Rapid Communication

Regulation of Smooth Muscle α-Actin Promoter by Vasopressin and Platelet-Derived Growth Factor in Rat Aortic Vascular Smooth Muscle Cells

Vicki Van Putten, Xiaomei Li, Judith Maselli, Raphael A. Nemenoff

Abstract Vasoconstrictors such as arginine vasopressin (AVP) and angiotensin II (Ang II) have been shown to increase protein and mRNA levels of smooth muscle α-actin (SM-α-actin) in vascular smooth muscle cells. In the same cells, platelet-derived growth factor (PDGF) decreased SM-α-actin protein and mRNA. The rat SM-α-actin promoter that has recently been isolated contains two E-boxes and three CC(A/T)6GG (CARG) elements. To examine regulation of the SM-α-actin promoter, a 765-bp region of the rat SM-α-actin gene was ligated into chloramphenicol acetyltransferase (CAT)-containing vectors and transfected into rat aortic vascular smooth muscle cells. Stimulation of cells with either AVP or Ang II increased CAT activity 5- to 10-fold. PDGF was able to completely block the AVP-induced increase in CAT activity. To identify regions of the promoter responsible for both the AVP stimulation and PDGF inhibition of promoter activity, a series of truncation mutants were prepared and transfected into vascular smooth muscle cells. Truncation of both E-boxes and the most distal CARG element did not qualitatively alter either AVP-induced stimulation of CAT activity or PDGF inhibition. However, removal of the middle CARG element resulted in a loss of AVP stimulation. These data suggest that the AVP-induced elevation and PDGF-induced inhibition of SM-α-actin levels in vascular smooth muscle cells are mediated at least in part through regulation of the SM-α-actin promoter. The critical region of the promoter mediating this effect involves at a minimum one of the CARG elements. (Circ Res. 1994;75:1126-1130.)

Key Words • smooth muscle α-actin • vasopressin • platelet-derived growth factor • vascular smooth muscle cells

Vascular smooth muscle cells (VSMCs) undergo distinct patterns of growth in response to stimulation by polypeptide growth factors and vasoconstrictors. Stimulation by growth factors that signal through tyrosine kinase receptors, such as platelet-derived growth factor (PDGF) or epidermal growth factor, results in cell proliferation. Stimulation by vasoconstrictors such as angiotensin II (Ang II) or arginine vasopressin (AVP), in the absence of serum, results in hypertrophy of the cells with little, if any, increase in cell number. The molecular mechanisms underlying these distinct growth responses have not been established. In fact, a number of postreceptor signaling events are stimulated by both classes of agents, including activation of phospholipase C and mobilization of intracellular Ca2+, activation of Na+–H+ exchange, and stimulation of mitogen-activated protein kinases. Recently, it has been shown that vasoconstrictors and PDGF have opposing effects on the levels of specific muscle proteins, including the smooth muscle isoform of α-actin (SM-α-actin). AVP and Ang II have been shown to increase both the protein and levels of steady-state mRNA for SM-α-actin. PDGF decreased both protein and mRNA levels. In the present study, we have extended these studies to directly examine regulation of the SM-α-actin promoter by AVP and PDGF in rat VSMCs.

The rat SM-α-actin promoter has recently been isolated. From examination of the sequence, two E-boxes and three CC(A/T)6GG (CARG) elements have been identified within 750 bases of the start of translation. E-boxes have previously been identified in the promoter region of skeletal muscle–specific genes and have been shown to be involved in the regulation of myogenic gene expression through binding of members of the MyoD family of transcriptional factors (see Reference 15 for review). In cardiac and skeletal muscle cells, activation of SM-α-actin promoter activity through MyoD has been shown to involve E-boxes. However, members of the MyoD family do not appear to be expressed in smooth muscle cells, and the cis-acting elements required for induction of SM-α-actin by vasoconstrictors in these cells are poorly defined. We have been able to establish that AVP and PDGF have opposing effects on promoter activity. Suppression of SM-α-actin expression by PDGF is dominant over AVP-induced stimulation. In addition, we have defined the region of the promoter required for both AVP stimulation and PDGF repression of activity.

Materials and Methods

VSMC Isolation and Culture

Rat VSMCs were isolated and cultured as previously described. Briefly, thoracic aortas were dissected from Sprague-Dawley rats (250 to 300 g) and incubated in Eagle’s MEM containing 2 mg/mL collagenase (Cooper Biomedical)
for 1 hour at 37°C. After removal of the adventitia, the aortas were minced and incubated in MEM containing collagenase for 2 hours at 37°C. The isolated cells were resuspended in culture medium (Eagle's MEM containing 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal calf serum [FCS]) and plated in 35-mm culture dishes at a density of 1 x 10⁶ cells per milliliter. All of the following experiments were done on passages 3 through 8.

Isolation of the Rat SM-α-Actin Promoter

Rat VSMC genomic DNA (100 to 150 kb in size) was obtained by using a modification of the procedure described by Blin and Stafford. The polymerase reaction (PCR) technique was used to isolate a genomic clone of the 765-base rat SM-α-actin promoter region. Oligonucleotides were custom-designed and obtained from DNA Express. The 5' sense oligonucleotide (5'-CCCAGCTTAGCTCTTTAGCAT-GATATC) included a HindIII site and 21 bases matching the published sequence of the rat SM-α-actin promoter region. The resulting PCR product was digested with HindIII and Xho I and ligated into the corresponding restriction sites of the pCAT basic vector (Promega, CATACT(-713/52)). Plasmids were grown in Escherichia coli DH5α and purified by an alkaline lysis procedure with purification over an anion-exchange resin (QIAGEN, Inc). The genomic sequence of the double-stranded cDNA was determined by use of the SEQUENASE version 2.0 kit from United States Biochemical. Comparison of the genomic sequence of the isolated SM-α-actin promoter with the published sequence of the rat SM-α-actin promoter region revealed a single base discrepancy. At position -289, a cytosine rather than a thymidine was present. This substitution does not occur in any of the putative regulatory sequences and corresponds to the cytosine present in the sequences for mouse, human, and chicken SM-α-actin promoters.

Truncations of the promoter were isolated by using PCR with the same anti-sense 3' oligonucleotide and different sense 5' oligonucleotide. All sense oligonucleotides contained a HindIII site, followed by 21 bases matching the published sequence. The following truncations were made: -287 to +52, pCATACT(-287/52); -247 to +52, pCATACT(-247/52); -202 to +52, pCATACT(-202/52); -152 to +52, pCATACT(-152/52); -102 to +52, pCATACT(-102/52); and -57 to +52, pCATACT(-57/52). PCR products were confirmed by sequencing and were ligated into pCAT basic vector as described above.

Transfection and Chloramphenicol Acetyltransferase Activity in VSMCs

VSMCs were transiently cotransfected with the full-length pCAT-α-actin construct [pCATACT(-713/52)], truncations of the pCATACT, the pCAT basic vector (promoterless negative control), or the pCAT control vector (constitutively active positive control, Promega) together with a cytomegalovirus (CMV)-β-galactosidase vector (Clontech) by use of electroporation. VSMCs were suspended in full culture media at 2 x 10⁶ cells per milliliter. By use of a geneZAPPER (IBI), 100 µL of cell suspension was cotransfected with 15 µg of the various pCAT vectors plus 5 µg of the CMV-β-galactosidase vector. The cells were then plated in culture medium with 10% FCS for 18 hours. At this time, the cells were placed in Eagle's MEM with 0.2% FCS and exposed to AVP, PDGF, Ang II, phorbol 12-myristate 13-acetate (PMA), or dibutyryl cAMP (dBcAMP) as described in "Results." Duplicate samples were electroporated for each treatment.

After exposure to the hormones, the cells were harvested by trypsinization, and cell pellets were frozen at -20°C. The chloramphenicol acetyltransferase (CAT) activity of the cell lysates was measured by using a modification of the thin-layer chromatographic method as described by Gorman et al.

Western Blots for SM-α-Actin Content

VSMCs were plated in 60-mm culture dishes at a density of 2 x 10⁶ cells per dish. The cells were exposed to AVP (10⁻⁷ mol/L) or PDGF (20 ng/mL) for 96 hours in Eagle's MEM containing 0.2% FCS. At this time, cells were lysed in 0.5 mL lysis buffer (10 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1% sodium deoxycholate, 1% Triton X-100, and 0.1% sodium dodecyl sulfate [SDS]). Samples were matched for protein and separated by SDS-polyacrylamide gel electrophoresis on a 9% acrylamide gel. Samples were then transferred to nitrocellulose and immunoblotted with anti-SM-α-actin antibody (Boehringer-Mannheim). Results were quantified by densitometry.

Statistical Analysis

Statistical analysis of the data was performed by Student's t test and one-way ANOVA.

Results

Effect of AVP and PDGF on SM-α-Actin Levels in VSMCs

The effects of AVP and PDGF on the steady-state levels of SM-α-actin protein were determined by immunoblotting cell lysates with an antibody specific for SM-α-actin. Ninety-six hours of exposure to AVP in the presence of low concentrations of serum caused a threefold to fourfold increase in the steady-state levels of SM-α-actin, normalized either to cell protein or cell number (n=3). Exposure to PDGF decreased levels to below the value of untreated cells. A representative autoradiograph is pictured in Fig 1. These studies confirm earlier work showing that AVP increases both protein and steady-state mRNA levels of SM-α-actin.
Fig 2. Bar graphs showing regulation of smooth muscle \( \alpha \)-actin (SM-\( \alpha \)-actin) promoter in vascular smooth muscle cells (VSMCs). A, Time course of arginine vasopressin (AVP) stimulation of the SM-\( \alpha \)-actin promoter. VSMCs were transfected with pCATACT(−713/52) as described in "Materials and Methods." Cells were then exposed to AVP (10\(^{-8}\) mol/L) or vehicle for the indicated time. Chloramphenicol acetyltransferase (CAT) activity was then measured in the cell extracts. CAT activity (in picomoles chloramphenicol acetylated per 4-hour assay) was normalized with \( \beta \)-galactosidase activity, and the percentage of control (cells receiving no hormonal stimulation) CAT activity was calculated. Results represent mean±SEM (\( n=5 \); *\( P<.05 \) vs control). B, Effect of growth-promoting agents on SM-\( \alpha \)-actin promoter activity. After transfection with pCATACT(−713/52), VSMCs were exposed to the various agents described for 72 hours. At that time, cell extracts were prepared, and CAT activity was measured and normalized with \( \beta \)-galactosidase activity. PDGF indicates platelet-derived growth factor; All, angiotensin II; cAMP, dibutyryl cAMP; and PMA, phorbol 12-myristate 13-acetate. Results represent mean±SEM (\( n=5 \); *\( P<.05 \), **\( P<.01 \), and ***\( P<.001 \) vs control).

whereas PDGF decreases both protein and mRNA levels of SM-\( \alpha \)-actin.\( ^{12} \)

**Hormone Regulation of SM-\( \alpha \)-Actin Promoter Activity**

To directly examine the regulation of SM-\( \alpha \)-actin transcription, the effect of vasoconstrictors and PDGF on the activity of the SM-\( \alpha \)-actin promoter was examined. A region coding 713 bases of \( 5' \) sequence of the rat SM-\( \alpha \)-actin promoter was isolated by PCR and ligated into a promoterless CAT vector [pCATACT(−713/52)]. This construct was transfected into VSMCs, and CAT activity was measured as a function of time after exposure to AVP. Within 24 hours, AVP stimulated CAT activity (2-fold, \( n=5 \)) (Fig 2A). Continued exposure of the cells to AVP resulted in a 10- to 12-fold increase in CAT activity by 72 hours. Additional stimulation by AVP for up to 96 hours did not further increase CAT activity (data not shown). In all subsequent studies, cells were examined after 72 hours of stimulation with the indicated agents.

VSMCs were exposed to AVP (10\(^{-8}\) mol/L), Ang II (10\(^{-8}\) mol/L), PDGF (20 ng/mL), PMA (10\(^{-8}\) mol/L), or dBcAMP (5\(^{-10}\) mol/L), as indicated, for 72 hours (\( n=5 \)). As shown in Fig 2B, both vasoconstrictors, AVP and Ang II, induced CAT activity within 72 hours. PMA, an activator of protein kinase C, consistently stimulated CAT activity but not to the same extent seen with AVP or Ang II. In contrast, PDGF lowered CAT activity to values below the control level. Treatment of cells with dBcAMP did not significantly alter CAT activity. We next examined the ability of PDGF and dBcAMP to inhibit AVP-stimulated CAT activity. VSMCs were exposed for 72 hours to AVP simultaneously with either PDGF or dBcAMP. Under these conditions, PDGF completely inhibited AVP-stimulated CAT activity. The combination of dBcAMP with AVP slightly decreased but did not block AVP-stimulated CAT activity.

**Identification of Regulatory Elements Required for Regulation**

To define the elements of the SM-\( \alpha \)-actin promoter required for stimulation by AVP and inhibition by PDGF, a series of truncation mutants of the pCATACT(−713/52) were examined. The constructs used were isolated by PCR and are shown in Fig 3A. CAT activity was examined 72 hours after transfection and exposure to either AVP, PDGF, or a combination of both agents (\( n=4 \) to 6). As shown in Fig 3B, removing all of the region upstream from the two E-boxes [pCATACT(−287/52)] did not affect either AVP-stimulated CAT activity or the ability of PDGF to inhibit this stimulation. Similarly, the truncation of both E-boxes [pCATACT(−202/52)] and the distal CarG-3 element [pCATACT(−152/52)] did not affect either AVP stimulation or PDGF repression of AVP-stimulated CAT activity. However, a subsequent truncation that removed the middle CarG-2 element but left the 3′ CarG-1 element [pCATACT(−102/52)] totally abolished AVP-stimulated CAT activity. Truncation of the final CarG-1 elements also abolished AVP-stimulated CAT activity.

**Discussion**

Our data and previous work have shown that vasoconstrictors and tyrosine kinase receptors have opposing effects on protein and steady state mRNA levels for SM-\( \alpha \)-actin in vascular smooth muscle.\( ^{12,11} \) In the present study, we have shown that this effect is mediated at least in part through regulation of the SM-\( \alpha \)-actin promoter. AVP increased promoter activity, with detectable increases observed within 24 hours and persisting for several days. The time course of promoter activation is relatively slow but is consistent with the time course of induction of SM-\( \alpha \)-actin, during which significant increases in protein levels are not seen before 48 hours of AVP treatment (data not shown). Consistent with its effect on lowering SM-\( \alpha \)-actin protein and mRNA levels, PDGF lowered CAT activity below control values. Importantly, PDGF was also capable of completely blocking AVP-induced stimulation of promoter activity. SM-\( \alpha \)-actin has been used as a marker...
for the contractile phenotype of VSMCs both in vivo and in vitro. Therefore, these data suggest that AVP promotes differentiation of these cells to the nonproliferating contractile phenotype characterized by elevated SM-α-actin, whereas PDGF promotes differentiation into the proliferative phenotype that is characterized by low levels of SM-α-actin. Since conversion to the proliferative phenotype has been shown to precede and be required for mitogenesis, the induction of SM-α-actin by AVP may play an important role in mediating cell hypertrophy, as distinct from cell division.

From the analysis of truncation mutants, we have identified the critical region of the SM-α-actin promoter required for AVP stimulation. This region, from −102 to +52, contains a putative CArG element that is likely to regulate promoter activity. Previous studies have shown that stimulation of muscle-specific gene expression in cardiac and skeletal muscle is mediated through members of the MyoD family acting through E-boxes. However, since members of the MyoD family have not been detected in smooth muscle cells, the role of the E-boxes in regulating SM-α-actin expression is not clear. Since loss of PDGF-induced repression of the AVP stimulation is not lost before this truncation, it is reasonable to speculate that this CArG element controls both stimulation and repression of SM-α-actin promoter activity. CArG elements have been shown to be targets for many different growth factors and are generally associated with growth stimulation. However, recent studies have shown that this element is under both positive and negative regulation and may in certain cases be associated with suppression of growth. In particular, the CArG elements found in the promoter of the skeletal muscle α-actin gene are under both positive and negative regulation during muscle cell differentiation.

Several studies have examined regulation of SM-α-actin promoter activity by serum in fibroblasts. Serum stimulated the promoter activity in Rat-2 cells transfected with human SM-α-actin promoter CAT constructs. In contrast, AKR-2B fibroblasts transfected with a 1063-bp region of the mouse promoter failed to show serum stimulation. However, this latter study demonstrated the existence of a repressor CArG element located upstream from the three CArG elements examined in our study (bases −194 to −203). Truncation of this element resulted in serum stimulation of CAT activity, which was also affected by CArG-2 and, to a lesser extent, CArG-1. The consensus sequence for this fourth CArG element is missing in the rat promoter because of a G to A change at position −194. The reason for the discrepancy in these results is not clear. Different fibroblast lines may regulate SM-α-actin expression in different ways. Alternatively, species-dependent regions of the promoter may result in distinct patterns of regulation. Finally, since serum contains polypeptide growth factors and vasoconstrictors, which have opposing effects on SM-α-actin promoter activity, the present study sought to examine regulation of promoter activity by specific agonists.

To our knowledge, only one other study has examined regulation of promoter activity by Ang II in VSMCs. In that study, human SM-α-actin promoter was transfected into rat VSMCs, and relatively modest levels of stimulation of CAT activity (2.5-fold) were found; truncation of the 5′ region blunted stimulation further. It is conceivable that the lower levels of stimulation observed are a result of using human SM-α-actin promoter as opposed to rat promoter, as used in the present study. In that regard, different patterns of basal promoter activity have been observed when truncation mutants of
the chicken SM-α-actin promoter are transected into either chicken or rat VSMCs. The postreceptor signaling pathways mediating the opposing effects of AVP and PDGF have yet to be determined. The time course of promoter stimulation is sufficiently slow, suggesting that synthesis of a transcriptional regulator is required. Recent studies have shown that overexpression of serum response factor (SRF) is able to overcome repression of SM-α-actin promoter activity in ras-transformed fibroblasts. It is conceivable that in VSMCs AVP stimulates and PDGF inhibits SRF production. Further studies examining the regulation of SRF levels will be able to address this issue. Both AVP and tyrosine kinase receptors have been shown to stimulate mitogen-activated protein kinase in VSMCs. The effects of AVP have been shown to require activation of protein kinase C, whereas activation by PDGF is protein kinase independent and is mediated through the activation of ras. AVP does not significantly stimulate ras or ras in these cells (X. Li, L.E. Heasley, and R.A. Nemenoff, unpublished data). Since expression of constitutively active ras suppresses SM-α-actin promoter activity in fibroblasts, we suggest that the ability of PDGF to activate ras underlies its suppression of SM-α-actin in VSMCs. Treatment of cells with PMA mimicked the stimulation of CAT activity seen with AVP. However, since PMA stimulation was consistently less than that achieved with AVP, it is likely that additional signaling pathways distinct from protein kinase C activation mediate the vasoconstrictor-induced activation of SM-α-actin promoter activity.

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References


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