

# Cellular signalling pathways mediating dilation of porcine pial arterioles to adenosine A<sub>2A</sub> receptor activation

Travis W. Hein<sup>1\*</sup>, Wenjuan Xu<sup>1</sup>, Yi Ren<sup>1</sup>, and Lih Kuo<sup>1,2</sup>

<sup>1</sup>Department of Surgery, Scott & White Healthcare, College of Medicine, Texas A&M Health Science Center, 702 Southwest H.K. Dodgen Loop, Temple, TX 76504, USA; and <sup>2</sup>Department of Medical Physiology, Scott & White Healthcare, College of Medicine, Texas A&M Health Science Center, Temple, TX, USA

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**Aims** Adenosine is a potent vasodilator contributing to cerebral blood flow regulation during metabolic stress. However, the distribution of adenosine receptor subtypes and underlying signalling mechanisms for dilation of pial arterioles remain unclear. The present study aimed at addressing these issues.

**Methods and results** Isolated porcine pial arterioles were subjected to study of vasomotor function, localization of adenosine receptors, and production of nitric oxide (NO). Concentration-dependent vasodilation to adenosine was inhibited by A<sub>2A</sub> receptor antagonist ZM241385 but not by A<sub>1</sub> receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine. A<sub>2A</sub> receptors were detected in endothelium and smooth muscle of pial arterioles via immunohistochemistry. Adenosine significantly increased arteriolar production of NO, and the induced dilation was insensitive to K<sub>ATP</sub> channel blocker glibenclamide but was attenuated by endothelial denudation, NO synthase inhibitor L-NAME, or guanylyl cyclase inhibitor ODQ in a similar manner. Both inward rectifier potassium (K<sub>ir</sub>) channel inhibitor barium and cAMP signalling inhibitor Rp-8-Br-cAMPS attenuated adenosine-induced dilation. In the presence of L-NAME or the absence of endothelium, addition of Rp-8-Br-cAMPS but not barium further reduced adenosine-induced responses. Barium diminished endothelium-independent vasodilation to NO donor sodium nitroprusside. Comparable to the adenosine-induced response, vasodilation to A<sub>2A</sub> receptor agonist CGS21680 was attenuated by endothelial removal, ZM241385, L-NAME, barium, or Rp-8-Br-cAMPS, but not by glibenclamide.

**Conclusion** Adenosine evokes dilation of porcine pial arterioles via parallel activation of endothelial and smooth muscle A<sub>2A</sub> receptors. Stimulation of endothelial NO production activates smooth muscle guanylyl cyclase for vasodilation by opening K<sub>ir</sub> channels. Adenosine also activates smooth muscle cAMP signalling leading to vasodilation.

**Keywords** Adenosine • Microcirculation • Nitric oxide • Potassium channels • Vasodilation

## 1. Introduction

The cerebral microvascular network, especially at the level of arterioles, plays an important role in regulating blood flow to the surrounding cerebral tissue for proper nutrition and function.<sup>1</sup> Since the seminal studies of Roy and Sherrington,<sup>2</sup> cerebral blood flow has been shown to be closely coupled to brain activity through a metabolic regulation mechanism.<sup>3</sup> In particular, the tissue metabolite adenosine is a potent vasodilator<sup>4</sup> that has been suggested to play a prominent role in the regulation of cerebral blood flow during metabolic stress such as hypotension, neural activation, hypoxia/ischaemia, and hypercapnia/acidosis.<sup>5</sup> Interestingly, recent experimental evidence

has shown that dilation of pial arterioles on the surface of the cerebral cortex in response to adenosine is attenuated following subarachnoid haemorrhage in rats.<sup>6</sup> Although this vasomotor dysfunction could be mediated in part via scavenging of nitric oxide (NO) by haemoglobin as reported for large cerebral arteries,<sup>7</sup> the underlying mechanism for arteriolar dysregulation in response to adenosine remains unclear. A better understanding of the fundamental signalling pathways responsible for adenosine-induced dilation of pial arterioles may help to shed light on how cerebral arterioles dilate to metabolic stress and on the possible mechanisms contributing to vasomotor dysfunction in disease states such as cerebral ischaemia in patients following subarachnoid haemorrhage.<sup>8</sup>

\* Corresponding author. Tel: +1 254 724 3550, fax: +1 254 742 7181, Email: thein@tamu.edu

Previous evidence from *in vivo* studies suggests that activation of adenosine  $A_{2A}$  receptors mediates dilation of pial arterioles in response to adenosine.<sup>9,10</sup> However, the cellular distribution of the  $A_{2A}$  receptors and the underlying signalling pathway responsible for the adenosine-induced dilation at the arteriolar level remain unclear. *In vivo* studies in the newborn pig have shown that the increased cerebral blood flow in response to adenosine is partly inhibited by pharmacological blockade of NO synthase (NOS) and cAMP signalling,<sup>11</sup> suggesting the putative roles of NO and the activation of the cAMP pathway in this process. Notably, the source of NO and the role of cyclic nucleotide signalling remain unclear because the potential neuronal and glial cell influences in this vasodilator response have not been established. Additionally, adenosine-induced activation of potassium channels has been implicated in contributing to the dilation of pial vessels *in vivo*,<sup>10–13</sup> but inconsistent results have been reported for the type of potassium channel involved. Because haemodynamic changes and neural–glial interactions are known to influence vascular function and to produce confounding effects on vasomotor responses to agonists *in vivo*, we focused on identifying the cellular mechanism for adenosine-induced dilation under a defined environment using an isolated and pressurized vessel preparation, along with immunohistochemical and biochemical approaches. The relative roles of adenosine receptor subtypes, endothelial NOS, smooth muscle potassium channels, and cyclic nucleotide signalling (cAMP and cGMP) in vasodilation to adenosine were determined.

## 2. Methods

Please see Supplementary material online for expanded Methods section.

### 2.1 Functional assessment of isolated pial arterioles

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). Pigs were sedated with Telazol (4.4 mg/kg, *i.m.*) and anaesthetized with 2–4% isoflurane. 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. A segment of brain tissue on the surface of the cerebral cortex was removed and placed in a cooled dissection chamber ( $\sim 8^{\circ}\text{C}$ ) containing a physiological salt solution (PSS) with 1% bovine serum albumin. Single pial arteriolar segments (20–60  $\mu\text{m}$  in internal diameter *in situ*, 0.6–1.0 mm in length without branches) were isolated under a dissection microscope from the surrounding neural/connective tissues. Vessels were cannulated and pressurized to 60  $\text{cmH}_2\text{O}$  intraluminal pressure without flow for measurement of the internal diameter using videomicroscopic techniques.

After vessels developed stable basal tone, the concentration–diameter relationships for adenosine (1 nM–10  $\mu\text{M}$ ),  $A_1$  receptor agonist  $N^6$ -chloro-cyclopentyladenosine (CCPA; 1 nM–10  $\mu\text{M}$ ), and  $A_{2A}$  receptor agonist 2-[p-(2-carboxyethyl)]phenylethyl-amino-5'-N-ethylcarboxamidoadenosine (CGS21680; 1 nM–10  $\mu\text{M}$ ) were independently established. After completing the control response, the vasodilation elicited by adenosine and by the adenosine receptor agonists was re-examined after a 30 min incubation of vessels with the selective  $A_1$  receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 0.1  $\mu\text{M}$ )<sup>14–16</sup> 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  $\mu\text{M}$ ; Tocris).<sup>14,17</sup>

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 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 (1 nM), and showed unaltered response to endothelium-independent vasodilator sodium nitroprusside (SNP, 1 nM–10  $\mu\text{M}$ ) were accepted for data analysis. Secondly, the involvement of endothelium-dependent signalling 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  $\mu\text{M}$ ),<sup>18</sup> indomethacin (10  $\mu\text{M}$ ),<sup>19</sup> and a combination of apamin (0.1  $\mu\text{M}$ ,  $SK_{Ca}$  block) and TRAM-34 (1  $\mu\text{M}$ ,  $IK_{Ca}$  block).<sup>20,21</sup> Thirdly, 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  $\mu\text{M}$ ),<sup>22</sup> ATP-sensitive potassium ( $K_{ATP}$ ) channels (glibenclamide, 2  $\mu\text{M}$ ),<sup>12</sup> or inward rectifier potassium ( $K_{ir}$ ) channels [barium chloride ( $Ba^{2+}$ ), 100  $\mu\text{M}$ ].<sup>3,10</sup> Fourthly, the role of cyclic nucleotide signalling 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  $\mu\text{M}$ )<sup>18</sup> and cAMP signalling inhibitor Rp-8-Br-cAMPS (100  $\mu\text{M}$ ).<sup>18</sup> In some vessels, adenosine-induced vasodilation was examined following exposure to  $Ba^{2+}$  or Rp-8-Br-cAMPS with L-NAME (10  $\mu\text{M}$ ). Fifthly, 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  $\mu\text{M}$ ),<sup>14,23</sup> NO-mediated agonist bradykinin (1 nM), soluble guanylyl cyclase/cGMP signalling activator SNP (1  $\mu\text{M}$ ),  $K_{ATP}$  channel opener pinacidil (10  $\mu\text{M}$ ), adenylyl cyclase/cAMP signalling activator forskolin (0.1  $\mu\text{M}$ ), and  $Ba^{2+}$ -sensitive  $K_{ir}$  channel opener  $K^+$  (10 mM) were examined, respectively. Sixthly, the mechanism contributing to the vasodilator response to CGS21680 (10  $\mu\text{M}$ ) 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 signalling on  $K_{ir}$  channel activation, the vasodilations to forskolin and SNP were examined in the presence of  $Ba^{2+}$  (100  $\mu\text{M}$ ). In some vessels, forskolin-induced vasodilation was repeated following exposure to L-NAME (10  $\mu\text{M}$ ) without or with  $Ba^{2+}$ .

### 2.2 NO assay

Basal and adenosine (1  $\mu\text{M}$ )-stimulated production of NO from pial arterioles was evaluated by measuring the levels of nitrite, a major breakdown product of NO, using a chemiluminescence NO analyser (Sievers Instruments) as described previously.<sup>18</sup>

### 2.3 Immunohistochemical analysis

Frozen sections (10  $\mu\text{m}$  thick) of pial arterioles were fixed in 4% paraformaldehyde and then immunolabelled with anti-adenosine  $A_{2A}$  receptor antibody (1:100, Alpha Diagnostic International) or anti-endothelial NOS (eNOS) antibody (1:100, BD Biosciences). Images were observed using fluorescence microscopy.

### 2.4 Data analysis

Diameter changes in response to vasodilator agonists were normalized to the maximum diameter change in response to 100  $\mu\text{M}$  SNP in ethylenediaminetetraacetic acid (1 mM)- $Ca^{2+}$ -free PSS and expressed as % maximum dilation. Data are reported as mean  $\pm$  SEM. Statistical comparisons were performed with Student's *t*-tests or ANOVA when appropriate and tested with the Bonferroni multiple-range test. A value of  $P < 0.05$  was considered significant.

### 3. Results

#### 3.1 Vasodilation of pial arterioles to adenosine and adenosine receptor agonists

In this study, all vessels ( $n = 157$ ) developed a similar level of basal tone (constricted to  $43 \pm 1\%$  of maximum diameter). The average resting and maximum diameters of the vessel were  $36 \pm 1$  and  $83 \pm 2 \mu\text{m}$ , respectively. Adenosine,  $A_1$  agonist CCPA, and  $A_{2A}$  agonist CGS21680 produced concentration-dependent dilation of pial arterioles (Figure 1). The relative order of potency for these agonists was adenosine = CGS21680 > CCPA.

#### 3.2 Role of adenosine receptors

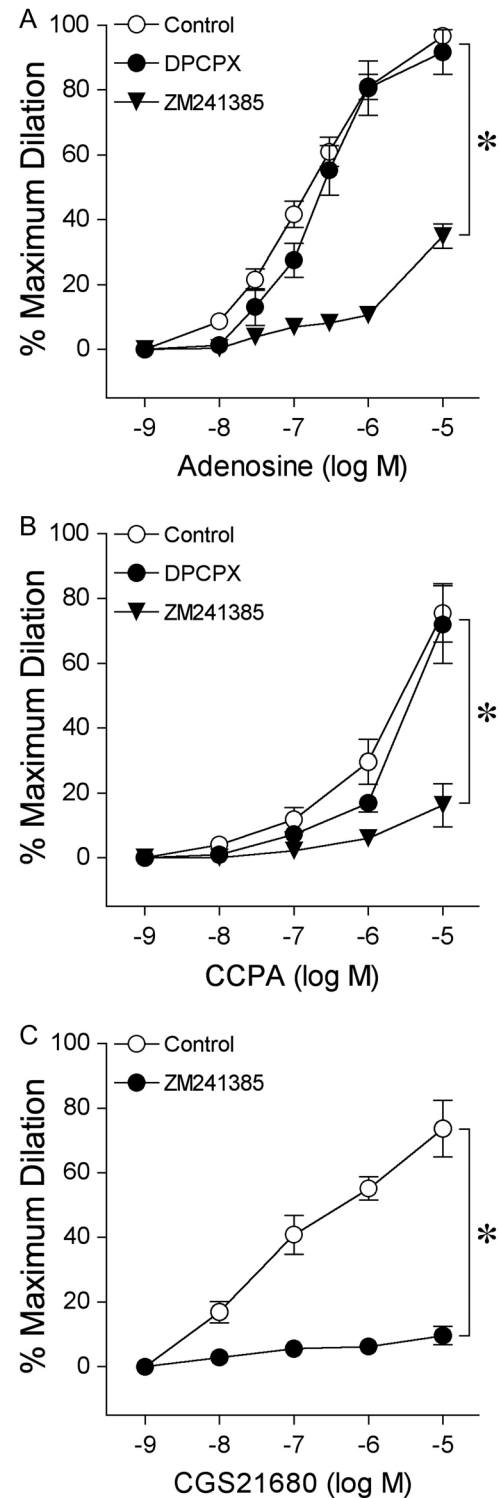
Blockade of  $A_1$  receptors by DPCPX had no effect on vasodilation to adenosine (Figure 1A). In contrast, the  $A_{2A}$  receptor antagonist ZM241385 nearly abolished vasodilation induced by the lower concentrations of adenosine ( $<1 \mu\text{M}$ ) and reduced the response to the highest concentration of adenosine ( $10 \mu\text{M}$ ) from 97% (Control) to 35% (Figure 1A). The dilation of arterioles to CCPA was not altered by DPCPX, but was almost completely inhibited by ZM241385 (Figure 1B). Pial arteriolar dilation to CGS21680 was nearly abolished by ZM241385 (Figure 1C). Notably, ZM241385 did not alter resting basal tone (Control:  $47 \pm 6\%$  vs. ZM241385:  $51 \pm 6\%$ ,  $n = 6$ ,  $P = 0.08$ ) or receptor-mediated vasodilation to 1 nM bradykinin ( $84 \pm 11\%$  dilation vs.  $89 \pm 6\%$  dilation with ZM241385 treatment,  $n = 5$ ,  $P = 0.39$ ).

#### 3.3 Localization of adenosine $A_{2A}$ receptors

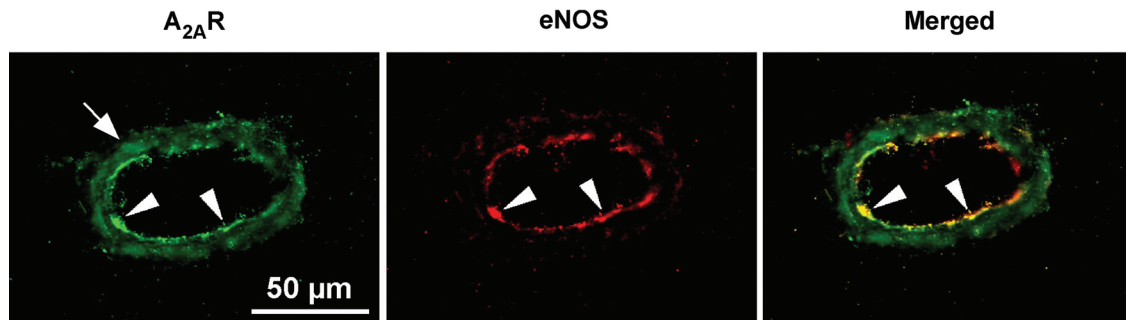
For cellular localization of proteins, we performed immunohistochemical analysis of  $A_{2A}$  receptors and eNOS in isolated pial arterioles. The  $A_{2A}$  receptor staining was detected in both smooth muscle and endothelial layers with the latter showing overlap with eNOS staining in the endothelium (yellow staining in merged image) (Figure 2).

#### 3.4 Roles of endothelium, NOS, and soluble guanylyl cyclase

Disruption of the endothelium attenuated adenosine-induced dilation (Figure 3A) and abolished bradykinin (1 nM)-induced dilation ( $88 \pm 4\%$  dilation vs.  $4 \pm 2\%$  dilation following denudation,  $n = 8$ ) of pial arterioles without significantly altering resting basal tone (Control:  $41 \pm 2\%$  vs. Denudation:  $48 \pm 3\%$ ,  $n = 8$ ,  $P = 0.07$ ) or dilation to SNP (Supplementary material online, Figure S1). In a similar manner, exposure to NOS inhibitor L-NAME did not significantly affect basal tone (Control:  $37 \pm 3\%$  vs. L-NAME:  $38 \pm 3\%$ ,  $n = 7$ ,  $P = 0.65$ ) but shifted the vasodilator response curve of adenosine to the right (Figure 3B). The L-NAME concentration was effective for blocking NOS because it significantly reduced the pial arteriolar dilation to endothelium-dependent NO-mediated agonist bradykinin ( $83 \pm 4\%$  dilation vs.  $10 \pm 2\%$  with L-NAME treatment,  $n = 7$ ). Furthermore, soluble guanylyl cyclase inhibitor ODQ significantly reduced the pial arteriolar dilation to adenosine (Figure 3C) without altering basal tone (Control:  $38 \pm 2\%$  vs. ODQ:  $33 \pm 3\%$ ,  $n = 5$ ;  $P = 0.34$ ). The efficacy of ODQ for soluble guanylyl cyclase inhibition was confirmed by significantly attenuating vasodilation in response to  $1 \mu\text{M}$  SNP ( $68 \pm 2\%$  dilation vs.  $26 \pm 4\%$  dilation with ODQ treatment,  $n = 8$ ). The cyclooxygenase-derived prostaglandins and  $SK_{Ca}/IK_{Ca}$ -dependent



**Figure 1** Effect of  $A_1$  and  $A_{2A}$  receptor blockade on dilation of isolated pial arterioles to (A) adenosine, (B)  $A_1$  receptor agonist CCPA, and (C)  $A_{2A}$  receptor agonist CGS21680. Concentration-dependent vasodilation was examined before (Control) and after incubation with  $A_1$  receptor antagonist DPCPX ( $0.1 \mu\text{M}$ ,  $n = 6$  for adenosine and  $n = 5$  for CCPA) or  $A_{2A}$  receptor antagonist ZM241385 ( $1 \mu\text{M}$ ,  $n = 6$  for adenosine and  $n = 5$  for CCPA and for CGS21680).  $n$  represents the number of vessels, one per animal. Data are expressed as mean  $\pm$  SEM. \* $P < 0.05$  vs. Control.



**Figure 2** Immunohistochemical detection of adenosine  $A_{2A}$  receptors in isolated pial arterioles. In the presence of anti- $A_{2A}$  receptor (green) or anti-eNOS (red) antibodies, high levels of immunostaining were detected in the endothelium and smooth muscle for  $A_{2A}$  receptors ( $A_{2A}R$ ) and in the endothelium for eNOS. The merged image shows overlap staining (yellow) in the endothelial layer. White arrowheads denote endothelial cells and arrows denote vascular smooth muscle cells. Data shown are representative of four separate experiments.

EDHF<sup>20,21</sup> were not involved in the adenosine-induced vasodilation because indomethacin and apamin plus TRAM-34 treatments did not alter the response (Supplementary material online, Figure S2).

### 3.5 Adenosine-stimulated NO production

In the absence of adenosine, the NO production from pial arterioles was  $72 \pm 10$  nmol/g protein. Adding adenosine (1  $\mu$ M) to the vessels increased NO production by more than four-fold (Figure 4). In the presence of L-NAME, the adenosine-stimulated NO production was reduced (Figure 4).

### 3.6 Role of $K_{ir}$ channels

As shown in Figure 5A,  $K_{ir}$  channel inhibitor  $Ba^{2+}$  (100  $\mu$ M) significantly reduced adenosine-induced vasodilation.  $Ba^{2+}$  did not affect basal tone (Control:  $42 \pm 3\%$  vs.  $Ba^{2+}$ :  $43 \pm 3\%$ ,  $n = 9$ ,  $P = 0.52$ ) or vasodilation to  $K_{ATP}$  channel opener pinacidil 10  $\mu$ M ( $91 \pm 7\%$  dilation vs.  $85 \pm 10\%$  dilation with  $Ba^{2+}$  treatment,  $n = 5$ ,  $P = 0.64$ ), but effectively blocked vasodilation to 10 mM  $K^+$  ( $95 \pm 3\%$  dilation vs.  $10 \pm 5\%$  dilation with  $Ba^{2+}$  treatment,  $n = 7$ ) and significantly attenuated vasodilation to 1  $\mu$ M SNP ( $56 \pm 4\%$  dilation vs.  $38 \pm 1\%$  dilation with  $Ba^{2+}$  treatment,  $n = 8$ ). Following the combined treatment of vessels with  $Ba^{2+}$  and L-NAME, there was no further reduction of the adenosine-induced dilation than to  $Ba^{2+}$  alone (Figure 5A). Similarly, in the absence of endothelium, vasodilation to adenosine was not further diminished by  $Ba^{2+}$  (Supplementary material online, Figure S3A). In another series of experiments, administration of  $K_{ATP}$  channel inhibitor glibenclamide or  $BK_{Ca}$  channel inhibitor iberiotoxin had no effect on the pial arteriolar dilation to adenosine (Supplementary material online, Figure S4) or basal tone (Control:  $43 \pm 2\%$  vs. Glibenclamide:  $46 \pm 2\%$ ,  $n = 7$ ,  $P = 0.08$ ; Control:  $38 \pm 2\%$  vs. Iberiotoxin:  $41 \pm 3\%$ ,  $n = 8$ ,  $P = 0.09$ ). The efficacy of glibenclamide to block  $K_{ATP}$  channels was confirmed by its ability to inhibit vasodilation to pinacidil ( $96 \pm 2\%$  vs.  $12 \pm 3\%$  dilation with glibenclamide treatment,  $n = 6$ ).

### 3.7 Role of cAMP signalling

The cAMP signalling inhibitor Rp-8-Br-cAMPS significantly reduced adenosine-induced vasodilation (Figure 5B) without altering basal tone (Control:  $40 \pm 2\%$  vs. Rp-8-Br-cAMPS:  $41 \pm 2\%$ ,  $n = 8$ ,  $P = 0.71$ ). Greater reduction in the adenosine-induced dilation was

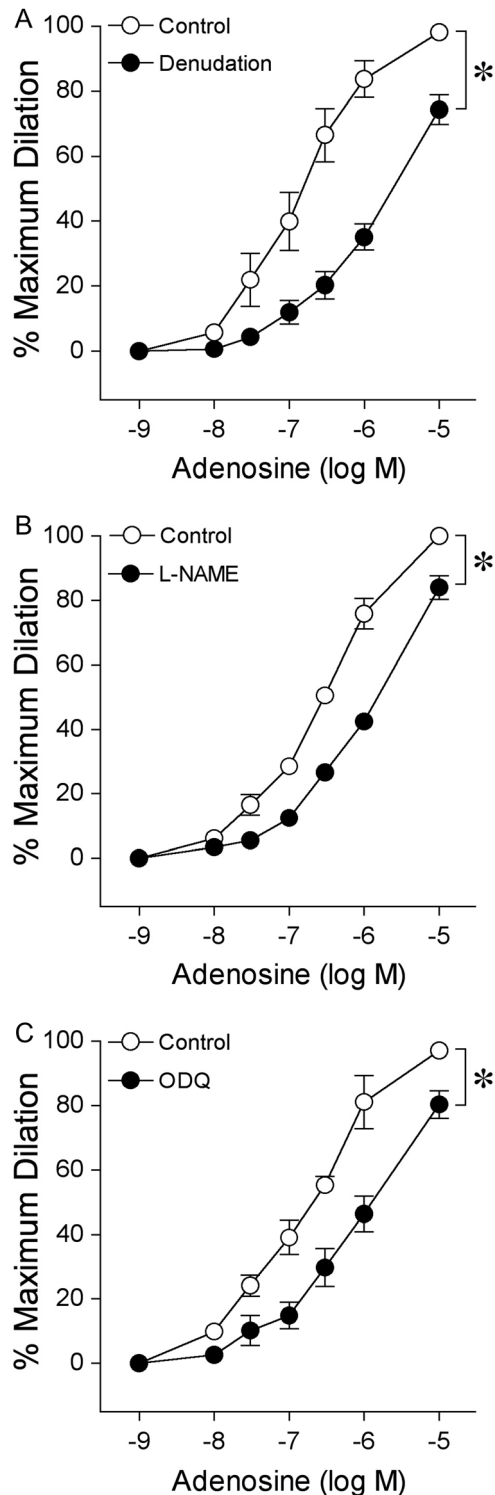
observed following the combined treatment with Rp-8-Br-cAMPS and L-NAME than to Rp-8-Br-cAMPS alone (Figure 5B). The diminished vasodilation to adenosine in the absence of endothelium was further attenuated following incubation with Rp-8-Br-cAMPS (Supplementary material online, Figure S3B). The concentration of Rp-8-Br-cAMPS used in this study effectively inhibited vasodilation in response to the adenylyl cyclase/cAMP signalling activator forskolin (Figure 5C). The forskolin-induced vasodilation was also partially blocked in a similar manner following removal of endothelium or treatment with L-NAME or  $Ba^{2+}$ , with no further reduction in the response when exposed to both L-NAME and  $Ba^{2+}$ , indicating that cAMP signalling is likely activated in both endothelium (i.e. NO) and smooth muscle by forskolin. Although Rp-8-Br-cAMPS did not alter vasodilation to 10 mM  $K^+$  ( $97 \pm 2\%$  dilation vs.  $91 \pm 4\%$  dilation with Rp-8-Br-cAMPS treatment,  $n = 5$ ,  $P = 0.12$ ), it significantly attenuated vasodilation to 1  $\mu$ M SNP ( $58 \pm 4\%$  dilation vs.  $36 \pm 3\%$  dilation with Rp-8-Br-cAMPS treatment,  $n = 5$ ). The combination of Rp-8-Br-cAMPS and  $Ba^{2+}$  treatment almost completely blocked SNP-induced dilation ( $64 \pm 5\%$  dilation vs.  $10 \pm 3\%$  dilation with Rp-8-Br-cAMPS and  $Ba^{2+}$  treatment,  $n = 7$ ).

### 3.8 $A_{2A}$ receptor-mediated vasodilation

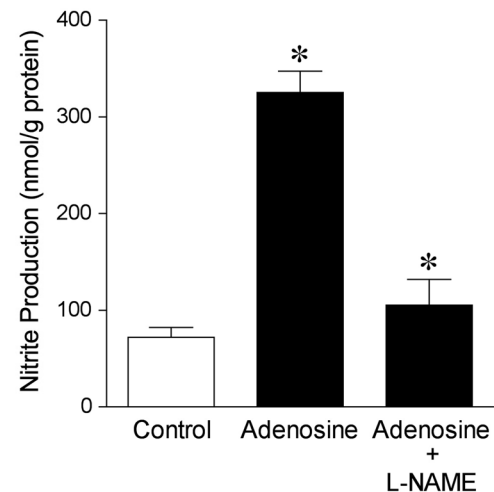
The signalling pathway contributing to the vasodilator response to  $A_{2A}$  receptor agonist CGS21680 (10  $\mu$ M) was also examined. Vasodilation to CGS21680 was unaffected by glibenclamide but was significantly attenuated in the absence of endothelium and following incubation with L-NAME,  $Ba^{2+}$ , or Rp-8-Br-cAMPS (Figure 6).

## 4. Discussion

A considerable volume of evidence has implicated that adenosine is an important mediator in the metabolic regulation of cerebral blood flow.<sup>5</sup> However, the mechanism contributing to the adenosine-induced dilation of arterioles in the pial circulation remains largely unclear in view that previous results were obtained from *in vivo* studies. The salient findings of the present study indicate that adenosine-induced dilation of isolated pial arterioles from the pig is mediated via parallel activation of endothelial and smooth muscle  $A_{2A}$  receptors. The endothelial release of NO linking cGMP activation to opening of smooth muscle  $K_{ir}$  channels and the cAMP signalling in



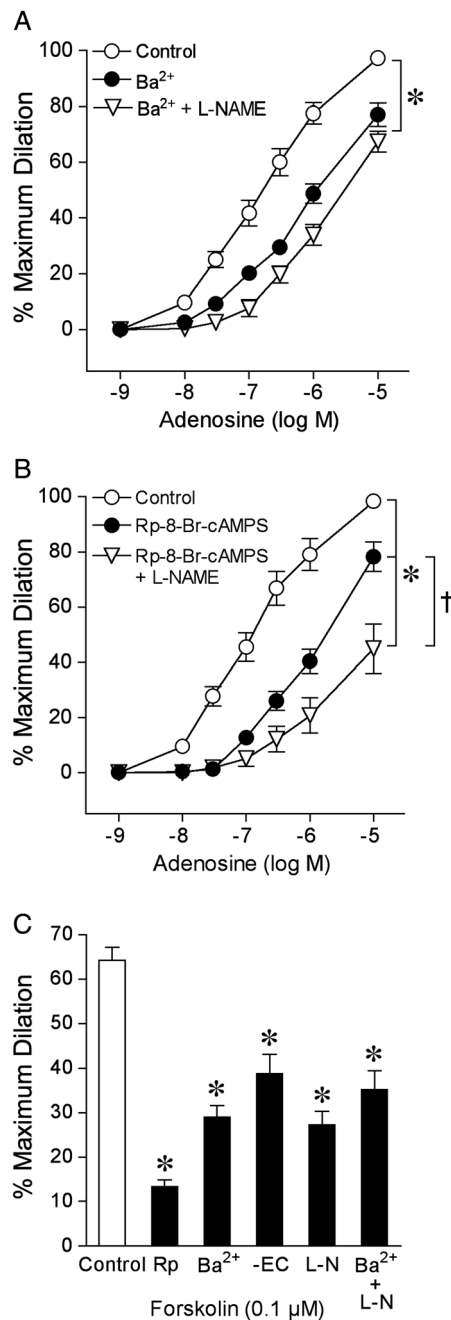
**Figure 3** Roles of endothelium, NOS, and soluble guanylyl cyclase in dilation of isolated pial arterioles to adenosine. Concentration-dependent vasodilation to adenosine was examined (A) in the presence (Control,  $n = 8$ ) or absence (Denudation,  $n = 8$ ) of endothelium. The contributions of NO and soluble guanylyl cyclase to adenosine-elicited vasodilation was assessed in endothelium-intact vessels before (Control) and after incubation with their specific inhibitor (B) L-NAME ( $10 \mu\text{M}$ ,  $n = 7$ ) and (C) ODQ ( $0.3 \mu\text{M}$ ,  $n = 5$ ), respectively.  $n$  represents the number of vessels, one per animal. Data are expressed as mean  $\pm$  SEM.  $*P < 0.05$  vs. Control.



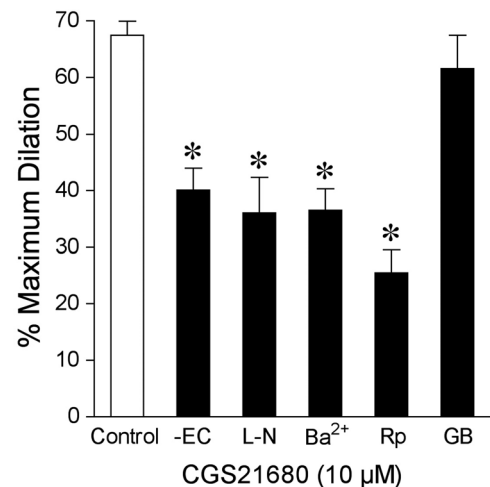
**Figure 4** Adenosine-stimulated NO production in isolated pial arterioles. Chemiluminescence detection of NO was measured in pial arterioles treated with vehicle (Control,  $n = 8$ ), adenosine ( $1 \mu\text{M}$ ,  $n = 8$ ), or adenosine and NOS inhibitor L-NAME ( $10 \mu\text{M}$ ,  $n = 4$ ) for 30 min.  $n$  represents the number of experiments, one per animal. Data are expressed as mean  $\pm$  SEM.  $*P < 0.05$  vs. Control.

the smooth muscle are key mechanisms contributing to the vasodilator response.

Experimental evidence from *in vivo* studies in the mouse<sup>9</sup> and rat<sup>10</sup> indicate that the activation of adenosine  $A_{2A}$  receptors mediates pial arteriolar dilation to adenosine. The present study extended these earlier findings to porcine pial arterioles because  $A_{2A}$  receptor antagonist ZM241385 but not  $A_1$  receptor antagonist DPCPX nearly abolished the dilation to adenosine. Moreover, to the best of our knowledge, we have provided the first immunohistochemical evidence of adenosine  $A_{2A}$  receptor expression in both the endothelial and smooth muscle layers of intact pial arterioles. The  $A_{2A}$  receptor antagonist ZM241385 is currently the most potent and selective antagonist for high affinity  $A_{2A}$  receptors available.<sup>17</sup> Ligand binding studies have demonstrated that ZM241385 has 6700-fold selectivity for  $A_{2A}$  over  $A_1$  binding sites.<sup>17</sup> In the present pial microvascular preparation, we also demonstrated that ZM241385 specifically inhibited vasodilation to selective  $A_{2A}$  receptor agonist CGS21680, but did not affect vasodilation to bradykinin, indicating that ZM241385 is a specific and selective antagonist for the  $A_{2A}$  receptor. It is possible that the residual dilation of porcine pial arterioles to the higher concentrations of adenosine was mediated via low affinity  $A_{2B}$  receptors because a potential role of these receptors in residual dilation of pial arterioles to adenosine in the presence of ZM241385 following hypoxia has been suggested in mice.<sup>9</sup> However, the direct role of  $A_{2B}$  receptors in pial arteriolar dilation to adenosine will have to be addressed when selective  $A_{2B}$  agonists and antagonists become available. Additional support for  $A_{2A}$  receptors was provided using CCPA and CGS21680, which are the most potent and selective agonists that are commercially available for  $A_1$ <sup>24</sup> and  $A_{2A}$ <sup>15</sup> receptors, respectively. We found that pial arterioles displayed relative order of potency for dilation with adenosine = CGS21680 > CCPA. Notably, pial arteriolar dilation to  $A_1$  agonist CCPA was not affected by its antagonist



**Figure 5** Roles of  $K_{ir}$  channels and cAMP signalling in isolated pial arteriolar dilation to adenosine and forskolin. Concentration-dependent vasodilation to adenosine was examined before (Control) and after incubation with (A)  $K_{ir}$  channel inhibitor  $Ba^{2+}$  (100  $\mu$ M,  $n = 9$ ) or (B) cAMP signalling inhibitor Rp-8-Br-cAMPS (100  $\mu$ M,  $n = 8$ ). Vasodilation was also examined following incubation with L-NAME and either  $Ba^{2+}$  ( $n = 14$ ) or Rp-8-Br-cAMPS ( $n = 6$ ). (C) Vasodilation to forskolin was examined in endothelium-intact vessels before (Control) and after incubation with Rp-8-Br-cAMPS (Rp, 100  $\mu$ M,  $n = 7$ ),  $Ba^{2+}$  (100  $\mu$ M,  $n = 6$ ), L-NAME (L-N, 10  $\mu$ M,  $n = 6$ ),  $Ba^{2+}$  (100  $\mu$ M) plus L-NAME ( $n = 5$ ), and in vessels following removal of endothelial cells (-EC,  $n = 7$ ).  $n$  represents the number of vessels, one per animal. Data are expressed as mean  $\pm$  SEM. \* $P < 0.05$  vs. Control. † $P < 0.05$  vs. Rp-8-Br-cAMPS.



**Figure 6**  $A_{2A}$  receptor signalling in dilation of isolated pial arterioles to CGS21680. Vasodilation to  $A_{2A}$  receptor agonist CGS21680 (10  $\mu$ M) was examined in the presence (Control,  $n = 6$ ) or absence of endothelial cells (-EC,  $n = 6$ ) or in endothelium-intact vessels before (Control) and after incubation with L-NAME (L-N, 10  $\mu$ M,  $n = 5$ ),  $Ba^{2+}$  (100  $\mu$ M,  $n = 6$ ), Rp-8-Br-cAMPS (Rp, 100  $\mu$ M,  $n = 5$ ), or glibenclamide (GB, 2  $\mu$ M,  $n = 6$ ).  $n$  represents the number of vessels, one per animal. Data are expressed as mean  $\pm$  SEM. \* $P < 0.05$  vs. Control.

DPCPX, but was effectively inhibited by ZM241385. Although CCPA has binding affinity for  $A_1$  receptors in the subnanomolar range, it is able to activate  $A_{2A}$  receptors in the micromolar range.<sup>24</sup> Therefore, our data suggest that the dilation elicited by 0.1–10  $\mu$ M CCPA is likely mediated by the activation of  $A_{2A}$  receptors and further imply the lack of functional  $A_1$  receptors in pial arterioles. Taken together, our results indicate that activation of both endothelial and smooth muscle  $A_{2A}$  receptors is responsible in large part for the adenosine-induced dilation of pial arterioles.

The contribution of endothelial adenosine  $A_{2A}$  receptors in pial arterioles was supported by the diminished vasodilations to adenosine and CGS21680 in denuded vessels. Activation of the  $A_{2A}$  receptors may stimulate the production and release of endothelium-derived factors such as NO,<sup>18,25,26</sup> prostaglandins,<sup>26</sup> and EDHF,<sup>21,26</sup> as suggested from recent reports in other vascular beds. In the present study, it does not appear that prostaglandins or EDHF are involved in pial arteriolar dilation to adenosine because blockade of cyclooxygenase or  $SK_{Ca}$  and  $IK_{Ca}$  channels did not alter the response. In newborn pigs, pial arteriolar dilation to topical application of adenosine was found to be reduced by 50% following NOS blockade ( $N^G$ -nitro-L-arginine, L-NNA),<sup>11</sup> suggesting the involvement of NO in this response. However, the exact source of NO for those observed vasomotor responses is uncertain because it is unclear whether the release of NO is a result of secondary changes in local haemodynamics (e.g. shear stress), metabolism, and/or neural-glial activities after adenosine application. In the present study, we report for the first time that pharmacological NOS blockade significantly attenuates adenosine- and CGS21680-induced dilations of isolated pial arterioles, suggesting the direct involvement of vascular NO in mediating the  $A_{2A}$  receptor-mediated response. A predominant role of endothelium-derived NO was apparent at the lower concentrations

of adenosine from 30 nM to 1  $\mu$ M because denudation and L-NAME both reduced the vasodilator response by nearly 50–60%. This contention is directly supported by the measurement of adenosine (1  $\mu$ M)-stimulated NO production from intact pial arterioles. In addition, we found that inhibition of the soluble guanylyl cyclase, a downstream target of NO in the vascular smooth muscle,<sup>27</sup> by ODQ attenuated the vasodilation to adenosine similar to that by L-NAME and endothelial denudation (Figure 3). The involvement of endothelium-derived NO/guanylyl cyclase/cGMP pathway in mediating adenosine-induced dilation as reported in the current study may explain the observed inhibition of pial arteriolar dilation<sup>11</sup> and cerebral hyperaemic response<sup>28</sup> to adenosine by NOS inhibitors, as well as the rise of cGMP level by adenosine in cortical periarachnoid fluid *in vivo*.<sup>11</sup>

Another signalling pathway that may be involved in pial arteriolar dilation to adenosine is via the opening of smooth muscle potassium channels. Experimental evidence from *in vivo* studies has provided disparate results supporting the contribution of  $K_{ATP}$ ,<sup>11,12</sup>  $BK_{Ca}$ ,<sup>13</sup> or  $K_{ir}$  channels<sup>10</sup> in adenosine-induced dilation of pial arterioles. In contrast, another *in vivo* study showed no role of  $K_{ATP}$  channels in the adenosine-induced dilation of pial arterioles.<sup>29</sup> In the present study, we found that only  $K_{ir}$  channel inhibitor  $Ba^{2+}$  reduced the adenosine-induced dilation of pial arterioles. The ability of  $Ba^{2+}$  to diminish vasodilation to CGS21680 extended support for  $A_{2A}$  receptor signalling via  $K_{ir}$  channels. Interestingly, in the presence of both  $Ba^{2+}$  and L-NAME, there was no greater reduction of the vasodilation to adenosine than by either  $Ba^{2+}$  or L-NAME alone, suggesting the pivotal role of NO in opening  $K_{ir}$  channels in vascular smooth muscle for vasodilation to adenosine. This idea is further supported by the ability of  $Ba^{2+}$  to diminish arteriolar dilation to NO donor SNP and by the lack of further reduction in dilation to adenosine in denuded vessels following exposure to  $Ba^{2+}$ . However, the putative role of  $K_{ATP}$  channels should be considered because it has been shown that  $Ba^{2+}$  inhibits not only  $K_{ir}$  channels but also possibly  $K_{ATP}$  channels in some preparations.<sup>30,31</sup> To address this issue, we treated the vessels with  $K_{ir}$  channel activator  $K^+$  (10 mM) and the  $K_{ATP}$  channel opener pinacidil, and found that  $Ba^{2+}$  only inhibited  $K^+$ -induced dilation, suggesting the specific  $K_{ir}$  channel inhibition by  $Ba^{2+}$  in the present study. The effectiveness of glibenclamide in blocking vasodilation to pinacidil, but not to adenosine or CGS21680, further suggests the absent role of  $K_{ATP}$  channels in pial arteriolar dilation to adenosine  $A_{2A}$  receptor activation. On the other hand, the early *in vivo* evidence in newborn pigs shows that glibenclamide diminishes the adenosine-induced dilation of pial arterioles by nearly 50%.<sup>11</sup> These disparate results may be related to the indirect influence of the inhibitor on  $K_{ATP}$  channels in brain neurons *in vivo*<sup>32</sup> and/or to differences in age/maturation (neonatal vs. juvenile) of the animals. Collectively, our data suggest that the activation of endothelial  $A_{2A}$  receptors leads to NO-dependent opening of smooth muscle  $K_{ir}$  channels, via guanylyl cyclase/cGMP pathway, for pial arteriolar dilation to adenosine.

In the present study, we found that the dilations of pial arterioles to adenosine, CGS21680, and forskolin were significantly reduced by the cAMP signalling inhibitor Rp-8-Br-cAMPS. It is important to note that treatment of pial arterioles with combined L-NAME and Rp-8-Br-cAMPS (Figure 5B) produced a significantly greater attenuation of the adenosine-induced dilation than treatment with either inhibitor alone, indicating the involvement of two independent vasodilation mechanisms. Because the dilation of pial arterioles to

forskolin was inhibited by at least 40–50% following L-NAME treatment or endothelial removal, it appears that this drug can promote cAMP-mediated activation of eNOS in porcine pial arterioles. This conclusion is consistent with previous evidence showing forskolin activates eNOS via cAMP signalling in cultured endothelial cells.<sup>33</sup> However, it does not appear that adenosine promotes a cAMP cascade to activate eNOS because in the absence of the endothelium Rp-8-Br-cAMPS further reduced the dilation to adenosine in a comparable manner to the combination treatment with L-NAME and Rp-8-Br-cAMPS. This finding indicates a component of smooth muscle cAMP signalling, independent of eNOS, in adenosine-induced dilation of porcine pial arterioles. Interestingly, we also found that Rp-8-Br-cAMPS had an inhibitory effect on the dilation of pial arterioles to SNP. This result may intuitively suggest the involvement of cAMP in mediating NO signalling for adenosine-induced vasodilation. It should be noted that the observed Rp-8-Br-cAMPS effect on SNP may be explained by a previous report that Rp-diastereomeric analogues of cAMP inhibit both cAMP- and cGMP-induced dilation of mesenteric arteries<sup>34</sup> or by evidence showing SNP, which is composed of both NO and iron, can activate cAMP signalling via release of iron rather than NO from its structure.<sup>35</sup> Our findings are consistent with the latter finding because the treatment of pial arterioles with  $Ba^{2+}$  or Rp-8-Br-cAMPS caused a comparable 40% reduction in dilation to SNP but the combined treatment with these two blockers nearly abolished the response. These results support the idea that SNP does not act solely as an NO donor in porcine pial arterioles. The activation of dual pathways, guanylyl cyclase/ $K_{ir}$  and cAMP, appear to mediate the dilation of pial arterioles to SNP. Further evidence to support the idea that NO and  $K_{ir}$  channels are linked in series for mediating dilation of pial arterioles was provided by similar attenuation of forskolin-induced vasodilation following exposure to  $Ba^{2+}$  or L-NAME with no further diminution during their combined treatment (Figure 5C). Collectively, our results indicate that the NO-independent dilation associated with smooth muscle adenosine  $A_{2A}$  receptor activation in the porcine pial microvessels is mediated via the adenylyl cyclase/cAMP pathway.

In summary, we found that dilation of porcine pial arterioles to adenosine is mediated by at least two parallel mechanisms. First, adenosine-induced activation of endothelial  $A_{2A}$  receptors leads to the production of NO with subsequent activation of smooth muscle soluble guanylyl cyclase and opening of  $K_{ir}$  channels for vasodilation. Secondly, activation of smooth muscle  $A_{2A}$  receptors evokes vasodilation through cAMP signalling. Notably, we previously found that dilation of porcine coronary<sup>18,23</sup> and retinal<sup>25</sup> arterioles to adenosine  $A_{2A}$  receptor activation is mediated in part via opening of smooth muscle  $K_{ATP}$  channels independent of cAMP signalling. It appears that the pial arterioles exhibit a unique mechanism for adenosine-induced dilation different from that of other metabolically active organs. Because  $K^+$  and adenosine released from neural-glial cells have been implicated as important factors contributing to neurovascular blood-flow regulation in the cerebral circulation,<sup>3,5</sup> our present results suggest that the opening of  $K_{ir}$  channels plays a central role in the vasodilator response of pial arterioles to both of these metabolites. Furthermore, the NO component of  $A_{2A}$  receptor activation of pial arterioles may be a target for impaired adenosine-induced dilation of these vessels following acute subarachnoid haemorrhage.<sup>6</sup> The NO scavenging role of haemoglobin<sup>36</sup> and/or elevated levels of endogenous eNOS inhibitor asymmetric dimethyl-L-arginine<sup>37</sup> may explain the attenuated adenosine response.

Identifying the molecular signalling pathways for adenosine-induced dilation of pial arterioles in the present study may help future mechanistic examinations on the role of this critical metabolite under pathophysiological conditions.

## Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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