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Role of JunB in Adenosine A_{2B} Receptor–Mediated Vascular Endothelial Growth Factor Production[S]

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ABSTRACT

Interstitial adenosine stimulates neovascularization in part through A_{2B} adenosine receptor-dependent upregulation of vascular endothelial growth factor (VEGF). In the current study, we tested the hypothesis that A_{2B} receptors upregulate JunB, which can contribute to stimulation of VEGF production. Using the human microvascular endothelial cell line, human mast cell line, mouse cardiac Sca1-positive stromal cells, and mouse Lewis lung carcinoma (LLC) cells, we found that adenosine receptor-dependent upregulation of VEGF production was associated with an increase in VEGF transcription, activator protein-1 (AP-1) activity, and JunB accumulation in all cells investigated. Furthermore, the expression of JunB, but not the expression of other genes encoding transcription factors from the Jun family, was specifically upregulated. In LLC cells expressing A_{2A} and A_{2B} receptor transcripts, only the nonselective adenosine agonist NECA (5′-*N*-ethylcarboxamidoadenosine), but not the selective A_{2A} receptor agonist CGS21680 [2-*p*-(2-carboxyethyl) phenylethylamino-5′-*N*-ethylcarboxamidoadenosine], significantly increased JunB reporter activity and JunB nuclear accumulation, which were inhibited by the A_{2B} receptor antagonist PSB603 [(8-[4-[4-((4-chlorophenzyl)piperazide-1-sulfonyl)phenyl]]-1-propylxanthine]. Using activators and inhibitors of intracellular signaling, we demonstrated that A_{2B} receptor-dependent accumulation of JunB protein and VEGF secretion share common intracellular pathways. NECA enhanced JunB binding to the murine VEGF promoter, whereas mutation of the high-affinity AP-1 site (−1093 to −1086) resulted in a loss of NECA-dependent VEGF reporter activity. Finally, NECA-dependent VEGF secretion and reporter activity were inhibited by the expression of a dominant negative JunB or by JunB knockdown. Thus, our data suggest an important role of the A_{2B} receptor-dependent upregulation of JunB in VEGF production and possibly other AP-1–regulated events.

Introduction

The endogenous nucleoside adenosine is an intermediate product of adenine nucleotide metabolism. It is accumulated at sites of tissue injury, inflammation and local hypoxia. This results in increased local adenosine concentrations in the interstitium, where it exerts its actions via binding to extracellular G protein–coupled adenosine receptors, namely, A_1, A_{2A}, A_{2B}, and A_3 (Fredholm et al., 2001). Adenosine has long been known to stimulate vascular endothelial growth factor (VEGF) production and angiogenesis (for reviews, see Adair, 2005; Feoktistov et al., 2009). In particular, we demonstrated that stimulation of A_{2B} adenosine receptors upregulated VEGF secretion in various cell types, including cardiac mesenchymal stem-like cells (Ryzhov et al., 2012), retinal and skin endothelial cells (Grant et al., 1999, 2001; Feoktistov et al., 2002), certain types of cancer cells (Zeng et al., 2003; Ryzhov et al., 2008a), tumor-infiltrating hematopoietic cells (Ryzhov et al., 2008a), and mast cells (Feoktistov and Biaggioni, 1995; Feoktistov et al., 2003; Ryzhov et al., 2008b).

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ABBREVIATIONS: AP-1, activator protein-1; CalDAG-GEF, calcium diacylglycerol guanine nucleotide exchange factor; CGS 21680, 2-*p*-(2-carboxyethyl) phenylethylamino-NECA; ChIP, chromatin immunoprecipitation; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; DNJunB, dominant negative mutant of JunB; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; Gö6983, 3-[1-[3-(dimethylamino)propyl]-5-methoxy-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione; H-89, *N*-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, dihydrochloride; HIF-1, hypoxia-inducible factor-1; HMC-1, human mast cell line; HMEC-1, human microvascular endothelial cell line; LLC, Lewis lung carcinoma; mCSC, mouse cardiac Sca-1^+CD31^- stromal cells; MEK, mitogen-activated protein kinase kinase; NECA, 5′-*N*-ethylcarboxamidoadenosine; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; PSB603, 8-[4-[4-((4-chlorophenzyl)piperazide-1-sulfonyl)phenyl]]-1-propylxanthine; Rp-cAMPS, Rp-adenosine 2′,5′-cyclic monophosphorothioate; Rap1, Ras-proximate-1; shRNA, short hairpin RNA; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene; U0124, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; U73122, 1-(6-{[17β-3-methoxyestra-1,3,5-(10)triene-17-yl]amino}hexyl)-1*H*-pyrrole-2,5-dione; U73343, 1-(6-{[17β-3-methoxyestra-1,3,5-(10)triene-17-yl]amino}hexyl)-2,5-pyrrolidine-dione; VEGF, vascular endothelial growth factor.

Although adenosine signaling through hypoxia-inducible factor-1 (HIF-1), a key transcription factor responsible for the tissue adaptation to ischemia, has been studied in detail (Merighi et al., 2005, 2006; De Ponti et al., 2007; Ramanathan et al., 2007, 2009; Alchera et al., 2008; Gessi et al., 2010), the role of other transcription factors in the adenosine-dependent VEGF production is not known. In addition to HIF-1, several transcription factor-binding sites for activator protein-1 (AP-1), AP-2, early growth response-1, specificity protein 1/3, and signal transducer and activator of transcription 3 have been identified within a 1.2 kb region of both mouse and human VEGF promoters (for review, see Pages and Pouyssegur, 2005). The AP-1 transcription factor family is composed of proteins belonging to the Jun (c-Jun, JunB, and JunD) subfamily proteins that can bind to AP-1 consensus sites either in their homodimeric forms or upon forming heterodimeric complexes with members of the Fos or the ATF subfamilies of AP-1 proteins (for review, see Karin et al., 1997).

There is growing evidence suggesting an important nonredundant role of JunB in the regulation of VEGF production and angiogenesis. When the different Jun members were deleted in mice, only the loss of JunB affected vascular development (Schorpp-Kistner et al., 1999). More recent studies have demonstrated that JunB can bind directly to an AP-1 consensus sequence within a 1.2 kb region of the mouse VEGF promoter and upregulate VEGF production in response to hypoxia or hypoglycemia independently of HIF-1 signaling (Textor et al., 2006; Schmidt et al., 2007). It is not known, however, whether JunB is involved in the adenosine-dependent regulation of VEGF production. To the best of our knowledge, the effects of adenosine on members of Jun subfamily of AP-1 proteins, including JunB, have not been reported yet. In our current study, we tested the hypothesis that A_{2B} adenosine receptors upregulate JunB, which can contribute to stimulation of VEGF production and possibly many other AP-1–dependent events.

Materials and Methods

Reagents. PSB603 (8-[4-[4-((4-chlorophenzyl)piperazide-1-sulfonyl) phenyl]]-1-propylxanthine) was from Tocris Bioscience (Bristol, UK). NECA (5′-*N*-ethylcarboxamidoadenosine) and CGS 21680 [2-*p*-(2-carboxyethyl) phenylethylamino-NECA] were purchased from Research Biochemicals, Inc. (Natick, MA). PMA (phorbol 12-myristate 13-acetate), forskolin, and Rp-cAMPS (Rp-adenosine 2′,5′-cyclic monophosphorothioate) were from Sigma-Aldrich (St. Louis, MO). H-89 (*N*-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, dihydrochloride), U73122 [1-(6-{[17β-3-methoxyestra-1,3,5-(10)triene-17-yl]amino}hexyl)-1*H*-pyrrole-2,5-dione], U73343 [1-(6-{[17β-3-methoxyestra-1,3,5-(10)triene-17-yl]amino}hexyl)-2,5-pyrrolidine-dione], U0126 (1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene), U0124, (1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene), and Gö6983 (3-[1-[3-(dimethylamino)propyl]-5-methoxy-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione) were purchased from Calbiochem (San Diego, CA). Cell culture media were purchased from Invitrogen Corporation (Carlsbad, CA). Mouse basic fibroblast growth factor was obtained from ProSpec-Tany Technogene, Ltd. (East Brunswick, NJ). Mouse interferon-γ was purchased from R&D Systems (Minneapolis, MN). Fetal bovine serum (FBS), nonessential amino acids, antibiotic-antimycotic solution, α-thioglycerol, and dimethyl sulfoxide were purchased from Sigma-Aldrich. When used as a solvent, final dimethyl sulfoxide concentrations in all assays did not exceed 0.1%, and the same dimethyl sulfoxide concentrations were used in vehicle controls.

Cell Culture. Human microvascular endothelial cells (HMEC-1), obtained from the Centers for Disease Control and Prevention/National Center for Infectious Diseases (Atlanta, GA), were grown in 199 medium containing 15% FBS and supplemented with 30 μg/ml endothelial cell growth supplement (BD Biosciences, San Jose, CA). The human mast cell line (HMC-1) was a gift from Dr. Joseph H. Butterfield (Mayo Clinic, Rochester, MN). HMC-1 cells were maintained in suspension culture at a density between 3 and 6 $\times$ 10^5 cells/ml by dilution with Iscove's medium supplemented with 10% FBS, 2 mM glutamine, and 1.2 mM α-thioglycerol. Lewis lung carcinoma (LLC) cells (CRL-1642) were obtained from the American Type Culture Collection (Manassas, VA) and maintained in 10% FBS Dulbecco's modified Eagle's medium (DMEM). Human embryonic kidney 293T cells were purchased from Thermo Fisher Scientific (Waltham, MA) and maintained in DMEM–high glucose supplemented with 10% FBS and 2 mM glutamine. Mouse cardiac Sca-$1^+$$CD31^-$ stromal cells (mCSC) were isolated as previously described elsewhere (Ryzhov et al., 2012). Cells were propagated on 0.1% gelatin-coated tissue culture dishes in DMEM–high glucose supplemented with 10% FBS, 2 mM glutamine, 10 ng/ml basic fibroblast growth factor, and 10 ng/ml interferon-γ at 33°C. Three days before the experiments, the cells were replated and cultured in the absence of interferon-γ at 37°C.

Real-Time Reverse-Transcription Polymerase Chain Reaction. Total RNA was isolated from cells using RNeasy Mini kit (Qiagen, Valencia, CA). One microgram of total DNase-treated RNA was used to generate cDNA with MMLV RT (Promega, Madison, WI) and random hexamers (Applied Biosystems, Foster City, CA). Reverse-transcription polymerase chain reaction was performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems), as described previously elsewhere (Ryzhov et al., 2012). Primer pairs for human and murine adenosine receptors (A_1, A_{2A}, A_{2B}, A_3) were obtained from Applied Biosystems (human AdoRs: Hs00181231, Hs00169123, Hs00386497, and Hs00181232; and murine AdoRs: Mm01308023, Mm00802075, Mm00839292, and Mm00802076). Primer sequences for human and mouse JunB, JunD, c-Jun, and β-actin are listed in Table 1.

Western Blot Analysis. Cells were lysed in radioimmunoprecipitation assay buffer containing protease inhibitors cocktail (Roche Diagnostics, Indianapolis, IN). Total protein concentrations were quantified with the Pierce BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Equal amounts of protein (30–60 μg/well) were resolved in NuPAGE Novex 4–12% Bis-Tris polyacrylamide gel in the presence 1$\times$ MES buffer (2-(*N*-morpholino)ethanesulfonic acid; Invitrogen) and transferred to a polyvinylidene fluoride membrane Immobilon-FL (Millipore Bioscience Research Reagents, Temecula, CA). Rabbit polyclonal anti-JunB (sc-73), anti–extracellular signal-regulated kinase (anti-ERK) (sc-154), goat polyclonal phospho-ERK antibody (sc-7976), and mouse monoclonal anti–β-actin (sc-69879) were used at 1:750, 1:500, 1:200, and 1:1000 dilutions, respectively (all from Santa Cruz Biotechnology, Santa Cruz, CA). Anti–β-tubulin (DM1B) antibody (Millipore) was used at a concentration of 0.2 μg/ml (1:5000 dilution). After treatment with an appropriate peroxidase-conjugated secondary antibody, the bands were visualized with an enhanced chemiluminescence method (Nesbitt and Horton, 1992). The intensity of protein bands was quantified by a densitometer using ImageJ 1.45s software (National Institutes of Health, Bethesda, MD). In some experiments, secondary anti-mouse IRDye@800CW (1:8000) and anti-rabbit IRDye@680LT (1:20,000) antibodies (Li-Cor Bioscience, Lincoln, NE) were used. The blots were scanned with LICOR Odyssey Infrared imager (Li-Cor Bioscience) to visualize the fluorescent immunocomplexes.

Immunofluorescence Microscopy. LLC cells were seeded into glass eight-chamber slides and grown to 90–95% confluency. Cells were incubated in the presence of the reagents indicated in *Results* for 6 hours and fixed in 4% paraformaldehyde containing 0.1% Triton X-100. Cells were permeabilized with 0.3% Triton X-100 and blocked with 3% bovine serum albumin in phosphate-buffered saline. Cells were incubated with rabbit anti-JunB (sc-73; Santa Cruz Biotechnology) antibody overnight at 4°C, washed, and incubated with AlexaFluor 488–conjugated donkey anti-rabbit IgG (Molecular Probes, Eugene OR)

TABLE 1
Primers for reverse-transcription polymerase chain reaction used in this study

Target	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
Mouse		
JunB	TAAAGAGGAACCGCAGACCGTA	AGTGTCTTCACCTTGTCCTCCA
JunD	AGTTGGACTCCACACATCCCAT	TGTCAGTTTCACAGGGTGGAGT
c-Jun	GGTGGCACAGCTTAAGCAGAAA	TCTCTGTCGCAACCAGTCAAGT
β-Actin	AGTGTGACGTTGACATCCGTA	GCCAGAGCAGTAATCTCCTTCT
Human		
JunB	TGGAACAGCCCTTCTACCAC	GGTTTCAGGAGTTTGTAGTC
JunD	GCCTCATCATCCAGTCCAAC	CCACCTTGGGGTAGAGGAAC
c-Jun	TGACTGCAAAGATGGAAACG	CAGGGTCATGCTCTGTTTCA
β-Actin	CGCCCCAGGCACCAGGGC	GGCTGGGGTGTTGAAGGT

at 1:1000 dilution for 2 hours. The slides were mounted with ProLong Gold Antifade reagent with DAPI (4′,6-diamidino-2-phenylindole; Invitrogen). Images were acquired with Olympus FV-1000 inverted confocal microscope (Olympus Corporation, Tokyo, Japan) using a 100×/1.40 SPlan-UApo oil immersion objective and excitation/emission wavelengths of 405 and 488, and processed using F10-ASW 1.6 Viewer. The corrected total cell fluorescence values were calculated as Integrated density − (Area of selected cell × Mean fluorescence of background readings) using ImageJ 1.45s software (NIH, Bethesda, MD).

Chromatin Immunoprecipitation Assay. The chromatin immunoprecipitation (ChIP) assay was performed using the Simple-ChIP Enzymatic Kit (Cell Signaling Technology, Danvers, MA) according to the manufacturer's instructions. Briefly, LLC cells (2×10^7 per immunoprecipitation) were cross-linked using formaldehyde. Cell nuclei were isolated after sonication (7 times with 20-second pulses on ice) using a Sonic Dismembrator 300 (Thermo Fisher Scientific). Equal amounts of chromatin were immunoprecipitated with JunB antibody (sc-73; Santa Cruz Biotechnology). Rabbit IgG was used as negative control. After reversal of crosslinks and DNA purification, the PCR amplification was performed using Mastercycler gradient thermal cycler (Eppendorf, Hamburg, Germany). Forward primer 5′-AGCTGGCCTACCTACCTTTCTGA-3′ and reverse primer 5′-CTTATCTGAGCCCTTGTCTG-3′ were used for amplification of the VEGF gene promoter region from −1140 to −733 harboring the putative AP-1 site (Schmidt et al., 2007), employing the following conditions: 94°C for 4 minutes; 30–35 cycles at 94°C for 30 seconds, 58.5°C for 30 seconds, and 72°C for 30 seconds; and final extension at 72°C for 10 minutes. The PCR products were analyzed on a 1.5% agarose gel.

Constructs and Luciferase Reporter Assays. To construct mouse JunB promoter-driven luciferase reporter, mouse genomic DNA was isolated using DNeasy Blood & Tissue kit (Qiagen). The JunB genomic DNA, comprising 5′ flanking −870 to +133 base pairs of the murine JunB gene, was generated using PCR. MluI and XhoI restriction sites were introduced into forward 5′-GAGGTACAGCCTCACGCGTACAA-3′ and reverse 5′-AGTTGGCTCGAGTGCGTAAAGGC-3′ primers. After digestion with *Mlu*I and *Xho*I, the 1.0-kb PCR fragment was ligated upstream of a promoterless luciferase gene into the *Kpn*I–*Xho*I sites of pGL2-basic vector (Promega). The sequence was verified against published database-accessible sequences. The pAP1-luc reporter was purchased from Agilent Technologies (Santa Clara, CA).

Mouse VEGF promoter-driven luciferase reporter, which encompasses 1.2 kb of the 5′-flanking sequence, the transcription start site, and 0.4-kb of corresponding 5′-UTR (Shima et al., 1996) was kindly provided by Dr. Patricia A. D'Amore (Harvard University, Boston MA). Mouse VEGF luciferase reporter with the putative AP-1 site at position −1093 to −1086 mutated from TGAATCA to AGGTTCC (Schmidt et al., 2007) was kindly provided by Dr. Marina Schorpp-Kistner (DKFZ-German Cancer Research Center, Heidelberg, Germany). Human VEGF promoter-driven luciferase reporter VEGF-P7 (Forsythe et al., 1996) was kindly provided by Dr. Greg L. Semenza (Johns Hopkins Hospital, Baltimore, MD). Dominant negative JunB mutant construct pcDNA3-JunBΔN (Ikebe et al., 2007), was a kind gift from Dr. Mitsuyasu Kato (University of Tsukuba, Japan).

Cells were grown to 50% confluency and transfected with 1 μg/well of plasmid DNA using FugeneHD reagent (Roche). To test the effect of dominant negative JunB mutant on VEGF reporter activity, pcDNA3-JunBΔN, pVEGF-luc, and pRL-SV40 vectors were cotransfected at ratio 80:20:1. Twenty-four hours after transfections, cells were incubated in the presence of the reagents indicated in *Results* for an additional 6 hours. Reporter activity was measured using a Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase reporter activities were normalized against *Renilla* luciferase activities from the coexpressed pRL-SV40 and expressed as relative luciferase activities over the basal level.

Stable Transfection of Dominant Negative JunB Mutant in LLC Cells. LLC cells were transfected with pcDNA3-JunBΔN or empty pcDNA3 plasmid. After 24 hours, the growth medium was replaced with a fresh medium containing 0.8 mg/ml G-418. One week later, the colonies of LLCs that survived after G-418 treatment were selected, and the expression of dominant negative JunB mutant was verified by Western immunoblotting using rabbit polyclonal anti-JunB (sc-73; Santa Cruz Biotechnology).

Production and Stable Transduction of Lentiviral Vectors Encoding Short Hairpin RNA in LLC Cells. MISSION pLKO.1-puro JunB short hairpin RNAs (shRNA1) (TRCN0000232241), shRNA2 (TRCN0000232242), shRNA3 (TRCN0000054488), shRNA4 (TRCN0000042698), shRNA5 (TRCN0000042699), empty vector (SHC001), and nontargeting control nonmammalian shRNA (SHC002) plasmids were purchased from Sigma-Aldrich. Lentiviral psPAX.2 packaging (cat. no. 12260) and pMD2.G (cat. no. 12259) envelope plasmids were obtained from Addgene (Cambridge MA). Human embryonic kidney 293T cells were cotransfected with MISSION pLKO.1-puro, psPAX.2, and pMD2.G plasmid constructs using Lipofectamine 2000 (Invitrogen). Virus-containing supernatants were harvested 48 hours later and frozen at −80°C.

For infection, LLC cells (2×10^5 per well) were seeded on six-well plates and 12 hours later were incubated with lentiviral particles diluted 1:1000 in DMEM containing 10% FBS and 8 μg/ml Polybrene (Sigma-Aldrich) for an additional 12 hours. After 36 hours' recovery, stably transduced LLC cells were selected by culturing in the presence of 2 μg/ml puromycin (Sigma-Aldrich).

Evaluation of Ras-Proximate-1 Activation. After overnight serum starvation, HMEC-1 cells were incubated in the absence or presence of 10 μM U73122 for 30 minutes followed by incubation in the presence of 10 μM NECA for 5, 10, or 15 minutes. Ras-proximate-1 (Rap1) activation was determined after pull-down of Rap1-GTP with GST-RalGDS-Rap1 binding domain using the Active Rap1 Pull-Down and Detection Kit (Thermo Fisher Scientific) following the supplier's instructions.

Analysis of VEGF Secretion. The VEGF protein level in culture media was measured using an enzyme-linked immunosorbent assay kit (R&D Systems).

Statistical Analysis. Data were analyzed using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA) and are presented as mean

± S.E.M. Comparisons between several treatment groups were performed using one-way analysis of variance, followed by appropriate post hoc tests. Comparisons between two groups were performed using two-tailed unpaired *t* tests. $P < 0.05$ was considered statistically significant.

Results

JunB Accumulation and Increase in AP-1 Activity Are Associated with Adenosine-Dependent Upregulation of VEGF Production in Different Human and Mouse Cell Types. To determine the potential involvement of JunB in adenosine-dependent stimulation of VEGF production, we chose two human cell lines, HMEC-1 and HMC-1, and two mouse cell lines, mCSC and LLC, in all of which the A_{2B} receptor dependent-regulation of VEGF secretion has been described (Feoktistov et al., 2002, 2003; Ryzhov et al., 2008a, 2012). Figure 1 demonstrates that stimulation of adenosine receptors with 10 μM NECA for 6 hours significantly increased VEGF release (Fig 1, A–D) from all cells studied. To determine whether the upregulation of VEGF production by NECA occurs at the transcriptional level, two mouse cell lines mCSC and LLC were transiently transfected with a mouse VEGF promoter-driven luciferase reporter, which encompasses 1.2 kb of the 5′-flanking sequence that includes

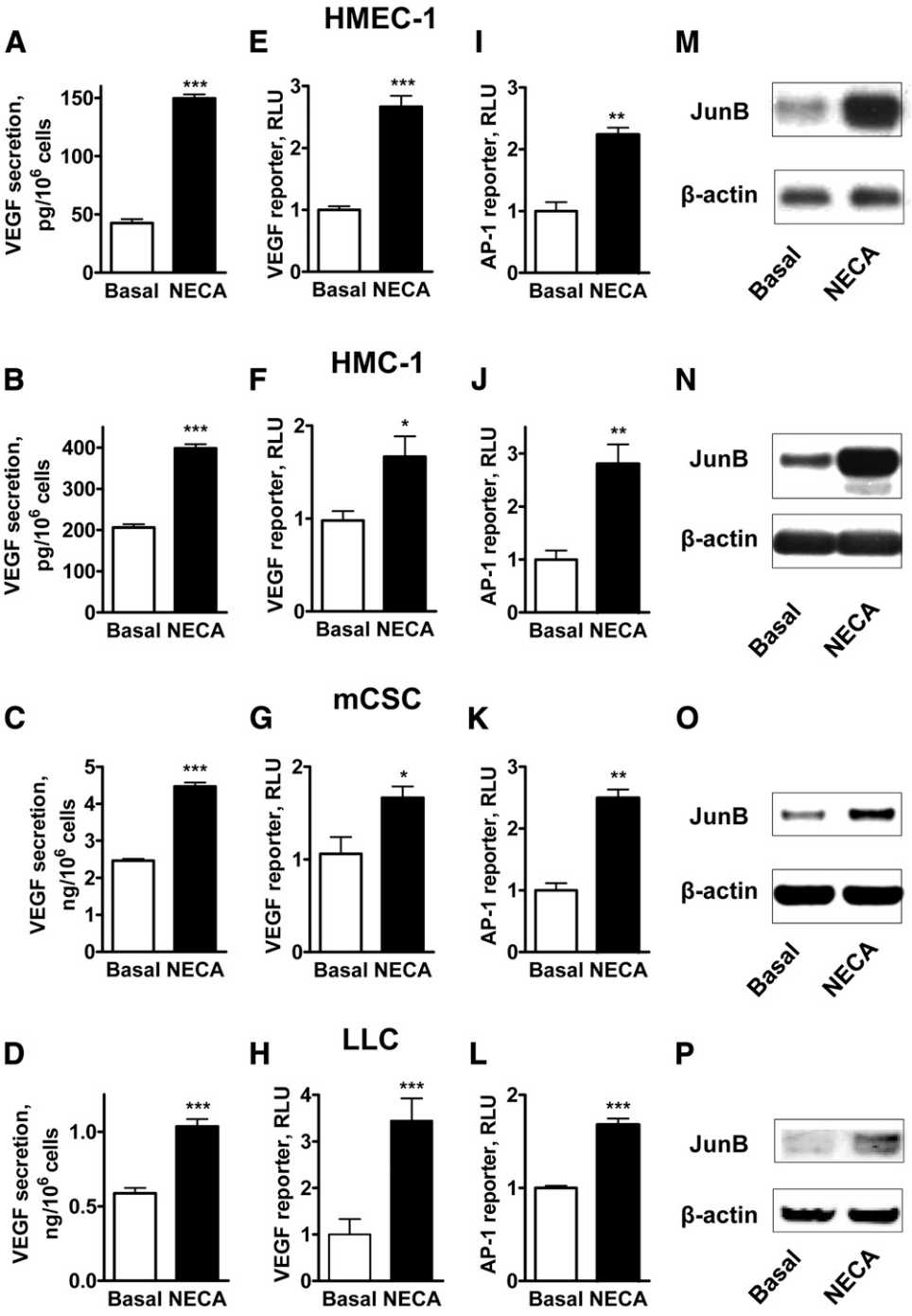

Fig. 1. NECA-dependent upregulation of VEGF production is associated with an increase in VEGF transcription, AP-1 activity, and JunB accumulation in various human and mouse cell types. HMEC-1 cells (A, E, I, M), HMC-1 cells (B, F, J, N), mCSC cells (C, G, K, O), and LLC cells (D, H, L, P) were either not transfected (A–D, M–P) or transfected with plasmids encoding VEGF (E–H) or AP-1 (I–L) luciferase reporters and were incubated in the absence (Basal) or presence of 10 μM NECA for 6 (A–L) or 3 (M–P) hours. VEGF release (A–D), VEGF (E–H), and AP-1 (I–L) reporter activities and JunB protein levels (M–P) were analyzed as described in *Materials and Methods*. Values are presented as mean ± S.E.M. (n = 3–10) in A–L. Asterisks indicate the statistical difference (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) compared with basal values by two-tailed unpaired *t* tests. Representative blots of 3–5 experiments are shown in M–P.

AP-1 binding sites previously proven to physically interact with JunB (Schmidt et al., 2007). Human cell lines HMEC-1 and HMC-1 were transiently transfected with a homologous human VEGF promoter-driven luciferase reporter. Figure 1, E–H, shows that stimulation of adenosine receptors with 10 μM NECA for 6 hours significantly increased luciferase expression in all cells investigated, indicating that upregulation of VEGF production by NECA can occur at the transcriptional level.

Next, we tested whether stimulation with NECA increases AP-1 reporter activity. We transiently transfected cells with the AP-1 luciferase reporter construct pAP1-luc that contains seven times repeated AP-1–binding sites. Figure 1, I–L, shows that stimulation of adenosine receptors with 10 μM NECA for 6 hours significantly increased luciferase expression in all cells investigated.

In parallel to the stimulation of the AP-1 and VEGF reporters, Western blot analysis of JunB protein content in cell lysates showed that stimulation of adenosine receptors with 10 μM NECA for 3 hours significantly increased JunB protein accumulation in all cells investigated (Fig. 1, M–P). Furthermore, we found that NECA significantly upregulated the expression of JunB but not the expression of other genes encoding transcription factors from the Jun family (Fig. 2).

A_{2B} Adenosine Receptors Promote JunB Expression at the Transcriptional Level and Increase Accumulation of JunB Protein in the Nucleus. The expression of A_{2B} receptors is a common feature of all cells investigated in this study. Figure 3A compares the results of real-time reverse-transcription polymerase chain reaction analysis of the expression of mRNA encoding subtypes of adenosine receptors in the HMEC-1, HMC-1, mCSC, and LLC cell lines. In addition to the expression of A_{2B}, all cells also expressed A_{2A} receptor transcripts with A_{2A}/A_{2B} receptor ratio lowest in LLC and highest in HMC-1 cells. No other receptor subtype transcripts were detected in these cells except for HMC-1 expressing A_3 receptors in addition to A_{2A} and A_{2B} receptors.

To determine whether stimulation of A_{2B} adenosine receptors can promote JunB expression at the transcriptional level, LLC cells were transiently transfected with a mouse JunB promoter-driven luciferase reporter, which encompasses 5′ flanking −870 to +133 base pairs. Figure 3B shows that only the nonselective adenosine agonist NECA (10 μM) but not the selective A_{2A} receptor agonist CGS 21680 (1 μM) significantly increased the reporter activity, and this effect was inhibited by the A_{2B} receptor antagonist PSB603 (Borrmann et al., 2009) at a selective concentration of 100 nM.

PSB603 inhibited JunB protein levels in a concentration-dependent manner in whole lysates of LLC cells stimulated with 10 μM NECA for 3 hours (Fig. 3C). Furthermore, stimulation of A_{2B} adenosine receptors promoted JunB accumulation specifically in the cell nucleus. As seen in Fig. 3D, only faint immunofluorescence staining with anti-JunB antibody was detected in the nuclei of control cells (Basal), contrasting with the strong fluorescence present in the nuclei of NECA-treated cells after 3 hours of treatment. No JunB staining was detected in the cytoplasm.

The NECA-induced nuclear accumulation of JunB was reduced to nearly basal levels in the presence of 100 nM PSB603. Quantitative analysis of the immunofluorescence data confirmed a significant increase in JunB staining in cells stimulated with NECA in the absence but not in the presence of PSB603 (Fig. 3E). Taken together, our data suggest that stimulation of A_{2B} receptors can activate the nuclear factor JunB at the transcriptional level, with subsequent accumulation of this protein in the nucleus.

A_{2B} Receptor-Dependent Upregulation of JunB Expression and VEGF Secretion Share Common Intracellular Pathways. We chose HMEC-1 cells to conduct pharmacologic analysis of the intracellular pathways involved in the adenosine-dependent regulation of JunB protein expression and VEGF secretion because of their robust responses to stimulation of adenosine receptors (Fig. 1, A and M). These cells express A_{2A} and A_{2B} but not A_1 or A_3 adenosine receptor

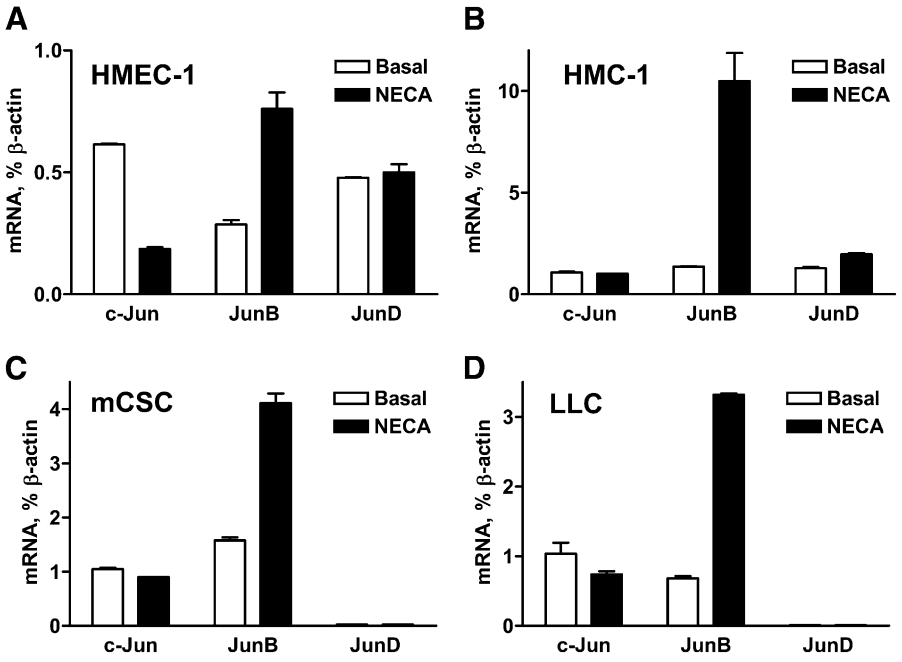

Fig. 2. Effects of NECA on the expression of members of the Jun family transcription factors in cells with adenosine-dependent regulation of VEGF. Real-time reverse-transcription polymerase chain reaction analysis of mRNA encoding c-Jun, JunB, and JunD in (A) HMEC-1 cells, (B) HMC-1 cells, (C) mCSC cells, and (D) LLC cells incubated in the absence (basal) or presence of 10 μM NECA for 60 minutes. Values are normalized to β-actin mRNA expression and are expressed as the average of two determinations made in triplicate.

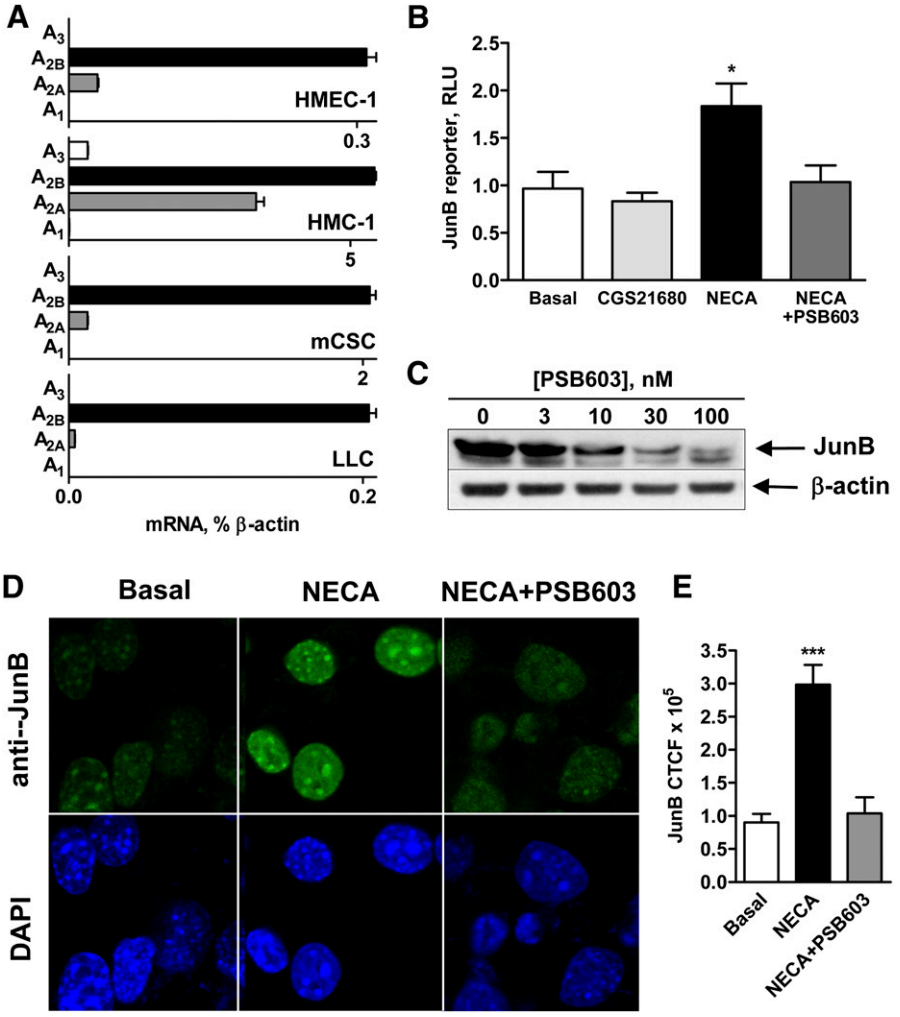

Fig. 3. A_{2B} adenosine receptors mediate an increase in JunB promoter-driven reporter activity and JunB protein accumulation in the nucleus. (A) Real-time reverse-transcription polymerase chain reaction analysis of mRNA encoding adenosine receptor subtypes in HMEC-1 cells, HMC-1 cells, mCSC cells, and LLC cells was performed as described in *Materials and Methods*. Values are expressed as average of two determinations made in triplicate. (B) Pharmacologic analysis of the role of A_2 adenosine receptor subtypes in regulation of JunB promoter-driven reporter activity in LLC cells. LLC cells were transiently transfected with JunB promoter-driven luciferase reporter and then incubated in the absence (basal) or presence of the selective A_{2A} receptor agonist CGS 21680 (1 μM), or the nonselective adenosine receptor agonist NECA (10 μM) in the absence or presence of the selective A_{2B} receptor antagonist PSB603 (100 nM) for 3 hours. Values are presented as mean ± S.E.M. (n = 3). An asterisk indicates the statistical difference ($^*P < 0.05$) compared with basal levels. (C) Effect of increasing concentrations of the selective A_{2B} receptor antagonist PSB603 on JunB protein levels in LLC cells stimulated with 10 μM NECA for 3 hours. A representative blot of three experiments is shown. (D) Pharmacologic analysis of the role of A_{2B} adenosine receptors in regulation of JunB protein accumulation in LLC nuclei. LLC cells were incubated in the absence (basal) or presence of the nonselective adenosine receptor agonist NECA (10 μM) in the absence or presence of the selective A_{2B} receptor antagonist PSB603 (100 nM) for 3 hours. Representative micrographs are shown of anti-JunB–stained LLC cells (green) and nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI; blue) from three experiments. (E) Quantification of the immunofluorescence data shown in D. Fluorescence intensity was measured in seven randomly chosen cells per slide using ImageJ, and the corrected total cell fluorescence (CTCF) values are presented as mean ± S.E.M. Asterisks indicate statistical difference ($^{***}P < 0.001$) compared with basal levels.

transcripts (Fig. 3A). The nonselective adenosine agonist NECA (10 μM) induced VEGF secretion (Fig. 4A) and increased JunB protein accumulation (Fig. 4, B and C). In contrast, the A_{2A} receptor agonist CGS 21680 had no effect when used at a selective concentration of 1 μM (Fig. 4, A–C). The A_{2B} receptors in HMEC-1 cells are known to couple to G_s and G_q proteins, resulting in accumulation of cAMP and diacylglycerol (DAG)/inositol trisphosphate (Feoktistov et al., 2002). As seen in Fig. 4, stimulation of cAMP-dependent pathways with 1 μM forskolin or DAG-dependent pathways with 10 nM PMA increased both JunB protein expression and VEGF secretion.

To explore the role of cAMP/protein kinase A (PKA) signaling pathways in adenosine-dependent upregulation of JunB and VEGF, HMEC-1 cells were stimulated with 10 μM NECA in the presence of PKA inhibitors. In ancillary experiments, we monitored cAMP/PKA signal transduction by the activity of luciferase reporter under control of tandem repeats of the cAMP-responsive elements transiently expressed in HMEC-1 cells. We estimated that the PKA inhibitor H-89 inhibited the activity of NECA-stimulated cAMP-responsive element reporter with an IC_{50} value of 3.2×10^{-7} M (Supplemental Fig. 1). However, 1 μM H-89 had little if any effect on VEGF release induced by 10 μM NECA (Fig. 5A).

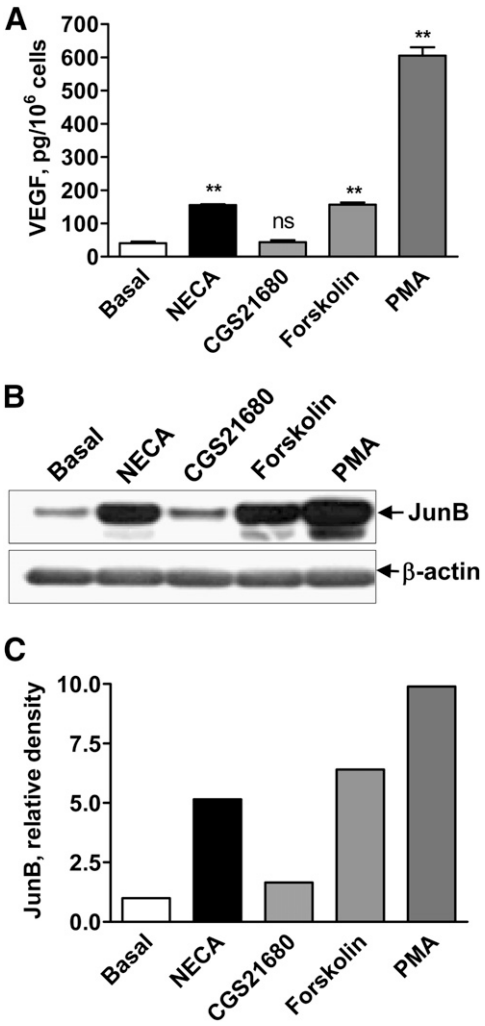

Fig. 4. Stimulation of A_{2B} receptor-linked signaling pathways increases VEGF secretion and JunB protein levels. (A) VEGF release from HMEC-1 cells incubated in the absence (basal) or presence of 10 μM NECA, 1 μM CGS 21680, 1 μM forskolin, or 10 nM PMA for 6 hours. Values are expressed as mean ± S.E.M. (n = 6). Asterisks indicate significant difference (**$P < 0.01$, one-way ANOVA with Dunnett's post test) and ns indicates nonsignificant difference compared with basal levels. (B) Western blot analysis of JunB protein levels in HMEC-1 cells incubated in the absence (basal) or presence 10 μM NECA, 1 μM CGS 21680, 1 μM forskolin, or 10 nM PMA for 3 hours. Arrows indicate positions of JunB and β-actin, the latter used as a loading control. A representative blot of three experiments is shown. (C) Graphic representation of data shown in B quantified by densitometry and expressed as relative density of JunB bands normalized to corresponding β-actin bands.

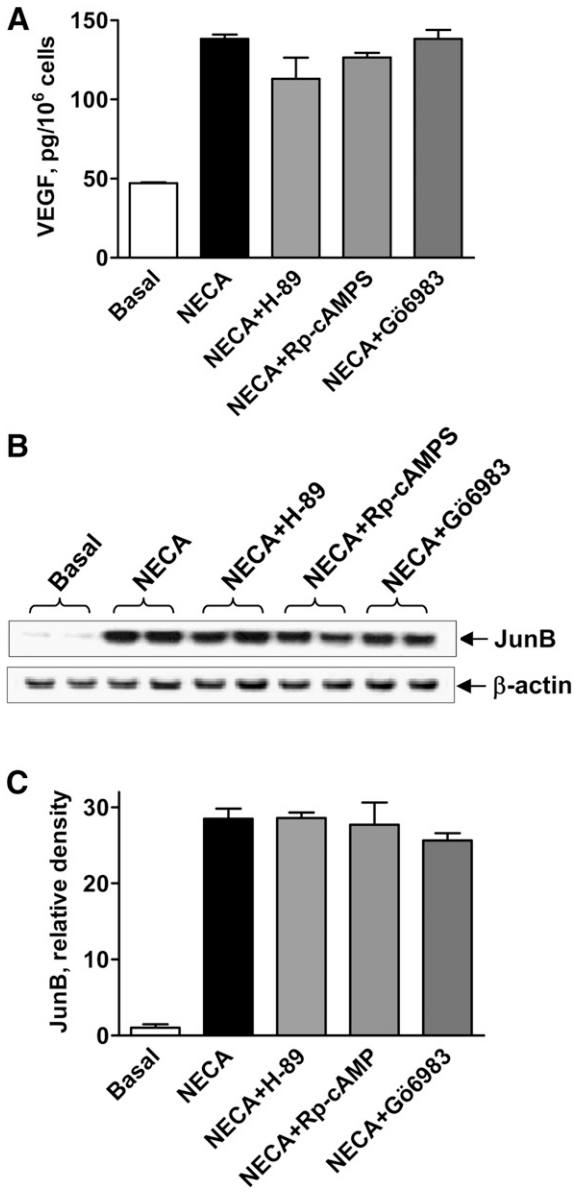

Fig. 5. Effects of inhibitors of protein kinases A and C on A_{2B} receptor-dependent increase in VEGF secretion and JunB protein levels. (A) VEGF secretion from HMEC-1 cells incubated in the absence (basal) or presence of 10 μM NECA, or in the presence of NECA and 1 μM H-89, 100 μM Rp-cAMPS, or 1 μM Gö6983 for 6 hours. Values are presented as mean ± S.E.M. (n = 3). (B) Western blot analysis of JunB protein levels in HMEC-1 cells incubated in the absence (basal) or presence of 10 μM NECA, or in the presence of NECA and 1 μM H-89, 100 μM Rp-cAMPS, or 1 μM Gö6983 for 3 hours. Arrows indicate positions of JunB and β-actin, the latter used as a loading control. A representative blot of three experiments is shown. (C) Graphic representation of data shown in B quantified by densitometry and expressed as relative density of JunB bands normalized to corresponding β-actin bands.

Furthermore, another PKA inhibitor Rp-cAMPS with a different mechanism of action and reported IC_{50} values in a low micromolar range (Rothermel et al., 1983) had also no effect on NECA-induced VEGF secretion at concentrations up to 10^{-4} M (Fig. 5A). These results suggest that adenosine actions on VEGF release are PKA-independent.

To explore a potential role of protein kinase C (PKC) signaling pathways in adenosine-dependent upregulation of JunB and VEGF, we tested effects of the broad-spectrum inhibitor of PKC Gö6983, with reported IC_{50} values for most isoforms in a low nanomolar range (Gschwendt et al., 1996), on NECA-induced VEGF secretion. We found that 1 μM Gö6983 had no effect on NECA-induced VEGF secretion (Fig. 5A). These results suggest that Gö6983-sensitive PKC isoforms play no role in NECA-induced VEGF secretion. In parallel to their lack of effects on NECA-induced VEGF release, the PKA inhibitors H89 (1 μM) and Rp-cAMPS (100 μM), and the PKC inhibitor Gö6983 (1 μM) had also no effect on NECA-induced JunB protein accumulation in HMEC-1 cells (Fig. 5, B and C).

We next evaluated a potential role of alternative signaling pathways in A_{2B} receptor-dependent VEGF production by using inhibitors of phospholipase C (PLC)-β (Fig. 6A) and mitogen-activated protein kinase kinase (MEK) 1/2 (Fig. 6B). From these

experiments we chose concentrations of inhibitors to test their effects on A_{2B} receptor-dependent JunB protein accumulation (Fig. 6, C and D). To explore the role of A_{2B} receptor-G_q–linked PLC-β pathways in adenosine-dependent upregulation of VEGF, we used the PLC inhibitor U73122 and its succinimido analog U73343 as a negative control (Bleasdale et al., 1990). Figure 6A shows that U73122 at concentrations of 1–10 μM produced a greater inhibition of NECA-stimulated VEGF release from HMEC-1 compared with U73343. A similar difference between U73122 and U73343 in their inhibitory actions on JunB protein accumulation was observed when these compounds were added at concentrations of 10 μM before stimulation of HMEC-1 cells with NECA (Fig. 6, C and D).

We have previously reported that inhibition of MEK1/2 with U0126 partially reduced A_{2B} receptor-dependent VEGF production in human and mouse mast cells (Ryzhov et al., 2008b). Therefore, we compared the effects of MEK1/2 inhibition on NECA-induced VEGF release and JunB protein accumulation in HMEC-1 cells. As seen in Fig. 6B, the MEK1/2 inhibitor U0126 at concentrations of 1–10 μM inhibited VEGF release by approximately 50%. In contrast, the inactive form of MEK1/2 inhibitor U0124 had no effect on NECA-dependent VEGF secretion at concentrations up to 10 μM. Similarly, 1 μM U0126 produced partial inhibition of NECA-induced JunB protein accumulation, whereas 1 μM U0124 had no effect (Fig. 6, C and D). However, U0126 at this concentration almost completely blocked ERK activity (Supplemental Fig. 2). Taken together, our data suggest that ERK1/2 activated by MEK1/2 may be partially responsible not only for NECA-induced VEGF release but also for JunB protein accumulation.

Calcium diacylglycerol guanine nucleotide exchange factor (CalDAG-GEF)–Rap1 pathway has been implicated in PMA-dependent ERK activation, thus providing a potential link between PLC and ERK (Stork and Dillon, 2005). To determine whether stimulation of adenosine receptors leads to PLC-dependent activation of Rap1 in HMEC-1, we stimulated cells with 10 μM NECA in the absence or presence of 10 μM U73122 and measured Rap1 activation. As seen in Fig. 7, stimulation of adenosine receptors with NECA indeed induces transient activation of Rap1. The NECA-induced activation of Rap1 was considerably lower in the presence of the PLC inhibitor U73122.

JunB Binding to the VEGF Promoter Is an Important Step in the A_{2B} Receptor-Dependent Stimulation of VEGF Production. Previous studies have identified the AP-1 recognition site located at position −1093 to −1086 of the murine VEGF promoter as the only high-affinity JunB-binding site and demonstrated that JunB acts primarily through this element to stimulate VEGF transcription during hypoxia (Schmidt et al., 2007). To prove that stimulation of A_{2B} receptors induces physical interaction of JunB with the VEGF promoter, ChIP analysis was performed in LLC cells incubated in the absence or presence of 10 μM NECA. Primers were designed to amplify the region of the VEGF promoter (−1140 to −733) containing the high-affinity AP-1 site. We found weak basal JunB binding to this promoter region in the absence of NECA, which was considerably enhanced in cells stimulated with NECA (Fig. 8A). To demonstrate the requirement of JunB binding to the high-affinity AP-1 site for the A_{2B} receptor-dependent stimulation of VEGF transcription, we used a luciferase reporter analysis. As seen in Fig. 8B, mutation of the high-affinity AP-1 site (−1093 to −1086) within the murine promoter fragment resulted in a loss of stimulation of luciferase activity by NECA. To further ascertain the role of JunB in the A_{2B} receptor-dependent regulation of VEGF transcription, a plasmid encoding dominant negative mutant of JunB (DNJunB) or an empty expression vector (mock transfection) was cotransfected with VEGF promoter-driven luciferase reporter in LLC cells. Twenty-four hours after transfection, cells were incubated in the presence or absence of 10 μM NECA for an additional 6 hours. Figure 8B shows that the expression of DNJunB significantly suppressed NECA-stimulated reporter activity.

To further demonstrate the role of JunB in A_{2B} receptor-dependent regulation of VEGF secretion, we established LLC colonies that either stably expressed DNJunB or underwent mock transfection with an empty vector. DNJunB protein

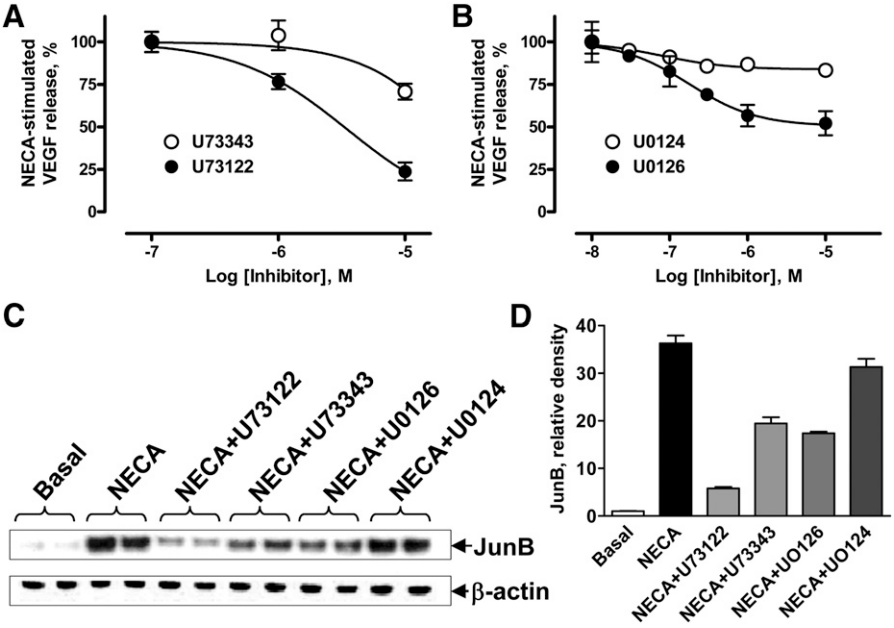

Fig. 6. Effects of PLC and MEK inhibition on VEGF secretion and JunB levels in HMEC-1 cells. (A) Effect of the PLC inhibitor U73122 (●) and its inactive control analog U73343 (○) on VEGF secretion from cells stimulated with 10 μM NECA. In the absence of inhibitors, 10 μM NECA increased concentrations of VEGF in the medium from 47 ± 1 to 138 ± 1 pg/10^6 cells. Values are presented as mean ± S.E.M. (n = 6). (B) Effect of increasing concentrations of the MEK inhibitor U0126 (●) and its inactive control analog U0124 (○) on VEGF secretion from cells stimulated with 10 μM NECA. Values are presented as mean ± S.E.M. (n = 3). (C) Western blot analysis of JunB protein levels in HMEC-1 cells incubated in the absence (basal) or presence of 10 μM NECA, or in the presence of NECA and 10 μM U73122, 10 μM U73343, 1 μM U0126, or 1 μM U0124 for 3 hours. Arrows indicate positions of JunB and β-actin, the latter used as a loading control. A representative blot of three experiments is shown. (D) Graphic representation of data shown in C quantified by densitometry and expressed as relative density of JunB bands normalized to corresponding β-actin bands.

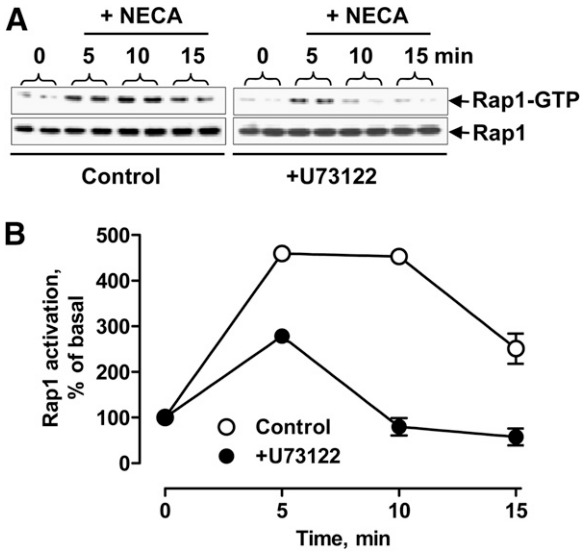

Fig. 7. Effect of PLC inhibition on adenosine-dependent Rap1 activation. (A) Western blot analysis of active GTP-bound form of Rap1 obtained by glutathione *S*-transferase-Ral-GDS pull-down before and 5, 10, and 15 minutes after stimulation of HMEC-1 cells with 10 μM NECA in the absence (control) and presence of 10 μM U73122. Total Rap1 protein levels in corresponding cell lysate aliquots are shown in the bottom panels. Representative blots of three experiments are shown. (B) Graphic representation of data shown in A quantified by densitometry and expressed as a percentage of the corresponding levels in resting cells normalized to total Rap-1 protein levels used as internal control.

expression was confirmed by Western blotting (Fig. 8C). Images of full-length gels are shown in Supplemental Fig. 3. We found that the stable expression of DNJunB resulted in significant suppression of NECA-induced VEGF secretion (Fig. 8C). It should be noted that the expression of DNJunB also affected basal VEGF secretion to some extent. However, a relative increase in VEGF secretion induced by NECA was still considerably lower in cells expressing DNJunB compared with mock-transfected control (1.5- and 2-fold, respectively).

Finally, we used an RNA interference approach to evaluate the effect of JunB knockdown on the A_{2B} receptor-dependent VEGF production. Based on Western blot analysis of efficacy of JunB silencing after stable lentiviral transfection of LLC cells with different JunB shRNA constructs (shRNA1-5; Fig. 8E), we selected cells expressing shRNA5 for further experiments. We confirmed that both basal and NECA-induced JunB mRNA levels were largely suppressed in LLC cells expressing shRNA5 compared with cells expressing nontargeting control shRNA (Fig. 8F). We found that JunB knockdown resulted in significant suppression of NECA-induced VEGF secretion (Fig. 8G) similar to that seen in cells expressing DNJunB (Fig. 8D). Taken together, our results suggest that JunB plays an important role in the A_{2B} receptor-dependent regulation of VEGF transcription.

Discussion

Our study demonstrated for the first time that JunB protein accumulation is induced in response to stimulation of A_{2B} adenosine receptors in different types of murine and human cells. A_{2B} receptor-dependent regulation of JunB abundance can occur at the transcriptional level, as evidenced from stimulation of a JunB reporter by the adenosine analog NECA, which was blocked by the selective A_{2B} receptor antagonist PSB603. Stimulation with NECA induced a robust increase in JunB transcripts in all cells under investigation. In contrast, the expression of c-Jun was rather downregulated by NECA, albeit to a different extent depending on the cell type. NECA had little if any effect on the expression of JunD. Thus, our results suggest that of all members of the Jun subfamily, JunB is the principal target of A_{2B} receptor signaling. Using an AP-1 reporter, we confirmed that adenosine-dependent increase in JunB results in formation of functional AP-1 proteins in these cells.

AP-1 proteins have been implicated in the induction of VEGF expression by UVB irradiation in fibroblasts (Dong et al., 2012), by hyperbaric oxygen in endothelial cells (Lee et al., 2006), and by tumor necrosis factor α in breast cancer cells (Yin et al., 2009). Because physical interaction of JunB with AP-1 binding sites of the VEGF promoter and subsequent stimulation of VEGF secretion have been previously demonstrated in fibroblasts and endothelial cells upon stimulation with hypoxia (Schmidt et al., 2007) or hypoglycemia (Textor et al., 2006), our new findings raised a possibility that JunB can be involved also in the upregulation of VEGF by A_{2B} receptor signaling. We investigated signaling pathways linked to A_{2B} receptors that may lead to JunB protein accumulation and VEGF secretion in HMEC-1 cells (Fig. 9). These cells express mRNA encoding A_{2A} and A_{2B} receptors, but not other adenosine receptor subtypes. Both A_{2A} and A_{2B} receptors are known to be coupled to G_s proteins linked to stimulation of adenylate cyclase and accumulation of cAMP (Fredholm et al., 2001). However, we have previously shown that only A_{2B} receptors are functionally coupled to adenylate cyclase in these cells because only NECA but not the selective A_{2A} agonist CGS 21680 stimulated cAMP accumulation (Feoktistov et al., 2002). In agreement with these data, CGS 21680 had no effect on either JunB or VEGF.

In various cells including HMEC-1, A_{2B} adenosine receptors were shown to not only stimulate adenylate cyclase via coupling to G_s, but also stimulate PLCβ via a GTP-binding protein of the G_q family resulting in accumulation of DAG and inositol trisphosphate (Feoktistov and Biaggioni, 1995; Linden et al., 1999; Feoktistov et al., 2002; Ryzhov et al., 2009). In the present study, we found that stimulation of adenylate cyclase by forskolin or activation of DAG-dependent pathways with PMA increased JunB protein levels and VEGF production. Further analysis employing inhibitors of PLC-linked pathways revealed their essential role in A_{2B} receptor-dependent regulation of both JunB and VEGF. Inhibition of PLC with U73122 reduced A_{2B} receptor-dependent JunB protein accumulation and VEGF secretion.

The most prominent intracellular targets of DAG and the functionally analogous phorbol esters belong to the PKC family (Ron and Kazanietz, 1999). However, the broad-spectrum PKC inhibitor Gö6983 had no effect on the A_{2B} receptor-dependent increase in JunB protein levels and VEGF production. In addition to stimulation of PKC, PMA is known to activate CalDAG-GEF–Rap1 pathway, eventually leading to activation of ERK (Stork and Dillon, 2005). In the current study, we demonstrated that this pathway is indeed activated by stimulation of A_{2B} receptors. Inhibition of NECA-induced Rap1 activation by the PLC inhibitor U73122 suggests that Rap1 and its activator CalDAG-GEF, which is known to respond to calcium and DAG (Kawasaki et al., 1998), are targets of A_{2B}

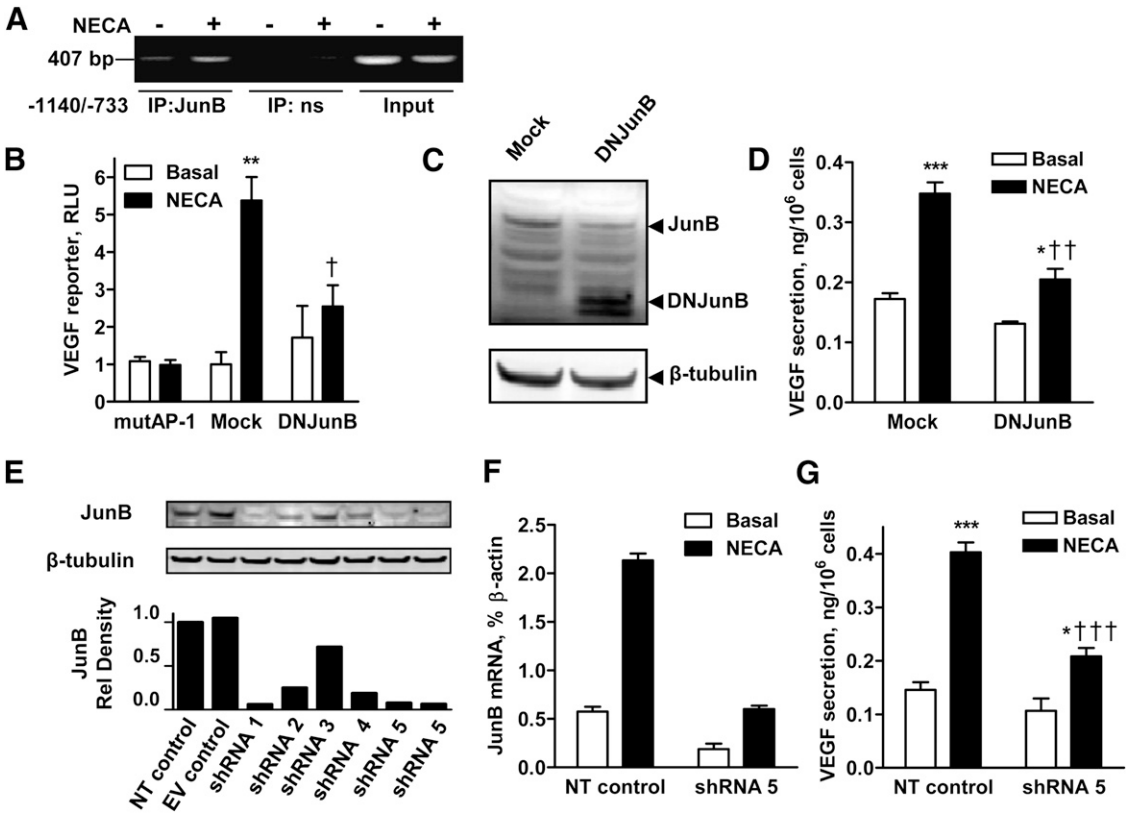

Fig. 8. Role of JunB in A_{2B} receptor-dependent VEGF production. (A) Effect of NECA on JunB binding to the VEGF promoter. ChIP analysis was performed using an antibody specific for JunB (IP: JunB) and IgG as a nonspecific control (IP: ns). Specific primer sets were used for the promoter region harboring the AP-1 site (−1140 to −733). JunB binding to the VEGF promoter region between −1140 and −733 was detected and was enhanced in LLC cells treated with 10 μM NECA for 3 hours. (B) Effects of AP-1 site mutation and dominant negative JunB on VEGF reporter activity. The role of JunB in A_{2B} receptor-dependent regulation of the VEGF gene promoter was studied in LLC cells transiently cotransfected with VEGF luciferase reporter together with plasmid encoding DNJunB or with an empty vector (mock). A set of LLC cells was also transfected with VEGF luciferase reporter containing the mutated AP-1 site at −1093 (indicated on graph as mutAP-1). Twenty-four hours after transfections, cells were incubated in the absence (basal) or presence of 10 μM NECA for an additional 6 hours. Values are presented as mean ± S.E.M. (n = 3). Asterisks indicate statistical difference (**$P < 0.01$) compared with basal levels, and a dagger indicates the statistical difference ($^{\dagger}P < 0.05$) compared with mock controls by one-way ANOVA with Bonferroni post-test. (C) Expression of JunB and DNJunB in LLC cells stably transfected with plasmid encoding DNJunB or with an empty vector (mock). Western blot analysis was performed using antibodies against a common epitope in the sequences of JunB and DNJunB. Arrows indicate positions of JunB and DNJunB. Immunostaining of β-tubulin was used as a loading control. (D) Effect of the expression of dominant negative JunB on VEGF secretion. The role of JunB in A_{2B} receptor-dependent regulation of VEGF secretion was studied in LLC cells stably transfected with plasmid encoding DNJunB or with an empty vector (mock) by measuring VEGF release from cells incubated in the absence (basal) or presence of 10 μM NECA for 6 hours. Values are presented as mean ± S.E.M. (n = 3). Asterisks indicate statistical difference (*$P < 0.05$; ***$P < 0.001$) compared with basal levels; daggers indicate statistical difference ($^{\dagger\dagger}P < 0.01$) compared with mock controls by one-way ANOVA with Bonferroni post test. (E) Efficacy of JunB silencing after stable lentiviral transfection of LLC cells with different shRNA constructs (shRNA1-5) compared with shRNA nontargeting (NT) and empty vector (EV) controls. Upper panels show representative Western blots of basal levels of targeted JunB protein and β-tubulin loading controls. The lower panel is a graphic representation of the data expressed as relative density of JunB bands normalized to corresponding β-tubulin bands and NT control. (F) Real-time reverse-transcription polymerase chain reaction analysis of JunB mRNA levels in LLC cells stably transfected with lentiviral vectors encoding shRNA5 or nontargeting shRNA (NT control). Cells were incubated in the absence (basal) or presence of 10 μM NECA for 60 minutes. Values are normalized to β-actin mRNA expression and expressed as the average of two determinations made in triplicate. (G) Effect of JunB knockdown on VEGF secretion. The role of JunB in A_{2B} receptor-dependent regulation of the VEGF secretion was studied in LLC cells stably expressing JunB silencing shRNA5 or nontargeting shRNA (NT control) incubated in the absence (basal) or presence of 10 μM NECA for 6 hours. Values are presented as mean ± S.E.M. (n = 3). Asterisks indicate statistical difference (*$P < 0.05$; ***$P < 0.001$) compared with basal levels; daggers indicate statistical difference ($^{\dagger\dagger\dagger}P < 0.001$) compared with NT controls by one-way ANOVA with Bonferroni post-test.

receptor-activated PLCβ. Although A_2 receptor-dependent stimulation of Rap1 has been previously reported in cells overexpressing A_2 receptors (Seidel et al., 1999; Schulte and Fredholm, 2003), this is the first evidence of PLC involvement in this process.

Further downstream, inhibition of A_{2B} receptor-dependent stimulation of ERK with U0126 resulted in a partial inhibition of both JunB protein accumulation and VEGF production. Because complete inhibition of A_{2B} receptor-dependent upregulation of JunB and VEGF was not achieved even at U0126 concentrations that almost entirely blocked ERK activity, it is likely that additional pathways are involved in this mechanism. Although stimulation of adenylate cyclase by forskolin also increased JunB protein levels and VEGF production, our data suggest involvement of PKA-independent pathways in this process because the PKA inhibitors with different mechanisms of action H89 and Rp-cAMPS had no effect on the A_{2B} receptor-dependent increase in JunB protein levels and VEGF production.

A_{2B} receptor-dependent stimulation of exchange proteins directly activated by cAMP, which are known to activate Rap1 (de Rooij et al., 1998), was recently demonstrated in

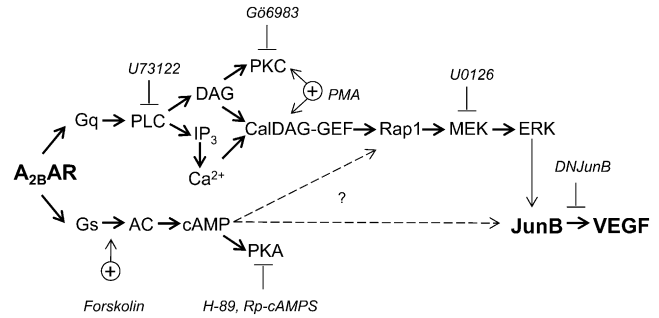

Fig. 9. Schematic representation of A_{2B} receptor-stimulated intracellular pathways involved in regulation of VEGF. A_{2B} adenosine receptors (A_{2B}AR) are coupled to adenylate cyclase (AC) via G_s proteins. Activation of this pathway results in accumulation of cAMP and stimulation of PKA. A_{2B}AR are coupled also to PLC via a GTP-binding protein of the G_q family. Activation of this pathway results in accumulation of DAG and inositol trisphosphate (IP_3), and the latter triggers mobilization of calcium from intracellular stores (Feoktistov and Biaggioni, 1995). In this study, we present evidence that A_{2B}AR increase JunB protein levels and VEGF production via stimulation of PLC and ERK, which are possibly linked by the CalDAG-GEF–Rap1 pathway. PMA, an activator of PKC and CalDAG-GEF, also increased JunB protein levels and VEGF production. However, the broad-spectrum PKC inhibitor Gö6983 had no effect on the A_{2B}AR-dependent increase in JunB protein levels and VEGF production. In contrast, the PLC inhibitor U73122 inhibited the A_{2B}AR-dependent Rap1 activation, the increase in JunB protein levels, and VEGF production. The MEK inhibitor U0126 blocked the A_{2B}AR-dependent stimulation of ERK and inhibited an increase in JunB protein levels and VEGF production. Stimulation of AC by forskolin also increased JunB protein levels and VEGF production. Broken arrows in the diagram signify potential effects of cAMP. These effects are PKA-independent because the PKA inhibitors with different mechanisms of action H-89 and Rp-cAMPS had no effect on the A_{2B}AR-dependent increase in JunB protein levels and VEGF production. Finally, the overexpression of a DNJunB inhibited A_{2B}AR-dependent increase in VEGF transcription and secretion, indicating an important role of JunB in this process.

endothelial cells (Fang and Olah, 2007). Whether this mechanism involved in A_{2B} receptor-dependent increase in JunB protein levels and VEGF production remains to be elucidated. Finally, the overexpression of dominant negative mutant of JunB or JunB knockdown with shRNA inhibited A_{2B} receptor-dependent increase in VEGF production, indicating an important role of JunB in this process. Thus, despite the apparent complexity and potential crosstalk between signaling pathways, our inhibitory analysis revealed a clear association and causal relationship between A_{2B} receptor-dependent regulation of JunB accumulation and VEGF secretion.

In conclusion, our study has demonstrated that A_{2B} adenosine receptor-dependent upregulation of JunB is a common feature shared by different cells known to be involved in angiogenesis. In addition to the regulation of VEGF production examined in this study, JunB has been implicated in cell cycle regulation (Hess et al., 2004), endothelial cell morphogenesis (Licht et al., 2006), osteoblast differentiation (Kenner et al., 2004), myeloid cell differentiation (Passegue et al., 2001), mast cell degranulation, and cytokine release (Textor et al., 2007). Almost all of these events are reportedly regulated also by adenosine via A_{2B} receptors (Feoktistov and Biaggioni, 1995, 1997; Auchampach et al., 1997; Grant et al., 2001; Novitskiy et al., 2008; Ryzhov et al., 2011; Carroll et al., 2012). Therefore, it would be interesting to determine whether upregulation of JunB represents a common and important step in A_{2B} receptor-dependent regulation of these cell functions by extracellular adenosine.

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Authorship Contributions

Participated in research design: Ryzhov, Biktasova, Dikov, Feoktistov.

Conducted experiments: Ryzhov, Biktasova, Goldstein, Zhang.

Performed data analysis: Ryzhov, Biktasova, Feoktistov.

Wrote or contributed to the writing of the manuscript: Ryzhov, Biktasova, Dikov, Biaggioni, Feoktistov.

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