ONLINE SUPPLEMENT

Cellular Signaling Pathways Mediating Dilation of Porcine Pial Arterioles to Adenosine A_{2A} Receptor Activation

Travis W. Hein , Wenjuan Xu, Yi Ren, Lih Kuo

Supplemental Methods

Animal Preparation. All animal procedures were approved by the Scott & White Institutional Animal Care and Use Committee and conform to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). Domestic, juvenile pigs (8-12 weeks old of either sex; 7-15 kg) purchased from Real Farms (San Antonio, TX) were sedated with Telazol (4.4 mg/kg, i.m.) and anesthetized with 2-4% isoflurane. Heparin (1,000 units/kg) was administered into the marginal ear vein to prevent clotting. After a left thoracotomy was performed, the heart was excised for exsanguination. Following exsanguination, the skull was removed and the brain was carefully isolated and placed in ice-cold saline.

Isolation and Cannulation of Pial Arterioles. A segment of brain tissue on the surface of the cerebral cortex was removed with a scalpel blade and placed in a cooled dissection chamber (~8°C) containing a physiological salt solution (PSS; in mM: NaCl 142.0, KCl 4.7, $CaCl_2$ 1.5, $MgSO_4$ 1.17, NaH_2PO_4 1.2, glucose 5.0, pyruvate 2.0, EDTA 0.02, MOPS 3.0) with 1% bovine serum albumin (Affymetrix, Cleveland, OH). Single pial arteriolar segments (20-60 μm in internal diameter in situ, 0.6 to 1.0 mm in length without branches) on the surface of the cerebral cortex were selected and carefully isolated under a dissection microscope from the surrounding neural/connective tissues. Each isolated arteriole was then transferred for cannulation to a Lucite vessel chamber containing PSS (pH = 7.4) without albumin equilibrated with room air at ambient temperature. Both ends of the arteriole were cannulated with glass micropipettes (tip outer diameter of 20-30 μm) filled with

PSS-albumin solution (pH = 7.4) and the outside of the arteriole was securely tied to the pipettes with 11-0 ophthalmic suture (Alcon, Fort Worth, TX). After cannulation, the vessel and pipettes were transferred to the stage of an inverted microscope (model CKX41, Olympus) coupled to a video camera (Sony DXC-190, Labtek, Campbell, CA) and video micrometer (Cardiovascular Research Institute, Texas A&M Health Science Center, College Station, TX) for continuous measurement of the internal diameter. The cannulating micropipettes were connected to independent pressure reservoirs. By adjusting the height of the reservoirs, the vessel was pressurized to 60 cmH_2O intraluminal pressure without flow. Arterioles with side branches and leaks were excluded from further study and all arterioles used developed basal tone.

Experimental Protocols for Vasomotor Assessment. After vessels developed stable basal tone (~60 minutes) at 36-37˚C, the concentration-diameter relationships for natural ligand adenosine (1 nM to 10 μM), A_1 receptor agonist N^6-chloro-cyclopentyladenosine (CCPA; 1 nM to 10 μM), and A_{2A} receptor agonist 2-[p-(2-carboxyethyl)]phenylethyl-amino-5'-N-ethylcarboxamidoadenosine (CGS21680; 1 nM to 10 μM) were independently established. The vessels were exposed to each concentration of agonist for 2-4 minutes until a stable diameter was maintained. The reproducibility of the vasodilator response to each adenosine analogue was confirmed in our pilot studies with no observation of tachyphylaxis. After completing the control response, the vasodilation elicited by adenosine and by the adenosine receptor agonists was re-examined after a 30-minute incubation of vessels with the selective A_1 receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 0.1 μM)[1-3] or the selective A_{2A} receptor antagonist, 4-(2-[7-amino-2-(2-furyl)[1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385, 1 μM; Tocris).[1, 4]

The following studies were performed to elucidate the possible cellular mechanisms involved in the pial arteriolar dilation to adenosine. First, the role of endothelium in the pial arteriolar dilation to adenosine and other agonists was evaluated in vessels following air bolus injection (2 minutes) to remove endothelial cells. Vasodilations to agonists were evaluated and

compared in intact and denuded vessels from the same animal. The denuded vessels that exhibited normal basal tone, showed no vasodilation to endothelium-dependent vasodilator bradykinin (10 nM), and showed unaltered response to endothelium-independent vasodilator sodium nitroprusside (SNP, 1 nM to 10 μM) were accepted for data analysis. Second, the involvement of endothelium-dependent signaling pathways such as activation of NOS, cyclooxygenase, and opening of small and intermediate calcium-activated potassium (SK_{Ca} and IK_{Ca}) channels in adenosine-induced dilation was examined by incubation of vessels with known effective concentrations of their specific inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 10 μM),[5] indomethacin (10 μM),[6] and combination of apamin (0.1 μM, SK_{Ca} block) and TRAM-34 (1 μM, IK_{Ca} block).[7, 8] Third, the involvement of various potassium channels in vasodilation to adenosine was examined by treating the vessels with selective inhibitors for large conductance K_{Ca} (BK_{Ca}) channels iberiotoxin, 0.1 μM),[9] ATP-sensitive potassium (K_{ATP}) channels (glibenclamide, 2 μM),[10] or inward rectifier potassium (K_{ir}) channels [barium chloride (Ba^{2+}), 100 μM].[11, 12] Experiments with effective concentrations (>100 μM) of 4-aminopyridine, the voltage-dependent potassium channel inhibitor, were not examined because they caused extensive vasomotion of pial arterioles, which precluded the reliable measurement of adenosine-induced vasodilation. Fourth, the role of cyclic nucleotide signaling was evaluated by incubating the vessels with soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ, 0.3 μM)[5] and cAMP signaling inhibitor Rp-8-Br-cAMPS (100 μM).[5] In some vessels, adenosine-induced vasodilation was examined following exposure to Ba^{2+} or Rp-8-Br-cAMPS with L-NAME (10 μM). Fifth, to confirm the efficacy of ZM241385, L-NAME, ODQ, glibenclamide, Rp-8-Br-cAMPS and Ba^{2+}, vasodilations to adenosine A_{2A} receptor agonist CGS21680 (10 μM),[1, 13] NO-mediated agonist bradykinin (1 nM), soluble guanylyl cyclase/cGMP signaling activator SNP (1 μM), K_{ATP} channel opener pinacidil (10 μM), adenylyl cyclase/cAMP signaling activator forskolin (0.1 μM) and Ba^{2+}-sensitive K_{ir} channel opener K^+ (10 mM) were examined, respectively. The extraluminal K^+ concentration (5 mM) was

increased to 10 mM by the addition of KCl to the vessel bath and balanced by equimolar decreases in the NaCl concentration to maintain constant osmolarity.[14] Sixth, the mechanism contributing to the vasodilator response to CGS21680 (10 μM) was examined in the absence or the presence of endothelium, L-NAME, Ba^{2+}, Rp-8-Br-cAMPS, or glibenclamide. Finally, to assess the impact of nucleotide signaling on K_{ir} channel activation, the vasodilations to forskolin and SNP were examined in the presence of Ba^{2+} (100 μM). In some vessels, forskolin-induced vasodilation was repeated following exposure to L-NAME (10 μM) without or with Ba^{2+}. Vessels were exposed to pharmacological inhibitors for 20-40 minutes.

NO Assay. Basal and adenosine-stimulated production of NO from pial arterioles was evaluated by measuring levels of nitrite, a major breakdown product of NO, using a chemiluminescence NO analyzer (Sievers Instruments) as described previously.[5] Porcine pial arterioles (5-7 vessels/sample) were isolated and placed in a microcentrifuge tube containing 100 μL PSS at 37°C. After a 30-minute initial incubation with or without L-NAME (10 μM), adenosine (10 μL, final concentration 1 μM) was added to the vessel bath. The bathing solution was then collected for measurement of nitrite after a 30-minute incubation with adenosine. In addition, a separate experiment was run as a control group by adding vehicle solution (PSS) instead of adenosine and L-NAME to the vessel sample for 30 minutes. The background levels of nitrite in the solutions were measured from a tube containing either PSS only or PSS plus adenosine (1 μM) without or with L-NAME, and then the corresponding values were subtracted from the sampled solution to obtain nitrite production. The protein levels in each vessel sample were quantified by bicinchoninic acid protein assay (Pierce) and were used to normalize the nitrite production.

Immunohistochemical Analysis. To identify and localize adenosine A_{2A} receptors, isolated pial arterioles were embedded and frozen in O.C.T. Compound. Frozen sections (10-μm-thick) were fixed in 4% paraformaldehyde, and then immunolabeled with anti-adenosine A_{2A} receptor antibody

(1:100, Alpha Diagnostic International) or anti-endothelial NOS (eNOS) antibody (1:100, BD Biosciences). We have previously confirmed specific binding of this A_{2A} antibody to A_{2A} receptors in the porcine corpus striatum via immunoblotting.[13] After labeling with primary antibodies, the slides were incubated with Cy3-conjugated or FITC-conjugated secondary antibodies (1:200, Jackson ImmunoResearch Laboratories). Staining control tissues were exposed for the same duration to non-immune serum in place of primary antibody. Slides were observed for red (Cy3) and green (FITC) images, and analyzed using a fluorescence microscope (Axiovert 200, Zeiss, Thornwood, NY). Merged images were created with ImageJ software (National Institutes of Health).

Chemicals. Drugs were obtained from Sigma Chemical Co., except as specifically stated. Adenosine, apamin, bradykinin, $BaCl_2$, iberiotoxin, L-NAME, and SNP were dissolved in PSS. Pinacidil, CCPA and ODQ were dissolved in ethanol, whereas CGS21680, glibenclamide, TRAM-34 and ZM241385 were dissolved in DMSO as stock solutions (10 mM). Subsequent concentrations of these drugs were diluted in PSS. The highest final concentration of ethanol or DMSO in the vessel bath was 0.1%. Vehicle control studies indicated that this final concentration of ethanol or DMSO had no effect on the arteriolar function.

Data Analysis. At the end of each functional experiment, the vessel was relaxed with 100 μM SNP in ethylenediaminetetraacetic acid (1 mM)-Ca^{2+}-free PSS to obtain its maximum diameter at 60 cmH_2O intraluminal pressure. Diameter changes in response to vasodilator agonists were normalized to this maximum vasodilation and expressed as % maximum dilation. Data are reported as mean ± SEM. Statistical comparisons of vasomotor responses and NO production under various treatments were performed with one-way or two-way ANOVA when appropriate and tested with the Bonferroni multiple-range test. Differences in resting diameter and in vasodilation to single concentration of agonist in the absence and presence of endothelium or pharmacological treatments

were compared by paired or unpaired Student's *t* tests when appropriate. A value of $P<0.05$ was considered significant. Taking into account the observed variability in responses (standard deviation) when applying ANOVA, the sample size for each protocol in the present study is sufficient to detect a 5% change in dilation with the power of at least 0.80 when $\alpha = 0.05$.

Supplemental References

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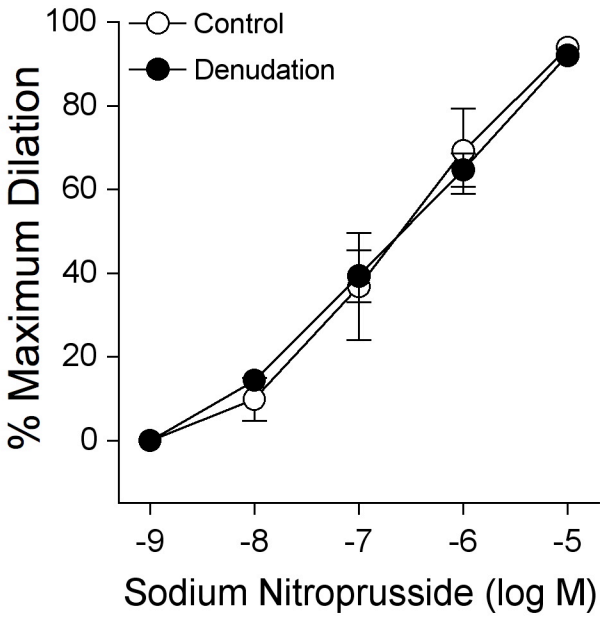
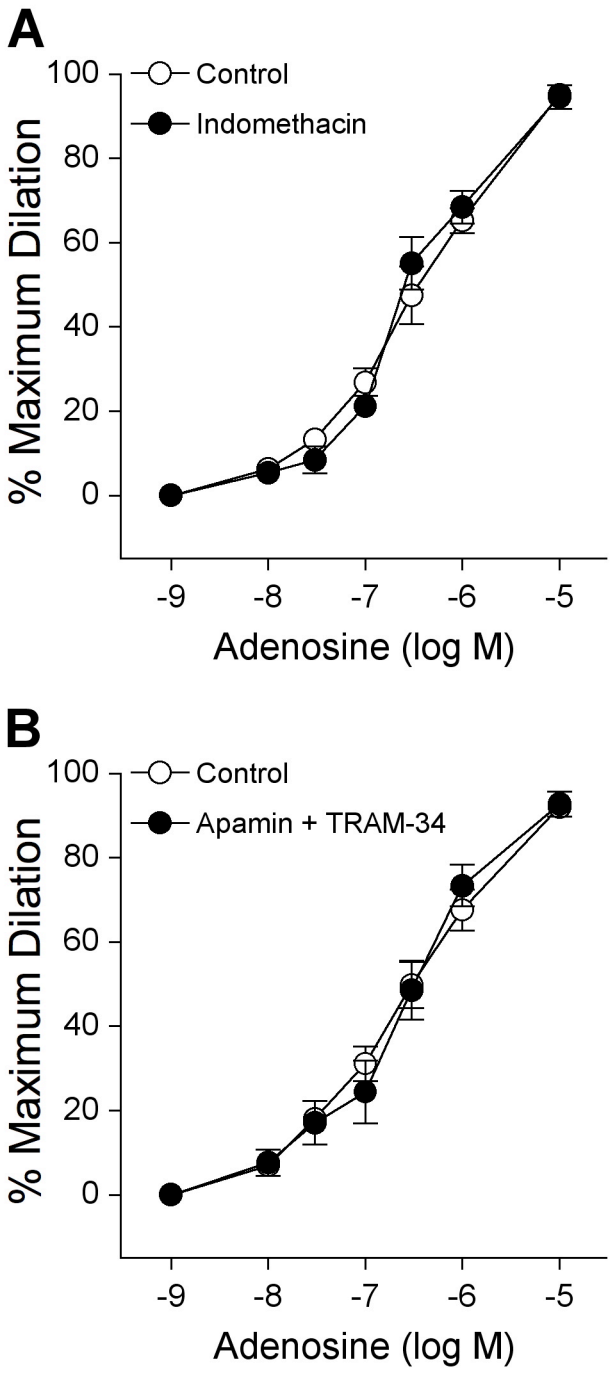
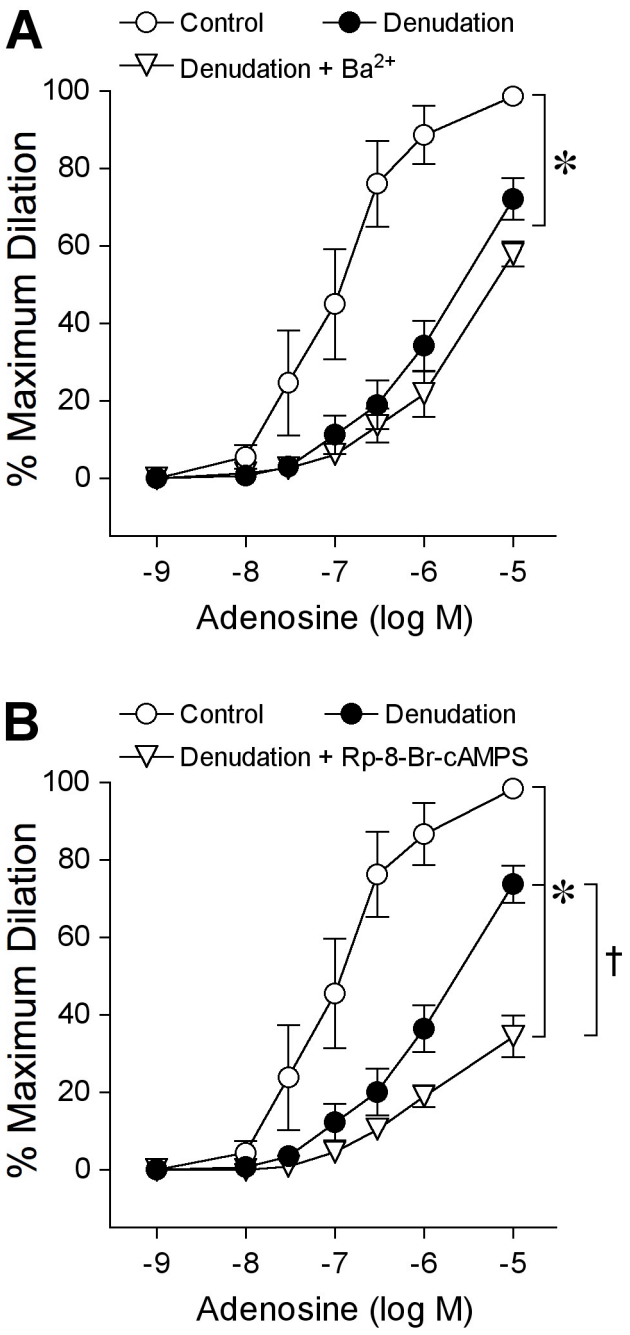


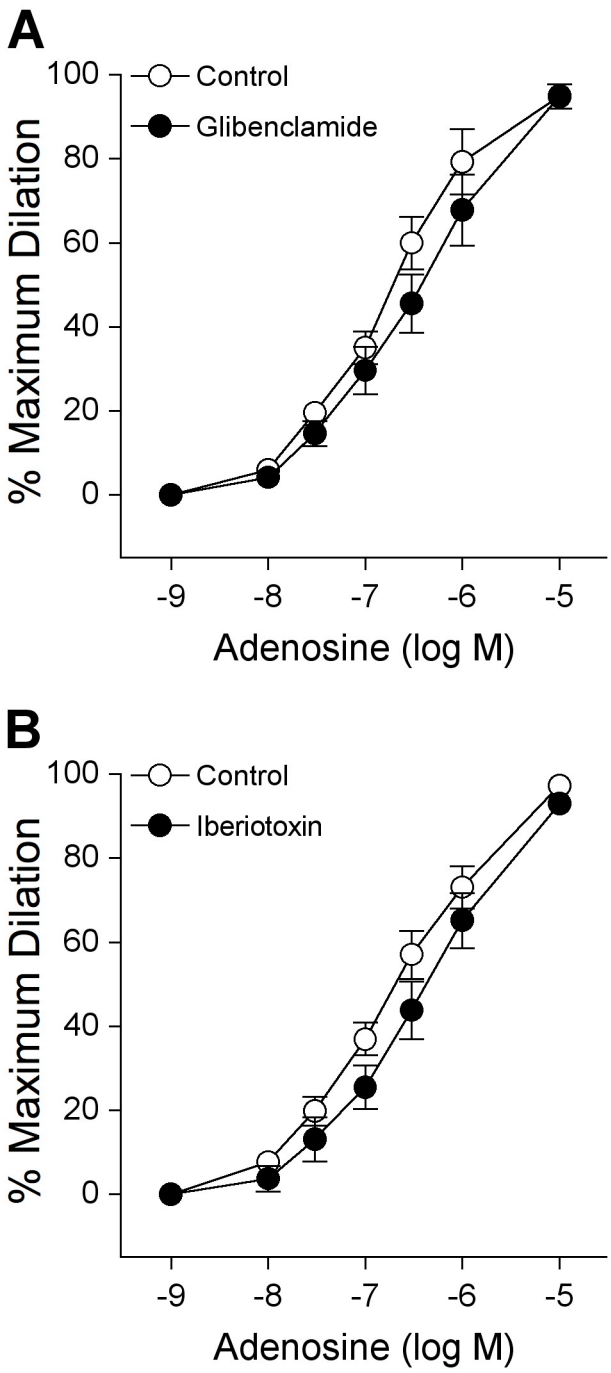
Supplemental Figure 1. Effect of endothelial removal on dilation of isolated pial arterioles to sodium nitroprusside. Concentration-dependent vasodilation to sodium nitroprusside was examined in the presence (Control, $n = 7$) or in the absence of endothelium (Denudation, $n = 7$). n represents number of vessels, 1 per animal. Data are expressed as mean ± SEM.



Supplemental Figure 2. Effect of cyclooxygenase and SK_{Ca}/IK_{Ca} channel inhibitors on dilation of isolated pial arterioles to adenosine. Concentration-dependent vasodilation to adenosine was examined before (Control) and after incubation with (A) indomethacin (10 μM, *n* = 6) or (B) combination of apamin (0.1 μM) and TRAM-34 (1 μM, *n* = 5). *n* represents number of vessels, 1 per animal. Data are expressed as mean ± SEM.



Supplemental Figure 3. Effect of endothelial removal and cAMP signaling blockade on dilation of isolated pial arterioles to adenosine. Concentration-dependent vasodilation to adenosine was examined in the presence (Control) or in the absence of endothelium (Denudation) before and after treatment with (A) Ba^{2+} (100 μM, *n* = 5) or (B) cAMP signaling inhibitor Rp-8-Br-cAMPS (100 μM, *n* = 5). *n* represents number of vessels, 1 per animal. Data are expressed as mean ± SEM. *P < 0.05 vs. Control. †P < 0.05 vs. Denudation.



Supplemental Figure 4. Effect of K_{ATP} and BK_{Ca} channel inhibitors on dilation of isolated pial arterioles to adenosine. Concentration-dependent vasodilation to adenosine was examined before (Control) and after incubation with (A) glibenclamide (2 μM, *n* = 7) or (B) iberiotoxin (0.1 μM, *n* = 8). *n* represents number of vessels, 1 per animal. Data are expressed as mean ± SEM.