Novel Mitogenic Effect of Adenosine on Coronary Artery Smooth Muscle Cells
Role for the A1 Adenosine Receptor

Jianzhong Shen, Stephen P. Halenda, Michael Sturek, Peter A. Wilden

Abstract—Adenosine is a vascular endothelial cell mitogen, but anti-mitogenic for aortic smooth muscle cells and fibroblasts when acting via the A2B adenosine receptor. However, we show that adenosine increases porcine coronary artery smooth muscle cell (CASMC) number, cellular DNA content, protein synthesis, and PCNA staining. RT-PCR analysis indicates that porcine CASMC express A1, A2A, A2B, and barely detectable levels of A3R receptor mRNAs. The mitogenic effect of adenosine is mimicked by NECA, CCPA, and R-PIA, but not by CGS21680 and 2-CI-IB-MECA, and is inhibited by DPCPX, indicating a prominent role for the A1 receptor. This interpretation is supported by the finding that adenosine- and CCPA-induced DNA synthesis is significantly inhibited by pertussis toxin, but substantially potentiated by PD81723, an allosteric enhancer of the A1 receptor. When a cDNA encoding the porcine A1 receptor was cloned and expressed in COS-1 cells, A1 receptor pharmacology is confirmed. Anti-sense oligonucleotides to the cloned sequence dramatically suppress the mitogenic effect of adenosine and CCPA. Conversely, over-expression of the cloned A1 receptor in CASMC increases adenosine- and CCPA-induced DNA synthesis. Furthermore, stimulation with adenosine or CCPA of intact coronary arteries in an organ culture model of vascular disease increases cellular DNA synthesis, which was abolished by DPCPX. We conclude that adenosine acts as a novel mitogen in porcine CASMC that express the A1 adenosine receptor, possibly contributing to the development of coronary artery disease. (Circ Res. 2005;96:000-000.)

Key Words: adenosine receptors • coronary artery smooth muscle cells • proliferation • molecular cloning • porcine

The diverse cellular actions of adenosine are mediated by a family of adenosine receptors (ARs), of which four subtypes (A1R, A2AR, A2BR, and A3R) have been cloned and pharmacologically characterized. In general, the A1R and A2AR are coupled with Gs proteins, whose activation causes a decrease of intracellular cAMP. In contrast, activation of the Gi-coupled A2BR and A3R increases intracellular cAMP. Thus, the end biological action of adenosine in a particular organ or cell population may depend on the relative expression level and signaling efficiency of the individual AR subtypes.

Adenosine, like many other vasodilators, historically has been thought to act as an inhibitor of the proliferation of vascular smooth muscle cells (VSMCs). This contention was supported by recent studies in cultured aortic VSMCs, showing that adenosine was antimitogenic through its activation of the A2B receptor. To the best of our knowledge, however, current studies of the long-term effects of adenosine on VSMC proliferation have been limited to aortic smooth muscle.

Given the heterogeneity of VSMCs and the diversity of the expression profile of ARs in cells from different blood vessels, it remains to be shown whether the antiproliferative action of adenosine can be extended to other VSMCs, particularly coronary artery smooth muscle cells (CASMCs).

In porcine CASMCs, previous work in our laboratory has demonstrated that ATP, a major precursor of adenosine, induced a proliferative response via activation of several intracellular signaling pathways, involving activation of the P2Y1 receptor. In the process of identifying the P2Y independent receptor mechanism(s) responsible for ATP-induced CASMC proliferation, we unexpectedly found that adenosine, but not most other nucleotides/nucleosides, mimicked the effects of ATP. This observation led us to propose that, in contrast to the previous observation in aortic VSMCs, adenosine might behave as a mitogen in CASMCs by a receptor mechanism not involving A2B. Indeed, ligand-binding assays and histological studies indicated that porcine coronary arteries express A1R. We therefore hypothesized that A1R activation causes CASMC proliferation.
Because no porcine AR has been cloned, in the present study, we have cloned and pharmacologically characterized the porcine A1R and explored its role in CASMC proliferation. Results presented here demonstrate that adenosine acts via the A1R as a mitogen in cultured porcine CASMCs and in intact coronary arteries.

Materials and Methods

CASMIC Isolation and Cell Culture

Porcine CASMCs dispersed from right coronary arteries of farm pigs were cultured as previously described in our laboratory. Smooth muscle cell lineage was confirmed by immunocytochemistry. Stock CASMC cultures were maintained in a subconfluent state and used between passage 4 and 10.

[^H]-Thymidine Incorporation Assays

CASMCs (35,000/well) were seeded in 12-well plates in DMEM/high-glucose medium containing 10% FBS for 48 hours, and then starved for 24 hours in serum-free DMEM containing 0.1% BSA before stimulation. Incorporation of [^H]-thymidine into DNA was performed as previously described.

Protein Synthesis Assays

CASMCs were seeded and starved as stated earlier. [^H]-leucine incorporation was used for measuring cellular total protein synthesis as previously described.

Determination of Cell Numbers

CASMCs were seeded in 96-well plates (2000 cells/well) in DMEM containing 10% FBS for 48 hours, and then serum-starved for 48 hours, after which the culture medium were replaced with fresh serum-free starvation medium and CASMCs were stimulated with agonists for 4 days. Cell numbers were determined by MTT Cell Growth Assay Kit (Chemicon) as previously described.

Immunocytochemistry for Proliferating Cell Nuclear Antigen

CASMCs at ~40% confluence were starved for 24 hours before stimulation with adenosine for 24 hours. Standard immunocytochemistry for proliferating cell nuclear antigen (PCNA) was performed using rabbit anti-PCNA (Santa Cruz Biotechnology).

Receptor cDNA Cloning and RACE Experiments

The full cDNA sequence of the porcine A1R and partial cDNA coding sequences for porcine A2aR, A2bR, and A3R were cloned by PCR using a Smart Race cDNA amplification kit (Clontech).

Heterologous Expression

The pCR3.1 plasmids containing the porcine A1R sequence were transfected into COS-1 cells using Effectene Transfection Reagent (Qiagen).

Effect of Adenosine on CASMC Proliferation

Stimulation of cultured porcine CASMCs with adenosine-induced increases of the cellular DNA content, protein synthesis, and cell number in a concentration-dependent manner, with an apparent EC[	extsubscript{50}] ~10 μmol/L (Figure 1A through 1C). On average, adenosine, at 100 μmol/L, induced ~5-fold and ~2.5-fold increases in cellular DNA content and protein synthesis respectively (Figure 1A and 1B). Consistent with this observation, a corresponding ~30% increase in the total cell number was induced after a 4-day incubation of CASMCs with adenosine (Figure 1C). In addition, adenosine stimulation (100 μmol/L, 24 hours) nearly doubled the

**A1R Overexpression in CASMCs**

CASMCs were transfected in 12-well plates using the Effectene Transfection Reagent according to the manufacturer’s instruction.

Antisense Oligonucleotide Experiments

Phosphorothioate-modified oligonucleotide (OGN) (antisense, 5’-GAGATGGAGGGCGCCATGTT-3’; sense, 5’-ACCATGCGGCC-CTCATCCTC-3’) were used for A1R knockdown as previously documented.

Western Blot Analysis

Cells were lysed and lysates Western blotted for the A1R (rabbit polyclonal antibody, 1:1000 dilutions, Novus Biologicals, Inc). Membranes were stripped and reprobed with anti-actin antibody (1:1000; Cytoskeleton).

Ex Vivo Coronary Artery Organ Culture and DNA Synthesis Assays

Pig hearts were isolated and arteries organ cultured as described in our laboratory. Incorporation of [^H]-thymidine into coronary arterial rings was performed as described previously.

Reverse-Transcription Polymerase Chain Reaction Analysis

Primers for reverse-transcription polymerase chain reaction (RT-PCR) were designed based on our original cloned sequences for the 4 porcine ARs (GenBank accession nos. Y772411, Y772412, Y772413, and Y772414). The following compounds were from TOCRIS: 5’-N-ethylcarboxamidoadenosine (NECA); 2-chloro-N’-cyclopropyladenosine (CPA); 2-[p-(carboxyethyl) phenylethylamino]-5’-N-ethylcarboxamidoadenosine (CGS21680); 2-chloro-N’-(3-iodobenzyl)-adenosine-5’-N-methylcarboxamide (2-Cl-IB-MECA); 8-cyclopentyl-1,3-dipropylyxanthine (DPCPX); 4-(2-[7-amino-2-[2-furyl]-1,2,4-triazolo[2,3-a]-1,3,5-triazin-5-yl-amino]ethyl)phenol (ZM241385); N-(4-acetyl-phenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]-acetamide (MRS 1706); N’-(2-methoxyphenyl)-N-[2-(3-pyridyl) quinolin-4-yl]urea (VU5574). Reagents from Sigma were as following: 3,7-dimethyl-1-propargylxanthine (DMXPA), (−)-N’-(2-phenilisopropyl)adenosine (R-PIA); platelet-derived growth factor (PDGF); and lysophosphatidic acid (LPA).

Analysis of Data

Data are expressed as mean±SEM. Means of 2 groups were compared using Student t test (unpaired, two tailed), and one-way ANOVA was used for comparison of more than 2 groups with P<0.05 considered to be statistically significant. An expanded Materials and Methods can be found in the online data supplement available at http://circres.ahajournals.org.

Results

Effect of Adenosine on CASMC Proliferation

Stimulation of cultured porcine CASMCs with adenosine-induced increases of the cellular DNA content, protein synthesis, and cell number in a concentration-dependent manner, with an apparent EC[	extsubscript{50}] ~10 μmol/L (Figure 1A through 1C). On average, adenosine, at 100 μmol/L, induced ~5-fold and ~2.5-fold increases in cellular DNA content and protein synthesis respectively (Figure 1A and 1B). Consistent with this observation, a corresponding ~30% increase in the total cell number was induced after a 4-day incubation of CASMCs with adenosine (Figure 1C). In addition, adenosine stimulation (100 μmol/L, 24 hours) nearly doubled the
number of PCNA-positive cells (Figure 1D through 1F). These results clearly indicate that adenosine is a CASMC mitogen.

**AR mRNA Expression in Cultured CASMCs and Intact Coronary Arteries**

Figure 2 shows that cultured CASMCs expressed high levels of the mRNAs for A1R, A2aR, and A3R, with a barely detectable mRNA level for A2bR. A similar expression pattern was found in native CASMCs isolated from pig right coronary arteries denuded of endothelium (Figure 2). Of note, although our RT-PCR approach only was able to amplify very faint bands for the A2bR in CASMCs, the same experimental condition resulted in a robust amplification for the A2bR mRNA in primary cultured porcine coronary endothelial cells (Figure 2B). Thus, our RT-PCR analysis suggests that porcine CASMCs primarily express A1R, A2aR, and A3R but not A2bR.

**Effect of AR Agonists on CASMC Proliferation**

Stimulation of CASMCs with NECA, a nonselective AR agonist, fully mimicked the mitogenic effect of adenosine as shown by increases in the cellular DNA/protein synthesis and cell number (Figure 3). As expected, NECA exhibited a similar efficacy to adenosine, yet it was more potent than adenosine in triggering cell proliferation as evidenced by one log-unit left shift of its dose-response curves (Figure 3A and 3B). In addition, less efficacious, but more potent, mitogenic effects were observed for R-PIA or CCPA, two A1R-selective agonists (Figure 3). In contrast, CGS21680 and 2-Cl-IB-MECA, A2AR and A3R-selective agonists, respectively, were essentially inactive up to 1 µmol/L (Figure 3). These results suggest a major role for A1R in mediating the mitogenic action of adenosine.

**Effect of AR Antagonists on CASMC Proliferation**

Figure 4 shows that adenosine-, NECA-, and CCPA-induced DNA/protein synthesis were dramatically inhibited in a concentration-dependent manner by DPCPX, a highly selective A1R antagonist. Interestingly, although CCPA-induced DNA/protein synthesis were nearly abolished by 0.1 µmol/L DPCPX, the effects of adenosine and NECA were only inhibited by ≈70% to 90% (Figure 4A and 4B). In addition, treatment of the cells with MRS1706 or VUF5574, A2bR- and
A3R-selective antagonists, respectively, had no significant impact on the synthesis of DNA (Figure 4C) and protein (Figure 4D) induced by adenosine, NECA, or CCPA. This indicates that A2BR and A3R may not be critical in the mitogenic action of adenosine. In analysis of A2AR, however, we observed an apparent conflicting result in that DMPX, a broad A2-selective antagonist even at 10 μmol/L, did not affect the DNA/protein synthesis induced by adenosine, NECA, or CCPA, whereas ZM241385, an A2AR-selective antagonist, unexpectedly inhibited not only the effects of adenosine and NECA, but also the effect of CCPA by ≈30% at the DNA level and ≈50% at the protein level (Figure 4C and 4D). Therefore, we question the selectivity of ZM241385 on the porcine A2AR versus A1R.

Molecular Cloning and Pharmacological Characterization of the Porcine A1R

To overcome the potential drawback of our pharmacological analysis that relied on the ligand-selectivity inferred from the A1R of other species, we cloned the porcine A1R and characterized its pharmacological properties. By degenerate PCR and RACE approaches, we successfully isolated the porcine A1R cDNA with an open reading frame of 981 bp (GenBank accession no. AY772411). Hydrophobicity analysis showed a deduced primary sequence of 326 amino acids containing 7 hydrophobic domains characteristic of G protein–coupled receptors (Figure 5A). Sequence alignment revealed that the porcine A1R had the highest amino acid identity with human A1R (94.2%), and like the A1R of other species,19 displayed very short N- and C-termini (Figure 5A). In addition, a possible palmitoylation site,20 2 putative N-glycosylation sites, and several protein kinase phosphorylation sites were conserved (Figure 5A).

The cloned cDNA was stably transfected into COS-1 cells devoid of ARs,21 and its binding properties were compared with the native receptor in CASMCs. Figure 5B shows membranes from the transfected COS-1 cells displayed high-affinity binding for [3H]-DPCPX (Kd=0.58±0.06 nmol/L) with a Bmax of 290.6±44.3 fmol/mg. A similar binding site was also determined in CASMC membranes (Kd=0.59±0.08 nmol/L) with a Bmax of 28.77±6.45 fmol/mg. No specific binding to [3H]-DPCPX was detected in the membranes of nontransfected COS-1 cells (data not shown). In addition,
competition binding assays revealed the rank order of potency for the agonists was CCPA \( \sim R \)-PIA > NECA > 2-Cl-IB-MECA > CGS21680, and of the antagonists was DPCPX \( \sim ZM241385 > MRS1706 > DMPX \sim VUF5574 \) (Figure 5C), reflecting characteristics of typical A1R.

Figure 5D shows that in A1R-transfected COS-1 cells, CCPA inhibited forskolin-induced cAMP elevation, and increased \( [Ca^{2+}]_i \) in a concentration-dependent manner. CCPA also inhibited forskolin-induced cAMP accumulation in cultured CASMCs, albeit in a lower efficacy (Figure 5D). We, however, did not observe any \( Ca^{2+} \) signaling on CCPA stimulation of CASMCs. Consistent with the literature,1 the A1R-mediated \( Ca^{2+} \) response in COS-1 cells was completely prevented by pertussis toxin (PTX) pretreatment (data not shown), indicative of coupling to \( G_{i/o} \) proteins.

**Effects of A1R Antisense OGN and Overexpression on CASMC Proliferation**

To extend our pharmacological analysis, we designed antisense OGN to the porcine A1R and tested their effects on cell proliferation. Figure 6A shows antisense, but not sense, OGN substantially decreased A1R protein expression as evaluated by Western blotting. Reduction of the A1R by antisense, but not sense, OGN decreased adenosine- and NECA-induced DNA synthesis by more than 80% and also nearly abolished the action of CCPA, with no effect on LPA-induced DNA synthesis (Figure 6B).

Figure 6D shows that A1R overexpression had no significant effect on the basal DNA synthesis as compared with mock transfection (Figure 6D). However, A1R overexpression potentiated adenosine- and CCPA-induced DNA synthesis by 53% and 61%, respectively, whereas the response to LPA was not affected (Figure 6D). A moderate increase of the transfected A1R protein expression was confirmed by Western blotting (Figure 6C).

**Effects of PTX and PD81723 on CASMC Proliferation**

Pretreatment of CASMCs with PTX (G, protein inhibitor, 100 ng/mL, 24 hours) significantly suppressed, but did not abolish, both adenosine- and NECA-induced DNA synthesis, whereas the action of CCPA was completely inhibited (Figure 7A). These results are consistent with our antagonist and antisense results.

PD81723 can allosterically enhance A1R binding and signaling.1 Treatment of the cells with PD81723 (3 \( \mu \)mol/L) significantly potentiated adenosine- and CCPA-induced cellular DNA synthesis, with no effect on the basal or LPA-induced DNA synthesis (Figure 7B). The opposite effects of PTX and PD81723 provided additional evidence that A1R can mediate CASMC proliferation.

**Dual Effects of Adenosine and CCPA on DNA Synthesis in Organ-Cultured Coronary Arteries**

We next extended our observation in cell culture into the ex vivo model of vessel cultures. Treatment of the endothelium-denuded porcine coronary artery rings with adenosine or CCPA induced a stimulatory effect on DNA synthesis at low concentrations and an inhibitory action at high concentrations (Figure 8A). Specifically, adenosine at 10 \( \mu \)mol/L and CCPA at 10 nmol/L significantly increased DNA synthesis by 34.7% and 40.3%, respectively, whereas at the highest concentrations, both inhibited DNA synthesis by 38.9% and 15.8%, respectively (Figure 8A). Strikingly, the magnitude of increase in DNA synthesis triggered by CCPA was comparable to the maximal action of PDGF (Figure 8A).

Finally, we examined whether the stimulatory effects of adenosine and CCPA are because of A1R activation. Figure 8B shows that pretreatment of the artery rings with DPCPX (0.1 \( \mu \)mol/L) abolished adenosine- and CCPA-induced increases of DNA synthesis, without affecting the basal and...
PDGF-induced DNA synthesis, indicative of a predominant role for the A1R.

**Discussion**

In the present study, we have demonstrated for the first time that adenosine can stimulate the proliferation of porcine CASMCs in culture and in intact coronary arteries. We also have demonstrated that the mitogenic effect of adenosine on CASMCs is predominately mediated by the A1R that has been cloned and pharmacologically characterized.

Historically, it has been thought that contractile vasoactive substances such as TxA2, angiotensin II, and endothelin-1 have stimulatory effects on VSMC growth, whereas smooth muscle dilators, eg, prostacyclin and nitric oxide, inhibit VSMC proliferation.7 In line with this concept, Jonzon et al22 observed nearly 2 decades ago that in cultured rat aortic VSMCs, adenosine inhibited cellular DNA synthesis. Recently, using the same model of cultured aortic VSMCs, Dubey et al reported a series of studies supporting the antimitogenic effect of adenosine and further highlighting a specific role for the A2BR.4–8 In addition, an A2BR-mediated proapoptotic effect of adenosine in cultured human aortic VSMCs has also been reported.23 Despite these consistent observations, it remained unknown whether the growth-inhibitory effect of adenosine on aortic VSMCs can be generalized to VSMCs from other locations. Raising this point is important because adenosine is mitogenic in vascular endothelial cells via activation of the same A2BR.24,25 In the present study, we found that stimulation of cultured porcine CASMCs with adenosine increased cellular DNA content, protein synthesis, cell number, and PCNA staining, indicating a mitogenic action of adenosine. Importantly, our data further
showed that this mitogenic effect of adenosine extends to intact coronary arteries in an organ culture model of vascular disease. Thus, our results are in sharp contrast to the aforementioned studies and challenge the conceptual view that vasodilators can only function as antimitogens in VSMCs. In this regard, it is tempting to postulate that at least in the porcine conduit coronary arteries, adenosine may be a unique vasodilator that also functions as a mitogen in smooth muscles.

How can we reconcile the contradictory results between our study and those of others? The precise reasons for this discrepancy are unclear, but several factors could potentially account for the difference, eg, the difference in experimental methodology and animal species. However, the most important factor that could explain such a difference is a possible differential receptor expression mechanism. The hypothesis we propose here is that VSMC A1R mediates mitogenesis, whereas A2BR mediates antimitogenic effects. Therefore, the end functional readout in response to adenosine with respect to proliferation versus antiproliferation would highly depend on the balance of the expression levels and the signaling efficiency of these two receptors. In the present study, we found porcine CASMCs expressed a high level of A1R as demonstrated by the high mRNA level, detectable ligand binding, positive immunoblotting, and potent cAMP modulation. In contrast, a barely detectable message for A2BR, together with the fact that the A2BR-selective antagonist did not affect adenosine-induced cell proliferation, strongly indicate that porcine CASMCs did not express a significant number of functional A2BR. Thus, lack of the growth-inhibitory A2BR could render porcine CASMCs proliferative in response to adenosine via activation of the highly expressed A1R. Evidence supporting this view includes the following: (1) A1R-selective agonists mimicked the mitogenic action of adenosine; (2) A1R-selective antagonist blocked adenosine-, NECA- and CCPA-induced DNA/protein synthesis; (3) adenosine- and CCPA-induced DNA synthesis was inhibited by PTX, but potentiated by PD81723, an allosteric A1R enhancer; (4) antisense knockdown of A1R decreased adenosine- and CCPA-induced DNA synthesis; and (5) conversely, A1R overexpression increased the magnitude of the responses to adenosine and CCPA. Thus, we have provided several lines of compelling data demonstrating that A1R plays a key role in adenosine-mediated CASMC proliferation, a finding that is consistent with a recent study showing stimulation of angiogenesis by A1R agonists.26

Figure 6. Effects of A1R antisense OGN and overexpression on the DNA synthesis of CASMCs. CASMCs were pretreated with vehicle (control), transfection reagent (Trans. Reagent), antisense oligonucleotides to porcine A1R (antisense OGN, 0.3 μg), or the sense OGN (0.3 μg) (A). Western blotting was performed to confirm the knockdown of endogenous A1R. Molecular weights of A1R and actin are 39 and 43 kDa, respectively. In parallel, pretreated CASMCs were stimulated with adenosine (100 μmol/L), NECA (10 μmol/L), CCPA (0.1 μmol/L), or LPA (100 μmol/L) for 48 hours, after which cellular DNA content was determined and the data were transformed to the percentage inhibition of the controls (B). In another experiment, CASMCs were transfected with vehicle (control), transfection reagent (Trans. Reagent), pCR3.1 containing the porcine A1R cDNA (Plasmid-A1R), or mock pCR3.1 (C). Western blotting was performed to confirm A1R overexpression. In parallel, the cellular DNA content was determined after stimulation of posttransfected CASMCs with adenosine (100 μmol/L), NECA (10 μmol/L), CCPA (0.1 μmol/L), or LPA (100 μmol/L) for 48 hours (D). Data shown are the mean±SEM from four independent experiments performed in triplicate. *P<0.01 relative to the respective control.
be noted that the antimitogenic effect of adenosine on rat aortic VSMCs was observed in the presence of serum or PDGF, both of which are potent mitogens that could mask the relatively weak growth-promoting signals from the A₁R that could be in a lower expression level than the A₂AR. In addition, it should also be emphasized that the antimitogenic effect of adenosine on rat aortic VSMCs was observed at low, but not standard, cell seeding density. Therefore, the contradictory results between our study and others could be reconciled by a differential receptor expression mechanism, because we found the following: (1) adenosine increased the DNA synthesis of CASMCs seeded in standard and low density, and further increased serum- and PDGF-induced DNA synthesis (see online Figures 1 and 2 available in the online data supplement); (2) in contrast, adenine has no or minor effects on the DNA synthesis of aortic VSMCs in the absence of serum (online Figure 3); and (3) importantly, overexpression of A₁R in aortic VSMCs changed the effect of adenosine from antimitogenic to mitogenic (online Figures 5 and 6).

Although there were no significant growth-inhibitory signals generated by adenosine in CASMCs, it should be noted that the A₂AR are highly expressed in porcine CASMCs and
intact coronary arteries, and it has been shown that adenosine-induced relaxation of coronary arteries from pig\textsuperscript{27,28} and mouse\textsuperscript{29} are mediated by A\textsubscript{2b}R, as opposed to A\textsubscript{3}R in rat coronary arteries.\textsuperscript{30} Although A\textsubscript{2b}R and A\textsubscript{3}R may similarly increase cAMP, perhaps the A\textsubscript{2b}R and A\textsubscript{3}R are more strongly coupled to relaxation and antiproliferation, respectively. Thus, adenosine-induced relaxation and proliferation in porcine CASMCs could be 2 unrelated cellular events driven by different receptors. From this point of view, adenosine is unique, because virtually all other vasodilators inhibit VSMC growth, perhaps because of their targeting on either one receptor, enzyme or ion channel in VSMCs.

The A\textsubscript{1}R have remarkable species differences in pharmacology. The bovine, canine,\textsuperscript{31} and guinea pig\textsuperscript{1} homologs are the most divergent as compared with human, rabbit, rat, and mouse with respect to the agonist binding affinity. Porcine CASMCs have been postulated to express A\textsubscript{1}R.\textsuperscript{14,15} However, the molecular and pharmacological properties of this receptor have not been conclusively documented. We now report the cloning of the cDNA for this receptor. Heterologous expression and functional analysis of this receptor demonstrated CCPA and DPCPX are the most potent agonist and antagonist, respectively. In addition, our ligand binding assay revealed the rank order of potency for the agonists/antagonists was similar to the A\textsubscript{1}R cloned from humans, rabbits, and rodents.\textsuperscript{1} The compound ZM241385, however, exhibits a moderate affinity to the porcine A\textsubscript{1}R that is much higher than in other species. Therefore, although this compound has been used as a highly selective A\textsubscript{2b}R antagonist, caution must be taken when used in pig tissues/cells to differentiate the A\textsubscript{2b}R versus A\textsubscript{3}R.

The fact that our results differ from results of prior work in VSMCs of other sites exemplifies the prediction made by Ross\textsuperscript{32} that VSMCs of different embryonic origins could respond differently to stimuli that generate atherosclerotic lesions in an artery segment–dependent manner. Although it is too early to conclude that adenosine is involved in the etiology of coronary artery diseases, several aspects of consideration pertaining to adenosine and the A\textsubscript{1}R point to that potential: (1) the A\textsubscript{1}R is upregulated in diabetes,\textsuperscript{33} hypertension,\textsuperscript{34} and by oxidative stress,\textsuperscript{35} all of which contribute to the development of coronary artery disease; (2) the A\textsubscript{1}R has higher affinity for adenosine than A\textsubscript{2b}R, and the desensitization of A\textsubscript{1}R is much slower than A\textsubscript{2b}R (t\textsubscript{1/2} \approx 10 hours versus \approx 20 minutes),\textsuperscript{36} both properties of which could imply a role for A\textsubscript{1}R in chronic effects on CASMC proliferation; and (3) adenosine is directly released from endothelial cells, stressed myocardial cells, and can also accumulate after catabolism of ATP/ADP released from activated platelets and inflammatory cells surrounding CASMCs after arterial injury.\textsuperscript{1} From these points of view, together with the fact that VSMC proliferation is involved in the development of hypertension and atherosclerosis as well as restenosis after angioplasty and bypass surgery,\textsuperscript{7,8,32} it is conceivable that identification of a novel mitogenic effect of adenosine and defining its receptor mechanism on CASMC will not only increase our understanding of the role of adenosine in the coronary circulation and disease, but also imply potential clinical applications via pharmacological intervention such as A\textsubscript{1}R antagonism for limiting the abnormal growth of CASMCs under the aforementioned diseases.

In summary, we report a novel mitogenic action of adenosine in cultured porcine CASMCs and in ex vivo coronary arteries. In addition, we demonstrate that this effect is predominantly mediated by the A\textsubscript{1}R. These results suggest that adenosine may make a previously unrecognized contribution in vascular diseases related to CASMC proliferation, thereby highlighting the A\textsubscript{1}R as a new potential target for further mechanistic study and pharmacological intervention.

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References


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Expanded Materials and Methods

CASMC Isolation and Cell Culture

Porcine CASMC were dispersed from right coronary arteries of farm pigs as previously described in our lab.\textsuperscript{1,2,3} Cultures of porcine CASMC or COS-1 cells were grown in DMEM/high-glucose medium that contained 10% fetal bovine serum (FBS) at 37°C with 5% CO\textsubscript{2}. Smooth muscle cell lineage was confirmed by \(\alpha\)-smooth muscle actin immunocytochemistry. Stock CASMC cultures were maintained in a sub-confluent state and used between passage 4 and 10.\textsuperscript{1,2,3}

\textsuperscript{[3]H}-Thymidine Incorporation Assays

Incorporation of \textsuperscript{[3]H}-thymidine into DNA was carried out as previously described.\textsuperscript{1,2,3} CASMC (35,000 cells/well) were seeded in 12-well plates in DMEM/high-glucose medium containing 10% FBS at 37°C for 48 h, and then starved for 24 h in DMEM containing 0.1% BSA before cell stimulation. Starved CASMC were treated with or without adenosine or its analogs for 24 h at
37°C in DMEM/high-glucose medium containing 0.1% BSA before the addition of 1 μCi of [methyl-3H]-thymidine (NEN, Wilmington, DE) for an additional 24 h at 37°C. All receptor antagonists were added 30 min before the cell stimulation. The cells were washed three times in ice-cold PBS and solubilized in 0.1% SDS. Trichloroacetic acid was added to a final concentration of 10%, and the precipitate was collected by filtration on glass-fiber disks for determination of radioactivity by liquid scintillation counting.

**Protein Synthesis Assays**

[3H]-leucine incorporation was used for measuring cellular total protein synthesis. The method is similar to that described in "[3H]-thymidine incorporation assay" with the exception that 1 μCi of [3H]-leucine instead of radioactive thymidine was added in the medium in the presence of adenosine receptor agonists/antagonists or LPA.

**Determination of Cell Numbers**

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**Immunocytochemistry for Proliferating Cell Nuclear Antigen (PCNA)**

CASMC were cultured in 8-channel slides and reached to ~40% confluence. After 24 h starvation, cells were stimulated with or without adenosine (100 µmol/L) for another 24 h. By the end of stimulation, cells were washed 3 times in PBS, fixed with -20°C methanol for 10 min, and blocked with 10% goat serum in PBS for 20 min. Fixed cells were treated with the primary
For 1 h, followed by the secondary antibody, Texas Red goat anti-rabbit IgG (1:200 dilution in 1.5% goat serum/PBS, Molecular Probes, Inc.) for another 1 h. Immunofluorescence was detected using a fluorescent microscope (Nikon ECLIPSE E600). Cells with a median pixel intensity ≥ 50 were considered to be PCNA positive as determined with Adobe Photoshop. The numbers of positive cells were normalized to total cell numbers in the same regions of interest counted under the phase contrast. Data were collected in a blinded manner to minimize potential bias.

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water. The second round of PCR was performed as semi-nested PCR, in which the same reverse primer was used, but used a new gene specific forward primer whose sequence is downstream of the original forward primer (5'-CATCGTCTATGCCTTCCGCATCCAGAAG-3'). The PCR conditions were as follows: jump start for 1 min at 94°C, denaturation for 1 min at 94°C, annealing for 1 min at 60°C, and extension at 72°C for 1 min, for 35 cycles.

To obtain the 5’-end of the coding sequence of the porcine A1R, we designed a degenerate forward primer based on the conserved sequence fragment located in the 5’-untranslated region (5’-UTR) of the human and bovine A1R. The degenerate forward primer (5’-CTCGTGCC/ACCTTGGT/CGCC-3’) was paired with gene specific reverse primer (5’-CGATGCAGTTAAGGATGTG-3’) to amplify new PCR product. After sequencing of several products with the predicted size, partial of the 5’-UTR sequence ahead of the start codon was achieved. Then, a final set of primers (forward: 5’-GTCTGCTGAAGTGCCCAGCCTGTG-3’, reverse: 5’-CAGGTGGGAGCAGGTGGA-3’), totally based on the 5’-UTR and 3’-UTR sequence but flanking the open reading frame, was designed, and used for amplification of the entire cDNA coding sequence of the porcine A1R. The PCR products on agarose gel were isolated using a QIAquick Gel Extraction Kit (QIAGEN), and the purified cDNA was ligated into the plasmid vector pCR3.1 (a modified form of pcDNA3.1, INVITROGEN). The ligation product was used to transform One Shot TOP10F’ competent cells (E. coli cells) from the TA cloning kit (INVITROGEN). The cDNA inserts from at least three different clones were sequenced in both directions using T7 and BGH primers. All DNA sequencing work was performed by the DNA Core of the University of Missouri-Columbia.

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were screened for positive expression by testing the Ca\(^{2+}\) response to A\(_1\)R-selective agonist (CCPA), and by RT-PCR detection of the mRNA of the transfected porcine A\(_1\)R.

**Intracellular [Ca\(^{2+}\)] Measurements**

Intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in stably transfected COS-1 cells was measured with the InCa\(^{++}\) calcium imaging system (INTRACELLULAR IMAGING INC.). Cells were grown on glass coverslips for two days and serum-starved for another 24 h and then used at about 40% confluence. After incubation with 2.5 \(\mu\)mol/L fura 2-AM (MOLECULAR PROBES) at 37°C for 30 min, the cells were superfused in a constant-flow superfusion chamber mounted on an inverted epifluorescence microscope (NIKON; model TMD) using physiological salt solution (PSS) containing (mmol/L) NaCl 138, KCl 5, CaCl\(_2\) 2, MgCl\(_2\) 1, HEPES 10, glucose 10, pH 7.4, in the presence or absence of indicated concentrations of CCPA. Fura-2 was excited at 340 nm and 380 nm, and emitted fluorescence (510 nm) was collected using a monochrome CCD camera (COHU). Data were collected as a ratio of emitted light intensity at 340 and 380 nm, and then converted to [Ca\(^{2+}\)]\(_i\) based on a standard curve.\(^3\)

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100 µl of the acetylated supernatant was used for a conventional ELISA assay followed the protocol of the kit. cAMP levels were normalized for protein content of each sample as determined by the Bradford assay.

Radioligand Binding Assays

Binding assays were performed with crude cell membranes prepared from either COS-1 cells stably expressing the porcine A1R or the cultured CASMC using [\textsuperscript{3}H]-DPCPX (130 Ci/mmol; AMERSHAM) as the radioligand as previously described. In brief, cells in confluence were starved for 24-hour, washed 3 times with Ca\textsuperscript{2+}-free PBS, scraped and harvested by a rubber policeman into a total volume of 30 ml of 25 mmol/L sodium phosphate (pH 7.4) and 5 mmol/L MgCl\textsubscript{2}. The suspended cells were lysed by 30-second homogenization and 10-second sonication on ice. The cell lysates were centrifuged at 30,000 g for 15 min at 4 °C. The pellet was resuspended and centrifuged again. The resulting crude cell membranes were suspended in an appropriate volume of the binding buffer (20 mmol/L HEPES-buffer pH 7.4, 10 mmol/L MgCl\textsubscript{2} and 100 mmol/L NaCl) to make aliquots concentration at 1 mg/ml and then stored at -80 °C until used.

For binding assays membranes were thawed, diluted 10 times in the binding buffer and 2 U/ml of adenosine deaminase (SIGMA) was added. Membranes (490 µl) were mixed with [\textsuperscript{3}H]-DPCPX (5 µl) in the presence or absence of the indicated concentrations of unlabeled ligands. Non-specific binding was measured in the presence of 10 µmol/L R-PIA. Binding reactions were incubated at room temperature for 2 h and stopped by rapid filtration through Whatman glass fiber (GF/C) filters using a Cell Harvester. Filters were washed 3-times with 3 ml of ice-cold washing buffer (10 mmol/L Tris-Cl, 1 mmol/L MgCl\textsubscript{2}, pH 7.4). The radioactivity in the filters was determined by liquid scintillation counting at an efficiency of 60%.
A1R Over-expression in CASMC

Porcine CASMC were transfected in 12-well plates using the **Effectene Transfection Reagent** according to the manufacturer’s instruction (QIAGEN). Briefly, 0.3 µg plasmid DNA (pCR3.1-pA1R) was added into the DNA-condensation buffer (buffer EC), mixed with 2.4 µl enhancer and incubated at room temperature for 3 min. Effectene Transfection Reagent of 6 µl were added to the above DNA-enhancer mixture and incubated at room temperature for 10 min to allow transfection-complex formation. CASMC were washed once with phosphate buffered saline (PBS), added fresh DMEM growth medium and overlayed with the transfection-complex diluted in 400 µl DMEM growth medium. To minimize cytotoxicity, the transfection-complex was removed after 6 h transfection by washing the cells with PBS and adding fresh DMEM growth medium. After 18 h post-transfection, CASMC were starved for additional 24 h, and then stimulated with agonists for DNA synthesis assay as described above.

Anti-sense Oligonucleotide Experiments

Phosphorothioate-modified oligonucleotides (OGN) designed according to the cDNA sequence of the porcine A1R cloned in this study were: anti-sense OGN (5’-GAGATGGAGGGCGGCATGGT-3’) and sense OGN (5’-ACCATGCCGCCCTCCATCTC-3’). Sequence uniqueness was checked across multiple databases using BLAST. An optimized concentration of 0.3 µg sense or anti-sense OGN was mixed with the Effectene Transfection Reagent and transfected the cultured CASMC as described above. Cellular uptake of OGN was verified using FITC-conjugated OGN under the same experimental conditions. Western blot analysis was performed in parallel to verify the suppression of A1R expression by anti-sense OGN.

Western Blot Analysis
After transfections, CASMC were serum deprived for 24 h to induce quiescence, washed with PBS, solubilized in Laemmli sample buffer with 200 mmol/L dithiothreitol, and boiled. Lysates were sonicated to disrupt DNA, and proteins were separated on 10% SDS-PAGE gels. The proteins were electrophoretically transferred to nitrocellulose in 25 mmol/L Tris, 192 mmol/L glycine, 20% methanol, and 0.02% SDS. The nitrocellulose was blocked with 5% nonfat dry milk in TBST buffer (20 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, and 0.01% Tween 20). The membranes were probed with a rabbit polyclonal antibody specific for the A1R (1:1000 dilutions, Novus Biologicals, Inc) overnight in 3% BSA in TBST buffer. The blots were washed with TBST buffer and the bound antibody was detected by a horseradish peroxidase-conjugated anti-rabbit IgG and enhanced chemiluminescence (Pierce, Rockford, IL). As a loading control, membranes were stripped of original antibodies, and re-probed with primary anti-actin antibody (1:1000; CYTOSKELETON).

**Ex Vivo Coronary Artery Organ Culture Model of Vascular Disease and DNA Synthesis Assays**

Pig hearts were isolated and prepared as described in our laboratory.³,⁵ Briefly, under sterile conditions, the left and right coronary arteries were dissected, denuded of endothelium and cut into about 10 segments of 3~5 mm per artery. The artery rings were randomly divided and placed in 24-well culture dishes containing 1 ml of DMEM medium without serum. This model of vascular disease is characterized by increased proliferation (bromodeoxyuridine incorporation into DNA), dedifferentiation of sarcoplasmic reticulum morphology, and increased expression of P2Y₂ nucleotide receptors.³,⁵

Incorporation of [³H]-thymidine into coronary arterial rings was performed as described previously.⁶,⁷ Briefly, after initial 2 h equilibration under a standard cell culture condition (37 °C, 5% CO₂), the arterial rings were stimulated with adenosine, CCPA, PDGF or the vehicle for
additional 24 h, during which 1 µCi of [methyl-\(^3\)H]-thymidine was added in the last 18 h of incubation. Of note, the antagonist, DPCPX, was added 40 min before the agonist challenge. By the end of stimulation, arterial rings were washed 3 times with ice-cold PBS, and further incubated with 1 ml of the unlabeled thymidine (1 mmol/L in PBS) for 30 min to displace [\(^3\)H]-thymidine of non-specific binding. Finally, the arterial rings were digested overnight in 1 ml of NaOH (0.5 mol/L) at 60 ºC. Aliquots of 200 µl were used for determination of radioactivity by liquid scintillation counting, and the data were normalized to protein concentrations.

**Reverse-Transcription Polymerase Chain Reaction (RT-PCR) Analysis**

Total RNA was isolated from CASMC cultured in quiescence or dispersed from freshly isolated porcine coronary conduit arteries and RT-PCR protocols were performed as described above in “Receptor cDNA Cloning and RACE Experiments”. Sets of primers used for detecting mRNAs for individual adenosine receptor subtypes were: porcine A\(_1\)R (Shen & Sturek, GenBank accession No. AY772411), forward: 5’-CCTCATCCTCTTCTTTGCCC-3’ and reverse: 5’-CTAGTCATCAGGCTCTTTGCTGCC-3’, yielding a 270 bp product; porcine A\(_2\)AR (Shen & Sturek, GenBank accession No. AY772412), forward: 5’-GATCAGCCTCGCCTCAACG-3’ and reverse: 5’-TCAGGACACTCCTGTCTGCTC-3’, yielding a 240 bp product; porcine A\(_2\)BR (Shen & Sturek, GenBank accession No. AY772413), forward: 5’-CCTCCAGCGGGAGATCCA-3’ and reverse: 5’-GCGGAAGTCTCGGTTCCG-3’, yielding a 220 bp product, and porcine A\(_3\)R (Shen & Sturek, GenBank accession No. AY772414), forward: 5’-TGCCAGTTCCGTTTGTACAGGAGG-3’ and reverse: 5’-TGACAGGCAGGCAGCCGACC-3’, yielding a 320 bp product. The PCR conditions were as described in “Receptor cDNA Cloning and RACE Experiments”. The resulting PCR products were resolved on a 2% agarose ethidium bromide gel and the amplified bands were visualized with ultraviolet light.

**Materials**

All the cell culture media were from INVITROGEN. The following compounds were purchased from TOCRIS: 5’-N-ethylcarboxamidoadenosine (NECA), 2-chloro-N6-cyclopentyladenosine.
(CCPA), 2-[p-(2-carboxyethyl) phenylethylamino]-5'-N-ethylcarboxamidoadenosine (CGS 21680), 2-chloro-N(6)-(3-iodobenzyl)-adenosine-5'-N-methyl-carboxamide (2-Cl-IB-MECA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo-{2,3-a}{1,3,5}-triazin-5-yl-amino]ethyl)phenol (ZM 241385), N-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]acetamide (MRS 1706), N-(2-methoxyphenyl)-N-[2-(3-pyridyl) quinazolin-4-yl]urea (VUF 5574). 3,7-dimethyl-1-propargylxanthine (DMPX) and (-)-N(6)-(2-phenylisopropyl)adenosine (R-PIA), Platelet-Derived Growth Factor (PDGF), and Lysophosphatidic Acid (LPA) were purchased from Sigma (St. Louis, MO). PCR primers and OGN were synthesized and purified by Integrated DNA Technologies (IDT).

Analysis of Data
Data are expressed as mean ± S.E.M. The concentration-response curves were fitted by logistic (Hill equation), non-linear regression analysis using GraphPad Prism 4.0 (San Diego, CA). Means of two groups were compared using Student’s t-test (unpaired, two tailed), and one–way ANOVA was used for comparison of more than two groups with p<0.05 considered to be statistically significant.

References


Supplemental Figure 1. Effect of cell density on adenosine- and CCPA-induced DNA synthesis of cultured porcine CASMC. CASMC in standard density (35,000 cells/well) or in low density (7,000 cells/well) were serum-starved for 24 h and then stimulated with indicated concentrations of adenosine (A) or CCPA (B) for 48 h, after which the cellular DNA content was determined. Data shown are the means ± S.E.M. from four independent experiments performed in triplicate. *, p < 0.05; **, p < 0.01 relative to the respective control.
Supplemental Figure 2. Effect of adenosine on serum- and PDGF-induced DNA synthesis of cultured porcine CASMC. CASMC in standard density (35,000 cells/well) were serum-starved for 24 h and then stimulated with 2.5% FBS (fetal bovine serum, A) or 100 ng/L PDGF (B) in the absence or presence of indicated concentrations of adenosine for 48 h, after which the cellular DNA content was determined. Data shown are the means ± S.E.M. from four independent experiments performed in triplicate. *, p < 0.05; **, p < 0.01 relative to the respective control (FBS or PDGF alone).
Supplemental Figure 3. Differential effects of adenosine on the DNA synthesis of coronary and aortic smooth muscle cells. Porcine coronary artery smooth muscle cells (CASMC) or aortic smooth muscle cells (SMC) or rat aortic SMC (A7r5 cell line) in standard density (35,000 cells/well) were serum-starved for 24 h and then stimulated with indicated concentrations of adenosine for 48 h, after which the cellular DNA content was determined. Data shown are the means ± S.E.M. from four independent experiments performed in triplicate. *, p < 0.05; **, p < 0.01 relative to the respective control.
Supplemental Figure 4. Effect of A₁R transfection on adenosine- and CCPA-induced DNA synthesis of COS-1 cells. Wild type COS-1 cells (devoid of adenosine receptors) or COS-1 cells stably transfected with the porcine A₁ adenosine receptor (COS-1-A₁R) or mock plasmid-transfected COS-1 cells (COS-1-pCR3.1) in standard density (35,000/well) were serum-starved for 24 h and then stimulated with 100 µmol/L adenosine or 0.1 µmol/L CCPA for 48 h, after which the cellular DNA content was determined. Data shown are the means ± S.E.M. from three independent experiments performed in triplicate. *, p < 0.01 relative to the control.
**Supplemental Figure 5.** Effect of A₁R over-expression on adenosine inhibition of serum-induced DNA synthesis of rat aortic SMC in low density. Wild type A7r5 cells (rat aortic SMC line) or A7r5 cells transiently transfected with the porcine A₁ adenosine receptor (Aorta SMC-A₁R) or mock plasmid-transfected A7r5 cells (Aorta SMC-pCR3.1) in low density (7,000 cells/well) were serum-starved for 24 h and then stimulated with 100 µmol/L adenosine or 2.5% FBS or both for 48 h, after which the cellular DNA content was determined. Data shown are the means ± S.E.M. from three independent experiments performed in triplicate. *, p < 0.05 relative to FBS stimulation alone.
Supplemental Figure 6. Effect of A$_1$R over-expression on adenosine-induced DNA synthesis of rat aortic SMC in standard density. Wild type A7r5 cells (rat aortic SMC line) or A7r5 cells transiently transfected with the porcine A$_1$ adenosine receptor (Aorta SMC-A$_1$R) or mock plasmid-transfected A7r5 cells (Aorta SMC-pCR3.1) in standard density (35,000 cells/well) were serum-starved for 24 h and then stimulated with 100 µmol/L adenosine for 48 h, after which the cellular DNA content was determined. Data shown are the means ± S.E.M. from three independent experiments performed in triplicate. *, p < 0.01 relative to the control.
Novel Mitogenic Effect of Adenosine on Coronary Artery Smooth Muscle Cells: 
Role for the A₁ Adenosine Receptor

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Expanded Materials and Methods

CASMC Isolation and Cell Culture
Porcine CASMC were dispersed from right coronary arteries of farm pigs as previously described in our lab.¹,²,³ Cultures of porcine CASMC or COS-1 cells were grown in DMEM/high-glucose medium that contained 10% fetal bovine serum (FBS) at 37°C with 5% CO₂. Smooth muscle cell lineage was confirmed by α-smooth muscle actin immunocytochemistry. Stock CASMC cultures were maintained in a sub-confluent state and used between passage 4 and 10.¹,²,³

[^H]-Thymidine Incorporation Assays
Incorporation of[^H]-thymidine into DNA was carried out as previously described.¹,²,³ CASMC (35,000 cells/well) were seeded in 12-well plates in DMEM/high-glucose medium containing 10% FBS at 37°C for 48 h, and then starved for 24 h in DMEM containing 0.1% BSA before cell stimulation. Starved CASMC were treated with or without adenosine or its analogs for 24 h at
37°C in DMEM/high-glucose medium containing 0.1% BSA before the addition of 1 µCi of [methyl-³H]-thymidine (NEN, Wilmington, DE) for an additional 24 h at 37°C. All receptor antagonists were added 30 min before the cell stimulation. The cells were washed three times in ice-cold PBS and solubilized in 0.1% SDS. Trichloroacetic acid was added to a final concentration of 10%, and the precipitate was collected by filtration on glass-fiber disks for determination of radioactivity by liquid scintillation counting.

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Radioligand Binding Assays

Binding assays were performed with crude cell membranes prepared from either COS-1 cells stably expressing the porcine A1R or the cultured CASMC using [³H]-DPCPX (130 Ci/mmol; AMERSHAM) as the radioligand as previously described. In brief, cells in confluence were starved for 24-hour, washed 3 times with Ca²⁺-free PBS, scraped and harvested by a rubber policeman into a total volume of 30 ml of 25 mmol/L sodium phosphate (pH 7.4) and 5 mmol/L MgCl₂. The suspended cells were lysed by 30-second homogenization and 10-second sonication on ice. The cell lysates were centrifuged at 30,000 g for 15 min at 4 °C. The pellet was resuspended and centrifuged again. The resulting crude cell membranes were suspended in an appropriate volume of the binding buffer (20 mmol/L HEPES-buffer pH 7.4, 10 mmol/L MgCl₂ and 100 mmol/L NaCl) to make aliquots concentration at 1 mg/ml and then stored at -80 °C until used.

For binding assays membranes were thawed, diluted 10 times in the binding buffer and 2 U/ml of adenosine deaminase (SIGMA) was added. Membranes (490 µl) were mixed with [³H]-DPCPX (5 µl) in the presence or absence of the indicated concentrations of unlabeled ligands. Non-specific binding was measured in the presence of 10 µmol/L R-PIA. Binding reactions were incubated at room temperature for 2 h and stopped by rapid filtration through Whatman glass fiber (GF/C) filters using a Cell Harvester. Filters were washed 3-times with 3 ml of ice-cold washing buffer (10 mmol/L Tris-Cl, 1 mmol/L MgCl₂, pH 7.4). The radioactivity in the filters was determined by liquid scintillation counting at an efficiency of 60%.
A1R Over-expression in CASMC

Porcine CASMC were transfected in 12-well plates using the *Effectene Transfection Reagent* according to the manufacturer’s instruction (QIAGEN). Briefly, 0.3 µg plasmid DNA (pCR3.1-pA1R) was added into the DNA-condensation buffer (buffer EC), mixed with 2.4 µl enhancer and incubated at room temperature for 3 min. *Effectene Transfection Reagent* of 6 µl were added to the above DNA-enhancer mixture and incubated at room temperature for 10 min to allow transfection-complex formation. CASMC were washed once with phosphate buffered saline (PBS), added fresh DMEM growth medium and overlayed with the transfection-complex diluted in 400 µl DMEM growth medium. To minimize cytotoxicity, the transfection-complex was removed after 6 h transfection by washing the cells with PBS and adding fresh DMEM growth medium. After 18 h post-transfection, CASMC were starved for additional 24 h, and then stimulated with agonists for DNA synthesis assay as described above.

**Anti-sense Oligonucleotide Experiments**

Phosphorothioate-modified oligonucleotides (OGN) designed according to the cDNA sequence of the porcine A1R cloned in this study were: anti-sense OGN (5'-GAGATGGAGGGCGGCATGGT-3') and sense OGN (5'-ACCATGCCGCCCTCCATCTC-3'). Sequence uniqueness was checked across multiple databases using BLAST. An optimized concentration of 0.3 µg sense or anti-sense OGN was mixed with the *Effectene Transfection Reagent* and transfected the cultured CASMC as described above. Cellular uptake of OGN was verified using FITC-conjugated OGN under the same experimental conditions. Western blot analysis was performed in parallel to verify the suppression of A1R expression by anti-sense OGN.

**Western Blot Analysis**
After transfections, CASMC were serum deprived for 24 h to induce quiescence, washed with PBS, solubilized in Laemmli sample buffer with 200 mmol/L dithiothreitol, and boiled. Lysates were sonicated to disrupt DNA, and proteins were separated on 10% SDS-PAGE gels. The proteins were electrophoretically transferred to nitrocellulose in 25 mmol/L Tris, 192 mmol/L glycine, 20% methanol, and 0.02% SDS. The nitrocellulose was blocked with 5% nonfat dry milk in TBST buffer (20 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, and 0.01% Tween 20). The membranes were probed with a rabbit polyclonal antibody specific for the A1R (1:1000 dilutions, Novus Biologicals, Inc) overnight in 3% BSA in TBST buffer. The blots were washed with TBST buffer and the bound antibody was detected by a horseradish peroxidase-conjugated anti-rabbit IgG and enhanced chemiluminescence (Pierce, Rockford, IL). As a loading control, membranes were stripped of original antibodies, and re-probed with primary anti-actin antibody (1:1000; CYTOSKELETON).

Ex Vivo Coronary Artery Organ Culture Model of Vascular Disease and DNA Synthesis Assays

Pig hearts were isolated and prepared as described in our laboratory. Briefly, under sterile conditions, the left and right coronary arteries were dissected, denuded of endothelium and cut into about 10 segments of 3~5 mm per artery. The artery rings were randomly divided and placed in 24-well culture dishes containing 1 ml of DMEM medium without serum. This model of vascular disease is characterized by increased proliferation (bromodeoxyuridine incorporation into DNA), dedifferentiation of sarcoplasmic reticulum morphology, and increased expression of P2Y2 nucleotide receptors.

Incorporation of [3H]-thymidine into coronary arterial rings was performed as described previously. Briefly, after initial 2 h equilibration under a standard cell culture condition (37 °C, 5% CO2), the arterial rings were stimulated with adenosine, CCPA, PDGF or the vehicle for
additional 24 h, during which 1 µCi of [methyl-3H]-thymidine was added in the last 18 h of incubation. Of note, the antagonist, DPCPX, was added 40 min before the agonist challenge. By the end of stimulation, arterial rings were washed 3 times with ice-cold PBS, and further incubated with 1 ml of the unlabeled thymidine (1 mmol/L in PBS) for 30 min to displace [3H]-thymidine of non-specific binding. Finally, the arterial rings were digested overnight in 1 ml of NaOH (0.5 mol/L) at 60 °C. Aliquots of 200 µl were used for determination of radioactivity by liquid scintillation counting, and the data were normalized to protein concentrations.

**Reverse-Transcription Polymerase Chain Reaction (RT-PCR) Analysis**

Total RNA was isolated from CASMC cultured in quiescence or dispersed from freshly isolated porcine coronary conduit arteries and RT-PCR protocols were performed as described above in “Receptor cDNA Cloning and RACE Experiments”. Sets of primers used for detecting mRNAs for individual adenosine receptor subtypes were: porcine A1R (Shen & Sturek, GenBank accession No. AY772411), forward: 5’-CCTCATCCTCTTCTTTCCTTTGGCCCT-3’ and reverse: 5’-CTAGTCATCAGGCTCCTCTCCTG-3’, yielding a 270 bp product; porcine A2AR (Shen & Sturek, GenBank accession No. AY772412), forward: 5’-GATCAGCCTCCGCTCAACGCGCC-3’ and reverse: 5’-TCAGGACACTCCTGCTGTCTCT-3’, yielding a 240 bp product; porcine A2BR (Shen & Sturek, GenBank accession No. AY772413), forward: 5’-CCTCCAGCGGGAGATCC-3’ and reverse: 5’-GCGGAAGTCTCGGTTCCG-3’, yielding a 220 bp product, and porcine A3R (Shen & Sturek, GenBank accession No. AY772414), forward: 5’-TGCCAGTTCCGTTCCGTCATGAGG-3’ and reverse: 5’-TGGGACAGCAGAGCCAAGTACAG-3’, yielding a 320 bp product. The PCR conditions were as described in “Receptor cDNA Cloning and RACE Experiments”. The resulting PCR products were resolved on a 2% agarose ethidium bromide gel and the amplified bands were visualized with ultraviolet light.

**Materials**

All the cell culture media were from INVITROGEN. The following compounds were purchased from TOCRIS: 5’-N-ethylcarboxamidoadenosine (NECA), 2-chloro-N6-cyclopentyladenosine
(CCPA), 2-[p-(2-carboxyethyl) phenylethylamino]-5'-N-ethylcarboxamidoadenosine (CGS 21680), 2-chloro-N(6)-(3-iodobenzyl)-adenosine-5'-N-methyl-carboxamide (2-Cl-IB-MECA), 8- cyclopentyl-1,3-dipropylxanthine (DPCPX), 4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo-{2,3-a}{1,3,5}- triazin-5-yl-amino]ethyl)phenol (ZM 241385), N-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6- dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]acetamide (MRS 1706), N-(2-methoxyphenyl)-N-[2- (3-pyridyl) quinazolin-4-yl]urea (VUF 5574), 3,7-dimethyl-1-propargylxanthine (DMPX) and (-)-N(6)-(2-phenylisopropyl)adenosine (R-PIA), Platelet-Derived Growth Factor (PDGF), and Lysophosphatidic Acid (LPA) were purchased from Sigma (St. Louis, MO). PCR primers and OGN were synthesized and purified by Integrated DNA Technologies (IDT).

**Analysis of Data**

Data are expressed as mean ± S.E.M. The concentration-response curves were fitted by logistic (Hill equation), non-linear regression analysis using GraphPad Prism 4.0 (San Diego, CA). Means of two groups were compared using Student’s t-test (unpaired, two tailed), and one–way ANOVA was used for comparison of more than two groups with p<0.05 considered to be statistically significant.

**References**


Supplemental Figure 1. Effect of cell density on adenosine- and CCPA-induced DNA synthesis of cultured porcine CASMC. CASMC in standard density (35,000 cells/well) or in low density (7,000 cells/well) were serum-starved for 24 h and then stimulated with indicated concentrations of adenosine (A) or CCPA (B) for 48 h, after which the cellular DNA content was determined. Data shown are the means ± S.E.M. from four independent experiments performed in triplicate. *, p < 0.05; **, p < 0.01 relative to the respective control.
**Supplemental Figure 2.** Effect of adenosine on serum- and PDGF-induced DNA synthesis of cultured porcine CASMC. CASMC in standard density (35,000 cells/well) were serum-starved for 24 h and then stimulated with 2.5% FBS (fetal bovine serum, A) or 100 ng/L PDGF (B) in the absence or presence of indicated concentrations of adenosine for 48 h, after which the cellular DNA content was determined. Data shown are the means ± S.E.M. from four independent experiments performed in triplicate. *, p < 0.05; **, p < 0.01 relative to the respective control (FBS or PDGF alone).
Supplemental Figure 3. Differential effects of adenosine on the DNA synthesis of coronary and aortic smooth muscle cells. Porcine coronary artery smooth muscle cells (CASMC) or aortic smooth muscle cells (SMC) or rat aortic SMC (A7r5 cell line) in standard density (35,000 cells/well) were serum-starved for 24 h and then stimulated with indicated concentrations of adenosine for 48 h, after which the cellular DNA content was determined. Data shown are the means ± S.E.M. from four independent experiments performed in triplicate. *, p < 0.05; **, p < 0.01 relative to the respective control.
Supplemental Figure 4. Effect of A₁R transfection on adenosine- and CCPA-induced DNA synthesis of COS-1 cells. Wild type COS-1 cells (devoid of adenosine receptors) or COS-1 cells stably transfected with the porcine A₁ adenosine receptor (COS-1-A₁R) or mock plasmid-transfected COS-1 cells (COS-1-pCR3.1) in standard density (35,000/well) were serum-starved for 24 h and then stimulated with 100 µmol/L adenosine or 0.1 µmol/L CCPA for 48 h, after which the cellular DNA content was determined. Data shown are the means ± S.E.M. from three independent experiments performed in triplicate. *, p < 0.01 relative to the control.
Supplemental Figure 5. Effect of A₁R over-expression on adenosine inhibition of serum-induced DNA synthesis of rat aortic SMC in low density. Wild type A7r5 cells (rat aortic SMC line) or A7r5 cells transiently transfected with the porcine A₁ adenosine receptor (Aorta SMC-A₁R) or mock plasmid-transfected A7r5 cells (Aorta SMC-pCR3.1) in low density (7,000 cells/well) were serum-starved for 24 h and then stimulated with 100 μmol/L adenosine or 2.5% FBS or both for 48 h, after which the cellular DNA content was determined. Data shown are the means ± S.E.M. from three independent experiments performed in triplicate. *, p < 0.05 relative to FBS stimulation alone.
Supplemental Figure 6. Effect of $A_1$R over-expression on adenosine-induced DNA synthesis of rat aortic SMC in standard density. Wild type A7r5 cells (rat aortic SMC line) or A7r5 cells transiently transfected with the porcine $A_1$ adenosine receptor (Aorta SMC-$A_1$R) or mock plasmid-transfected A7r5 cells (Aorta SMC-pCR3.1) in standard density (35,000 cells/well) were serum-starved for 24 h and then stimulated with 100 µmol/L adenosine for 48 h, after which the cellular DNA content was determined. Data shown are the means ± S.E.M. from three independent experiments performed in triplicate. *, $p < 0.01$ relative to the control.