Regulation of the A3 Adenosine Receptor Gene in Vascular Smooth Muscle Cells: Role of a cAMP and GATA Element

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ABSTRACT

In previous studies, we reported that the level of expression of the adenylyl cyclase inhibitory A3 adenosine receptor (AR) impacts vascular tone and that rat vascular smooth muscle cells (VSMCs) coexpress the A3 AR and the adenylyl cyclase stimulatory A2a- and A2b-type ARs. In the current study, we investigated the regulation of expression of the A3 AR gene, focusing on sequences conserved in the mouse and human promoters. Transient transfection of primary cultures of rat VSMCs, using the mouse A3 AR promoter, shows that mutation of a conserved cAMP response element (CRE) significantly up-regulates promoter activity in first passage cells, whereas mutation of a conserved GATA site reduces promoter activity. This suggests that an inhibitory protein binds the CRE, whereas an enhancing factor binds the GATA sequence. Electrophoretic mobility shift assays (EMSAs) indicate that the putative CRE and GATA sites indeed bind cAMP response element modulator 1/c-Jun and the GATA6 protein, respectively. A3 AR promoter activity is significantly up-regulated in the presence of forskolin, the nonselective agonist 5’-(N-ethylcarboxamido)adenosine, or the A2a AR agonist 4-[2-[6-amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid (CGS21680), reaching levels similar to those of the A3 AR promoter bearing a mutated CRE. EMSA indicates that in the presence of forskolin the binding to the CRE is inhibited, suggesting that cAMP elevation disturbs the formation of an inhibitory complex on the CRE. Further, semiquantitative reverse transcription-polymerase chain reaction analysis reveals that endogenous A3 AR mRNA is elevated in response to forskolin. Our findings suggest the presence of a mechanism by which cAMP might control its own level in cells via regulation of genes involved in modulation of adenylyl cyclase activity.

In addition to its biological role in cellular metabolism, it has become increasingly clear that adenosine plays a major role in cardiovascular function (Belardinelli et al., 1989). The various physiological effects of adenosine are mediated through its activation of specific cell surface receptors. Adenosine receptors (ARs), members of the superfamily of G protein-coupled receptors, have been classified into subtypes based on 1) primary structure, 2) second messenger systems to which they are coupled, and 3) differential affinities for a number of AR agonists and antagonists (Olah and Stiles, 1995). Each receptor subtype is distributed in a distinct pattern through the body. Analysis of mRNA from rat, bovine, and human tissue by Northern blotting and in situ hybridization shows AR to be highly expressed in brain, spinal cord, kidney, heart, adipose tissue, and testis (Mahan et al., 1991; Reppert et al., 1991; Weaver and Reppert, 1992; Olah and Stiles, 1995). Abundant expression of A2a AR has been reported in the rat and human striatum, heart, kidney, and lungs (Fink et al., 1992; Linden et al., 1993). In contrast to A2a AR expression, Northern analysis of rat mRNA re-

ABBREVIATIONS: AR, adenosine receptor; VSMC, vascular smooth muscle cell; CRE, cAMP response element; ATP, activating transcription factor; NOS, 5’-(N-ethylcarboxamido)adenosine; HGH, human growth hormone; PCR, polymerase chain reaction; FBS, fetal bovine serum; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; CMV, cytomegalovirus; EMSA, electrophoretic mobility shift assay; TBST, Tris-buffered saline/Tween 20; CREM, mutated A3 promoter partial CRE; CREB, cAMP response element binding protein; CREM, cAMP response element modulator; bp, base pair(s); kb, kilobase(s); CGS21680, 4-[2-[6-amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid.
veals the greatest expression of the A2b AR transcript in the large intestine, cecum, and urinary bladder, with lesser amounts in brain, spinal cord, and lungs (Stehle et al., 1992). The most abundant expression of rat A3 AR mRNA is found in testis, although the profile of distribution for human A3 AR mRNA is lung = liver >> brain = aorta > testis > heart (Salvatore et al., 1997).

Recently, we have demonstrated the presence of A3 AR mRNA in rat vascular smooth muscle cells (VSMCs; Zhao et al., 1997). Analysis of A3 AR knockout mice revealed that this receptor serves as a brake for the A2-type AR with respect to blood pressure regulation and cAMP elevation (Zhao et al., 2000). Hence, changes in A3 AR levels impact cAMP maintenance. It then became important to study the regulation of expression of the A3 AR gene in VSMCs. We cloned the mouse A3 AR gene and characterized it by Northern blotting and transient transfection studies. It was determined that the mouse A3 AR gene contains 1 kb of coding sequence, composed of two exons separated by a single 2-kb intron and 0.3 kb of 5'-noncoding region (Zhao et al., 1999). The mouse A3 AR promoter contains a putative cyclic AMP response element (CRE) at position −1299 to −1303 and a putative GATA site at position −16 to −27, both of which are conserved in the human A3 AR promoter.

It has been established that the factors that bind to the CRE belong to a diverse family of leucine zipper transcriptional activators collectively known as activating transcription factor (ATF) factors (Yamamoto et al., 1990), whereas GATA binding proteins are a family of zinc finger transcription factors that regulate tissue-specific gene expression in a variety of cell types, including differentiated VSMCs (Morrissey et al., 1996; Walsh and Takahasi, 2001). In the current study, we investigated regulatory elements of the mouse A3 AR promoter, focusing on the CRE and GATA regions. We conclude that GATA6 binds to the GATA site of the mouse A3 AR promoter, enhancing promoter activity. Furthermore, an inhibitory protein, belonging to the family of CRE binding proteins, binds the CRE, whereas cAMP elevation both reduces formation of this complex and increases A3 AR promoter activity. Accordingly, the endogenous A3 AR mRNA is elevated when cAMP is increased in VSMCs. Our findings are novel in that they suggest the presence of a mechanism by which cAMP might control its own levels in cells via regulation of genes involved in modulation of cAMP levels.

Materials and Methods

Materials. All oligonucleotide primers were acquired from Invitrogen (Carlsbad, CA), as were the original TA Cloning kit, DH5α cells, TRizol, all tissue culture reagents, and all reverse transcription reagents, except dNTPs, which were acquired from Roche Applied Science (Indianapolis, IN). The FuGENE 6 transfection reagent and Protease Inhibitor Cocktail tablets were also acquired from Roche Applied Science. TagDNA polymerase was from Promega (Fitchburg, WI). Costar brand 75-cm² tissue culture flasks were acquired from Corning (Cornning, NY), whereas six-well tissue culture plates and dishes were acquired from BD Biosciences (Franklin Lakes, NJ). All restriction enzymes, T4 polynucleotide kinase buffer, T4 polynucleotide kinase, and bovine serum albumin were acquired from New England Biolabs (Beverly, MA). Forskolin was acquired from Calbiochem (La Jolla, CA), whereas 5'-(-N-ethylcarboxamido)adenosine (NECA) and CGS21680 were acquired from Sigma-Aldrich (St. Louis, MO), as was Ponceau S dye. Radioactive compounds were acquired from PerkinElmer Life Sciences (Boston, MA), as was the Renaissance chemiluminescence reagent plus, and Kodak X-OMAT AR film. The human growth hormone (hGH) assay kit was acquired from Nichols Institute (San Juan Capistrano, CA). The Bradford reagent was acquired from Bio-Rad (Hercules, CA). Microspin G-50 columns and poly(dI-dC) were acquired from Amersham Biosciences (Piscataway, NJ). The monoclonal smooth muscle α-actin antibody (catalog no. A2547) was from Sigma-Aldrich, and von Willebrand factor antibody has been described previously (Frojmovic et al., 1997). All remaining primary and secondary antibodies were either a generous gift of Dr. Judith Foster (Dept. of Biochemistry, Boston University School of Medicine, Boston, MA) and/or purchased from Santa Cruz Biotechnology (Santa Cruz, CA), as follows: anti-CREB-1 (sc-186x), anti-CREB2 (sc-200x), anti-CREM1 (sc-440x), anti-ATF1 (sc-243x), anti-ATF2 (sc-6233x), anti-c-Jun (sc-45x), anti-GATA1 (sc-266x), and anti-GATA6 (sc-7244x). Sprague-Dawley rats were acquired from Charles River Laboratories, Inc. (Wilmington, MA). All animals were handled in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals. All other reagents were the highest quality available from Bioanalytical (Natick, MA), unless otherwise noted.

Site-Directed Mutagenesis of the Partial CRE and GATA Sites in the Mouse A3 AR Promoter. Three base substitutions of the CRE were generated by a two-step symmetrical PCR procedure using a pair of complementary, template-mismatched central primers containing the desired mutated sequence (Higuchi et al., 1988). Briefly, the first PCR was performed with primers A3U14 (outer, sense) and pA3CRE-AS (5'-AAA GGT CTT CGT GCA TTT GTT A-3'), which includes a mutation of the CRE element. A second reaction was performed with primers 129svA3R13 (outer, antisense) and pA3CRE-S (5'-TAA ACA AAA CAT GGA CCC AGA CCT TG-3'), the products of both reactions were combined for use as template in a third reaction, carried out with the outer primers. The full-length product was digested with BsaAI and BstBI and cloned into the pA3hGH plasmid (also digested with BsaAI and BstBI), generating the plasmid pA3CREmBGH2. A similar procedure was used to generate pA3GATAmBGH, a plasmid identical to the pA3hGH plasmid, except for a specific mutation of two bases of the GATA consensus sequence immediately upstream of the transcriptional start of the A3 AR promoter. In this case, the two primer pairs used in the first round of amplification were A3H3-GATAm-F (5'-GAG TGC TAA AAT TGA TGG AGC TCG AAA GTT-3') with HGH2R (5'-CGA AAC CAG AGG GCA AC-3') and A3H3-GATAm-R (5'-GCT CCA TCT ATT TTA GCA CTC TTC TTT AGA GTC TTT-3') with A3U2 (5'-CGT CTA GGA AAT TGC TGG-3'). pA3GH was used as template in both cases. The primer pair of HGH2R and A3U2 was subsequently used for a second round of amplification with the combined products of the first round as template. The resulting, full-length PCR product was cloned directly into the pCR2.1 vector using the Original TA Cloning kit, and transformed into DH5α cells, as desired by the manufacturer. This plasmid was digested with DraII to release a 1.3-kb fragment containing the GATA mutation of the mouse A3 AR. This fragment was subcloned into the pA3hGH plasmid (also digested with DraIII), generating the plasmid pA3GATAmBGH2. All mutations and cloning steps were verified by sequencing.

Cell Culture for Transient Transfection Experiments. VSMCs, isolated from aortae of 2- to 3-day-old Sprague-Dawley rats, were seeded in 75-cm² flasks at a density of 2 × 10⁴ cells/cm² in 20 ml of DMEM, containing 10% fetal bovine serum (FBS), and incubated in a humidified atmosphere at 37°C, 5% CO₂, as we and others have described previously (Barone et al., 1988). Media were changed twice weekly for 2 weeks to expand the harvested cells. After 2 weeks, cells were trypsinized by the addition of 5 ml of trypsin/EDTA to each flask and either used (first passage) or seeded, at a density of 4.0 × 10⁵ cells/ml, and allowed to grow for an additional 3 to 4 days before use (second passage). Purity of VSMC cultures was determined by immunohistochemistry and morphology. Although 98% of
cultured cells were positive for smooth muscle α-actin, none were positive for von Willebrand factor, an endothelial cell marker (antibodies used are specified under “Materials”).

RBL-2H3 cells (American Type Culture Collection, Manassas, VA) were grown in 75-cm² flasks containing 10 ml of mast cell culturing media (Eagle’s minimum essential medium/15% FBS with 2 mM l-glutamine, 1.5 g/l sodium bicarbonate, 0.1 mM nonessential amino acids, and 1.0 mM sodium pyruvate). Media were changed twice weekly.

Cell Culture and Reverse Transcription for Semiquantitative PCR Analysis. First passage cells were seeded in 75-cm² flasks in 10 ml of VSMC culturing media (DMEM with 10% FBS, 1× sodium pyruvate, 1× nonessential amino acids, and 1× penicillin/streptomycin) at a density of 4.0 × 10⁵ cells/ml. Cells were allowed to settle overnight in a humidified incubator at 37°C, 5% CO₂ before addition of ligands. Eighteen hours after seeding, VSMC culturing media were removed and replenished with 10 ml of fresh VSMC culturing media containing 2 μM forskolin. Twenty-four hours after addition of agonist, cells were processed for RNA isolation. For RNA isolation, media were removed and cells were trypsinized by adding 5 ml of trypsin/EDTA to each flask and incubated at 37°C, 5% CO₂ for 5 min. Cells were collected by centrifugation at 1200 rpm, washed in 1× phosphate-buffered saline (PBS) prepared in diethyl pyrocarbonate-treated doume media. RNA pellets were resuspended in diethyl pyrocarbonate-treated double-distilled H₂O, quantitated by absorbance at 260 nm, and stored at −80°C until use. Reverse transcription of VSMC RNA was performed in a total volume of 30 μl, as directed by the manufacturer. RNA (4.5 μg) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase in a reaction that included 10 units of cloned RNase inhibitor. Reactions were incubated at 42°C for 2 h and subsequently boiled for 2 min to inactivate the enzymes. Reactions were stored at −20°C until use.

Semiquantitative PCR. PCR reactions were performed in a total volume of 50 μl using a Stratagene Rotocycler Gradient 40 PCR machine (Stratagene, La Jolla, CA). Each PCR reaction contained 0.2 pmol each of sense and antisense A3 AR or GAPDH primers (GAPDH: sense 5’-TCA CCA TCT TCG AGG AG-3’ and antisense 5’-GCT TCA CCA CCT TCT TG-3’; A3 AR: sense 5’-TCC ATG TCC TTG CTG-3’ and antisense 5’-GCA CAT GAC AAC CAG GGA-3’), 0.2 mM dNTP plus 0.2 μCi of [α-32P]dCTP, and 2.5 units of TaqDNA polymerase. The program for amplification was 95°C for 1 min, followed by repeating cycles of 95°C for 1 min, annealing temperature for 1 min, and 72°C for 2 min. Annealing temperatures of 58 and 55°C were used for amplification of A3 AR and GAPDH, respectively. Samples (15 μl) were removed at cycle number 29, 32, and 35 for A3 AR amplifications and at cycle number 20, 23, and 26 for GAPDH amplifications, and added to 5 μl of non-denaturing stock dye (30% glycerol, 0.25% bromphenol blue, and 0.25% xylene cyanol). PCR products were analyzed on a 5% non-denaturing acrylamide gel buffered in 1× Tris borate-EDTA. After separation, gels were dried for 1 h, under vacuum, at 80°C and exposed to Kodak X-OMAT AR film at either room temperature or at −80°C, with intensifying screens.

Transfection Experiments and hGH Transient Expression Assay. VSMCs were transfected using the FuGENE 6 transfection reagent, as described by the manufacturer, using circular plasmid DNA (4 μg) purified by CsCl density gradient centrifugation. For transfections, first passage cells were plated in six-well plates at a density of 3.0 × 10⁵ cells/well, in VSMC culturing media, and allowed to settle overnight before transfection (at which point the cells were about 70–80% confluent). In situ analysis of VSMCs transfected with pCMVβ-gal (Escherichia coli β-galactosidase gene driven by the cytomegalovirus promoter; Ravid et al., 1991), revealed that approximately 10 to 15% of cells were transfected by this method (data not shown). After transfection, the cell cultures were incubated for up to 72 h (cells were then confluent), after which the supernatant was removed and used for hGH assay. The level of hGH produced by the cells was determined by using an hGH assay kit, as described by the manufacturer and in Ravid et al. (1991). Simian virus thymidine kinase promiscuous promoter driving hGH expression was used as a positive control in all transfection experiments. Transfection efficiency was assessed by cotransfection with 2 μg of pCMVβ-gal and by measuring the resultant β-galactosidase activity in transfected cell lysates (Herbomel et al., 1984). hGH levels were normalized to β-galactosidase activity in each sample, thus correcting for differences in the efficiency of transfection. When agonists were included in the culture medium they were added at the time of transfection. Treatments included the addition of dimethyl sulfoxide (DMSO) carrier, 2 μM forskolin, 10 μM NECA, or 1 μM CGS21680. pCMVβ-gal was not cotransfected during these experiments, because agonist addition alters the activity of the CMV promoter (our unpublished observation). Instead, total protein derived from cell lysates was used to normalize hGH levels. Each well of VSMCs was trypsinized, collected by centrifugation, washed in 1× PBS, and resuspended in 50 μl of protein lysate buffer (50 mM Tris-Cl, pH 7.4, 250 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor, and 0.5% Nonidet P-40). Protein lysates were incubated on ice for 15 min, cleared of debris by centrifugation at 10,000 rpm for 5 min, and stored at (−20°C until analysis. Then 5 to 20 μl of each sample was assayed for protein using the Bradford reagent and protein concentration determined by comparison with a bovine serum albumin standard curve.

RBL-2H3 cells were transfected by electroporation. Cells were trypsinized, washed in mast cell culturing media lacking FBS, and resuspended at a concentration of 1 × 10⁵ cells/0.8 ml of mast cell culturing media lacking FBS. Then 50 μg/0.8 ml of pA3hGH was added, and the cells were transferred to cuvettes and held on ice for 10 min. Cells were electroporated using a GenePulser II (Bio-Rad) at 250 V/500 μF and held on ice for an additional 10 min. Then, cells were transferred to 10 ml of mast cell culturing media containing carrier or 10 μM forskolin. After a 48-h incubation, hGH levels were determined from the media and corrected for cell number determined for each sample.

Generation of Nuclear Extracts. First passage VSMCs were plated onto round tissue culture dishes (100 × 20 mm) at a density of 2 × 10⁴ cells/cm². Cells were grown under standard culturing conditions (100% humidity, 5% CO₂, 37°C) in 10 ml of VSMC culturing media. Cells were grown for 2 weeks, changing media every 3 days. VSMCs were then treated with 1 μM forskolin, dissolved in DMSO, or with DMSO alone, in 10 ml of VSMC culturing media [DMSO final concentration 0.02% (v/v)] for 24 h, under standard culturing conditions. Cells were then transferred to 4°C where media was immediately exchanged with 10 ml of ice-cold 1× PBS. All subsequent steps were performed at 4°C. The 1× PBS was aspirated and cells were scraped into 5 ml of fresh, ice-cold 1× PBS. Cells were collected by centrifugation for 5 min at 350g, washed twice with 30 ml of ice-cold 1× PBS, and resuspended in 5 ml of Nonidet P-40 lysis buffer (10 mM Tris-Cl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, and 0.5% Nonidet P-40), and incubated on ice for 5 min. Nuclei were collected by centrifugation for 5 min at 500g, resuspended in 5 ml of Nonidet P-40 lysis buffer, and immediately centrifuged for 5 min at 500g. Nuclei were then resuspended in an equal volume of extraction buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, and 25% glycerol), containing a 2× final concentration of Protease Inhibitor Cocktail. Resuspended nuclei were incubated at 4°C, with gentle rotation, for 20 min, after which they were centrifuged at 14,000 rpm in a Microfuge (BD Biosciences) for 30 min. The top, liquid layer was removed and protein concentration determined by the Bradford method. Samples were aliquoted, flash-frozen in liquid nitrogen, and stored at −80°C.

Generation of Double-Stranded Oligonucleotides. Oligonucleotides were annealed at a concentration of 20 pmol/μl in 1× Tris kinase buffer by boiling for 4 min and then allowing the water bath to cool at room temperature for 2 h. One microliter of annealed oligonucleotides was radiolabeled in a volume of 25 μl containing 1×
T4 kinase buffer, 10 units of T4 polynucleotide kinase, and 16.5 pmol of [γ-32P]ATP (specific activity >1500 Ci/mmol). Reactions were incubated at 37°C for 1 h. Double-stranded oligonucleotides were purified using a MicroSpin G-50 column, and their specific activity was determined by scintillation counting. Samples were diluted to a specific activity of 10⁵ cpm/μl. Cold competitors for labeled oligonucleotides were generated by diluting the annealed stock solution in TES (10 mM Tris-Cl, pH 8.0, and 0.1 mM EDTA) to 25x the concentration of the labeled oligonucleotides. The oligonucleotides used in the gel mobility shift assays were as follows: A3 promoter partial CRE (A3CRE), A3-CREBb-F (25 mer), 5'-AAA CAT GGA CCG TCA CCT TGT TTC T-3' and A3-CREBBb-R (25 mer), 5'-AGA AAC AAG GTG ACG GTC CAT GTT T-3'; Mutated A3 promoter partial CRE (CREm), A3-CREBb-Bm-F (25 mer), 5'-AAA CAT GGA CCC AGA AGA GTC CAT GTT T-3' and A3-CREBBb-Bm-R (25 mer), 5'-AGA AAC AAG GTG ACG GTA TCT CAT TTT C-3'; Consensus CRE (CRE), A3-CREBFull-F (28 mer), 5'-AAA CAT GGA CGT ACA TCG TGA CTT TGC TCT T-3' and A3-CREBFull-R (28 mer), 5'-AGA AAC AAG GTG ACG TCA GTC CAT GTT T-3'; A3 promoter GATA site (A3GATA), A3-GATA-F (26 mer), 5'-AGG AGT GCT TAT CCT GAT GTA GCT CG-3' and A3-GATA-R (26 mer), 5'-CGA CCT TCA AGA TAG ACG CTC CT-3'; Mutated A3 promoter GATA site (GATAm), A3-GATAm-F (26 mer), 5'-AGG AGT GCT AAA ATT GAT GGA GCT CG-3' and A3-GATAm-R (26 mer), 5'-CGA CCT TCA ATT TTA GCA CTC CT-3'; Consensus GATA site (GATA), GATA-F (26 mer), 5'-GCC CAA CTA GTA GAT AAG ATG TGA GCT GT-3' and GATA-R (26 mer), 5'-ACT CAC ATC TTA TCT ACT AGT TGC GG-3'; and Consensus TATA site (TATA), TATA-F (26 mer), 5'-ACT CAC GAT ACG TAT AA AGT TGC GG-3' and TATA-R (26 mer), 5'-GCC CAA CTT TTA TAC GTA CCG TCA GT-3'.

Electrophoretic Mobility Shift Assay (EMSA). Ten micrograms of nuclear extract was added to a mixture containing 4 μl of 5× binding buffer (20 mM Tris-Cl, pH 7.9, 60 mM HEPES, pH 7.9, 300 mM KCl, 5 mM EDTA, 5 mM dithiothreitol, and 60% glycerol), 1 μg of double-stranded poly(dI-dC) and double-distilled H₂O. For competition experiments, 2 μl of 25× cold competitor, or 2 μg of specific antibody, was added. The final volume after adding nuclear extract was always 19 μl. Samples were incubated at room temperature for 30 min. After 30 min, 1 μl (10⁶ cpm/μl) of radiolabeled oligonucleotide was added and incubation continued, on ice, for another 30 min. Samples were then loaded onto a 4.5% nondenaturing polyacrylamide gel (39:1 acrylamide/bisacrylamide ratio; 0.5 mM oligonucleotide was added and incubation continued, on ice, for an additional 30 min. After 30 min, 1 μl (10⁶ cpm/μl) of radiolabeled oligonucleotide was added and incubation continued, on ice, for another 30 min. Samples were then loaded onto a 4.5% nondenaturing polyacrylamide gel (39:1 acrylamide/bisacrylamide ratio; 0.5× Tris borate-EDTA buffer) and separated at 4°C, for 3 to 4 h, at 6 V/cm, using a PROTEAN II electrophoresis device (Bio-Rad). Gels were run at 6 V/cm for 30 min at 4°C before loading. After separation, gels were dried for 1 h under vacuum at 80°C and exposed to X-Omat AR film for 40 h at either room temperature, or at -80°C with intensifying screens.

Western Blot Analysis. Forty micrograms of nuclear extract in a volume of 10 μl was combined with 10 μl of 2× sample running buffer [0.125 M Tris-Cl, pH 6.8, 20% glycerol, 4% SDS, 2% betamercaptoethanol, and 0.001% (w/v) bromphenol blue], incubated for 5 min in a boiling water bath, and allowed to cool for 5 min at room temperature. Twenty microliters of each sample was then separated at 25 V/cm by SDS-polyacrylamide gel electrophoresis (10% separating gel, 3.9% stacking gel), at room temperature, and transferred overnight at 4°C to an OPTITRAN nitrocellulose membrane (Schleicher & Schuell; Keene, NH) using a Mini Trans-blot Cell (Bio-Rad), as directed by the manufacturer. To confirm equal loading of protein, the membranes were stained for 15 min in a 0.1% Ponceau S/1% acetic acid solution, rinsed several times with water, and photographed to determine equal loading of protein. Membranes were washed for 1 h with TBST [100 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20 containing 5% (w/v) Carnation nonfat dry milk (Nestle; Glendale, CA)], followed by a 5-min wash in fresh TBST containing 5% milk. Membranes were then incubated for 1 h with a 1:200 dilution of primary antibody against CREM1 or c-Jun in TBST containing 5% milk. After four, 10-min washes with TBST containing 5% milk, membranes were incubated for 1 h with 1:2000 dilution of secondary antibody conjugated to horseradish peroxidase in TBST containing 1% milk. After four, 10-min washes with TBST containing 1% milk, the membranes were rinsed twice with Tris-buffered saline (100 mM Tris-Cl, pH 7.5, and 150 mM NaCl). Horseradish peroxidase-antibody complexes were visualized using the Renaissance chemiluminescence reagent plus, as directed by the manufacturer, and detected using Kodak X-Omat AR film.

Results

Mouse A3 AR Promoter and Effect of cAMP Elevation on A3 AR Promoter Activity. The mouse A3 AR promoter does not contain traditional TATA and CAAT box sequences, but does share several regions of distinct homology with the human A3 AR promoter (GenBank accession numbers AF069778 and S81868, respectively). Of note are the partial CRE located at -1299 to -1303, and a putative GATA binding site located at -16 to -27 relative to the transcriptional start site (Fig. 1A). The corresponding nucleotide positions for the human partial CRE are -401 to -397, and for the GATA element, -47 to -42. To determine whether the partial CRE, located within the mouse A3 AR promoter, is critical for promoter activity and for mediating the effects of cAMP, the A3 AR CRE was modified using site-directed mutagenesis. Three point mutations were introduced into the CRE, which was subsequently cloned into an hGH reporter construct (Fig. 1B). hGH reporter constructs containing a wild-type A3 AR promoter or a promoter containing a mutated CRE were transfected into either first or second passage VSMCs. Experiments demonstrated that second passage cells were significantly less responsive to agonists compared with first passage cells (Fig. 2A). A variety of potential mechanisms may lead to a loss of an effect upon passing cultured cells, the exploration of which was not the focus of our study. This was presented, however, to alert of future studies. Filled circles indicated the location of a mutated base.

![Fig. 1](https://example.com/figure1.png)

Fig. 1. Schematic of the mouse A3 AR promoter. A, scale diagram of 2.3 kb of mouse genomic DNA 5' upstream of the A3 AR coding region. The location of the conserved GATA site, as well as a conserved partial CRE, in the mouse and human genes, is indicated. B, nucleotide sequences surrounding the CRE and GATA sites as well as distances from the transcriptional start. Boxed nucleotides are identical between mouse and human A3 AR promoters. Also shown are mutations of the sites used in these studies. Filled circles indicated the location of a mutated base.
potential variability in results when using VSMC of different passages for the study of A3 AR gene regulation.

The A3 AR promoter containing a mutated CRE was significantly more active than the wild-type A3 AR promoter, as measured by hGH production in first passage cells (Fig. 2A). Wild-type A3 AR promoter activity increased significantly upon nonselective activation of AR (NECA) or upon activation of just A2a AR (CGS21680) or upon direct activation of adenyl cyclase by forskolin. All of these ligands elevated cAMP levels in first passage cells, as we have described previously (Zhao et al., 1997; data not shown). Reporter constructs containing a mutated CRE showed significantly higher basal promoter activity, compared with the wild-type promoter, with no additional increase in the presence of ligands (Fig. 2A). We have been able to detect both the A2a and A2b AR in VSMCs by PCR (data not shown). The fact that these confluent VSMCs responded similarly to NECA, a nonselective AR agonist, and to CGS21680, an A2a AR-selective agonist, suggests that A2a AR activation may be sufficient for A3 AR promoter stimulation. Forskolin also enhanced A3 AR promoter activity, suggesting that the effect is mediated by cAMP.

Activation of Adenylyl Cyclase Increases Levels of Endogenous A3 AR mRNA. To confirm that activation of adenylyl cyclase results in up-regulation of expression from the endogenous A3 AR promoter, RNA, isolated from VSMCs cultured in the absence or presence of forskolin, was subject to reverse transcription-PCR using primers specific for the A3 AR. Because the isolation of large quantities of first passage cells needed for RNA preparation is technically challenging, and because the effect on A3 AR promoter activity is considerable by direct activation of adenyl cyclase, we focused on repetitions of mRNA measurements in forskolin-treated cells. We resorted to determination of mRNA levels because there are no reliable antibodies to the A3 AR, and because studies indicated that the density of A3 AR in VSMCs (and some other cell types) is too low to be reliably determined by binding studies (performed essentially as in Linden et al., 1999; data not shown). The level of A3 AR mRNA expressed in VSMCs was significantly up-regulated in response to forskolin (Fig. 3). A3 AR levels in forskolin-treated cells increased 2.27 ± 0.49-fold over untreated cell (average of four determinations from three different RNA samples). GAPDH mRNA expression was equivalent within the linear range of its amplification and treatment with forskolin did not result in its up-regulation. These results confirm our transient transfection data and support a mechanism by which elevation of cAMP leads to enhanced A3 AR mRNA expression in VSMCs.

CRE binding protein (CREB)/CRE modulator (CREM) Family Proteins Bind the Partial cAMP Response Element of the Mouse A3 AR Promoter and Are Reduced Upon Treatment with Forskolin. To determine what protein factors bind the CRE, we performed EMSA

![Fig. 2. Effect of cAMP elevation on mouse A3 AR promoter activity. An hGH reporter plasmid containing the wild-type mouse A3 AR promoter (pA3hGH) or containing a mouse A3 AR promoter with a mutated CRE (pA3CREmGH) were transfected into either first (A) or second (B) passage VSMCs. Transfected cells were incubated in DMSO carrier (control), 1 μM forskolin, 10 μM NECA, or 1 μM CGS21680 for 72 h, and hGH levels in the media were determined. Average corrected expression (nanograms of hGH per microgram of protein) in first and second passage pA3hGH-transfected cells was 12.52 and 13.49 ng/μg, respectively. Results shown are averages and standard deviations of triplicate samples from two independent experiments, all normalized to hGH levels from control cells.](image)

![Fig. 3. Treatment of VSMCs with forskolin increases levels of A3 AR message. Semiquantitative reverse transcription-PCR was performed on RNA harvested from first passage VSMCs treated with DMSO carrier (−) or with 2 μM forskolin (+) for 24 h. Primers specific for the A3 AR or GAPDH were used for PCR amplification and samples were taken during the linear phase of amplification. Quantitation using the Kodak 1D Image Analysis Software revealed that A3 AR levels in forskolin-treated cells increased 2.27 ± 0.49-fold over untreated cell. Results are the average and standard deviation of four determinations from three different RNA samples.](image)
A CREB/CREM family protein binds the partial CRE of the mouse A3 AR promoter. EMSA was performed with a \(^{32}\)P-labeled, double-stranded oligonucleotide probe containing the partial CRE of the mouse A3 AR promoter (A3CRE) using 10 \(\mu\)g of nuclear extract from cultured, first passage VSMCs. Competition was performed with a 50-fold molar excess of unlabeled A3CRE, a double-stranded oligonucleotide containing a full CRE (CRE), or with 2 \(\mu\)g of the indicated antibody. Specific binding is indicated (horizontal line). Duplicate experiments performed with different preparations of nuclear extract are shown.

Fig. 4. A CREB/CREM family protein binds the partial CRE of the mouse A3 AR promoter. EMSA was performed with a \(^{32}\)P-labeled, double-stranded oligonucleotide probe containing the partial CRE of the mouse A3 AR promoter (A3CRE) using 10 \(\mu\)g of nuclear extract from cultured, first passage VSMCs. Competition was performed with a 50-fold molar excess of unlabeled A3CRE, a double-stranded oligonucleotide containing a full CRE (CRE), or with 2 \(\mu\)g of the indicated antibody. Specific binding is indicated (horizontal line). Duplicate experiments performed with different preparations of nuclear extract are shown.

using nuclear extract from VSMCs. This nuclear extract showed specific binding to the partial CRE located within the A3 AR promoter (A3CRE), which was competed well with a 50-fold excess of cold A3CRE or with a probe containing the full CRE (CRE; Fig. 4, lane 5). Full or partial CRE are often occupied by protein homo- or heterodimers, which are members of the CREB family. To further identify protein(s) that may bind the partial CRE located within the wild-type A3 AR promoter, EMSA was performed using antibodies against CREM/CREB family members (Fig. 4). Antibodies against ATF1, ATF2, CREB1, and CREB2 displayed no effect on nuclear protein binding to A3CRE. Anti-CREM1, an antibody reactive with CREM1 and also weakly reactive with other ATF/CREB proteins and other CREM isoforms, and to a lesser extent anti-c-Jun, both inhibited binding. Because specific antibodies to ATF/CREB did not affect the binding, the data suggest that the protein complex that binds to A3CRE consists of a member of the CREM family of proteins and c-Jun.

EMSA experiments also indicate that treatment of VSMCs with forskolin significantly reduced protein binding to the A3CRE probe (Fig. 5A). These results suggest that when cAMP levels are elevated, the binding of protein from VSMC nuclear extract to the partial CRE within the A3 AR promoter is reduced. This binding was competed by cold A3CRE, but not by the same probe with a mutation of the CRE (CREm). This suggests that the detected binding depends exclusively on the partial CRE located within the A3 AR promoter. Accordingly, no binding was detected when CREM was used as a probe (data not shown). The same nuclear extract was also used in EMSA analysis with the full CRE probe, which typically binds predominantly to CREB1, but also to other CREB/CREM family members. We observed binding to the full CRE probe, albeit with a different migration profile than the one observed for A3CRE. A weak upper band (I) and a prominent lower band (II) were observed by EMSA, of which band I was reduced upon forskolin treatment, whereas band II was slightly increased (Fig. 5B). This experiment also demonstrates that reduced binding to the A3CRE in forskolin-treated cells is not due to a general reduction in levels of all binding proteins in the nuclear extract.

Several mechanisms could account for decreased binding to the A3CRE site in nuclear extract from forskolin-treated cells, including a reduction in levels of the CREM protein. A Western blot of nuclear extract from forskolin-treated cells demonstrated that the level of a 35-kDa protein, recognized by the CREM1 antibody and corresponding to the size of CREM1, is reduced by nearly one-half compared with nuclear extract of control cells (Fig. 6). No other proteins were detected on the Western blot reacted with this antibody. There are no commercially available antibodies specific to phosphorylated CREM proteins. The level of c-Jun, another potential binding protein at the A3 AR CRE, was not significantly altered in the nuclear extract of forskolin-treated cells compared with nuclear extract from control cells (Fig. 6). These results suggest that a CREM family member (most likely CREM1) is involved in transcription factor binding to the CRE of the A3 AR promoter, and that elevations in cellular cAMP may enhance expression from this promoter by reducing levels of this CREM family member.

**cAMP Elevation and A3 AR Promoter Activity in a Rat Mast Cell Line.** We wanted to extend our findings on increased activity of the A3 AR promoter in response to cAMP elevation to other systems. Therefore, we transfected RBL-2H3 cells, a rat mast cell line that is known to express high levels of endogenous A3 AR (Ramkumar et al., 1993), with an hGH reporter construct containing a wild-type A3 AR promoter. These cells were subsequently treated with carrier or 10 \(\mu\)M forskolin, which resulted in a 4- to 5-fold elevation in cAMP (data not shown). Wild-type A3 AR promoter activity increased significantly upon treatment with forskolin, suggesting an activation of the A3 AR promoter as a result of elevated cAMP levels (Fig. 7). These results support the concept that cAMP elevation specifically activates the A3 AR promoter through a general mechanism found in cells expressing A3 AR.

Fig. 5. Specific binding to the partial CRE of the mouse A3 AR promoter is reduced upon treatment with forskolin. EMSA was performed with a \(^{32}\)P-labeled, double-stranded oligonucleotide probe containing the partial CRE of the mouse A3 AR promoter (A3CRE) (A) or a full CRE (CRE) (B). Ten micrograms of nuclear extract from cultured, first passage VSMCs treated for 24 h with DMSO carrier (–FORSKOLIN) or with 10 \(\mu\)M forskolin (+FORSKOLIN) was used for EMSA. Competition was performed with a 50-fold molar excess of the unlabeled A3CRE, CRE, or an unlabeled, double-stranded oligonucleotide containing a mutation of the CRE of the mouse A3 AR promoter (A3CREm). Specific binding is indicated (horizontal lines I and II).
Putative GATA Site Is Essential for Full A3 AR Promoter Activity and Binds GATA6. We investigated the role of the putative GATA site, conserved between mouse and human promoters, by transient transfection of hGH reporter constructs containing either the wild-type mouse A3 AR promoter, or a promoter with a mutation of the conserved GATA site. Mutation of the GATA site reduced the activity of the A3 AR promoter by 50% (Fig. 8). EMSA was performed to determine binding of nuclear extract from VSMCs to oligonucleotide probes corresponding to the putative GATA sequence of the A3 AR (A3GATA) or a consensus GATA sequence (GATA). Proteins contained within VSMC nuclear extract efficiently bound the putative GATA sequence located within the A3 AR promoter (Fig. 9A). This binding was effectively competed by cold oligonucleotides corresponding to A3GATA or a consensus GATA sequence, but not by those corresponding to either a consensus TATA sequence or a mutated GATA sequence. Moreover, binding of nuclear extract from VSMCs to A3GATA was reduced in the presence of antibodies to CREB/CREM family proteins.
GATA6, but not antibodies to GATA1 (Fig. 9B). VSMCs are known to express GATA6 (Walsh and Takahasi, 2001) and our results suggest that this protein enhances A3 AR promoter activity by binding to the conserved GATA site.

Discussion

In a previous study, we described the role played by the A3 AR in blood pressure response to intravenously administered adenosine and in determination of steady-state levels of cAMP in the vasculature, using A3 AR knockout mice (Zhao et al., 2000). We have focused, in this study, on defining the transcriptional regulation of the A3 AR gene by analyzing its promoter in rat VSMCs. Sequence analysis of the 2.3-kb 5'-flanking region of the A3 AR gene reveals a number of potential transcription factor binding sites, including a partial CRE located at −1299 bp, and a putative GATA site located at −16 bp, relative to the transcriptional start site (Zhao et al., 1999). Studies revealed that the in vitro system we use is highly sensitive to the effects of passage, leading us to focus exclusively on first passage cells.

Cell specific regulation of A3 AR promoter activity may be conferred by protein binding either to the core promoter or to upstream regulatory regions. Mutation of the partial CRE resulted in significant enhancement of A3 AR promoter activity. This suggests a major role for factors binding at this site in mediating the suppression of core promoter activity by the upstream regulatory region. Other studies have demonstrated that the molecular mechanisms involved in cAMP-stimulated gene transcription are complex (Roessler et al., 1988). Two classes of cis-acting elements, the CRE and the AP2 element, have been identified in other cAMP-regulated genes. Although sequence analysis revealed that the A3 AR promoter region also contains an AP2 element at −290 bp, mutation of this element increased promoter activity less than 2-fold in VSMCs (data not shown). We, therefore, conclude that the AP2 element does not significantly contribute to the suppression of A3 AR core promoter activity.

The consensus CRE is an 8-bp palindromic sequence (TGACGTCA) with greater conservation in the 5' half of the palindrome than in the 3' half (Borelli et al., 1992). The A3 AR CRE differs from the consensus CRE by 3 bp at the 3' end (Fig. 1B). Others have demonstrated that single-base changes may influence the binding properties and function of this site (Holmberg et al., 1995). We have compared the binding of nuclear extract from VSMCs treated with forskolin to a labeled oligomer, containing the A3 AR partial CRE or a mutated A3 AR partial CRE, to the binding of nuclear extract from untreated cells. Antibody supershift assay coupled to EMSA suggests that proteins, members of the CREB/CREM family of nuclear transcription factors, bind the CRE. However, Western blotting of nuclear extract with an antibody to CREM proteins revealed a single 35-kDa protein, the level of which is reduced upon forskolin treatment. This indicates that the bands identified by EMSA are a result of binding of a CREM isoform in association with other, unidentified proteins, of varying molecular weights. Our EMSA data indicate that c-Jun may be one of the proteins in this complex. Interestingly, protein binding to the CRE in the A3 AR promoter was reduced in forskolin-treated cells. This suggests that increased levels of cAMP alter the pattern of transcription factor binding to the CRE of the A3 AR promoter. Taken together with the transient transfection data, we conclude that the partial CRE of the A3 AR promoter binds proteins that repress transcription. This supports the contention that elevations in cellular cAMP could enhance expression from the A3 AR promoter by reducing the level of a CREM protein that represses transcription through binding to the partial CRE within the A3 AR promoter (Fig. 10). Levels of endogenous A3 AR mRNA, in VSMCs, increased upon elevation of cAMP by forskolin, further supporting this proposed mechanism of transcriptional regulation (Fig. 3). Finally, increased A3 AR promoter activity, in response to elevation in cAMP, was observed in a rat mast cell line, RBL-2H3, which is known to express high levels of the A3 AR. This suggests that the mechanism of cAMP activation of the A3 AR promoter may be generalized to other cells that express A3 AR.

Transcription factors that bind to CRE are varied and diverse. The first CRE-binding factor to be characterized was the CREB. At least two more genes encode cAMP responsive transcription factors in mammals: the CREM and ATF-1 (Sassone-Corsi, 1995; Montminy, 1997). These factors all belong to the basic domain-leucine-zipper class of proteins. These factors form homodimers and heterodimers that bind to the CRE, but only in certain combinations. Some ATF/CREB factors can heterodimerize with Fos and Jun, and this may change the specific affinity of binding to a CRE, but binding to other yet unidentified factors has not been ruled out (Hai and Curran, 1991). Among the different isoforms of CREB and CREM, some proteins act as activators and some as repressors of cAMP-responsive transcription (Molina et al., 1993; Nakajima et al., 1997). It is important to note that cAMP has been demonstrated to mediate repression of the Ecto-5'-nucleotidase promoter. The inhibition depends on the presence of a functional ATF-1 site (Spychala et al., 1999). A second example of inhibition of transcription by cAMP involves the malic enzyme gene in chick embryo hepatocytes. Studies with this gene have suggested a major role for c-Fos and ATF-2 in glucagon-induced inhibition of transcription (Mounier et al., 1997). It seems, then, that in different cell
types, different factors are involved in cAMP-mediated repression of gene expression.

To further characterize the regulation of the A3 AR promoter, the effect of mutating the putative GATA sequence on basal transcription was also analyzed in transient transfection studies. Mutation of the GATA site of the A3 AR promoter resulted in a reduction of promoter activity by 50%. Further analysis revealed that GATA6, but not GATA1 binds to this site. This demonstrates that the GATA site of the A3 AR promoter is essential for proper A3 AR expression, and that this, in turn, may be activated by the binding of GATA6 to the A3 AR promoter. There are six GATA transcription factors that have been found to be essential coordinators of spatial as well as temporal gene expression during embryogenesis, differentiation, and development. Their role in the development of the hematopoietic system has been well characterized (Weiss and Orkin, 1995), whereas the role of GATA transcription factors in the cardiovascular system has only recently begun to be elucidated (for review, see McBride and Nemer, 2001). GATA6 gene expression is found in quiescent arterial smooth muscle cells as well as other tissues in the adult mouse (Morrisey et al., 1996; Molkentin, 2000) and is a key coordinator of VSMC proliferation and differentiation (Walsh and Takahasi, 2001). GATA6 expression is found in quiescent arterial smooth muscle cells as well as other tissues in the adult mouse (Morrisey et al., 1996; Molkentin, 2000) and is a key coordinator of VSMC proliferation and differentiation (Walsh and Takahasi, 2001). GATA6 expression is down-regulated in response to both mitogen-activated proliferation in VSMCs (Suzuki et al., 1996), and in response to VSMC balloon injury in vivo (Mano et al., 1999). Additionally, GATA6 has been found to activate transcription from the smooth muscle-specific myosin heavy chain promoter (Wada et al., 2000), suggesting a potential role of GATA6 in maintaining the differentiated state of VSMCs. Taken together, these data support the notion that GATA6 is an important transcription factor involved in regulating VSMC gene expression and differentiation. Our current study indicates that GATA6 binding to the A3 AR promoter is important for full promoter activity, suggesting a role for this transcription factor in promoting A3 AR-induced effects on the vasculature (Fig. 10). Because GATA6 is down-regulated in response to VSMC balloon injury in vivo, it would be interesting to examine the level of A3 AR mRNA under these conditions.

In summary, our data are novel in that a role for the GATA protein in determining A3 AR levels is suggested. In addition, our study is novel in that it is the first to suggest a mechanism by which cAMP may control its own level in cells, by regulating genes involved in cAMP modulation. Future studies into the consequences of overexpression of the A2-type AR on A3 AR gene expression and activation will further examine this contention.

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References
