ref. 7). Second messenger activity has been implicated in the negative control of mRNAs encoding some cell surface G protein-coupled receptors, including muscarinic (8), thyrotropin-releasing hormone (9), and α_1-adrenergic receptors (10). Studies by Malbon *et al*. (11, 12) suggest the β_2-adrenergic receptor mRNA is degraded *in trans* as a long term response (>24 hr) to cAMP elevation. This is correlated with the induction of cytoplasmic mRNA binding proteins interacting with AU-rich mRNA motifs in the β_2-adrenergic receptor mRNA (13–15). However, in other studies, β_2-adrenergic receptor mRNA destabilization has not been observed under the conditions of short term agonist

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ABBREVIATIONS: VSMC, vascular smooth muscle cell; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; Bt2, dibutyryl; AT_1-R, type I angiotensin II receptor; PKA, protein kinase A; DMSO, dimethylsulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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exposure (16, 17), although the confounding influence of transcriptional control of the gene by the same signals may obfuscate any early effects of agonist on mRNA stability. Similar complications were found in studies of regulation of the mRNAs encoding the plasminogen activator inhibitor (18) and tyrosine aminotransferase (19), which together represent the few known examples in which changes in cAMP levels appear to be capable of destabilizing specific mRNAs.

Recent studies of the regulation of VSMC AT_1-R reveal it to be an interesting model system for the study of regulated mRNA decay processes. VSMC AT_1-R gene expression is down-regulated by a diverse group of extracellular signals, include angiotensin II and other agonists of related G_q-coupled receptors; by growth factors; and by several synthetic agents used as activators or even inhibitors of signal transduction cascades (20–22). The current data indicate that down-regulation of the AT_1-R mRNA in response to growth factors and G_q-coupled receptor agonists is, like the β_2-adrenergic receptor, mediated by superimposed processes involving both transcriptional repression and enhanced mRNA decay. Although there is some evidence in the literature that AT_1-R gene expression can be down-regulated by cAMP-elevating agents, it has been unclear whether this effect is a consequence of transcriptional repression, mRNA destabilization, or both (22, 23). In this report, we show that cAMP-elevating agents do not affect AT_1-R gene transcription in VSMC, yet they rapidly induce a loss of steady state mRNA levels. These observations provide clear evidence of a role for PKA activity in the control of VSMC AT_1-R mRNA stability by extracellular signals.

Experimental Procedures

Materials. [α-^{32}P]UTP, [α-^{32}P]dCTP, [γ-^{32}P]ATP, [^{3}H]adenine, and Hybond N membranes were from Amersham (Arlington, Heights, IL). TRI-reagent was from Molecular Research Center (Cincinnati, OH). Ro20–1724 was from Research Biochemicals (Natick, MA). Isoproterenol, DRB, forskolin, Bt_2-cAMP, Bt_2-cGMP, salts, and buffers were from Sigma Chemical (St. Louis, MO) or Life Technologies (Gaithersburg, MD). Antibiotics, serum, and cell culture media were from Life Technologies. Isoproterenol, Bt_2-cAMP, and Bt_2-cGMP were dissolved in sterile water. DRB was prepared as 10 μg/μl stock in highly purified DMSO. Forskolin was prepared as a 10 mM stock and also diluted in DMSO. The same concentration of solvent was used as vehicle in all experiments.

Rat VSMC culture. Rat thoracic aorta VSMCs are kept as a continuous cell line and were kindly provided by Dr. R. W. Alexander (Emory University); they were cultured in Dulbecco's modified Eagle's medium with 3.7 g/liter of $NaHCO_3$, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated calf serum in an incubator in 5% CO_2 at 37°. After confluence, cells were growth-arrested before experiments through culture in serum-free media for 24 hr. Cells were used between passages 10–25 after primary explant.

cAMP production assay. VSMCs in 35-mm-diameter six-well plates were incubated for 2 hr at 37° with 1 ml of [^{3}H]adenine (5 μCi/ml) prepared in Dulbecco's modified Eagle's medium with 10 mM HEPES, pH 7.4, before exposure to agonists for 5 min in the presence of the phosphodiesterase inhibitor Ro20–1724 (0.1 mM). Production of cAMP was terminated by aspiration and the addition of 1 ml of 5% trichloroacetic acid to the wells. [^{3}H]cAMP was isolated by sequential Dowex and alumina chromatography and measured by scintillation counting (24). Production of cAMP was calculated as a percentage of conversion, with the cpm in [^{3}H]cAMP fractions divided by the sum of cpm in the [^{3}H]cAMP and [^{3}H]ATP fractions.

PKA assay. The protocol used in this study is a modification of one provided by Dr. Lee Graves (University of North Carolina, Chapel Hill) (25). VSMCs grown onto 35-mm dishes were rinsed and exposed for 5 min at 37° to the indicated concentrations of isoproterenol in the absence of phosphodiesterase inhibitors. Next, the dishes were aspirated and rinsed twice with 1 ml of ice-cold phosphate-buffered saline, pH 7.4. After an additional 1 ml rinse in homogenization buffer (50 mM β-glycerophosphate, pH 7.4, 1.5 mM EGTA, 1 mM dithiothreitol, 0.1 mM Ro20–1724), the cells were scraped, collected, and sonicated briefly in 300 μl of homogenization buffer before centrifugation for 10 min at 10,000 $\times$ *g* and 4° to remove debris. Aliquots of the supernatant (10 μl) were assayed in triplicate for PKA activity over 15 min at 30° in a buffer (30 μl final volume) containing 25 mM β-glycerophosphate, pH 7.4, 1.25 mM EGTA, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.17 mM Kemptide, and 2 μCi of [γ-^{32}P]ATP (3000 Ci/mmol). The reactions were terminated by spotting with 25 μl of the reaction mix on squares (2 $\times$ 2 cm) of P-81 phosphocellulose paper. The paper was washed four or five times in 300 ml of 150 mM phosphoric acid and once in ethanol, dried to air, and then counted by scintillation. PKA phosphorylation is defined as the difference in radioactive counts between aliquots assayed in the absence or presence of protein kinase I (5–22) inhibitor peptide (15 μM). Total PKA activity was determined in each experiment by measuring Kemptide phosphorylation in the presence of 50 μM cAMP.

RNA isolation and hybridization. Confluent 2- to 3-day cultures on 35- or 60-mm dishes were grown in serum-free medium for an additional 24 hr before challenge with vehicle or agonists for the indicated time. Total RNA was extracted using TRI reagent and quantified by measuring absorbancy at 260 nm. In some experiments, RNA (10 μg) was separated by formaldehyde-agarose gel electrophoresis before transfer to Hybond nylon membranes. In other experiments, using a slot-blot apparatus, duplicate samples of total RNA (5–10 μg) was denatured by mixing with equal volumes of formaldehyde (37%) and 20× SSC (1× contains 150 mM sodium chloride, 15 mM sodium citrate, pH 7.4) and heating at 65° on a heating block for 15 min. Samples were then diluted in 0.5 ml of diethylpyrocarbonate-treated 10× SSC and transferred to a nylon membrane under gentle vacuum. RNA was cross-linked onto the membrane under ultraviolet light (1200 kJ) in a Stratalinker 2400 (Statagene, La Jolla, CA).

An [α-^{32}P]dCTP-labeled AT_1-R cDNA probe was made from a 824-base pair fragment of AT_1-R cDNA (20). The nylon membranes were first prehybridized at 42° for 2 hr in solution containing 50% deionized formamide, 6× SSC, 0.5% SDS, 5× Denhardt's solution, and 10 μg/ml denatured salmon sperm DNA. The radiolabeled probe was then added to a hybridization solution (50% formamide, 0.5% SDS, 6× SSC, and 10 μg/ml denatured salmon sperm DNA) and hybridized with the blot for 16 hr at 42°. The blot was washed sequentially in 2× SSC for 15 min at room temperature and 2× SSC/0.1% SDS for 15 min at 50°, rinsed briefly in 2× SSC at room temperature, exposed to PhosphorImager cassette overnight, and quantified by a volume integration protocol in ImageQuant (Molecular Dynamics, Santa Clara, CA). The volume of the rectangle covering the hybridization signal was subtracted with that of a randomly chosen background rectangle of the same area. For comparison of the treatment and control, these hybridization signals were normalized as a percent of the value from samples derived from vehicle-treated cells.

Nuclear run-on. The nuclear run-on method was modified from that reported by others (26) as described previously (20). Cells in 150-mm-diameter dishes were grown to confluence in growth media and then for an additional 24 hr in serum-free conditions before treatment for either 45 min or 4 hr with either vehicle or the indicated concentration of forskolin. The cells were harvested and nuclei were isolated as described previously (20). Nuclei (1–3 $\times$ 10^7/reaction) were mixed with 450 μCi of [α-^{32}P]UTP (>3000 μCi/mmol) and 100 μM concentrations of ATP, CTP, and GTP in a final volume of 300

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μl. The run-on reaction took place at 30° for 30 min before the radiolabeled transcripts were extracted with 800 μl of TRI-reagent and resuspended in 50 μl of diethylpyrocarbonate-treated water.

Plasmid reporter slot-blots were prepared by denaturing and cross-linking with 5 μg each of plasmids containing cDNAs for GAPDH in pIBIP, the AT_1-R in pBSKS$^+$Ca18b (27), or pBSKS$^+$ alone as a negative control; they were prehybridized for 2 hr in a solution containing 50% formamide, 0.3 M NaCl, 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.4, 250 μg/ml yeast tRNA (heat denatured), 10 μg/ml denatured salmon sperm DNA, 0.2% SDS, and 5× Denhardt's reagent before hybridization with radiolabeled nuclear RNA (1×10^7 cpm) in 1 ml of hybridization solution (prehybridization solution lacking 5× Denhardt's reagent). After 12 hr of hybridization at 42°, the blots were washed twice in 2× SSC for 10 min each, 2× SSC/0.1% SDS at 42° for 10 min, and 1 μg/ml RNase A in 2× SSC for 10 min at room temperature. After brief rinsing in 2× SSC, the blots were wrapped in Saran wrap and exposed in a PhosphorImager cassette overnight.

Results

The time-dependent effect of cAMP-elevating agents on AT_1-R mRNA levels in VSMC were examined by treating the cells for various periods of time with a single concentration (10 μM) of forskolin, as a direct activator of adenylyl cyclase. This effect was compared with that for vehicle (0.1% DMSO) alone and for cells pretreated for 30 min with 50 μg/ml DRB, an inhibitor of transcriptional elongation (28) to block AT_1-R mRNA synthesis, in the absence or presence of forskolin. The results of a representative hybridization slot-blot are shown in Fig. 1, and the cumulative results of several experiments are shown in Fig. 2. Forskolin induces a time-dependent loss of AT_1-R mRNA levels that is evident as early as 2 hr after agonist treatment and continues until ~6 hr after treatment, after which AT_1-R mRNA levels approach a new, lower steady state.

In the presence of DRB, AT_1-R mRNA steady levels are reduced to ~50% of the level in vehicle-treated cells within 6 hr, which is consistent with previous observations with the use of actinomycin D instead (20, 21). This treatment reveals the intrinsic decay rate of the AT_1-R mRNA under nonstimulated conditions, but the possibility that DRB treatment is also affecting components of the processes involved in AT_1-R mRNA decay cannot be excluded. Treatment with forskolin

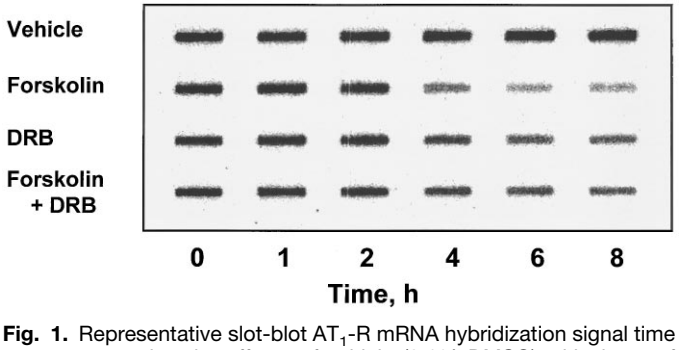

Fig. 1. Representative slot-blot AT_1-R mRNA hybridization signal time course comparing the effects of vehicle (0.1% DMSO) with those of forskolin (10 μM), DRB (50 μg/ml), and forskolin after a 30-min pretreatment of VSMCs with DRB. Each slot contains 5 μg of total VSMC RNA. Reproduction of a PhosphorImage file that has been sharpened to remove background grayness and improve contrast. The AT_1-R transcripts detected in this hybridization protocol have been extensively characterized in other Northern hybridization studies by formaldehyde gel electrophoresis (20, 21).

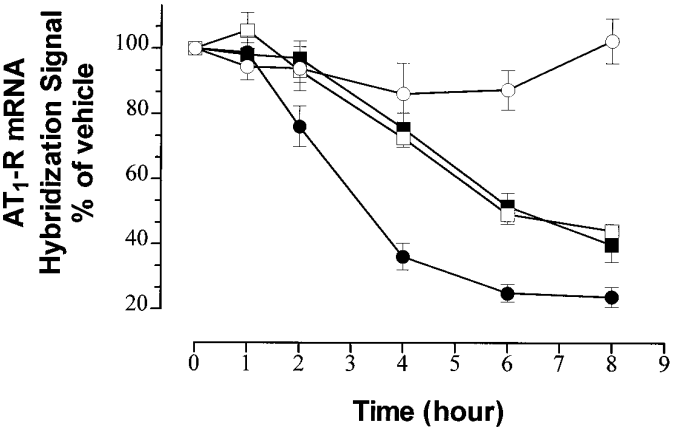

Fig. 2. Destabilization of AT_1-R mRNA by forskolin and the effect of a transcriptional inhibitor. VSMCs were administered a single dose of forskolin (10 μM; ●) or vehicle (0.1% DMSO; ○). At the zero-hour time point, VSMC and RNA samples were subsequently collected at the indicated times. Other cells were pretreated for 30 min with 50 μg/ml DRB before the addition of 10 μM forskolin (■) or vehicle (□) at the zero-hour time point and collection of RNA samples at the indicated times. *Points*, relative hybridization signal normalized to the zero-hour vehicle time point and is expressed as mean ± standard error of five independent experiments. All treatments were performed in parallel.

induces a faster rate of decay of AT_1-R mRNA levels than that observed in the presence of DRB, in which mRNA levels are reduced to ~50% of the control level within 3 hr after the addition of forskolin. Because the rate of forskolin-stimulated AT_1-R mRNA decay is greater than the rate of intrinsic decay, this comparison alone suggests that forskolin treatment enhances the degradation of, or destabilizes, the AT_1-R mRNA. DRB pretreatment abolishes the effect of forskolin. One interpretation of this observation is that forskolin stimulates the transcription of a gene or genes that mediate AT_1-R mRNA destabilization.

Nuclear run-on assays were performed to assess whether forskolin can repress transcription of the AT_1-R gene. Confluent VSMCs were treated with vehicle or 10 μM forskolin for 45 min or with 1 or 30 μM forskolin for 4 hr before nuclear harvest and *in vitro* run-on transcription. As shown in Fig. 3, forskolin treatment under any of these conditions does not affect significantly transcription of the AT_1 receptor gene. On the basis of these results, the reductions in the levels of the intact cell AT_1-R mRNA after treatment with forskolin are entirely attributable to destabilization of the mRNA. For each of these nuclear run-on experiments, GAPDH was used as an internal control only to ensure the integrity of the *in vitro* transcription system. However, in each run-on experiment we have conducted after a 4-hr forskolin treatment, GAPDH transcription is enhanced significantly, reaching >200% of control levels.

Isoproterenol was used as an agonist of β_2-adrenergic receptors; it activates adenylyl cyclase by coupling through heterotrimeric $G_{\alpha s}$ and is used to evaluate the effect of receptor-stimulated adenylyl cyclase on AT_1-R mRNA levels. The expression level of β_2-adrenergic receptors in VSMCs is very low (only at 10 fmol/mg of membrane protein).[1] However, when VSMCs are treated with isoproterenol, a dose-dependent rise of cAMP production is seen, with an EC_{50} value of 355 ± 59 nM (mean ± standard error; nine experi-

[1] X. Wang, G. Nickenig, and T. J. Murphy, unpublished observations.

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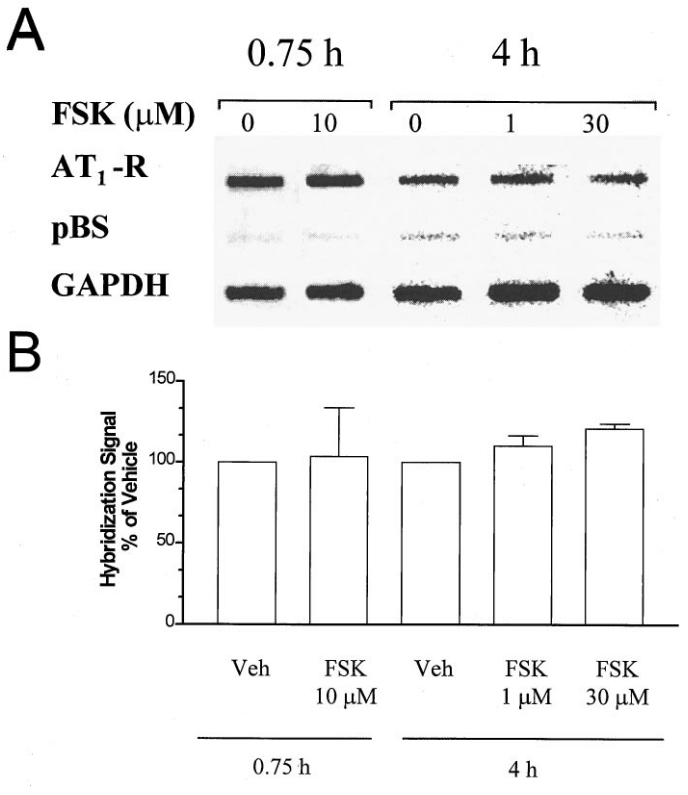

Fig. 3. Forskolin does not affect AT_1-R gene transcription. VSMCs were treated with vehicle (0.1% DMSO) (*Veh*) or 10 μM of forskolin (*FSK*) for 45 min or for 4 hr with vehicle, 1 μM forskolin, or 30 μM forskolin before isolation of nuclei. Radiolabeled nuclear RNA was isolated after a 30-min *in vitro* run-on in the presence of [^{32}P]UTP and used as a probe for hybridization to blots cross-linked with 5 μg each of plasmids for the AT_1-R cDNA Ca18b in pBluescript (AT1-R), pBluescript alone as negative control (*pBS*), or GAPDH in pIBIP as positive control for the run-on assay. A, Collage of PhosphorImages from representative 45-min or 4 hr run-on assays. B, Results of quantification of the hybridization signals for all assays conducted for each conditions expressed as a percentage ± range of the response to vehicle. Three run-on assays were performed after a 45-min treatment, and two run-on experiments were performed for each concentration of forskolin in the 4-hr treatments.

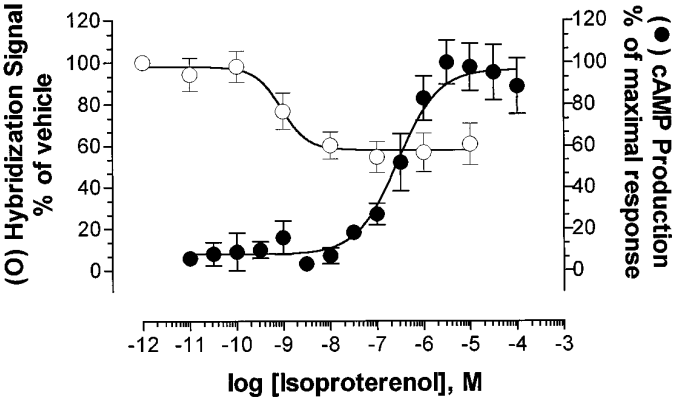

Fig. 4. Dose-dependent effects of isoproterenol on cAMP production and AT_1-R mRNA level in VSMCs. For the cAMP production assays, the VSMCs were prelabeled with [^{3}H]adenine and exposed to indicated concentrations of isoproterenol for 5 min before fractionating radiolabeled nucleotides. cAMP production (●) was calculated as a percentage of the conversion rate of [^{3}H]ATP to [^{3}H]cAMP normalized to the highest response to isoproterenol (100%) within each separate experiment. *Points*, mean ± standard error of nine independent experiments in which maximal percent conversion was 16 ± 4-fold over basal. AT_1-R mRNA levels (○) were analyzed after a 4-hr treatment with either vehicle or the indicated concentration of isoproterenol. *Points*, relative hybridization signal (mean ± standard error) normalized to the treatment with vehicle (100%) from six independent experiments.

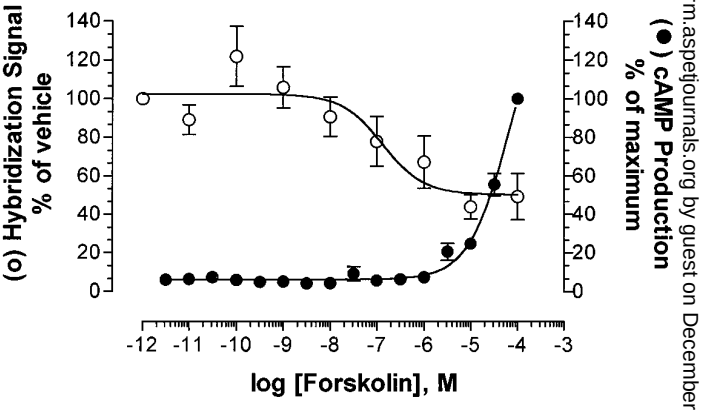

Fig. 5. Dose-dependent effects of forskolin on cAMP production and AT_1-R mRNA level in VSMCs. For the cAMP production assays, the VSMCs were prelabeled with [^{3}H]adenine and exposed to indicated concentrations of forskolin for 5 min before fractionating radiolabeled nucleotides. cAMP production (●) was calculated as a percentage of the conversion rate of [^{3}H]ATP to [^{3}H]cAMP normalized to the highest response to forskolin (100%) within each separate experiment. *Points*, the relative hybridization signal normalized to the treatment with vehicle (100%; mean ± standard error of four independent experiments) in which maximal percent conversion was 20 ± 5-fold over basal. AT_1-R mRNA levels (○) were analyzed by Northern hybridization after a 4-hr treatment with vehicle or the indicated concentration of forskolin.

ments) (Fig. 4). Maximal levels of cAMP production are achieved with 10 μM isoproterenol, which represents a 16-fold response over basal levels of production. In contrast, isoproterenol is much more potent than this in reducing AT_1-R mRNA levels, with an EC_{50} value of 0.4 ± 0.3 nM (six experiments). As shown in Fig. 4, the maximal reduction of isoproterenol treatment over 4 hr in AT_1-R mRNA levels represents 60 ± 6% (six experiments) of the levels in control cells. Attenuated hybridization signals are evident at ≥10 nM concentrations of isoproterenol. Interestingly, this concentration of isoproterenol does not yield cAMP production levels that are significantly greater than those observed with vehicle-treated controls.

No upper plateau of cAMP production in response to forskolin is observed in dose-effect stimuli, which is typical of its effect, so an EC_{50} value cannot be calculated for its ability to produce cAMP. Similar to isoproterenol, forskolin reduces VSMC AT_1-R mRNA levels in a dose-dependent manner (Fig. 5). Forskolin treatment for 4 hr down-regulates AT_1-R mRNA levels with an EC_{50} value of 66 ± 28 nM (four experiments). Similar to isoproterenol-stimulated cells, AT_1-R mRNA levels are clearly attenuated at forskolin concentrations (0.1 μM) that fail to elicit a detectable increase in cAMP production. AT_1-R mRNA levels are maximally reduced at 1.0 μM forskolin, a concentration that yields only a ~2-fold increase over basal levels of cAMP production. The data shown in Fig. 6 indicate that Bt_2-cAMP application down-regulates AT_1-R mRNA in a dose-dependent manner, whereas Bt_2-cGMP does not reduce AT_1 receptor mRNA levels at concentrations as high as 1 mM. These data suggest that cAMP- but not cGMP-dependent protein kinases are involved in down-regulation of AT_1-R mRNA levels in

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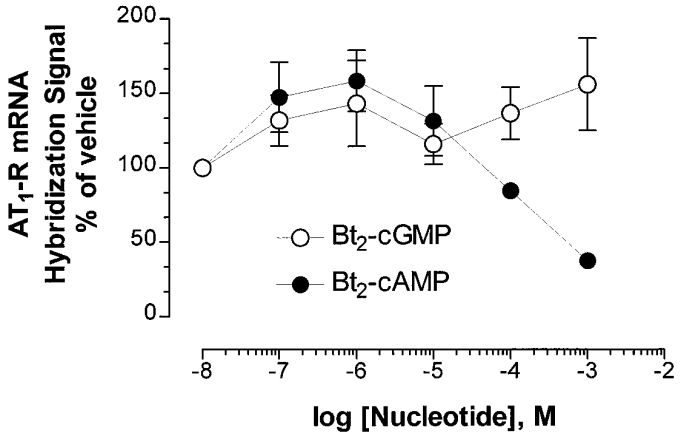

Fig. 6. Bt_2-cAMP but not Bt_2-cGMP decreases VSMC AT_1-R mRNA levels in VSMCs. Cells were treated for 4 hr with either vehicle or the indicated concentrations of Bt_2-cAMP or Bt_2-cGMP. Total RNA was isolated, and AT_1-R mRNA levels were quantified by Northern analysis. The data are normalized to the response observed in the vehicle-treated samples. *Points*, mean ± standard error of the effects observed in three independent experiments.

VSMCs. Taken together, these dose-effect results suggest that the levels of AT_1-R mRNA are very sensitive to minor increments in the levels of cAMP in VSMCs and suggest a role for PKA in the modulation of AT_1-R mRNA stability.

Assays were performed to determine whether PKA is activated at concentrations of isoproterenol and forskolin that reduce mRNA levels but do not elicit detectable cAMP production. VSMCs were stimulated for 5 min with vehicle or various doses of the drugs before preparation of extracts to assess PKA activity. As shown in Fig. 7, increased PKA activity is observed at 1 nM isoproterenol and maximally at ≥10 nM isoproterenol. Similarly, 1 μM forskolin is sufficient to stimulate PKA maximally. Thus, low concentrations of isoproterenol and forskolin that do not lead to detectable increases in cAMP production clearly affect both PKA activity and AT_1-R mRNA levels (compare data in Fig. 7 with that in Figs. 4 and 5). It is of interest to note, however, that the maximal level of PKA activation by isoproterenol and forskolin is only 50% greater than the activity seen in unstimulated cells. Furthermore, the maximal levels of PKA activity in extracts of the stimulated cells are equivalent to that which can be stimulated with 50 μM cAMP *in vitro*. These observations demonstrate that although basal levels of PKA activity are quite high in these cells, relatively modest increases in PKA activity by cAMP-elevating agents are sufficient to induce AT_1-R mRNA decay and disrupt the steady state for AT_1-R gene expression.

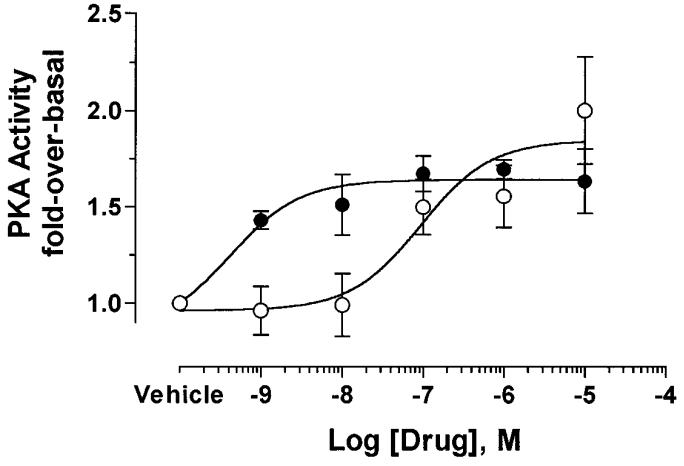

Fig. 7. PKA activity is stimulated maximally in VSMCs by low doses of isoproterenol and forskolin. VSMCs were stimulated for 5 min with the indicated concentrations of isoproterenol (●) or forskolin (○) before assay for PKA activity in 10 μl of cell extracts as described in the text. PKA-dependent phosphorylation of Kemptide substrate is defined as the difference between that in the absence and presence of 15 μM protein kinase I(5–22) peptide. *Points*, mean ± standard error of triplicate phosphorylation determinations from three experiments. Basal PKA activity is 36,711 ± 8,736 cpm/min/ml, whereas PKA activity in cells treated with 10 μM isoproterenol is 57,107 ± 9,642 cpm/min/ml.

Discussion

Because there is no effect of cAMP-elevating agents on transcription of the AT_1-R gene, the present data provide clear evidence that degradation of an mRNA can be controlled by cAMP-mediated signaling. The known systems in which evidence exists for either positive or negative mRNA stability control by cAMP-elevating agents tend to be complicated by redundant or competing transcriptional control processes. Such examples include studies of mRNAs for the β_2-adrenergic receptor (11, 16, 17), follicle-stimulating hormone receptor (29), hepatic enzymes (30), c-*myc* protooncogene (31), some T cell-derived cytokines (31), and pulmonary surfactant proteins (32).

Of these, regulation of the β_2-adrenergic receptor mRNA in hamster smooth muscle cells by cAMP signaling is among the most thoroughly studied. As shown in this study, forskolin-induced destabilization of the AT_1-R mRNA in VSMCs occurs within the first several hours of treatment. In contrast, destabilization of the β_2-adrenergic receptor mRNA is evident only after a more prolonged exposure to cAMP-elevating agonists (11, 12, 15, 33) but not within the first several hours after stimulation with cAMP-elevating agents (16, 17). It has been suggested (15) that the *trans*-acting mRNA binding proteins implicated in regulated β_2-adrenergic receptor mRNA turnover (13, 14) might also target other receptor mRNAs, including that for the AT_1-R. Although cAMP-induced AT_1-R mRNA binding proteins in VSMCs are incompletely characterized at this time,[2] angiotensin II stimulation induces a group of AT_1-R mRNA binding proteins that differ in several characteristics from the β_2-adrenergic receptor mRNA binding proteins (21). A role for PKA in this response to the hormone is a matter of current investigation, but it is of interest that angiotensin II stimulates cAMP production by 2–3-fold in these VSMCs.[2] Thus, it is unclear whether a relationship exists between the cAMP-dependent processes regulating β_2-adrenergic receptor mRNA stability and those regulating AT_1-R mRNA levels.

The dose-effect studies (Figs. 4 and 5) reveal an almost 3-order of magnitude discrepancy between the EC_{50} values of the isoproterenol-mediated mRNA effect and the cAMP production response. The sensitivity of the AT_1-R mRNA to these agonists is consistent with long-standing observations in many systems in which downstream PKA-dependent hormonal responses can occur without detectable changes in cAMP levels relative to basal (see, for example, Refs. 34–36) and are reminiscent of a classic signal-amplification process

[2] X. Wang and T. J. Murphy, data not shown.

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typical for a PKA-activated catalytic cascade (37). However, the tight correlation between the PKA activity and mRNA dose-response curves implicates PKA as a mediator of the mRNA decay response to isoproterenol and argues against the presence of additional catalytic mediators downstream of PKA. The failure to observe detectable changes in cAMP production at agonist concentrations that clearly have an effect on both AT_1-R mRNA levels and PKA activity is most likely explained by our use of a relatively insensitive assay for cAMP production. The finding of maximal activation of PKA at low concentrations of agonist explains how cAMP-elevating agents do not further decrease AT_1-R mRNA levels beyond those observed with low concentrations of agonists, even as cAMP production is further increased, and argue that PKA mediates the AT_1-R mRNA destabilization response after treatment with cAMP-elevating agents.

A paradox exists in that although this study demonstrates that activation of PKA triggers AT_1-R mRNA decay, the basal levels of PKA activity in the cells are already elevated to ~60% of maximum in unstimulated cells. If control of AT_1-R mRNA stability is a dominant pathway, one would expect AT_1-R mRNA expression levels to be quite low in this environment under unstimulated conditions; yet the mRNA is expressed robustly. AT_1-R gene expression has clearly adapted to these unique conditions in the cells. It is of interest to note that this high level of AT_1-R expression is quite unusual and is rarely observed in primary cultures of VSMCs. One explanation for the paradox is that high basal levels of PKA activity may also serve to facilitate AT_1-R mRNA synthesis indirectly, through effects on other pathways of AT_1-R gene expression control. Stimulation of cell surface receptors coupled to phospholipase C down-regulates transcription of the AT_1-R gene (20, 22), and PKA activation inhibits the mitogen-activated protein kinase cascade in VSMCs (25). Perhaps basal mitogen-activated protein kinase cascade activity serves normally to attenuate AT_1-R gene transcription, and the high levels of PKA activity provide an inhibitory tone on this pathway, thus allowing for stronger transcription than would occur if basal PKA activity were lower. This is consistent with the observation that treatment of these cells with cAMP-elevating agents does not induce an unchecked obliteration of the AT_1-R mRNA levels but rather stimulates a readjustment toward a new steady state in which ongoing mRNA synthesis is matched by the now more active mRNA decay process. In addition to maximal stimulation of PKA activity and enhancement of the rate of AT_1-R mRNA decay, other reasons may account for the approach to this new steady state asymptote ~6 hr after stimulation. It may represent destruction of the agonists or desensitization of the cells to their effects.

A previous study suggested that both PKA and protein kinase C activities are involved in down-regulation of AT_1-R gene expression in smooth muscle cells (23), but this conclusion is drawn largely from an analysis that used isoquinoline-based protein kinase inhibitors. It has been shown recently that the isoquinoline compound H-7 effectively inhibits gene transcription by blocking phosphorylation of the carboxyl-terminal domain of RNA polymerase II (38). This observation renders as inappropriate this and related compounds for use in studies of signaling mechanisms involved in gene expression control. We observed a broad dose-dependent reduction of AT_1-R mRNA levels in VSMCs using the isoquinoline derivative H-89 (data not shown), a compound purported to have cAMP-dependent kinase selectivity (39). The net responses observed with these kinase inhibitors on mRNA levels likely represent a composite of effects in which they act as general transcriptional inhibitors, as inhibitors of kinases such as PKA and protein kinase C, and also perhaps as inhibitors of related kinases (40). The results of the current study resolve at least one aspect of the issue for a role of kinases in regulation of VSMC AT_1-R gene expression by demonstrating mRNA destabilization as a mechanism for down-regulation of AT_1-R gene expression in response to cAMP-elevating agents. Previous observations have shown a significant repression of VSMC AT_1-R gene transcription in response to stimulation with growth factors and angiotensin II and that these effects are superimposed on apparent mRNA destabilization processes also elicited by these agonists (20–22). It will now be important to return to these other classes of agonists to understand how components of their more complex signaling pathways control AT_1-R gene expression.

To the extent that other mRNA molecules encoding for proteins involved in the differentiated contractile function in VSMCs may also serve as substrates for this regulated cAMP-dependent mRNA decay process, PKA-mediated mRNA destabilization may play a central role in orchestrating patterns of gene expression in VSMCs in response to persistent or skewed exposure to many classes of neurotransmitters, hormones, and growth factors. In this regard, mRNA destabilization may play a role in facilitating phenotypic rearrangements in VSMCs and, thus, vascular remodeling. In addition, the cardiovascular effects of angiotensin II are mediated exclusively by the cell surface AT_1-R. Inhibitors of AT_1-R function are widely effective in the treatment of cardiovascular diseases such as hypertension and congestive heart failure, but there is little incontrovertible evidence for genetic defects in any components of the renin-angiotensin system. It is possible that subtle defects in the cellular processes involved in regulation of the expression of a normal AT_1-R gene can exacerbate whatever role angiotensin II may play in disease progression. Establishment of the mechanisms involved in the control of VSMC AT_1-R gene expression can thus specifically contribute to understanding of the role played by VSMCs and the AT_1-R in the development of cardiovascular disease and generally is more important as a model system with which to elucidate mechanisms of mRNA stability control processes regulated by extracellular signals.

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