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Inhibition of Cyclic AMP-Dependent Kinase by Expression of a Protein Kinase Inhibitor/Enhanced Green Fluorescent Fusion Protein Attenuates Angiotensin II-Induced Type 1 AT_1 Receptor mRNA Down-Regulation in Vascular Smooth Muscle Cells

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ABSTRACT

Expression of the angiotensin II type 1 receptor (AT_1-R) mRNA in vascular smooth muscle cells (VSMC) is down-regulated by a variety of agonists, including growth factors, agonists of $G\alpha_q$ protein-coupled receptors, and activators of adenylyl cyclase. To determine whether cAMP-dependent protein kinases (PKA) participates in AT_1-R mRNA down-regulation controlled by multiple classes of receptors, a PKA inhibitor peptide (PKIα) was developed and expressed in rat VSMC as a fusion with the enhanced green fluorescent protein (eGFP). PKA activity elicited both by forskolin and angiotensin II is suppressed in cells expressing this fusion protein (PKIα-eGFP), but platelet-derived growth factor-BB does not stimulate PKA activity in this preparation. PKIα-eGFP expression fully inhibits the forskolin-stimulated down-regulation of AT_1-R mRNA levels and blocks 50% of the effect elicited by angiotensin II. This indicates that PKA plays a substantial role in angiotensin II-stimulated AT_1-R mRNA down-regulation. However, inhibition of PKA has no effect on AT_1-R mRNA down-regulation caused by platelet-derived growth factor-BB. These findings show how agonists such as angiotensin II that are not normally considered as activators of PKA can use PKA-dependent processes to modulate gene expression. These findings also provide definitive evidence that PKA-dependent pathways are involved in modulation of AT_1-R mRNA levels in VSMC.

An emerging concept from throughout the signal transduction literature now suggests that any single receptor class can modulate several gene expression control mechanisms by stimulating a variety of second messengers and signaling enzymes (Hill and Treisman, 1995). The control of AT_1-R mRNA levels in rat thoracic aorta VSMC provides an interesting model system for a pleiotropic effector response likely controlled by multiple signaling cascades. Previous studies have shown that the AT_1-R mRNA in VSMC is down-regulated similarly by growth factors, by agonists of G_q-coupled receptors, and by agonists of G_s-coupled receptors (Nickenig and Murphy, 1994, 1996; Lassègue *et al.*, 1995; Wang *et al.*, 1997).

Each of these diverse agonist classes has the same apparent effect on AT_1-R mRNA levels, raising the possibility that they may share common mechanisms of action to mediate this response. Growth factor and G_q-coupled receptor activation is associated with suppressed transcription of the AT_1-R gene (Nickenig and Murphy, 1994; Lassègue *et al.*, 1995), but transcriptional repression accounts for only a part of the down-regulation of AT_1-R mRNA levels because additional evidence suggests an mRNA destabilization mechanism may be superimposed on this effect (Nickenig and Murphy, 1996). In contrast, agents that elevate cAMP have no effect on AT_1-R gene transcription and seem to down-regulate the AT_1-R mRNA solely by post-transcriptional mechanisms (Wang *et al.*, 1997). Further, angiotensin II and PDGF have been shown to activate adenylyl cyclase and PKA in other VSMC preparations (Graves *et al.*, 1996; Rainey *et al.*, 1991). Taken together, these observations form the basis to suggest that PKA-mediated AT_1-R mRNA destabilization may represent a shared pathway invoked by various classes of agonists to down-regulate AT_1-R mRNA levels.

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ABBREVIATIONS: VSMC, vascular smooth muscle cells; AT_1-R, type-1 angiotensin II receptor; PKIα, heat stabile cAMP-dependent protein kinase inhibitor; PKA, cAMP-dependent protein kinase; eGFP, enhanced green fluorescent protein; tTA, tetracycline transactivator; LTR, long terminal repeat; PKG, cGMP-dependent protein kinase; DMEM, Dulbecco's modified Eagle's medium; FACScan, fluorescent activated cell scan analysis; FACS, fluorescent activated cell sorting; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PDGF-BB; platelet-derived growth factor, BB isoform.

Selective inhibition of PKA using the pseudosubstrate inhibitor polypeptide PKIα provides the best means of testing this hypothesis (Walsh *et al.*, 1990). Although the cellular functions of PKIα remain enigmatic, its selectivity for PKA over other kinases is unmatched by other PKA inhibitors. In particular, although some forms of the commonly used isoquinoline-based kinase antagonists inhibit PKA with some selectivity, they also inhibit a variety of other kinases that share with PKA a highly conserved ATP-binding domain that is targeted by these drugs (Engh *et al.*, 1996). The use of such compounds in gene expression assays is particularly problematic because among their known "nonspecific" effects is an ability to block gene transcription by inhibiting the phosphorylation of the RNA polymerase II carboxyl-terminal domain (Dubois *et al.*, 1994).

PKIα thus provides a superior choice for selective inhibition of PKA, but its use on intact cell systems has been limited by its inability to cross the plasma membrane and thus be supplied as a pharmacological reagent. One means of surmounting this limitation is to express PKIα as a recombinant gene in plasmid expression vectors (Grove *et al.*, 1987). Although this approach has great use in commonly used surrogate cells that are readily transfected with plasmids, it has not been extended to study questions in cells more difficult to transfect. This report describes an approach to inhibit PKA in cultured VSMC through expression of PKIα as a fusion with the eGFP using a tetracycline-regulated retroviral vector system. This design surmounts a series of technical barriers associated with efficient expression of recombinant genes in VSMC. Most importantly, the expression of a PKIα-eGFP fusion protein reveals a differential role for PKA in down-regulation of the AT_1-R mRNA by different classes of VSMC agonists.

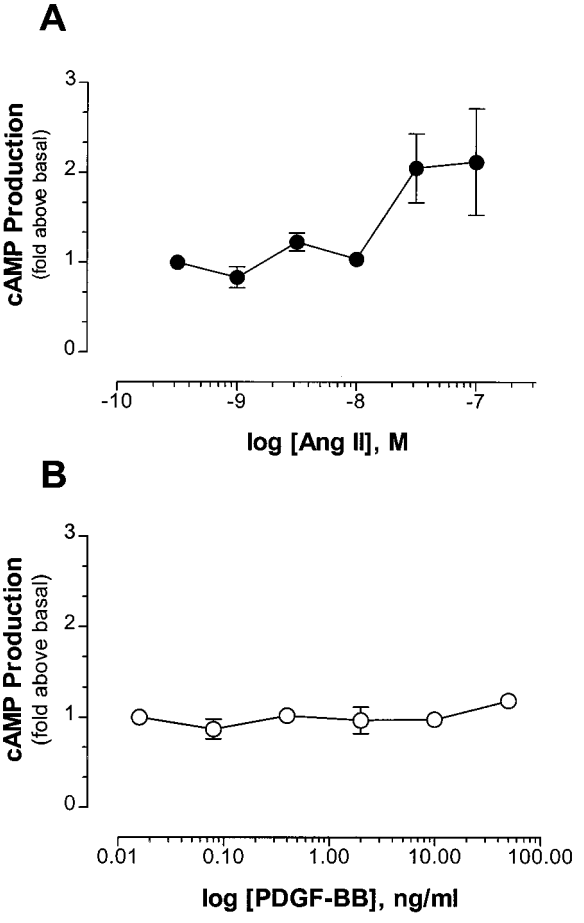

Fig. 1. Angiotensin II and PDGF-BB have different effects on cAMP production in VSMC. Wild-type VSMC were loaded with 5 μCi/ml [^{3}H]adenine and stimulated with the indicated concentrations of angiotensin II (*Ang II*) (A) or PDGF-BB (B) for 5 min at 37C in the presence of 0.1 mM Ro20–1724. cAMP production was measured as described in Experimental Procedures. *Points*, mean ± standard error of three experiments performed in duplicate.

Experimental Procedures

Materials

[α-^{32}P]dCTP (3000 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol), [^{3}H]adenine (26.8 Ci/mmol), [α -^{32}P]UTP (800 Ci/mmol), Na-[^{125}I] (2000 Ci/mmol), 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 International (Natick, MA). PDGF-BB was purchased from Calibiochem (San Diego, CA). Forskolin, angiotensin II, salts, and buffers were from Sigma Chemical (St. Louis, MO) or Life Technologies (Gaithersburg, MD). Anhydrotetracycline (Antet) was purchased from Acros Organics (Pittsburgh, PA). Antibiotics, serum, and cell culture media were from Life Technologies. Forskolin was prepared as a 10 mM stock and diluted in high purity dimethylsulfoxide (Sigma). The same concentration of solvent was used as vehicle in all experiments.

Methods

Cell culture. Rat thoracic aorta smooth muscle cells are kept as a continuous cell line and are kindly provided by Dr. R. W. Alexander (Emory University). Cells were cultured in DMEM with 3.7 mg/ml $NaHCO_3$, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated calf serum in 37° CO_2 incubator with 5% CO_2. After confluence, cells were growth-arrested before experiments by culture in serum-free media for 24 hr. Cells were used between passages 10 to 25 after primary explant. Cells infected with retrovirus were maintained with 1 μg/ml Antet continuously in the growth media unless otherwise stated. The retroviral producer cell line Bing CAK8 (American Type Culture Collection, Rockville, MD) was cultured in DMEM with 3.7 mg/ml $NaHCO_3$, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum in 37° CO_2 incubator with 5% CO_2. During retrovirus production and collection, Bing CAK8 cells were transferred into a 32° CO_2 incubator with 5% CO_2.

Plasmid construction. Retroviral vectors were constructed by standard protocols. pTSO5 contains the tTA cDNA derived from pUHD15–1 (Gossen and Bujard, 1992) and cloned into the retroviral vector pLXSH (a gift from A. D. Miller, Fred Hutchinson Cancer Center, Seattle, WA) (Miller *et al.*, 1993). The vector pXF40 is a modification of pLNCX, also kindly provided by Dr. A. D. Miller, wherein the tetracycline operator sequence in association with a minimal CMV promoter (tetO-CMV) is derived from pUHD10–3 (Gossen and Bujard, 1992), and a SV40 poly(A)$^+$ signal sequence is derived from pCDNA3. pXF40-LUC, pXF40-eGFP, and pXF40-PKIα-eGFP were all constructed on the basis of pXF40, as illustrated in Fig. 2. An eGFP cDNA was derived from peGFP-N1 purchased from Clontech, and the PKIα cDNA was cloned from rat skeletal muscle by RT-PCR using primers 5′-GGCTGTAAAGAGCACTGTTCC-3′ and 5′-GGTGTTGATGACCGTCAGAAT-3′. A luciferase cDNA was derived from poLUC (Brasier *et al.*, 1989), kindly provided by Dr. A. Brasier (University of Texas Medical Branch, Galveston, TX). Plasmids and their complete sequences are available on request.

Retroviral transfection protocol. The retrovirus production and transfection protocols are modifications of previously described methods (Kotani *et al.*, 1994). The retrovirus producer cell line Bing-

CAK8 was seeded at a density of 7.5×10^6 cells/100-mm-diameter dishes 1 day before transfection with 30 μg of retroviral vector DNA prepared in a buffered Ca^{2+} solution (Chen and Okayama, 1987) in growth media containing 25 μM chloroquine. Transfection was terminated 10 hr later by replacing the DNA-containing media with 15 ml of fresh growth media, and the cells were cultured for another 12 hr. The growth media was then replaced with 9 ml of fresh media, and the cells were cultured at 32C in a 5% CO_2 incubator. Conditioned media containing retrovirus was collected twice at 24- and 12-hr intervals after the first change to 6 ml by passing through a sterile 0.45-μm filter, snap-freezing in a dry ice-ethanol bath, and storage at −80° until use.

For infections, VSMC ($2 \sim 4 \times 10^5$ cells) grown on 35-mm-diameter wells in six-well plates were exposed to thawed retrovirus producer cell supernatant supplemented with 8 μg/ml polybrene. The plates then were centrifuged at 2500 rpm for 30 min at 32° in a GS-6 Beckman bench top centrifuge. After returning the cells to a 37° incubator for 8 hr, they were treated with another aliquot of thawed retroviral media, and the centrifugation was repeated. After a third such round of retroviral infection, the cells were placed in their standard growth media, and selective drugs were added to the cultures 24 hr after the last retroviral infection.

PKA assay. PKA activity was measured with a protocol modified slightly from that provided by Dr. Lee Graves (Chapel Hill, NC) (Graves *et al.*, 1996). After agonist treatment, cells confluent in 35-mm-diameter dishes were rinsed twice with ice-cold phosphate-buffered saline (137 mM NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) containing 0.1 mM of the phosphodiesterase inhibitor Ro20–1724 (Research Biochemical International), once with homogenization buffer (50 mM β-glycerolphosphate, 10 mM Tris·HCl, pH 7.4, 1 mM dithiothreitol, 0.1 mM Ro20–1724), and collected in 300 μl of homogenization buffer by scraping. These cells were homogenized by passing through a 21-gauge needle 10 times with a syringe and centrifuged at 15,000 × g for 15 min at 4°. Aliquots of supernatant were removed for PKA assays and for protein measurements using a BioRad Bradford-based kit and γ-globulin as standard. *In vitro* PKA assay reactions contain 10 μl of the cell lysate (5–10 μg of protein), 20 μl of the assay buffer (25 mM β-glycerolphosphate, 10 mM Tris·HCl, pH 7.4, 0.5 mM dithiothreitol, 0.1 mM Ro20–1724) with either buffer A, B, C, or D. The components of each buffer with final assay concentrations are buffer A, 100 μM ATP, 25 μCi/ml [γ-^{32}P]ATP (3000 Ci/mmol), 0.17 mM kemptide; buffer B, buffer A plus 1 μM PKIα (6–22) peptide; buffer C, buffer A plus 5 μM cAMP; and buffer D, buffer A plus 1 μM PKIα (6–22) peptide and 5 μM cAMP. The mixture was placed in a 30° water bath for 15 min before 25 μl of the sample was removed and spotted onto 25-mm-diameter circles of P81 cellulose filter to stop the reaction. The filters were washed four times in a 300 ml of 0.1 mM *o*-phosphoric acid in water for 5 min, rinsed briefly in 95% ethanol, and then dried in air. The filter paper was placed in 7 ml of scintillation fluid (Ecoscint A; National Diagnostics, Atlanta, GA) and counted in a Beckman LS6500 scintillation counter. PKA-dependent phosphorylation is defined as the difference in radioactivity associated with the filters in the absence and presence of PKIα (6–22). Conversion of cpm to pmol was based on the specific activity of [γ-^{32}P]ATP calculated in each assay.

cAMP assay. VSMC in 6 × 35-mm-diameter plates were incubated for 2 hr at 37° in 1 ml of DMEM with 10 mM HEPES, pH 7.4, containing 5 μCi of [^{3}H]adenine before being exposed to agonists for 5 min in the presence of 0.1 mM Ro20–1724. Aspiration of the media and the addition of 1 ml of 5% trichloroacidic acid terminated production of cAMP. [^{3}H]cAMP and [^{3}H]ATP fractions were isolated by sequential chromatography on dowex and alumina before scintillation (Shimizu *et al.*, 1969). cAMP production is derived as the percent conversion of [^{3}H]ATP to[^{3}H]cAMP (cpm [^{3}H]cAMP/cpm [^{3}H]cAMP + cpm [^{3}H]ATP).

eGFP detection and cell sorting. The eGFP used in this study is an enhanced version of wild-type GFP that has mutations shifting the emission wavelength to 409 nm to increase fluorescent intensity (Cormack *et al.*, 1996). To observe expression, cells infected with either eGFP or PKIα-eGFP retroviruses were washed twice with growth media lacking phenol red to remove Antet, which was used to suppress expression of the proteins, and then cultured for 24 hr. Images were obtained using a fluorescent microscope with a fluorescein isothiocyanate filter and recorded by a digital camera and processed by a Scion graph capture program. To prepare cells for sorting, eGFP or PKIα-eGFP was allowed to express for 24 hr by removing Antet before dispersion by treatment with trypsin. The

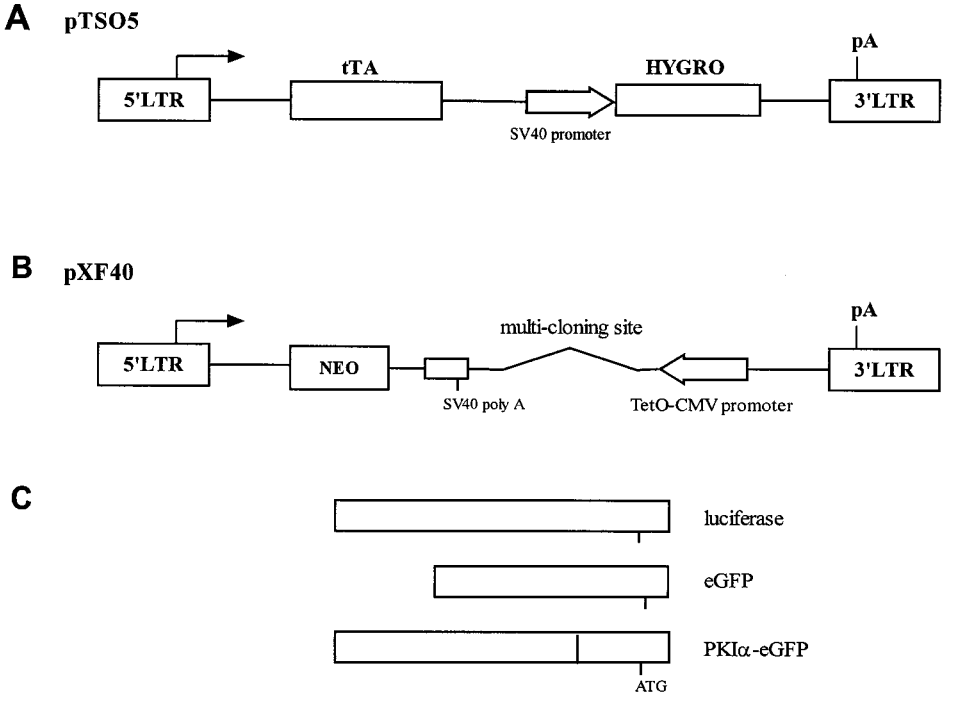

Fig. 2. Diagrams of retroviral vectors used in this study. A, pTSO5 expresses tTA from the viral 5′-LTR promoter. B, pXF40 drives transcription of inserts placed downstream of the tetO-minimal CMV promoter toward the 5′-LTR. C. Inserts placed into pXF40 include cDNAs for either luciferase, eGFP, or a PKIα-eGFP fusion protein. *pA*, polyadenylation signal in 3′-LTR. *NEO and HYGRO*, resistance genes to G418 and hygromycin, respectively.

samples were passed through a 70-μm filter to remove cell clusters. FACScan and FACS were done at the flow cytometry facility at the Emory University Hospital using FACScan and FACS Vantage flow cytometry systems, respectively (Becton Dickinson, San Jose, CA). After initial FACS, the percentage of cells recombinant for fluorescent proteins were monitored continuously by FACScan to ensure the studies were performed on highly (>80%) recombinant cells.

Luciferase assay. Cells were grown to confluence in wells on 12 × 20-mm-diameter culture plates containing the indicated concentrations of Antet. To measure luciferase induction, Antet was removed by washing the cells twice with growth media at various times before the luciferase assays. These assays were performed as described previously (Takeuchi *et al.*, 1993).

Northern hybridization. Cell growth and treatment, RNA extraction, and AT_1-R mRNA hybridization were performed as described previously (Wang *et al.*, 1997). Nylon slot blots were hybridized with an α-^{32}P-dCTP-labeled AT_1-R cDNA probe made from a 824-bp fragment from AT_1-R cDNA, which is described in detail elsewhere (Nickenig and Murphy, 1994). The eGFP mRNAs were detected using a similarly labeled 778-bp *Hin*dIII/*Not*I fragment derived from the peGFP-N1 plasmid. AT_1-R mRNA levels were quantified using images collected by a PhosphorImager and a volume integration protocol in the ImageQuant program from Molecular Dynamics (Santa Clara, CA). The volume of the rectangle covering the hybridization signal was subtracted from that of a background rectangle of the same size. For comparison of the treatments, hybridization signals were normalized as a percentage of the value from samples derived from vehicle-treated cells (100%).

Saturation binding of AT_1-R. The AT_1-R binding protocol has been reported elsewhere (Murphy *et al.*, 1991). In brief, confluent cultures on 100-mm-diameter dishes were harvested by scraping in phosphate-buffered saline and pelleted. Crude particulate membrane fractions were prepared by homogenizing the cells in ice-cold 50 mM Tris·HCl, pH 8.0. Saturation binding assays were conducted on membranes in a buffer containing 50 mM Tris·HCl, pH 7.4, 5 mM $MgCl_2$, and 0.1% bovine serum albumin, and mixed with increasing concentrations of ^{125}I-labeled [Sar^1,Ile^8]angiotensin II ([^{125}I]Sarile). Dup753 (10 μM) was used to define nonspecific binding. After incubation at room temperature for 1 hr, the samples were filtered through GF-B glass fiber filters which were soaked in 0.1% polyetheyleneimine. The data were analyzed using nonlinear regression with one-site binding equation in Prism software (GraphPAD, San Diego, CA) to derive K_D and B_{max} values.

Nuclear run-on. The nuclear run-on method is described elsewhere (Nickenig and Murphy, 1994; Wang *et al.*, 1997). Untransfected VSMC and VSMC expressing eGFP or PKIα-eGFP were cultured in 150-mm-diameter dishes to confluence in growth media and then for an additional 24 hr in serum-free conditions before treatment for 4 hr with either vehicle or 100 nM angiotensin II. After this, the nuclei were harvested, *in vitro* run-on assays were performed in the presence of [^{32}P]UTP, and the samples were hybridized to nylon membranes containing cross-linked AT_1-R and control plasmids as described in detail previously (Wang *et al.*, 1997).

Results

Although angiotensin II is an agonist for a G_q-coupled receptor, stimulation of VSMC with this hormone leads to a modest enhancement of cAMP production in the presence of phosphodiesterase inhibitors, indicating the response is due to new synthesis of cAMP (Fig. 1A). PDGF-BB does not elicit a detectable increase in cAMP production during this same time period (Fig. 1B) or after more prolonged periods of exposure to PDGF-BB (data not shown). These data predict that angiotensin II, but not PDGF-BB, should stimulate PKA activity in VSMC. However, low concentrations of more typical cAMP-elevating agonists such as forskolin and isoproterenol that do not give rise to detectable cAMP production in this assay have pronounced effects on both PKA activity and on AT_1-R mRNA down-regulation (Wang *et al.*, 1997). Because this previous study emphasized the sensitivity of PKA to agonist challenge, we investigated a potential role for PKA in the effects of both angiotensin II and PDGF-BB on AT_1-R mRNA levels, as described below.

Retroviral-tetracycline repressible expression system in VSMC. PKA has been implicated as a regulator of cell cycle progression (Grieco *et al.*, 1996). Reasoning that constitutive expression of a PKA inhibitor might inhibit VSMC growth, we exploited the tetracycline regulated expression system to control its expression. To obtain this control along with efficient transfection of VSMC with the gene, we reconstructed the tetracycline-regulated expression system (Gossen and Bujard, 1992) in retroviral vectors. The efficiency of plasmid DNA transfection of the VSMC used in this study is extremely low. In contrast, histochemical staining of cells infected with a retrovirus coding for β-galactosidase reveals the efficiency of retroviral-mediated gene transfer ranges from 70% to >99% in this preparation (data not shown). Retrovirus transfection has the additional advantage of more efficient integration of the transfected gene into the genome of the host cells than can be achieved using plasmids, allowing for more prolonged maintenance of the transgene in culture (Temin, 1986).

Two vectors comprise the retroviral-based tetracycline-repressible system developed for this study (Fig. 2). The plasmid pTSO5 is designed for constitutive expression of the tTA from the viral 5′-LTR promoter, which co-segregates with a hygromycin resistance gene, transcribed from a second promoter in the vector. The plasmid pXF40 has a minimal CMV promoter with an upstream oligomer of the tetracycline operator enhancer element driving the expression of the genes of interest. In this study, luciferase, eGFP, and PKIα-eGFP cDNAs were constructed behind this promoter. This latter expression cassette is oriented on the opposite strand relative to the 5′-LTR and is designed to segregate with a gene conferring cellular resistance to the neomycin analog G418.

The kinetics of tetracycline-repressible gene expression and Antet dosing conditions were tested by sequential infection of VSMC with virus prepared with the pTSO5 and pXF40-Luc vectors. A stable line of population-selected cells was established after G418 and hygromycin treatment in which only modest amounts of cell death were noted compared with noninfected controls (data not shown). Full suppression of luciferase expression occurs in these cells using 10 ng/ml Antet with an IC_{50} value of 0.34 ng/ml (Fig. 3A). On withdrawal of Antet, the expression of luciferase reaches a maximum within 24 hr (Fig. 3B). The ratio of fully expressed to fully suppressed luciferase levels is 37-fold (as a mean of three such experiments). These dose and time dependences of luciferase expression were used as guidelines in subsequent studies to regulate expression of the other proteins from pXF40-based vectors.

Regulated expression of eGFP and a PKIα-eGFP fusion protein in VSMC. A cDNA for PKIα was coupled to eGFP to monitor directly the level of PKIα expressed and the number of positive cells after viral infection. As a negative control, we used eGFP expressed from a similar vector as that used to express PKIα-eGFP (Fig. 2). Stable lines of population-selected cells expressing either eGFP or PKIα-

eGFP were established by sequential infection of VSMC with virus prepared from the pTSO5 and either pXF40-eGFP or pXF40-PKIα-eGFP, and cells resistant to both G418 and hygromycin were studied further. As above, only modest cell death was noted during this selection process.

The Antet-dependent expression of eGFP and PKIα-eGFP was determined by northern blot analysis. Antet was removed 24 hr before RNA extraction to allow for expression of eGFP and PKIα-eGFP. The levels of eGFP and PKIα-eGFP mRNA are very low but detectable in cells treated with Antet, which seems to represent transcriptional leak from the tetO promoter. On removal of Antet, both mRNAs are expressed more robustly at sizes predicted by the recombinant gene (Fig. 4). The PKIα-eGFP mRNA (~1.1 kb) is larger in size compared with eGFP mRNA (~0.8 kb) but not as abundant. The size of the transcript migrating at ~4 kb in the absence of Antet is consistent with one that escapes polyadenylation by the SV40-derived poly(A)$^+$ signals and instead is polyadenylated by the downstream poly(A)$^+$ signals present on this strand of the 5′-LTR.

After the removal of Antet for 24 hr, the cells were analyzed microscopically. Fluorescence was observed in both the eGFP- and the PKIα-eGFP-infected VSMC (Fig. 5), indicating both the eGFP and the PKIα-eGFP fusion protein were expressed in VSMC to detectable levels. The distribution of the two proteins in VSMC differs, however. In cells expressing eGFP alone, fluorescence is distributed throughout the cell body. In contrast, the PKIα-eGFP fusion protein is excluded from nucleus, which may reflect that the eGFP fusion exposes the nuclear export signal located at the carboxyl-terminus region of the PKIα (Fantozzi, *et al.*, 1994; Wen *et al.*, 1995). In the presence of Antet, low levels of eGFP expression can still be seen in some cells. This might reflect low levels of transcriptional leak and accumulation of the eGFP protein, which seems to be quite stable in these cells.

However, after retroviral infection, microscopic analysis indicates that only a fraction of the cells express eGFP, even though the cultures were resistant completely to both G418 and hygromycin and thus clearly infected by both viruses. FACScan analysis was used to obtain quantitative estimates of the expression and induction of eGFP and PKIα-eGFP. As shown in Fig. 6, A and B, ~70% of the cells in the cultures show a low level of fluorescence intensity, representing autofluorescence from nonexpressing cells in both the uninduced eGFP and PKIα-eGFP VSMC cell lines. When both cell lines are grown in Antet, ~30% of the cells are present in a second, higher intensity peak representing cells expressing escape levels of eGFP or PKIα-eGFP. Withdrawal of Antet has little or no effect on the distribution of cells in the peak representing autofluorescence, but the number of cells in the

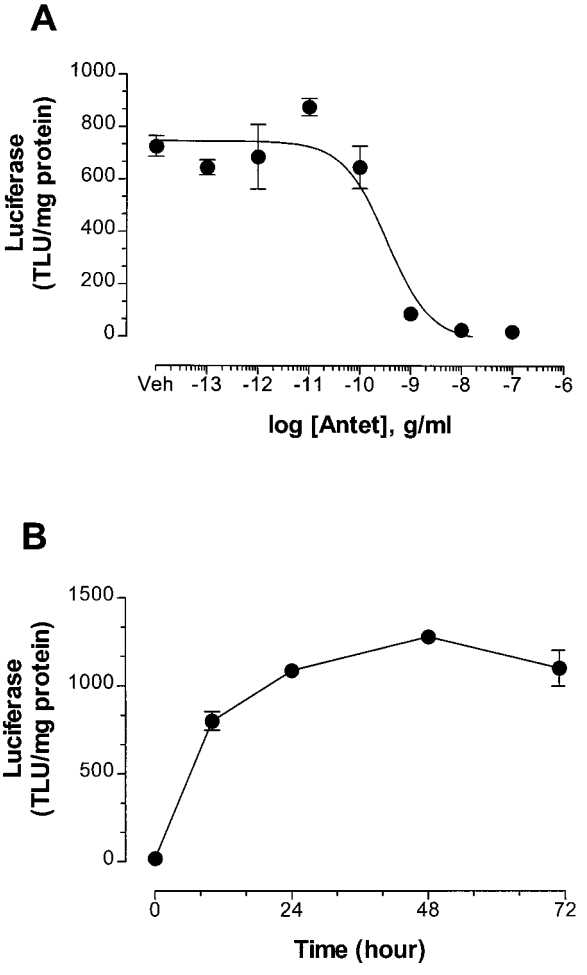

Fig. 3. Regulation of luciferase expression from the pXF40 expression vector. VSMC were infected with viruses prepared from pTSO5 and pXF40-Luc vectors. A, The cells were exposed to the indicated concentrations of Antet for 24 hr followed by measurement of luciferase activity in the cell lysate. B, Cells grown in 100 ng/ml Antet were washed with growth media to remove Antet and cultured for the indicated times before measurement of luciferase. Luciferase levels are expressed as arbitrary Turner light units normalized to protein content in each sample. *Data points*, mean ± standard error of quadruplicate determinations from a single experiment, with similar results obtained in two additional experiments.

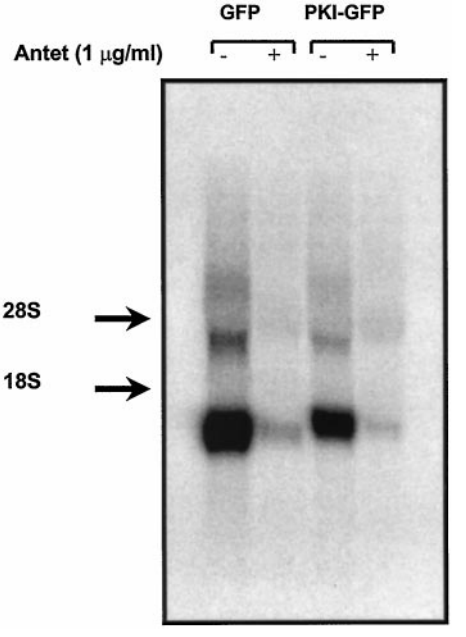

Fig. 4. Induction of eGFP or PKIα-eGFP mRNAs in VSMC. VSMC infected sequentially with retroviruses prepared from pTSO5 and either pXF40-eGFP or pXF40-PKIα-eGFP were plated in culture media containing 0.1 μg/ml Antet until confluence before washing with media without (−) or with (+) 1 μg/ml Antet and cultured an additional 24 hr. RNA was extracted from the cells 24 hr after the wash. The RNA was separated on formaldehyde gel and analyzed by Northern blot hybridization using a [α-^{32}P]dCTP-labeled eGFP cDNA probe. *Arrows*, positions of 18S and 28S ribosomal RNAs.

escape peak are reduced and an additional high intensity fraction appears in both cell lines. This latter peak represents the fraction of cells expressing Antet-regulated protein.

FACS was used to enrich for these particular cells, setting the collection window around the peak of cells in which expression is induced by withdrawal of Antet. After expansion through one passage, the enriched cells were reanalyzed using FACScan. As shown in Fig. 6, C and D, ~90% of the sorted cells express some level of eGFP or PKIα-eGFP and >50% of the cells express a higher level of eGFP or PKIα-eGFP when Antet is removed. This pattern of expression remains stable for several passages (data not shown). The following experiments were conducted on cells derived from three such iterations of retroviral infection through sorting. In the cultures used below, the percentages of cells expressing fluorescent proteins ranged from 80% to 90%.

Expression of PKIα inhibits PKA activity in VSMC. The ability of PKIα-eGFP expression to inhibit VSMC PKA activity was determined by adding exogenous cAMP to extracts prepared from cells grown with various doses of Antet (Fig. 7). As a control, similar measurements were obtained in extracts from cells recombinant for the eGFP gene. Although basal levels of PKA are consistently lower in the PKIα-eGFP cell line compared with the eGFP cell line, the difference is not significant across several assays (Table 1). A dose of exogenous cAMP (50 μM final) that was shown in preliminary dose-responses experiments to stimulate PKA activity maximally in wild-type VSMC extracts also stimulates PKA activity in both recombinant VSMC lines when grown in a high dose of Antet to suppresses expression of the inhibitor or its control. This activity is dose-dependently reduced with lower concentrations of Antet only in extracts prepared from the PKIα-eGFP cells and not in extracts from the cells expressing eGFP. Under conditions in which PKIα-eGFP expression is fully permissive, cAMP-stimulated PKA activity is not significantly different from basal PKA activity (Table 1). These results indicate that full expression of the PKIα-eGFP protein results in complete inhibition of cAMP-dependent kinase activity in VSMC extracts.

To determine whether induction of PKIα-eGFP expression can inhibit agonist-stimulated PKA activity, cells cultured in the absence or presence of 0.1 μg/ml Antet were treated with agonists for various times and PKA activity was measured in the cell extracts. Unlike for the previous set of experiments that measure maximal possible PKA activity, the PKA activity in the experiments shown in Fig. 8 depends on the amount of cAMP that is produced by the stimulus and present in the cells on disruption (Corbin, 1983). Under conditions where PKIα-eGFP expression is suppressed, forskolin activates PKA in VSMC 4.8 ± 0.2 fold-over-basal (mean ± standard error, three experiments) within 1 min of treatment and declines to basal levels in 5 min (Fig. 8A). Angiotensin II also elicits an increase in VSMC PKA activity to a maximal, 2.0 ± 0.1 fold-over-basal (mean ± standard error, three experiments) at 10 min (Fig. 8B). However, PKA is not activated by PDGF-BB treatment (Fig. 8C). The differential ability of these agonists to stimulate PKA is consistent with their ability to elicit cAMP production in VSMC (Fig. 1). These

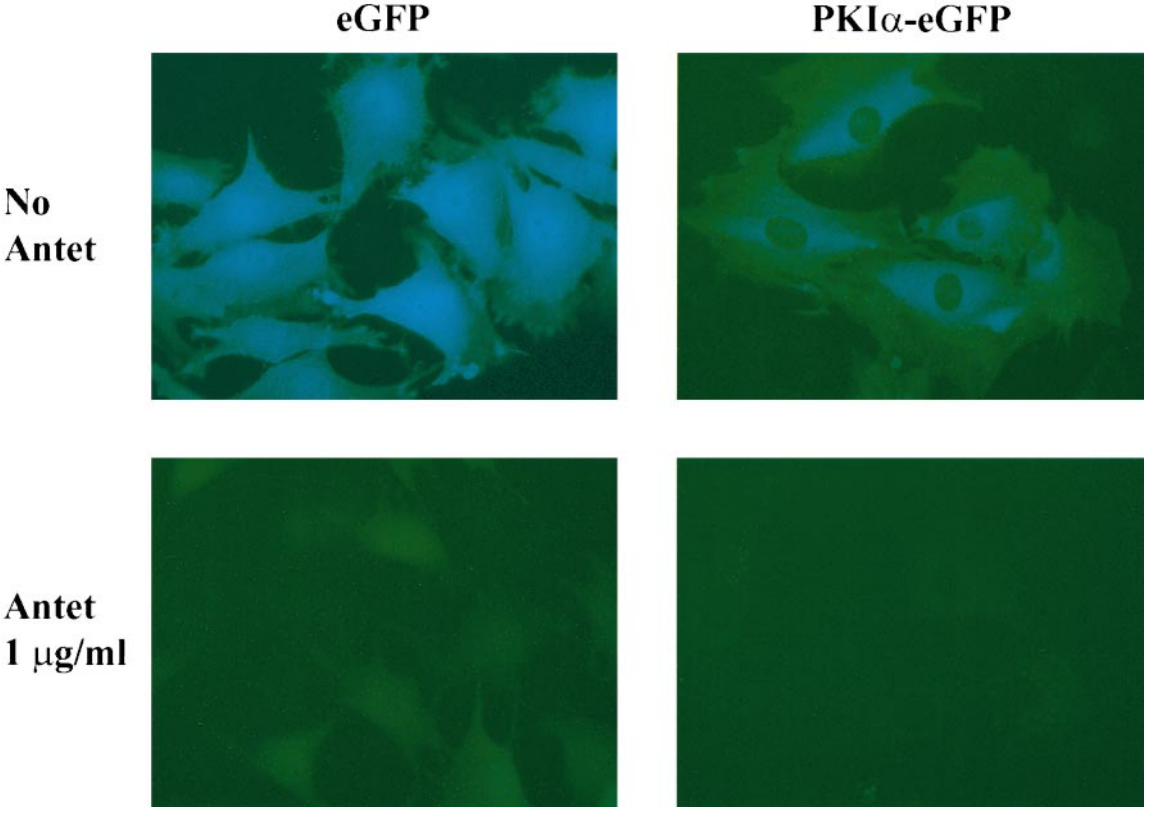

Fig. 5. Antet-regulated expression of eGFP and PKIα-eGFP protein in VSMC. Unsorted VSMC infected with retroviruses prepared from either pXF40-eGFP or pXF40-PKIα-eGFP were plated in 10-cm-diameter dishes in the absence or presence of 1.0 μg/ml Antet. The next day, cells at ~30% confluency were photographed using a microscope equipped with a fluorescein isothiocyanate filter. *Left*, VSMC expressing eGFP, cultured without (A) or with (B) 1.0 μg/ml Antet. *Right*, VSMC expressing PKIα-eGFP, cultured in media without (C) or with (D) 0.1 μg/ml Antet.

data show that full expression of PKIα-eGFP blocks agonist-stimulated PKA activation in VSMC but that suppression of PKIα-eGFP expression permits regulation of this enzyme.

Expression of PKIα-eGFP attenuates down-regulation of AT_1-R mRNA by forskolin and angiotensin II but not PDGF-BB. To determine the role of PKA in agonist-regulated AT_1-R mRNA levels, the time-dependent reductions in AT_1-R mRNA elicited by forskolin, angiotensin II, and PDGF-BB were measured in recombinant VSMC permissive for PKIα-eGFP expression. As control, these responses were compared with those in cells expressing eGFP alone. As shown in Fig. 9A, the reduction in AT_1-R mRNA elicited by 10 μM forskolin in cells expressing eGFP is abolished in cells expressing PKIα-eGFP. These data provide direct evidence that PKA mediates forskolin-induced down-regulation of the vascular AT_1-R mRNA. Expression of PKIα-eGFP also significantly attenuates but does not abolish the angiotensin II-induced down-regulation of vascular AT_1-R mRNA (Fig. 9B) but has no effect on the response to PDGF-BB (Fig. 9C).

PKIα-eGFP has no effect on steady state AT_1-R mRNA expression levels in VSMC. The effect of PKA inhibition on steady state levels of AT_1-R mRNA also was examined. Northern hybridization was performed to compare the basal levels of AT1-R mRNA in wild-type VSMC to those in cells expressing eGFP or PKIα-eGFP. As shown in Table 2, Antet *per se* has no effect on AT_1-R mRNA levels in wild-type VSMC or in the sorted recombinant cells. Compared with the parental cell line, AT_1-R mRNA levels are not significantly different in the sorted cells expressing either eGFP or PKIα-eGFP. Similarly, radioligand binding analysis indicates there are no significant differences in AT_1-R levels in either cell line under permissive or suppressive conditions. These observations suggest that basal levels of PKA activity may not play a significant role in specifying AT_1-R gene expression levels in VSMC.

PKIα-eGFP does not affect transcriptional inhibition of the AT_1-R gene by angiotensin II. Stimulation of VSMC with angiotensin II reduces transcription of the AT_1-R gene (Lassègue *et al.*, 1995). Nuclear run-on assays were conducted to determine whether this effect is mediated by PKA. Wild-type VSMC and VSMC expressing either eGFP or PKIα-eGFP were treated with vehicle or 100 nM angiotensin II for 4 hr before the nuclei were isolated for run-on transcription. As shown in Fig. 10, angiotensin II treatment reduces AT_1-R gene transcription in wild-type VSMC to 54% of control level, consistent with previous observations (Lassègue *et al.*, 1995). This response is slightly less in cells expressing eGFP or PKIα-eGFP, where angiotensin II inhibits AT_1-R gene transcription to 78% and 72% of vehicle control, respectively. Although it remains unclear why angiotensin II-mediated inhibition of AT1-R gene transcription in the recombinant cells is attenuated relative to wild-type cells, this effect occurs clearly by a PKA-independent pathway. Thus, the ability of PKIα-eGFP to attenuate angiotensin II-mediated down-regulation of the AT_1-R mRNA is most likely explained by interference with PKA-dependent mRNA destabilization stimulated by angiotensin II.

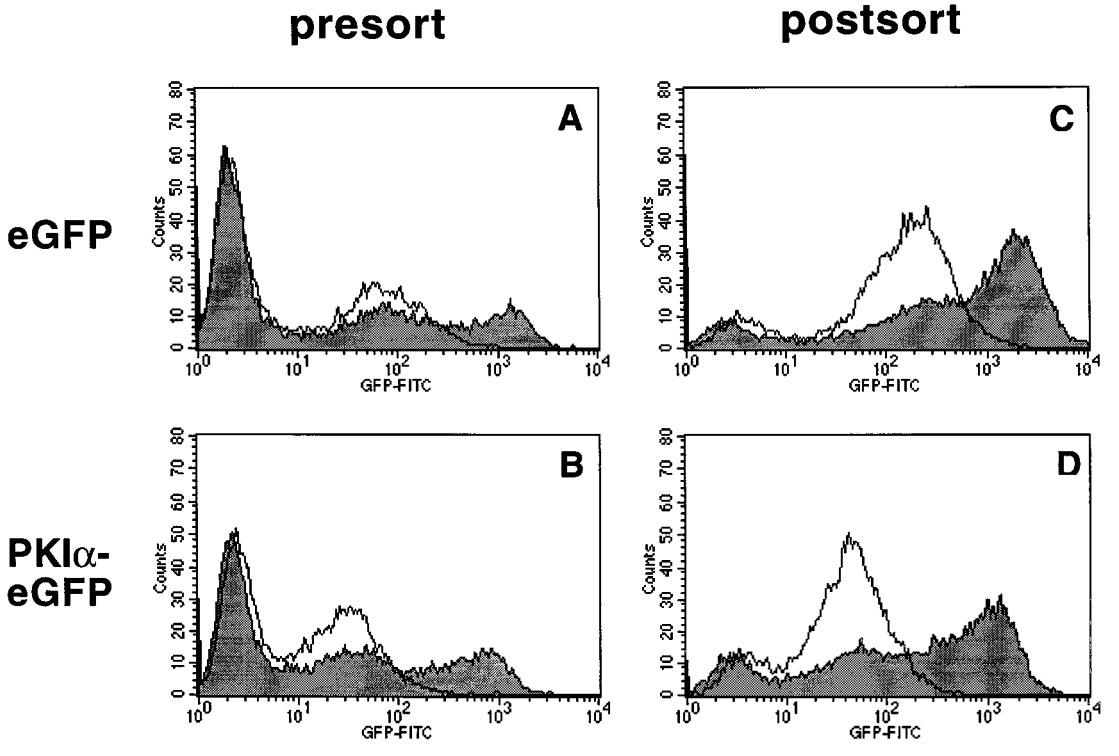

Fig. 6. Enrichment of VSMC expressing eGFP or PKIα-eGFP by FACS. VSMC were selected for dual stable integration of retrovirus prepared from pTSO5 and either pXF40-eGFP or pXF40-PKIα-eGFP by selection with G418 and hygromycin. FACS analysis was conducted after confluent monolayers were cultured for an additional 24 hr in growth media lacking (*shaded area*) or containing (*open area*) 0.1 μg/ml Antet. *Left*, VSMC expressing eGFP (A) or PKIα-eGFP (B) before enrichment by cell sorting. *Right*, VSMC expressing eGFP (C) or PKIα-eGFP (D) after enrichment by cell sorting. *Abscissa*, fluorescence intensity in arbitrary units. *Ordinate*, cell numbers (counts), also in arbitrary units. Values are from a single experiment representative of three.

Discussion

Our principal finding is that approximately half of angiotensin II-stimulated down-regulation of the AT_1-R mRNA in this preparation is mediated by PKA. Formally, nonselective inhibition of some unknown cAMP-independent kinase by PKIα-eGFP could also explain the attenuation of angiotensin II-stimulated AT_1-R mRNA down-regulation that we observe. For the following reasons, however, this seems unlikely. Although small peptide derivatives of PKIα have been shown to inhibit PKG, which is more homologous to PKA than any known kinase, they are nonetheless >2 orders of magnitude more selective for PKA over PKG (Glass *et al.*, 1986). To inhibit PKG, we suspect that the level of PKIα-eGFP fusion protein expression would likely need to be much higher than what we achieve here. Even if this were the case, because a cell-permeable cGMP analog has no effect on VSMC AT_1-R mRNA expression (Wang *et al.*, 1997), it is unlikely that inhibition of PKG would complicate the interpretation of the responses observed in this report. Except for PKG, there is no evidence in the literature indicating that PKIα or its derivatives are capable of inhibiting any other known kinases. Because hundreds of kinases are known and because it also is possible that as many or more await to be discovered, to completely rule out the possibility of cAMP-independent kinases to explain our observations would be a foreboding task.

Furthermore, our PKA assay provides a measure for the level of PKA-independent kinase activity that occurs after agonist stimulation because a crucial measured parameter is agonist-stimulated phosphorylation of the kemptide substrate *in vitro* in the absence of any PKA inhibitor (see Methods). Analysis of the PKA assay data show that angiotensin II and PDGF-BB can stimulate a significant degree of kemptide phosphorylation resistant to PKIα (6–22) inhibition. Most importantly, this PKIα (6–22)-resistant kemptide phosphorylation induced by the two agonists does not differ between extracts of cells expressing eGFP and PKIα-eGFP (data not shown). This indicates that expression of the fusion protein is not affecting the ability of these agonists to stimulate other kinases in the cells that share the kemptide substrate with PKA. Additionally, the fact that AT_1-R mRNA down-regulation by PDGF-BB is unaffected in cells expressing PKIα-eGFP (see Fig. 9) also argues further against nonselective effects of the inhibitor on other putative kinases. Taken together, it is reasonable to assert that effects of PKIα-eGFP expression on factors controlling AT_1-R mRNA levels reflect PKA-dependent processes.

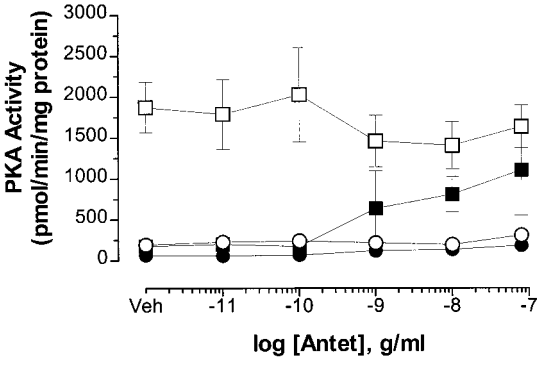

Fig. 7. Inhibition of cAMP-stimulated PKA activity in extracts of VSMC expressing PKIα-eGFP. VSMC expressing eGFP (○, □) or PKIα-eGFP (●, ■) were cultured in growth media containing the indicated concentrations of Antet for 24 hr before measurements of PKA activity in the cell lysate were taken either without (○, ●) or with 50 μM cAMP (□, ■). *Data points*, mean ± standard error of three independent experiments each performed with triplicate determinations.

A recently described lipid-modified PKIα peptide derivative provides an alternate approach to study the role of PKA in cell-based systems (Harris *et al.*, 1997). This approach was not taken here initially because of concerns about the possible instability of these reagents within the cells over the several hours of time needed to conduct gene expression studies (Fernandez *et al.*, 1991). Microinjection of PKI peptides has been employed in numerous studies to evaluate PKA-dependent processes, but it is useful only for responses that can be assessed on a cell-by-cell basis. Avruch and coworkers have pioneered the use of plasmid vectors expressing inhibitor peptides derived from PKIα as an approach to inhibit PKA in cultured cells (Grove *et al.*, 1987). Unfortunately, the VSMC used in this study are transfected poorly, when at all, by any of several standard plasmid DNA delivery techniques.

Although retroviral mediated gene transfer surmounts this obstacle, it has inherent peculiarities that can limit expression efficiency. For instance, bicistronic retroviral vectors such as those created here are subject to epigenetic suppression of one of the two transcriptional units encoded within the viral LTRs (Emerman and Temin, 1984). FACS was exploited to enrich for positive cells when it became apparent that epigenetic suppression of the fusion protein was likely occurring in a large fraction of the cells resistant to antibiotics and thus clearly recombinant for the retroviruses. These technical solutions hold considerable promise if applied to similar cell systems.

Vascular AT_1-R gene expression is sensitive to many classes of agonists (Lassègue *et al.*, 1995; Nickenig and Murphy, 1994; Nickenig and Murphy, 1996; Wang *et al.*, 1997). Understanding the pathways involved in this seemingly

TABLE 1

Comparison of basal and cAMP-stimulated protein kinase A activity in VSMC extracts prepared from cells expressing either eGFP or PKIα-eGFP

The cells were grown in the absence (−) or presence (+) of 100 nM Antet for 24 hr and not otherwise stimulated with agonists before preparing extracts and measuring PKA activity. Basal activity thus results from endogenous cAMP levels in the extracts, whereas experiments not shown indicate that *in vitro* PKA activity is stimulated maximally by 50 μM cAMP in this preparation. The data were inspected statistically using one-way analysis of variance and Tukey's multiple comparison test using the GraphPAD suite of software. Each value represents the mean ± standard error of three independent experiments, each performed in triplicate.

	PKA activity[a]			
	eGFP		PKIα-eGFP	
	(−)-Antet	(+)-Antet	(−)-Antet	(+)-Antet
	pmol/min/mg of protein			
Basal	197 ± 23[d]	315 ± 28[d]	65 ± 28[d]	187 ± 63[d]
+ 50 μM cAMP	1875 ± 310[b]	1643 ± 261[b]	178 ± 82[c,e]	1113 ± 555[b]

[a] Defined as total kemptide kinase activity observed in the extracts less the activity found when 1 μM PKI(6–22) is added to the assay buffer.
[b] Significantly ($p < 0.05$) different from basal activity in the respective extract.
[c] Significantly different ($p < 0.05$) from 50 μM cAMP-stimulated PKA activity in all other extracts.
[d] Not significantly different ($p > 0.05$) from basal PKA activity in all other extracts.
[e] Not significantly different ($p > 0.05$) from basal activity in the respective extract.

pleiotropic control of AT_1-R expression can give insights into the general problem of the mechanisms used by downstream effector responses to decode information within complex receptor-mediated signaling cascades. The current set of experiments were designed to test the simplest possible model to explain this system; a model that states that signals from several classes of receptors converge on a single mechanism to regulate AT_1-R mRNA levels. The results of the current study indicate that PKA activation does not represent an obligate pathway for controlling AT_1-R mRNA levels by all classes of receptors. Our data instead show that PKA activity is essential for the responses to forskolin and by inference other G_s-coupled receptors, is responsible for ~50% of the

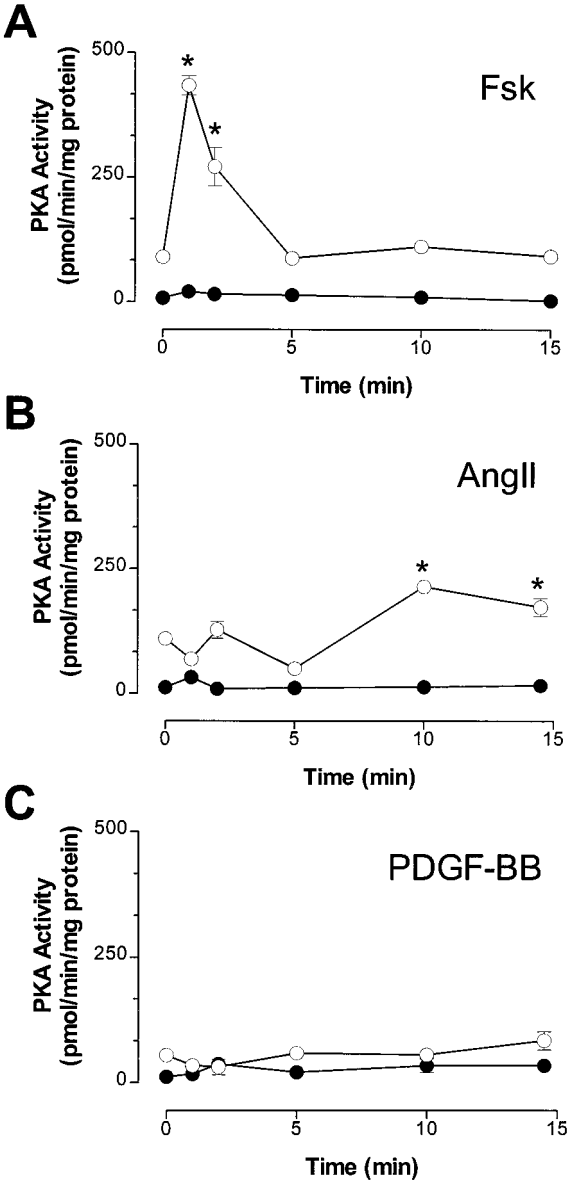

Fig. 8. PKIα-eGFP expression abolishes PKA activation by forskolin and angiotensin II. FACS-enriched VSMC expressing PKIα-eGFP were cultured in media containing 0.1 μg/ml Antet to suppress expression (○) or without Antet to induce expression (●) for 24 hr before exposure for the indicated times to 10 μM forskolin (A), 100 nM angiotensin II (B), or 50 ng/ml PDGF-BB (C) The level of PKA activity measured in these cell lysates is dependent on the amount of cAMP present in the extracts on cell lysis. *Data points*, mean ± standard error of triplicate determinations from a single experiment, which was repeated twice with similar results. *, Significantly different ($p < 0.05$) from basal levels of activity (zero hour time point) by one-way analysis of variance and Dunnett's multiple comparison test using the GraphPAD Prism software.

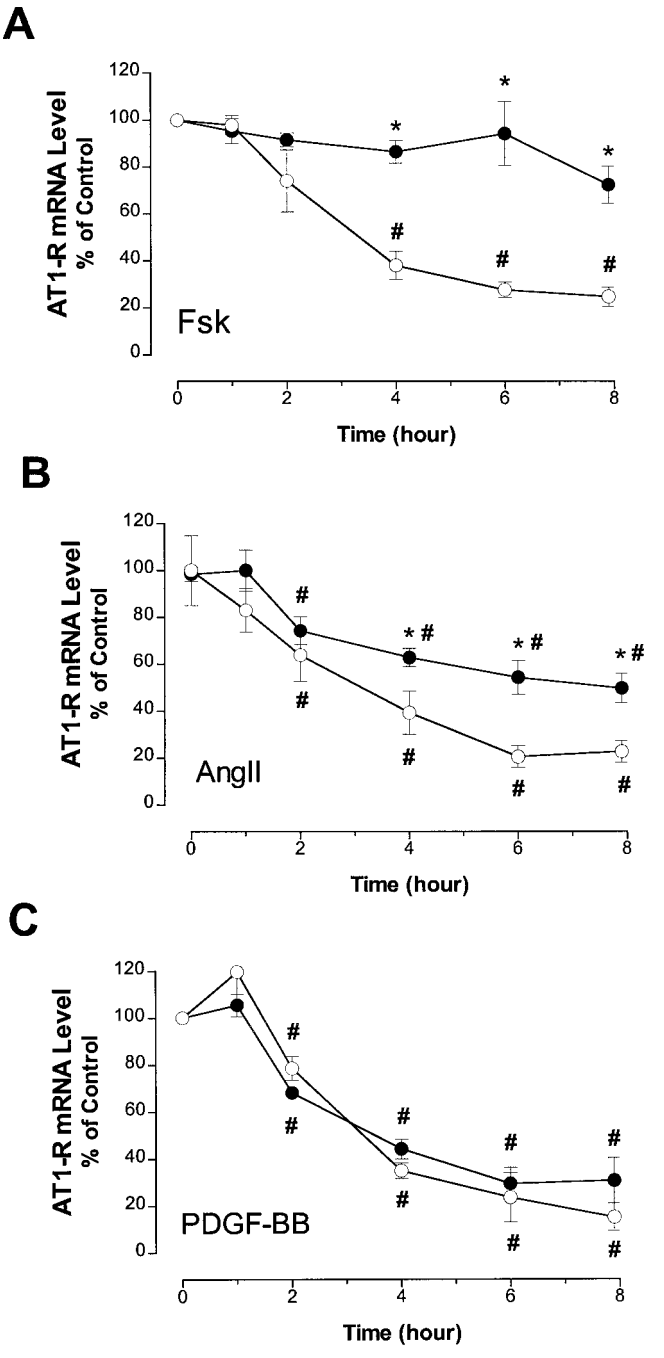

Fig. 9. Expression of PKIα-eGFP inhibits AT_1-R mRNA down-regulation by forskolin and angiotensin II but not PDGF-BB. FACS-enriched VSMC expressing either eGFP (○) or PKIα-eGFP (●) were cultured in Antet-free media to induce expression for 24 hr after reaching confluence. The cells were then treated with single bolus doses of 10 μM forskolin (A), 100 nM angiotensin II (B), or 50 ng/ml PDGF-BB (C) for the indicated period of time. AT1-R mRNA Northern hybridization signals were quantified using a PhosphorImager and normalized as percentage to the signal in untreated cells (zero hour time point). *Data points*, mean ± standard error of three to five experiments with duplicate mRNA determinations per treatment. *, Significant difference ($p < 0.05$) from corresponding time points in cells expressing eGFP alone. #, Significant difference from untreated cells. Both tests used one-way analysis of variance with Dunnett's multiple comparison test.

TABLE 2

PKIα expression does not affect AT_1-R mRNA or protein levels in VSMCs

Wild-type VSMCs and cells expressing either GFP or PKIα-eGFP were cultured with (+) or without (−) 0.1 μg/ml Antet for 24 hr before cell membranes were prepared and subjected to saturation binding with ^{125}I-sarile as described in Experimental Procedures. B_{max} and K_D were determined by 5-point nonlinear regression using one-site binding equation from GraphPAD Prizm software. AT_1-R mRNA levels were determined by Northern hybridization of 10 μg of total RNA separated on a formaldehyde gel with radiolabeled AT_1-R cDNA probe. AT_1-R mRNA levels are normalized as percentage of that in wild-type VSMCs without Antet. One-way analysis of variance indicates there are no significant differences ($p > 0.05$) in the measured parameters across the various cell lines.

	Wild-Type		eGFP		PKIα-eGFP	
Antet	(+)	(−)	(+)	(−)	(+)	(−)
AT_1-R mRNA levels Mean ± standard error (n)	100 ± 9 (2)	100 (3)	103 ± 24 (3)	66 ± 15 (3)	108 ± 22 (3)	66 ± 14 (3)
AT_1-Receptor levels B_{max} (fmol/mg of protein)	177	181	161 ± 32	141 ± 42	120 ± 18	150 ± 24
K_D (nM) Mean ± standard error (n)	0.27	0.30	0.30 ± 0.05 (3)	0.30 ± 0.06 (3)	0.35 ± 0.09 (3)	0.28 ± 0.08 (3)

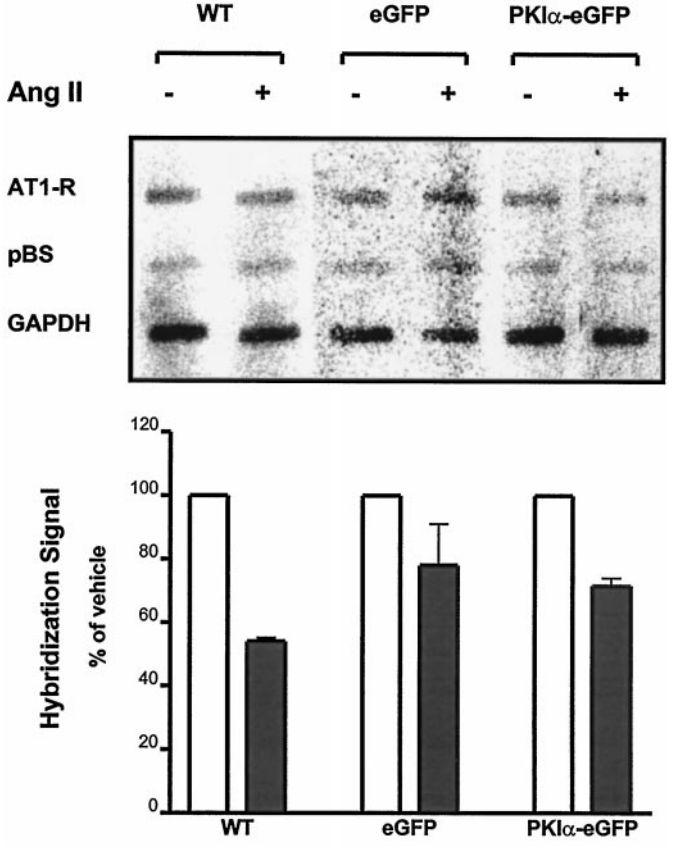

Fig. 10. PKIα-eGFP expression does not affect angiotensin II-stimulated inhibition of AT_1-R gene transcription. Confluent wild-type VSMC (*WT*) and either VSMC expressing eGFP or PKIα-eGFP were cultured for 24 hr in Antet-free media before exposure to either vehicle (−) or 100 nM angiotensin II (+) for 4 hr. Run-on transcription assays were performed on nuclei isolated from these cells as described in Experimental Procedures. The radiolabeled nuclear RNA was used as probe for hybridization with blots cross-linked with 10 μg of AT_1-R cDNA in pBlueScript (AT1-R), pBlueScript alone (*pBS*) as a negative control, or a glyceraldehyde-3-phosphate dehydrogenase cDNA as a positive control for transcription. Representative blots of two independent experiments are shown. Values are average ± range of two determinations. Because of significant background hybridization to the pBlueScript vector, the hybridization signal of AT_1-R slot is first subtracted by that of pBlueScript and then divided by the glyceraldehyde-3-phosphate dehydrogenase signal for normalization. The effect of angiotensin II is expressed as percentage of untreated control (100%) calculated as above.

effect of angiotensin II but is not involved in the response to the growth factor PDGF-BB.

The current data on the effects of angiotensin II are entirely consistent with recently published observations showing that AT_1-R mRNA levels in this preparation are exquisitely sensitive to elevation of cAMP levels (Wang *et al.*, 1997). Low concentrations of forskolin or the β-adrenergic receptor agonist isoproterenol that do not yield detectable increases in cAMP production using this assay are nonetheless very efficacious for down regulating the AT_1-R mRNA. Frequently, these types of findings lead to suggestions that agents such as forskolin or isoproterenol are modulating downstream effects through cAMP-independent pathways. The current study clarifies this issue in this preparation because direct inhibition of PKA completely abolishes forskolin-mediated AT_1-R mRNA down-regulation. Further, low strength cAMP signals such as those elicited by angiotensin II often are dismissed as physiologically irrelevant. The current findings provide yet another example that such judgments can be premature when not directly tested. The finding that PKIα-eGFP expression attenuates angiotensin II-induced AT_1-R mRNA down-regulation provides a definitive case study for showing that even very modest level of cAMP production by agonists can have significant consequences.

Our previous studies have shown that forskolin neither inhibits nor stimulates AT_1-R gene transcription and that it most likely down-regulates AT_1-R mRNA levels solely through destabilization of the mRNA (Wang *et al.*, 1997). By inference, the extent to which PKIα-eGFP abrogates AT_1-R mRNA down-regulation caused by forskolin or any other agonist provides a measure of the contribution of PKA-dependent mRNA destabilization to that response. From the results presented here, we infer that the component of angiotensin II-induced AT_1-R mRNA down-regulation that is sensitive to PKIα-eGFP quite likely reflects a PKA-mediated mRNA destabilization process. This conclusion is supported by the nuclear run-on experiments showing no difference in angiotensin II-inhibited AT_1-R gene transcription between cells expressing eGFP or PKIα-eGFP.

In contrast, PKA is not activated by PDGF-BB, nor do we observe any evidence that PKA is involved in the control of AT_1-R gene expression by this growth factor. This is somewhat surprising given that PDGF-BB has been shown to stimulate PKA activity strongly in human VSMC in a mechanism that seems to involve autocrine release of prostaglan-

dins (Graves *et al.*, 1996). Simple species differences do not seem likely to account for the discrepancy between this and the current study because others have shown that PDGF-BB can induce prostaglandin synthesis in rat VSMC (Li *et al.*, 1997). If PDGF-BB stimulates prostaglandin release similarly in the cells used in this study, the refractoriness might be explained by the absence in our particular cell line of receptors for these putative autocrine factors.

The reductions in AT_1-R mRNA levels elicited by angiotensin II and PDGF-BB that are resistant to PKIα-eGFP expression may not represent a regulated mRNA decay process and may simply reflect a consequence of transcriptional inhibition of the AT_1-R gene. If so, this would imply that two distinct mechanisms are responsible for AT_1-R mRNA degradation. One might involve a default decay process that is revealed only when synthesis of the AT_1-R mRNA is attenuated. The current data provide additional evidence that a second mechanism of AT_1-R mRNA decay exists in these cells and seems to be regulated by PKA-mediated signaling. Further studies are necessary to determine whether any other kinases stimulated by cell surface receptors control this latter process.

Some of this can be clarified better after identifying the molecular components induced by PKA that actually mediate AT_1-R mRNA decay. Once identified, it should be possible to determine more definitively than is now possible whether they are targeted by other classes of kinases that are activated by AT_1 and PDGF-BB receptors. Indeed, one of the rationales for performing this study was to derive some predictable features of the putative AT_1-R mRNA destabilization factors, as efforts are now in progress to isolate and identify them. The evidence presented in this report allows for at least one firm prediction, which is that a crucial component of the pathway leading to AT_1-R mRNA decay in VSMC is most likely phosphorylated by PKA.

Acknowledgments

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