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B-Myb Regulates the A_{2B} Adenosine Receptor in Vascular Smooth Muscle Cells

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Abstract

The A_{2B} adenosine receptor (A_{2B}AR) has been described to control various vascular functions, including inhibition of smooth muscle cell proliferation. Here, we sought to understand the regulation of *A_{2B}AR* gene expression in aortic vascular smooth muscle cells (VSMCs), with a focus on the proliferation phase. Assays with A_{2B}AR-β-gal aortic VSMCs, in which the endogenous *A_{2B}AR* gene promoter drives the expression of prokaryotic β-galactosidase (β-gal) instead of the endogenous *A_{2B}AR* gene, show that β-gal expression is upregulated when the cells are induced to exit from cell cycle arrest. Similarly, the level of A_{2B}AR mRNA is upregulated in proliferating primary aortic VSMCs. In search of related mechanisms, it was noted that the *A_{2B}AR* gene promoter contains several putative binding sites for the proliferation-inducing transcription factor, B-Myb. Using a clone of the 5′ region upstream of the mouse *A_{2B}AR* gene linked to a reporter gene, B-Myb site deletion mutants were generated. It was determined that B-Myb upregulates the *A_{2B}AR* gene promoter, and specific promoter binding sites were identified as functional. In accordance, B-Myb also elevates endogenous A_{2B}AR mRNA and receptor activity, and this activity decreases cell proliferation. Our data are novel in that they show that this proliferation-inhibiting A_{2B}AR is itself an inducible receptor regulated by B-Myb.

Keywords

A_{2B} adenosine receptor; B-Myb; vascular smooth muscle cells

Adenosine has been coined a “retaliatory metabolite”, and as such is an autocoid signaling molecule released or generated extracellularly by cells injured or under stress to act in an autoregulatory loop protecting cells and tissues [Hasko and Cronstein, 2004; Linden, 2005; Sitkovsky and Ohta, 2005]. This extracellular adenosine binds to adenosine receptors (AR), and once activated, AR initiate signaling to elicit functions in the regulation of the cardiovascular [Shryock and Belardinelli, 1997], central nervous [Sebastiao and Ribeiro, 1996], and immune systems [Ohta and Sitkovsky, 2001], cell growth and proliferation [Dubey et al., 2002], as well as antimitogenic effects [Dubey et al., 2001], and apoptosis [Jacobson et al., 1999; Peyot et al., 2000]. Adenosine is released from cells exposed to hypoxic or ischemic conditions. For example, an ischemic event in the heart results in a hypoxic environment that inhibits adenosine kinase, resulting in an increase of adenosine [Decking et al., 1997]. Additionally, with less oxygen available for oxidative phosphorylation, the cell generates less ATP and breaks down AMP via 5′-nucleosidase-I into intracellular adenosine [Sala-Newby et al., 1999]. The secretion of adenosine via

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nucleotide transporters allows intracellularly-generated adenosine to act as a stress signal to the cells in the immediate environment [Pastor-Anglada et al., 2001]. The “danger model” of immunity states that a tissue must signal that it is distressed for a full immunogenic response [Matzinger, 2002]. This idea was expanded, identifying adenosine as a “secondary danger signal” that attenuates the immune response such that it does not damage tissues with an overly robust activity [Sitkovsky and Ohta, 2005]. This has been seen in regards to the role of the A_{2B}AR in inhibiting tumor necrosis factor-α (TNF-α) release from macrophages [Kreckler et al., 2006], and augmentation of interleukin-10 (IL-10) production in macrophage [Nemeth et al., 2005].

ARs were initially classified based on the ability to inhibit (A_1AR and A_3AR) or activate (A_{2A}AR and A_{2B}AR) adenylyl cyclase activity, and A_{2B}AR has the lower affinity of the A_2ARs [Jacobson and Gao, 2006]. The *A_{2B}AR* gene was first cloned in 1992 [Pierce et al., 1992]. Our recent studies with an A_{2B}AR knock-out/β-galactosidase knock-in mouse (A_{2B}AR-β-gal) show that the distribution of the A_{2B}AR expression is selective; within the vasculature, the A_{2B}AR is expressed mainly in vascular smooth muscle cells (VSMCs), but only in a subset of vessels [Yang et al., 2006]. Additionally, the A_{2B}AR-β-gal mouse shows low-grade inflammation compared with wild type, as well as altered expression of cytokines in aortic VSMC [Yang et al., 2006].

Considering the important roles of the A_{2B}AR in the vascular system, the current study sought to understand the regulation of the *A_{2B}AR* gene in VSMC. Our preliminary search indicated that the mouse, rat, and human *A_{2B}AR* gene sequences contain several putative binding sites for the proliferation-induced transcription factor, B-Myb. In addition, a previous study involving gene display analysis of avian cells infected with a Myb protein found A_{2B}AR mRNA upregulation compared to control [Kattmann and Klempnauer, 2002]. Therefore, in this study we examined the contention that the mammalian *A_{2B}AR* gene is regulated by B-Myb and sought to identify Myb functional sites within the *A_{2B}AR* gene. Our investigation shows that the A_{2B}AR is an inducible receptor, the level and activity of which are upregulated by B-Myb. This is intriguing, since previous studies have shown A_{2B}AR to inhibit the proliferation of human aortic VSMC [Dubey et al., 1998]. Here, we show that B-Myb-induced proliferation and consequent upregulation of A_{2B}AR acts as a feedback regulatory mechanism to halt continued proliferation.

MATERIALS AND METHODS

Cloning of the A_{2B}AR Gene Promoter

Bacterial artificial chromosome (BAC) DNA clone # 27543 [GenBank accession number AL596110, identified using Incyte Genomics (Wilmington, DE)] contains the entire mouse genomic sequence surrounding the *A_{2B}AR* gene (Genbank accession number NM_007413). 5.81 kb upstream of the translation start site was used for promoter studies via cloning into pPF4GH plasmid [Ravid et al., 1991], by removal of PF4 promoter and inserting 5.81 kb of the A_{2B}AR promoter such that it drives the expression of the reporter gene human growth hormone (hGH) (named −5.81-hGH). To generate the −4.00-hGH promoter construct, the −5.81-hGH construct was digested with restriction enzymes *Sca*I and *Psh*AI, purified and religated resulting in truncation of 1.78 kb from the 5′ end of the −5.81-hGH construct. All plasmid constructions were confirmed by restriction digest and sequencing. Our cloned promoter has putative binding sites similar to those in the human *A_{2B}AR* gene promoter [Kong et al., 2006], including around the ATG and the transcriptional start, as well as B-Myb sites at further upstream, as described in the current paper (searches were carried out using the transcriptional factor data base outlined in: http://www.cbrc.jp/research/db/TFSEARCH.html).

Vascular Smooth Muscle Cell Dispersion and Cell Culture Conditions

Aortic VSMCs were isolated from neonatal and adult rat and from A_{2B}AR-β-gal mice [Yang et al., 2006] as previously described [Zhao et al., 1997; Yang et al., 2006]. Rat and mouse aortic smooth muscle cells were seeded at a density of 7.5×10^4 cell/ml in 2 ml medium per well of a six-well plate. VSMC were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% bovine calf serum (BCS), 1 mM sodium pyruvate (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), and 1% penicillin/streptomycin (Invitrogen). Cell counting was carried out in triplicates using the trypan blue exclusion method, using a hemocytometer. Once the phenomena of A_{2B}AR upregulation by a proliferative state (10% BCS) was confirmed in both neonatal and adult rat VSMC, studies were continued with neonatal VSMC.

Transient Transfection of Plasmid DNA and Viral Transduction

Plasmids were transfected into VSMC with FuGENE6® reagent (Roche, Indianapolis, IN), using 2 μg of construct plasmid DNA and 0.5 μg of pCMV-β-gal (used to ensure equal transfection efficiency between samples) in six-well plate samples according to manufacturer's instructions. The adenoviruses used for transduction of cells (Ad-GFP and Ad-B-Myb) were a generous gift from Dr. Scott A. Ness, and used at similar titer range as in [Rushton et al., 2003] after testing it in our system as well. In promoter construct transfection assays, adenoviral particles were added first and 3–5 h later the medium was replaced with a fresh one followed by DNA transfection as above.

hGH Quantification

Three to 4 days post-transfection, 200 μl media were collected and quantification of hGH secreted into media was determined using the hGH ELISA kit® (Roche) according to manufacturer's instructions. Absorbance was read with μQuant microplate reader (Bio-Tek Instruments, Winooski, VT).

β-Galactosidase Assay

β-Gal assay was performed 3–4 days post-transfection based on the method described by Craven et al. [1965].

X-gal Stain

X-gal stain for cells in culture is based on the method of Sanes et al. [1986] and used in a paper concerning the generation of A_{2B}AR-β-gal mice [Yang et al., 2006].

Western Blot Analysis

Cultured primary cells were transduced with either Ad-GFP or Ad-B-Myb while in 2% BCS supplemented medium. Two days post-transduction, whole cell extracts were prepared by lysing cells with a solution of phosphate buffered saline (PBS) containing 50 mM Tris–HCl pH 7.4, 150 mM, 1% Triton X-100 (American Bioanalytical, Natick, MA), 1% sodium deoxycholate (Sigma, St. Louis, MO), 0.1% sodium dodecyl sulfate (SDS) (American Bioanalytical) containing 1× complete® protease inhibitor (Roche). Lysates were vortexed for 5 min at 4°C. The supernatant was collected after the sample was centrifuged at 10,000 rpm for 10 min at 4°C. Equal amounts of lysate protein (25 μg) were loaded into each well of 10% SDS–polyacrylamide gels (SDS–PAGE). Proteins were transferred to a PVSF membrane (Bio-Rad Laboratories, Hercules CA), and the membrane was blocked with 5% non-fat milk in 1× PBS for 4 h at 4°C. Immunoblots were probed with a 1:1,000 dilution of rabbit polyclonal anti-B-Myb antibody followed by goat anti-rabbit antibody in blotting buffer (1:2,000 dilution) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immune complexes were visualized by incubation with ECL Western Blotting Detection Reagents

(GE Healthcare Life Sciences, Pittsburg, PA) and autoradiography. Membranes were blotted with 1:2,000 dilution of β-actin-specific antibody (Santa Cruz Biotechnology, Inc.) to confirm equal loading.

Transcription Factor Binding Sites

The mouse, rat, and human *$A_{2B}AR$* gene promoter sequences were searched for transcription factor binding sites using nucleic acid subsequences search on MacVector 9.0 (Accelrys, San Diego, CA). For design of electromobility shift assay (EMSA) oligonucleotides, the Web-based TFSEARCH program was used to identify potential transcription factor binding sequences, http://www.cbrc.jp/research/db/TFSEARCH.html. Searches were done using the vertebrate matrix with a homology threshold of 90% or more [Heinemeyer et al., 1998].

Measurement of A_{2B}AR Expression and Activity

Total RNA from aortic VSMCs was prepared with Trizol® (Invitrogen) according to the manufacturer's instructions as described previously [Chomczynski and Sacchi, 1987]. Reverse transcription polymerase chain reaction (RT-PCR) consisting of: 1 μg RNA, M-MLV reverse transcriptase, 1× first strand buffer, 0.5 mM dNTP, 5 mM DTT, 0.5 U/μl RNase inhibitor, 5 μM oligo dT primers, (Invitrogen), was used to measure A_{2B}AR mRNA expression. To ensure that the procedure yields consistent amounts of cDNA in different samples, glyceraldehyde phosphate dehydrogenase (GAPDH) was also amplified. The primer sets for A_{2B}AR were designed to produce a 746 bp fragment (A_{2B}AR primer sense: 5′ ATG CAG CTA GAG ACG CAA GA 3′, antisense: 5′ GGA GCC AAC ACA CAG AGC AA 3′), GAPDH primer set produced a band at 554 bp (sense 5′-TCA CCA TCT TCC AGG AG-3′ and antisense 5′-GCT TCA CCA CCT TCT TG-3′). To determine A_{2B}AR protein activity, cells were pretreated with 10 μM 8-[4-[((4-Cyanophenyl)carbamoyl-methyl)oxy]phenyl]-1,3-di(*n*-propyl)xanthine (MRS 1754) [Ji et al., 2001] (Sigma) for 10 min at 37°C in a 5% CO_2 incubator and subsequently treated with 10 μM 5′-(*N*-ethylcarboxamido)-adenosine (NECA), (Sigma) for another 10 min at the same conditions. The amount of cAMP produced was determined by using the Direct cAMP Correlate-EIA kit (Assay Designs, Inc., Ann Arbor, MI) according to manufacturer's instructions.

Polymerase Chain Reaction (PCR) Mutagenesis

Generation of mutations in the *$A_{2B}AR$* gene promoter was accomplished by three-step PCR mutagenesis, using the following primers: 1F 5′-TGG AGG CAA GGT GAC TCT AAA-3′; 1R 5′-GTG CTG CGT AGG GTG AGG GGC AGA CCA GCT CTC CTA CCA CCT ACG AGG GGT-3′; 2F 5′-ACC CCT CGT AGG TGG TAG GAG AGC TGG TCT GCC CCT CAC CCT ACG CAG CAC-3′; 2R 5′-TTG ACA AAT GGA TCC TGA AAT G-3′. Primers 1R and 2F are reverse complements and the underlined base pairs illustrate mutations corresponding to EcoRV or MluI restriction enzyme sites, where putative B-Myb binding sites occur in the wild type. First round PCR used −5.81-hGH as a template along with primers 1F and 1R to generate the fragment 1. Second round of PCR used −5.81-hGH as a template along with primers 2F and 2R to generate fragment 2. Fragments 1 and 2 were purified using GENECLEAN® Turbo spin columns (Q-Biogene, Irvine, CA) according to manufacturer's instructions. In the third round of PCR, purified fragments 1 and 2 were subjected to PCR reaction for four cycles without any primers, thus extending the fragments generating fragment 3. On the 5th cycle, primers 1F and 2R were added to amplify fragment 3. Within fragment 3 are BamHI sites that were utilized to clone fragment 3 into −5.81-hGH and −4.00-hGH, generating mut-5.81-hGH and mut-4.00-hGH, respectively. DNA sequencing was routinely explored to confirm procedures.

Nuclear Protein Extract Preparation

Primary cultures of neonatal rat VSMC were transduced with Ad-B-Myb, grown to subconfluent state and then trypsinized, pelleted, and washed twice with ice cold PBS. Cells were resuspended in a solution of 10 mM Tris–HCl pH 7.6, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40 (American Bioanalytical) and lysed on ice for 5 min, after which cells were centrifuged at 500*g* for 5 min and washed with 1 ml of the lysis buffer. Nuclei were then centrifuged and snap frozen in liquid nitrogen in freezing buffer consisting of 50 mM Tris–HCl pH 8.3, 5 mM $MgCl_2$, 0.1 mM EDTA, 40% glycerol, at approximately 10^7 nuclei/100 μl. An equal volume of a solution of 20 mM Hepes pH 7.9 (American Bioanalytical), 0.4 M NaCl, 15 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 μg/ml DTT supplemented with 1× complete® protease inhibitor (Roche) was added to the frozen nuclei and the extraction stirred gently with a cut pipette tip and incubated on ice for 20 min. The mixture was then centrifuged at 10,000*g* for 30 min at 4°C and supernatant recovered. The protein concentration was determined using Bio-Rad protein assay kit (Bio-Rad Laboratories). Extract aliquots were stored at −80°C

Electromobility Shift Assay (EMSA)

Ten microgram of each oligomer indicated in the figure legends were annealed by heating in a solution of 10 mM Tris pH 8.0 and 10 mM $MgCl_2$ to 95° for 5 min, and slowly cooling to 4°C. Annealed oligomers were labeled with (γ-^{32}P)-ATP using T4 polynucleotide kinase (New England Biolabs, Beverly, MA) for 2 h at 37°C; the enzyme was heat-inactivated at 65°C for 10 min. After chilling samples on ice, 75 μl of STE (10 mM Tris–HCl, pH 7.8, 10 mM NaCl, 1 mM EDTA) were added to the sample and then run through a Sephadex G-50 column (Amersham Biosciences, UK) twice to remove unbound (γ-^{32}P)-ATP. Samples were prepared by combining 10 μg of nuclear extracts, 5 μg poly(dIdC) (Amersham Biosciences), binding buffer (10 mM HEPES, pH7.9, 1 mM DTT, 0.1% Triton-X, 0.5% glycerol) and 5 μl of labeled oligomer diluted 1:10 and incubating for 0.5–1 h at room temperature. Protein-DNA complexes were resolved on a 4% native PAGE gel and run in TBE buffer at 150 V for 1.5–2 h. The gel was fixed in a solution of 10% methanol, 10% acetic acid, dried on a gel dryer and analyzed by autoradiography. For competition assays, cold oligomer was added to the mixture and incubated for 30 min prior to addition of radiolabeled oligomer. For competition with B-Myb specific antibody, nuclear extracts and 4 μg antibody were pre-incubated for 0.5–1 h. Wild type oligonucleotides, 5′-CTGTGTATTTGCTCTCC-CAAGTGCTGCAACTGGTGAGGGGCAGACC-AGCTCTCCTACCACAGTTGAGGGGTGAGG-CCAGCTCAGCACACTG-3′, and mutant oligonucleotides 5′-CTGTGTATTTGCTCTCCCAA-GTGCTGGATATCGTGAGGGGCAGACCAGC-TCTCCTACCAACGCGTAGGGGTGAGGCCA-GCTCAGCACACTG-3′ used as oligomers in EMSA were purchased from Invitrogen and designed using sequence surrounding B-Myb sites in −5.81-hGH and the mut-5.81-hGH.

Statistical Analysis

Statistical significance was determined by a two-tailed Student's *t*-test for unpaired variables assuming equal variance. Data were considered significant at $P \leq 0.05$.

RESULTS

A_{2B}AR Expression Is Suppressed in Vascular Smooth Muscle Cells (VSMCs) Arrested at G_0 of the Cell Cycle

The generation of an A_{2B}AR knock-out/β-galactosidase knock-in mouse (A_{2B}AR-β-gal) by our group has created a tool to determine which tissues and individual cells express the

$A_{2B}AR$ gene in vivo [Yang et al., 2006]. Noting the patchy expression of A_{2B}AR in VSMCs within the vasculature [Yang et al., 2006], factors that regulate *$A_{2B}AR$* gene expression in these cells were sought. VSMCs in fully differentiated aorta are typically cell cycle arrested or proliferate at a very low rate, though cells retain plasticity and can rapidly switch and proliferate under circumstances such as injury. Along with this regressive switch from fully differentiated to a proliferative state come changes in gene expression and upregulation of transcription factors not active and/or present in fully differentiated cells, including those that regulate the G1 phase of the cell cycle [Kumar and Owens, 2003]. To first look at how the transition from cell cycle arrest to proliferation effects *$A_{2B}AR$* gene expression in aortic VSMCs, these cells were isolated from A_{2B}AR-β-gal mice. These primary VSMCs were cultured in media containing 0.1% serum, which has been shown to arrest VSMC at G_0 phase of the cell cycle, thus mimicking the in vivo, non-proliferative state [Peiro et al., 1995] (also confirmed in our system by flow cytometry analysis; data not shown). As shown in Figure 1A, proliferating A_{2B}AR-β-gal VSMCs have an increase in expression of the *$A_{2B}AR$* gene, as reflected by an augmented fraction of cells that express β-gal. RT-PCR of control aortic VSMCs from rat mirrors this effect, showing increased A_{2B}AR mRNA in proliferating cells compared to G_0 arrested cells (Fig. 1B).

Overexpression of B-Myb in Vascular Smooth Muscle Cells

The data above indicate that exit from cell cycle arrest has a positive effect on *$A_{2B}AR$* gene expression in VSMCs. B-Myb is a transcription factor that is expressed in many cells at the G1 phase of the cell cycle and is an inducer of proliferation [Bessa et al., 2001]. Western blot analysis illustrates that B-Myb protein levels in VSMCs are also lower in cell cycle-arrested cells (Fig. 2A). To investigate more directly the effects of B-Myb on *$A_{2B}AR$* gene expression, we sought to identify potential functional binding sites for B-Myb in the *$A_{2B}AR$* gene promoter, as well as to examine effects of upregulated B-Myb on this gene promoter. To this end, adenovirus vectors carrying the CMV promoter to over-express both B-Myb and green fluorescent protein (GFP) (Ad-Myb) or GFP alone (Ad-GFP) were used. The adenovirus transduces approximately 90% of the cells (Fig. 2B), and the level of B-Myb protein produced was augmented in the transduced cells (Fig. 2C). As anticipated, cell counts showed that B-Myb overexpression significantly increases the proliferation of VSMCs (Fig. 2D).

B-Myb Upregulates A_{2B}AR Promoter Activity

The genomic sequence containing the mouse *$A_{2B}AR$* gene (Genbank accession number NM_007413) on BAC DNA clone #27543 contains 5.81 kb upstream of the ATG start site. This 5.81 kb 5′ non-coding region was first tested for promoter activity by engineering it such that it drives the expression of the hGH gene for use as a reporter (referred to as −5.81-hGH). As shown in Figure 3A, this A_{2B}AR promoter region has activity in VSMC in the range seen for a known, non-tissue specific, strong promoter (SVTK), and this A_{2B}AR promoter activity is greatly reduced in cell cycle-arrested cells compared to proliferating cells. In a sequence search of the promoter region we found putative B-Myb sites with a perfect match to the classical B-Myb sequence CAACTG, including a site at −4510 (on − strand from translation start), at −3276 (+ strand) and at −3243 (− strand) (Fig. 3B). The latter two adjacent sites in the A_{2B}AR promoter were mutated in the context of the full promoter (mut-5.81-hGH) (Fig. 3C), or in a deletion plasmid that contains only 4 kb (hence, lacking B-Myb putative site −4,510) of the promoter driving hGH (referred to as −4.00-hGH and mut-4.00-hGH). VSMCs were transduced with Ad-GFP or Ad-B-Myb and then transfected with −5.81-hGH, mut-5.81-hGH, -4.00-hGH or mut-4.00-hGH. Figure 3D shows a significant decrease in -4.00-hGH activity compared to -5.81-hGH, and a difference between basal expression levels of the -5.81-hGH and mut-5.81-hGH constructs, such that the latter and -4.00-hGH display comparable activity. Mutation of the two adjacent B-Myb

sites in the context of the -4.00-hGH construct (mut-4.00-hGH) resulted in significant reduction of promoter activity, compared with control non-mutated -4.00-hGH construct. Most importantly, Figure 3D also illustrates an upregulation of -5.81-hGH and -4.00-hGH by B-Myb, and B-Myb lost its ability to induce A_{2B}AR promoter activity in the mut-5.81-hGH or mut-4.00-hGH constructs, suggesting that the B-Myb adjacent sites mediate a B-Myb-induced upregulation of the A_{2B}AR promoter.

B-Myb Binds at Sites on the A_{2B}AR Promoter

To test if B-Myb interacts with the putative B-Myb binding sites −3,276 and −3,243, EMSA was used with the wild type oligomer (oligo). EMSA data illustrate that there is binding to the labeled wild type oligo, and that this binding is reduced, but not eliminated, when the oligo is mutated in the above Myb sites (Fig. 4A), suggesting that Myb sites are important for protein binding. Residual binding to the mutated oligo indicates additional complexes on sites other than Myb. The band observed with the labeled wild type oligo is large, further suggesting multiple complexes that might also bind to neighboring sequences, other than B-Myb. In accordance, cold Myb-mutated oligo competed only partially with the wild type oligo. Binding to the wild type oligo is effectively competed for by cold wild type oligo (Fig. 4B), to demonstrate specificity of binding. Antibody-induced supershifted or diminished complexes are indicative of specific protein binding. In our experiment, as the nuclear extracts and antibody were incubated together prior to the addition of labeled oligo, the antibody could possibly block and/or inhibit the binding of protein to the oligo, thus resulting in a diminished band intensity, as is seen in Figure 4A. Anti-B-Myb antibody, but not anti-crem1 antibody, used as a control, reduced this binding. These results suggest that the above B-Myb sites are crucial for protein binding, in addition to being functional (i.e., responsive to B-Myb ectopic expression), as demonstrated by the mutation assays described in Figure 3D.

Overexpression of B-Myb Upregulates A_{2B}AR mRNA and Protein Activity

B-Myb activation of the *A_{2B}AR* gene promoter transfected into VSMC might not be indicative of upregulation of endogenous A_{2B}AR mRNA. To examine this, RNA was extracted from control and B-Myb overexpressing cells, and subjected to reverse transcription and PCR (RT-PCR) to measure RNA levels. As shown in Figure 5A, A_{2B}AR mRNA is upregulated by B-Myb. To determine if this increase of endogenous A_{2B}AR mRNA upon B-Myb induction leads to increased receptor activity, cAMP was quantified, as it reflects adenylyl cyclase activity (Fig. 5B). Cells were treated with Ad-GFP or Ad-B-Myb and upon confirmation of overexpression (as above), cells were treated with the A_2AR agonist NECA or pretreated with MRS 1754 [Ji et al., 2001], a selective A_{2B}AR antagonist, prior to NECA treatment. As seen in Figure 5B, cells overexpressing B-Myb show a large increase in cAMP when treated with NECA. Murine VSMCs also express the A_{2A} and A_3 adenosine receptors [Yaar et al., 2002]. This cAMP assay indicates that the most abundant signaling from NECA occurs via the A_{2A}AR and/or A_{2B}AR; had A_1AR or A_3AR been abundant and elicited the predominant signal, there would not be a drastic increase in cAMP. To distinguish if the increase in cAMP due to NECA was through the A_{2A}AR or A_{2B}AR, we used MRS 1754, which does not bind A_{2A}AR [Ji et al., 2001]. We conclude that B-Myb-induced augmentation in cAMP is due primarily to the activity of the A_{2B}AR, as shown by the inhibition of this increase by MRS 1754, that is, the inhibition by the A_{2B}AR antagonist is greater in the B-Myb expressing cells compared to control cells treated with NECA and MRS 1754.

Activation of A_{2B}AR Restrains Cellular Proliferation

To understand the role of upregulation and activation of A_{2B}AR in proliferating cells we cultured VSMCs to about 50% confluency and transduced them with Ad-B-Myb, followed

by treatment with NECA or with MRS 1754 and NECA. As shown in Figure 6, when the A_2AR are activated by NECA there is a significant decrease in cell number. This decrease is restored when cells are co-treated with MRS 1754, indicating that effects seen can be attributed to the A_{2B}AR. As shown above, cells not transduced with Ad-B-Myb and at low serum display halted proliferation and low level of the A_{2B}AR (Figs. 2D and 5A). Under these conditions NECA has no significant inhibiting effect on the cell number (data not shown).

DISCUSSION

In the cardiovascular system, A_{2B}AR has been pharmacologically linked to coronary flow rates [Rose'Meyer et al., 2003], angiogenesis [Feoktistov et al., 2003], and vasodilation [Hinschen et al., 2003]. In advanced cardiovascular disease, apoptosis of macrophages and VSMCs has been linked to plaque destabilization [Kavurma et al., 2005]. Adenosine has been implicated in both developmental and disease initiated apoptosis [Jacobson et al., 1999], and in this regard A_{2B}AR has been shown to induce apoptosis in human arterial smooth muscle cells [Peyot et al., 2000]. As to the role of A_{2B}AR in proliferation, it has been shown to have dichotomous effects. Pharmacological studies in porcine and rat arterial endothelial cells showed A_{2B}AR to have a proliferative effect [Dubey et al., 2002], while an antimitogenic effect was observed in human and rat aortic VSMCs under mitogenic conditions [Dubey et al., 1998, 2001].

VSMCs invade the intima in atherosclerosis owing to cellular migration and proliferation. This process is regulated via signaling by growth factors, immune cells, proteolytic components, and extracellular matrix components causing a phenotypic switch from the normal contractile to a synthetic phenotype, allowing for the invasion and proliferation of VSMCs [Thyberg et al., 1995]. The plasticity of VSMCs was originally attributed to their ability to revert to a reparative and/or proliferative phenotype, however, recent research more precisely states that VSMCs are heterogeneous populations that include multipotent populations capable of reparative functions [Stenmark and Frid, 1998]. In line with the heterogeneity of VSMCs is the expression of A_{2B}AR in these cells [Yang et al., 2006]; could the presence of A_{2B}AR correlate with a distinct function of the cell expressing it? As A_{2B}AR has been shown to inhibit proliferation of human VSMCs [Dubey et al., 1998], could its activation during exposure to mitogenic stimuli inhibit VSMCs proliferation? The first step in addressing these inquiries requires better understanding of the regulation of the *A_{2B}AR* gene in VSMCs under proliferative versus synthetic conditions. Our data show that the murine *A_{2B}AR* gene is up-regulated and receptor activity level increased in the proliferative state and that A_{2B}AR protein activity inhibits cellular proliferation. This is intriguing, since as mentioned above, previous studies have shown A_{2B}AR to inhibit the proliferation of human and rat VSMCs [Dubey et al., 1998, 2001]. The upregulation of A_{2B}AR, therefore, might serve as an autocrine feedback regulatory mechanism to decrease proliferation.

B-Myb is a transcription factor that is expressed in a wide range of cells and is induced at the G1 phase of the cell cycle transition. It is activated in S-phase by cdk2 where it can then bind co-activators or bind directly to DNA [Bessa et al., 2001]. Moreover, Myb protein is upregulated in cells exposed to mitogenic stimuli [Rosenberg, 1993; Bloch et al., 1995]. Our study shows that when B-Myb is over-expressed in VSMCs, A_{2B}AR expression and activity are increased. In Figure 1A the assay reflects endogenous *A_{2B}AR* gene promoter activity, while in Figure 5, we determined the level of A_{2B}AR mRNA to be increased. Of note, the murine *A_{2B}AR* gene promoter cloned and examined here shows a response to B-Myb that follows the same trend as the endogenous *A_{2B}AR* gene promoter (compare Figs. 1A and 3A). To explore the significance of putative B-Myb binding sites within the murine *A_{2B}AR* gene

promoter, the effects of mutating two putative sites in close proximity to each other was explored. As illustrated in Figure 3C,D, compared to the full-length promoter, mutation of these B-Myb binding sites caused a significant decrease in basal levels of expression. Mutations of these B-Myb sites in the context of the mut-4.00-hGH or mut-5.81-hGH promoters eliminated the upregulatory effects of B-Myb, indicating that these putative B-Myb binding sites are required for optimum expression of A_{2B}AR in a proliferative state. Moreover, with mutation of these Myb sites, overexpression of B-Myb proved ineffective in upregulating the A_{2B}AR promoter. Mutations in these B-Myb sites did not completely ablate *A_{2B}AR* gene promoter activity, suggesting that B-Myb possibly enhances expression via interaction with weaker transcription factors present. Hence, our study adds the A_{2B}AR to a family of inducible receptors. Among the ARs, however, A_{2B}AR is not the only receptor reported to be induced at the transcriptional level. For instance, the A_3AR is regulated by cAMP levels [Yaar et al., 2002].

Relevant to our findings in mammalian cells is a report by Worpenberg et al. [1991] who used an inducible system of overexpression of v-Myb, a retroviral version of a mutated and truncated form of the c-Myb proto-oncogene, in a chicken macrophage cell line. These authors then performed a differential display procedure to isolate clones of a particular mRNA using PCR [Worpenberg et al., 1997]. Using this method, the authors found chicken *A_{2B}AR* gene mRNAs to be induced by v-Myb. Kattmann and Klempnauer [2002] later showed that a chicken *A_{2B}AR* gene promoter construct is upregulated in vitro by binding of v-Myb proteins to Myb binding domains. The base consensus sequence used in the two papers regarding v-Myb is PyAAC^{G}/T^{G}, a sequence that is the core of all Myb proteins. In addition to the mouse, regions 5′ to the translational start site in both rat (GenBank NM_017161) and human (GenBank NM_000676) *A_{2B}AR* gene promoters contain putative B-Myb binding sites, at similar locations, suggesting that the ability of B-Myb to induce expression of A_{2B}AR during cellular proliferation is conserved across these species (data not shown). For instance, in the mouse, putative B-Myb sites of CAACTG are located at −4,510, −3,276, and −3,243 bp from the ATG start site. To compare rat and human A_{2B}AR promoter, we scanned −6,000 bp from the ATG start site. Rat A_{2B}AR promoter has two putative B-Myb sites of CAACTG at −4,947 and −3,663 bp; these sites are similar in distance from the translational start site as in the mouse, however their location to each other is further apart. Human A_{2B}AR promoter contains four putative B-Myb sites of CAACTG at −3,883, −3,708, −1,868, and −1,769 bp. The human, it seems, has two doublets of CAACTG sites in close proximity, similar to that of the mouse. The former sites are in relatively the same location from the ATG start site as in the mouse. It is also interesting to note that the A_{2B}AR gene promoter contains several other interesting transcription factor binding sites involved in cellular proliferation, including C/EBP, c-Myc, and AP-1/JunB, and differentiation Nkx3.2, Sox and Ikaros (identified based on a search with the TFSEARCH program (http://mbs.cbrc.jp/research/db/tfsearchj.html) and MacVector 9.0 Nucleic Acids Subsequences search).

It is interesting to consider the potential effects of a cell cycle-dependent transcription factor upregulating expression of A_{2B}AR. At the site of vascular injury, cells may be induced to proliferate to repair damage, yet, as is known in the case of atherosclerosis or during smooth muscle cell thickening after vascular injury induced by angioplasty, this proliferation may result in deleterious effects [Roque et al., 2000; Hao et al., 2003]. If a cell is proliferating in a region under hypoxic conditions, due to lack of blood flow or immune cell activities, adenosine may accumulate in the extracellular area [Eltzschig et al., 2004], and if in a high enough concentration, signals through A_{2B}AR, which would be upregulated via B-Myb during this proliferation stage. This could potentially initiate signaling to halt continued proliferation, limiting vascular thickening. As shown in Figures 5 and 6, A_{2B}AR is elevated in B-Myb expressing cells, and receptor activation leads to reduction in cell number.

Together, these results suggest an autocrine loop by which upregulation of the A_{2B}AR under mitogenic conditions is important for attenuating a proliferative response.

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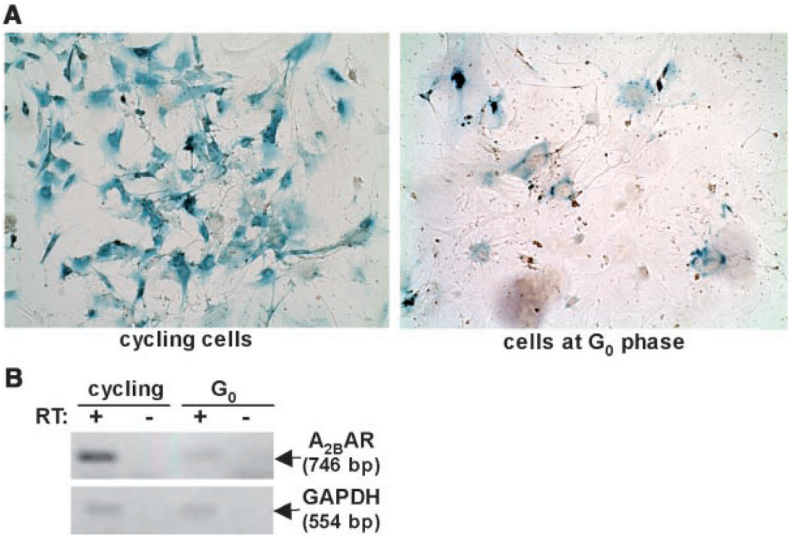

Fig. 1.

$A_{2B}AR$ expression is suppressed in vascular smooth muscle cells (VSMCs) arrested at G_0 of the cell cycle. **A**: Aortic VSMCs isolated from whole aorta of $A_{2B}AR$ knock-out/β-galactosidase knock-in ($A_{2B}AR$-β-gal) mice [Yang et al., 2006] were arrested at G_0 of the cell cycle or allowed to proliferate by incubation in 0.1% and 10% BCS-supplemented media for 48 h, respectively, followed by staining for β-gal activity reflective of *$A_{2B}AR$* gene promoter activity. Cell cycle arrest at G_0 was confirmed by flow cytometry analysis as in Nagata et al. [2005] (data not shown). **B**: Reverse transcription and PCR of endogenous $A_{2B}AR$ mRNA or GAPDH mRNA performed using primary cultures of neonatal rat aortic VSMC arrested at G_0 or proliferating (as in panel A). PCR of GAPDH cDNA was used to determine equal cDNA in different samples, and this was followed by PCR using $A_{2B}AR$ primers at the same conditions, except for the number of cycles (20 for GAPDH and 27 for $A_{2B}AR$). Reverse transcriptase (RT) was omitted as a control. Similar results were obtained with adult aortic VSMC (data not shown).

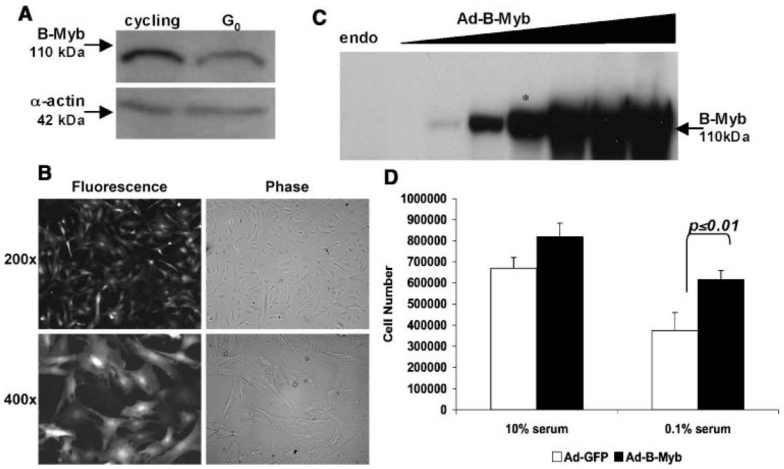

Fig. 2.

Overexpression of B-Myb in VSMCs. **A**: Western blot analysis of endogenous B-Myb in 20 μg lysate of G_0-arrested (serum starved) or proliferating VSMCs. **B**: Primary aortic VSMC at 48 h post-transduction with Ad-B-Myb, the vector system used to co-express Myb and GFP. Cells were visualized with an Olympus IX70 inverted microscope using an appropriate filter and a 20× or 40× objective. Approximately 90% of the cells are GFP-positive, as concluded from three experiments. **C**: Primary VSMCs transduced with increasing amount of Ad-B-Myb. Transduction was pursued as described in Materials and Methods Section. Protein was isolated 48 h post-transduction and subjected to western blot analysis with anti-B-Myb antibody. The asterisk denotes the transduction conditions used in subsequent experiments. Upon prolonged exposure of the film, endogenous (endo) B-Myb was also noted. **D**: Cell counting. Cells were plated at a density of 1.5×10^5 cells per well of a six-well plate, and transduced as above. At 48 h post-transduction, cells were trypsinized, stained with trypan blue and counted using the improved Neubauer hemocytometer. Results shown are means ± standard deviation (n = 9).

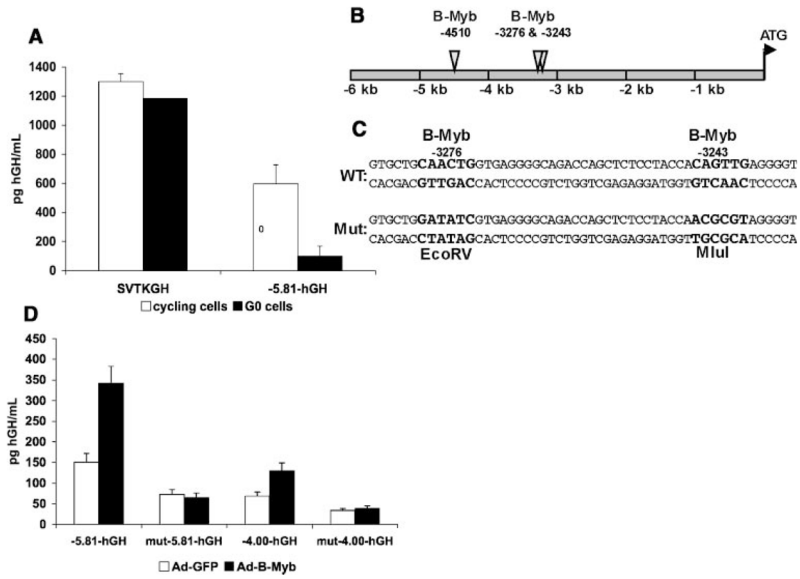

Fig. 3.

Overexpression of B-Myb upregulates *$A_{2B}AR$* gene promoter activity. **A**: VSMC were cell cycle arrested (by serum starvation) or allowed to proliferate as described in Figure 1. They were transfected with control plasmid SVTKGH or -5.81-hGH reporter plasmid on day 1. Media were changed at approximately 15 h post-transfection and hGH assay was performed on day 4. **B**: Classical B-Myb sites in the mouse *$A_{2B}AR$* gene promoter. The putative B-Myb site CAACTG was identified at −3,243, −3,276, and −4,510 bp upstream to the ATG start site. The bars in the scheme denote 1 kb. **C**: Sequence of mouse *$A_{2B}AR$* gene promoter containing adjacent putative B-Myb binding sites −3,243 and −3,276. These sites were mutated in constructs mut-5.81-hGH and mut-4.00-hGH and further used in experiments outlined in **panel D**. D: Primary cultures of VSMCs were transfected with the indicated A_{2B}AR promoter constructs and transduced, as indicated, with Ad- GFP or Ad-B-Myb adenovirus and cultured in 0.2% BCS, as detailed under Materials and Methods Section. Concentration of hGH was determined 4 days post-transfection. Results shown are means ± standard deviation (n=6).

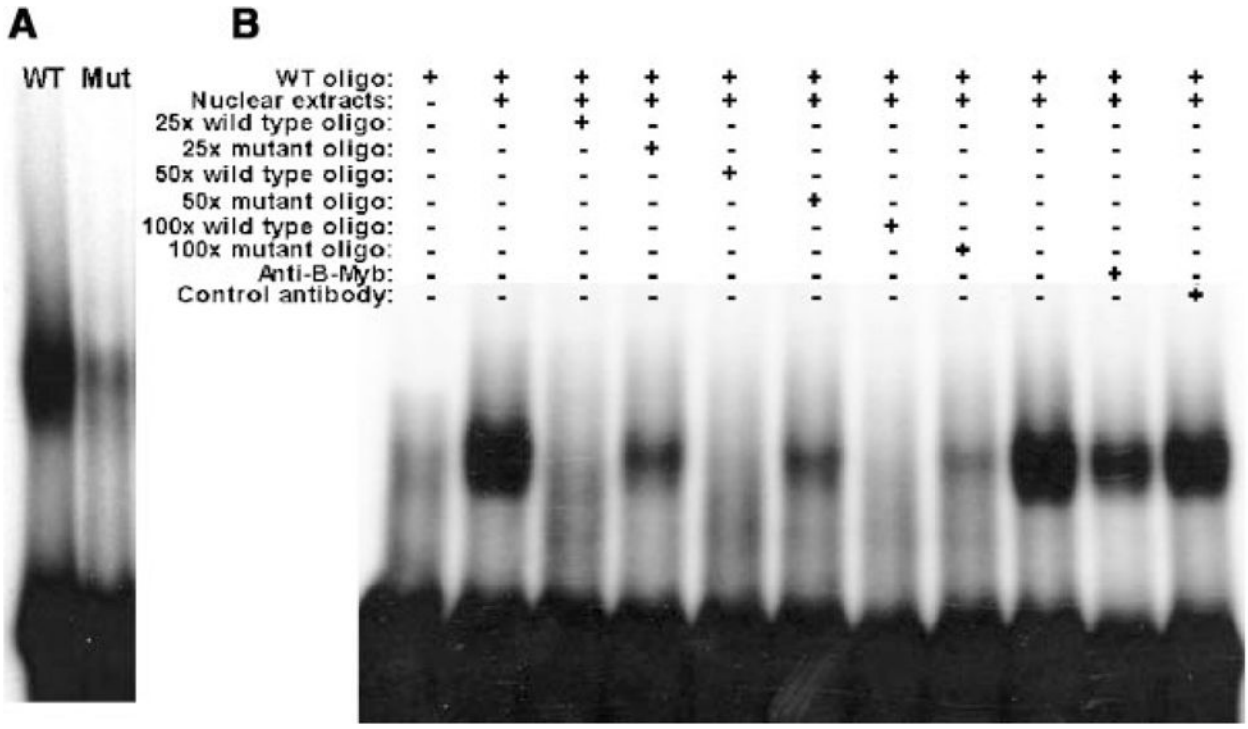

Fig. 4.

Electromobility shift assay shows specific binding at putative B-Myb sites. **A**: Nuclear extracts were prepared from primary cultures of aortic VSMCs transduced with Ad-B-Myb (as in Fig. 2) and incubated with ^{32}-P-wild type (WT) oligomer or ^{32}-P-mutated (Mut) oligomer (mutations as in Fig. 3C). Protein binding to the WT oligo was very weak in similar experiments performed with nuclear extracts derived from cells not over-expressing B-Myb (data not shown), suggesting the importance of B-Myb for protein binding to the this sequence. **B**: Nuclear extracts were incubated with WT oligomer. In lanes with antibody or cold competitors (WT or mutated), nuclear extracts were preincubated with the compound indicated in the figure.

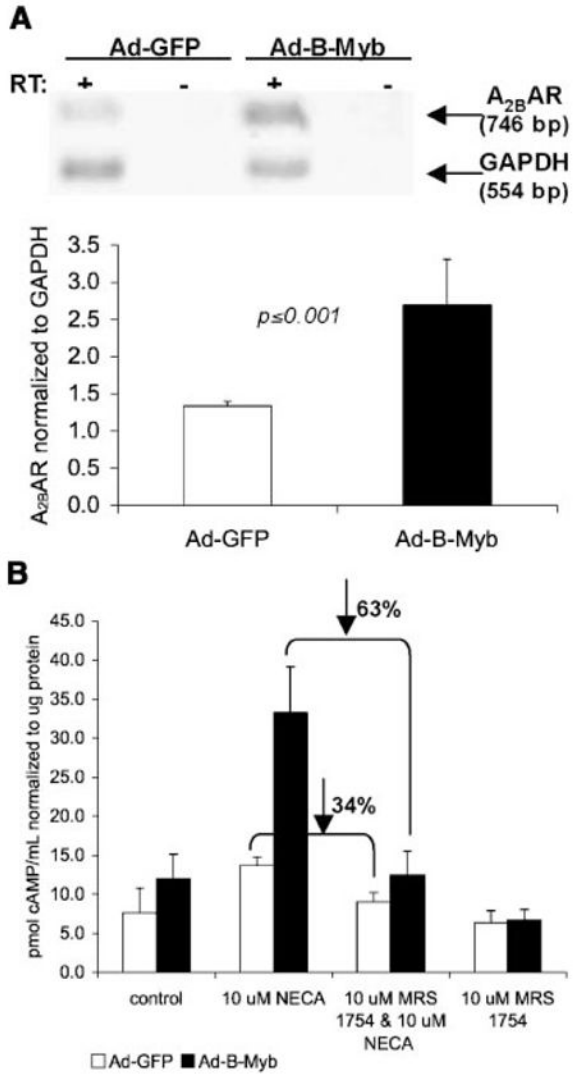

Fig. 5.

Overexpression of B-Myb in VSMCs upregulates A_{2B}AR mRNA and receptor activity. **A**: RT-PCR of non-proliferating VSMC transduced with control vector or B-Myb expressing vector, using GAPDH primers as control. Reverse transcriptase (RT) was also omitted, as indicated, as an additional control. Shown is a representative gel as well as quantitation of the data using ImageJ software (http://rsb.info.nih.gov/ij/). The data are means (n =3) ± standard deviation and subjected to Student's *t*-test. **B**: Ad-GFP or B-Myb overexpressing aortic VSMC were treated with 10 μM of the A_2AR agonist NECA or pretreated with 10 μM of the A_{2B}AR-specific antagonist MRS 1754 prior to treatment with 10 μM NECA. Data shown are means (n=5) ± standard deviation and subjected to Student's *t*-test.

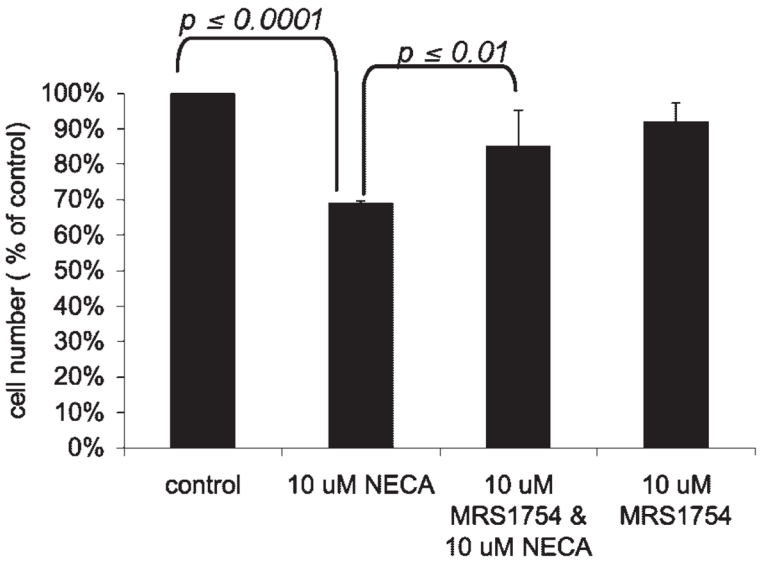

Fig. 6.

Activation of A_{2B}AR in B-Myb-overexpressing cells inhibits cellular proliferation. VSMCs were transduced with Ad-B-Myb under low serum concentration as detailed under Materials and Methods Section, followed by treatment with DMSO (control), 10 μM NECA alone, 10 μM MRS 1754 alone, or 10 μM NECA and 10 μM MRS 1754 at the same time. Cells were treated for 48 h prior to monitoring (as in Fig. 2D). Data shown are means of two experiments (n =4 each experiment) ± standard deviation.