

Adenosine A_{2A} Receptors Mediate Coronary Microvascular Dilation to Adenosine: Role of Nitric Oxide and ATP-Sensitive Potassium Channels¹

TRAVIS W. HEIN, LUIZ BELARDINELLI, and LIH KUO

Department of Medical Physiology, Cardiovascular Research Institute, Texas A&M University System Health Science Center, College Station, Texas (T.W.H., L.K.); and CV Therapeutics, Palo Alto, California (L.B.)

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ABSTRACT

Adenosine is a potent vasodilator that plays an important role in the regulation of coronary microvascular diameter. Although multiple adenosine receptor subtypes have been recently cloned, the specific adenosine receptor subtypes and the underlying mechanisms responsible for the vasodilation to adenosine in the coronary microcirculation remain unknown. Therefore, in the present study we determined the receptor subtypes for coronary arteriolar dilation to adenosine and investigated the role of nitric oxide (NO) and ATP-sensitive potassium (K_{ATP}) channels in this vasodilatory response. Pig coronary arterioles (50–100 μm in situ) were isolated, cannulated, and pressurized without flow for in vitro study. Arterioles developed basal tone and dilated in a concentration-dependent manner to adenosine and to adenosine receptor agonists (2S)-N⁶-[2-endo-norbornyl]adenosine (A₁), 2-[p-(2-carboxyethyl)]phenylethyl-amino-5'-N-ethylcarboxamidoadenosine (CGS21680; A_{2A}), N⁶-(3-iodobenzyl)adenosine-5'-N-methyluronamide (A₃), and N-ethyl-

carboxamidoadenosine (nonselective adenosine receptor activation). 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-yl amino]ethyl)phenol attenuated vasodilation to adenosine and to all adenosine receptor agonists tested, suggesting that the vasodilatory responses were primarily mediated by A_{2A} receptors. Adenosine- and CGS21680-induced dilations were attenuated in a similar manner by endothelial removal and by the NO synthase inhibitor N^G-nitro-L-arginine methyl ester. In denuded vessels, both adenosine- and CGS21680-induced dilations were nearly abolished by the K_{ATP} channel inhibitor glibenclamide. The selective A_{2A} agonist CGS21680 mechanistically mimics the vasodilation in response to adenosine. Collectively, our results suggest that the dilation of coronary arterioles to adenosine is mediated predominantly by A_{2A} receptors. Activation of this receptor subtype elicits vasodilation by endothelial release of NO and by the smooth muscle opening of K_{ATP} channels.

Adenosine is a potent coronary vasodilator that is implicated in the control of coronary blood flow during increases in metabolic demand or stress of myocardium (Berne, 1980). This vasodilatory response is mediated by the activation of cell surface adenosine receptors. Four adenosine receptor subtypes (A₁, A_{2A}, A_{2B}, and A₃) have been cloned from various cell types, tissues, and species (Fredholm et al., 1994), and coronary vascular cells have been shown to exhibit pharmacological activity of A₁ and A₂ receptors (Mills and Gewirtz, 1990). Pharmacological activation of A₁ or A₂ receptors in vivo (Nekoeian and Tabrizchi, 1996), in isolated

perfused hearts (Nakhostine and Lamontagne, 1993; Akatsuka et al., 1994; Shryock et al., 1998; Belardinelli et al., 1998), and in isolated vessels (Vials and Burnstock, 1993; Felsch et al., 1994) causes coronary vasodilation. However, the identity and relative importance of receptor subtypes responsible for vasodilation to adenosine in the coronary microcirculation have not been directly characterized. It is worth noting that the above-mentioned in vivo and in vitro studies of vasodilation associated with adenosine receptor activation are generally confounded by various factors. For instance, the limitations of in vivo or in situ study of the intact heart (Nakhostine and Lamontagne, 1993; Akatsuka et al., 1994; Nekoeian and Tabrizchi, 1996) are evident from influences on vasomotor function by neurohumoral, hemodynamic, and local control mechanisms (Jones et al., 1995a). On

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ABBREVIATIONS: NO, nitric oxide; CGS15943, 5-amino-9-chloro-2-(2-furyl)1,2,4-triazolo[1,5-c]quinazoline; CGS21680, 2-[p-(2-carboxyethyl)]phenylethyl-amino-5'-N-ethylcarboxamidoadenosine; CPX, 8-cyclopentyl-1,3-dipropylxanthine; ENBA, (2S)-N⁶-[2-endo-norbornyl]adenosine; AB-MECA, 4-aminobenzyl-5'-N-methylcarboxamidoadenosine; IB-MECA, N⁶-(3-iodobenzyl)adenosine-5'-N-methyluronamide; K_{ATP}, ATP-sensitive potassium; L-NAME, N^G-nitro-L-arginine methyl ester; CCPA, 2-chloro-N⁶-cyclopentyladenosine; DMSO, dimethyl sulfoxide; MRS1191, 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate; NECA, N-ethylcarboxamidoadenosine; PSS, physiological salt solution; ZM241385, 4-(2-[7-amino-2-(2-furyl)[1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-yl amino]ethyl)phenol.

the other hand, the *in vitro* studies (Vials and Burnstock, 1993; Felsch et al., 1994) were primarily performed in large conduit vessels that were precontracted with various pharmacological agents due to the lack of basal tone. These vasoconstrictor agents have the potential to influence the adenosine receptor response by activating and superimposing different contractile mechanisms (Herlihy et al., 1976; Jiang et al., 1991). Most importantly, the study on large vessels (Vials and Burnstock, 1993; Felsch et al., 1994; Belardinelli et al., 1998; Shryock et al., 1998) has minimal relation to the blood flow regulation that takes place in the resistance vessels, and thus the results cannot be extrapolated to the microcirculation. Furthermore, it is well recognized that many receptors in the coronary circulation are not equally distributed in large versus small blood vessels (Foulkes et al., 1991; Jones et al., 1993). Therefore, the specific receptor subtypes and its coupling mechanism responsible for vasodilation to adenosine in the coronary microcirculation remain unknown.

Regardless of the aforementioned experimental limitations, studies using intact heart preparations and isolated conduit vessels have suggested that coronary vasodilation to adenosine is mediated by the production of nitric oxide (NO; Vials and Burnstock, 1993; Abebe et al., 1995) and/or by the activation of smooth muscle ATP-sensitive potassium (K_{ATP}) channels (Akatsuka et al., 1994; Niiya et al., 1994; Randall, 1995). Although these vasodilatory pathways have not been unequivocally demonstrated in coronary conduit vessels (Vials et al., 1993; Akatsuka et al., 1994; Niiya et al., 1994; Abebe et al., 1995; Randall, 1995), it remains unclear whether coronary microvessels use similar pathways for vasodilation in response to adenosine. Moreover, the adenosine receptor subtype responsible for activation of the NO pathway and K_{ATP} channels has not been determined, in particular at the microvascular levels. Although a recent patch-clamp study of coronary artery smooth muscle cells suggested that adenosine might act at an A_1 receptor to activate K_{ATP} channels (Dart and Standen, 1993), there is no functional evidence to support this conclusion. Therefore, the goal of the present study was to determine the adenosine receptor subtypes responsible for the dilation of coronary microvessels to adenosine. In addition, the roles of NO and K_{ATP} channels in vasodilation associated with adenosine receptor activation were investigated. The present study was performed in the isolated coronary arterioles with basal tone. The luminal pressure and flow were precisely controlled to eliminate the possible confounding factors associated with *in vivo* and perfused heart preparations.

Materials and Methods

General Preparation. Pigs (8–12 weeks old of either sex) were sedated with an *i.m.* injection of Telazol [tiletamine and zolazepam (1:1) 4.4 mg/kg] and xylazine (2.2 mg/kg) and then anesthetized and heparinized with an *i.v.* administration of pentobarbital sodium (20 mg/kg) and heparin (1000 U/kg), respectively, via the marginal ear vein. Pigs were intubated and ventilated with room air. After a left thoracotomy was performed, the heart was electrically fibrillated, excised, and immediately placed in cold (5°C) saline solution.

Isolation and Cannulation of Microvessels. The techniques for identification and isolation of porcine coronary microvessels were described previously (Kuo et al., 1988). In brief, a mixture of India ink and gelatin in physiological salt solution (PSS) containing 145.0 mM NaCl, 4.7 mM KCl, 2.0 mM $CaCl_2$, 1.17 mM $MgSO_4$, 1.2 mM

NaH_2PO_4 , 5.0 mM glucose, 2.0 mM pyruvate, 0.02 mM EDTA, and 3.0 mM 3-(*N*-morpholino)propanesulfonic acid was perfused into the left anterior descending coronary artery (0.3 ml) and the circumflex artery (0.4 ml) to enable visualization of coronary microvessels. Subepicardial arteriolar branches (50–100- μ m diameter and 0.6–1.0 mm in length *in situ*) from the left anterior descending coronary or circumflex arteries were selected and carefully dissected from the surrounding cardiac tissue under cold (5°C) PSS containing 1% BSA (Amersham, Arlington Heights, IL) at pH 7.4. Each isolated arteriole was then transferred for cannulation to a Lucite vessel chamber containing PSS-albumin equilibrated with room air at ambient temperature. One end of the microvessel was cannulated with a glass micropipette (40 μ m in tip diameter) filled with filtered PSS-albumin, and the outside of the microvessel was securely tied to the pipette with 11-0 ophthalmic suture (Alcon, Fort Worth, TX). The ink-gelatin solution inside the vessel was flushed out. Then, the other end of the vessel was cannulated with a second micropipette and tied with suture. We have previously shown that the ink-gelatin solution has no detectable detrimental effect on either endothelial or vascular smooth muscle function (Kuo et al., 1988).

Instrumentation. After cannulation of a blood vessel, the chamber was transferred to the stage of an inverted microscope (model IM35; Zeiss, Thornwood, NY) coupled to a CCD camera (KP-161; Hitachi) and video micrometer (Microcirculation Research Institute, Texas A&M University Health Science Center, College Station, TX). Internal diameters of the vessel were measured throughout the experiment using video microscopic techniques (Kuo et al., 1988). The micropipettes were connected to independent reservoir systems, and intraluminal pressures were measured through side-arms of the two reservoir lines by low-volume displacement strain-gauge transducers (Statham P23Db; Gould, Cleveland, OH). The isolated vessels were pressurized without flow by setting both reservoirs at the same hydrostatic level. Leaks were detected by differences between reservoir pressure and intraluminal pressure. Preparations with leaks were excluded from further study.

Experimental Protocols. Cannulated arterioles were bathed in PSS-albumin at 36–37°C to allow development of basal tone. After vessels developed a stable basal tone (~30–40 min), concentration-dependent vasodilations to the natural ligand adenosine (0.1 nM to 10 μ M), the A_1 receptor agonist (2*S*)- N^6 -[2-endo-norbornyl]adenosine (ENBA; 1 nM to 10 μ M), the A_{2A} receptor agonist 2-[*p*-(2-carboxyethyl)]phenylethyl-amino-5'-*N*-ethylcarboxamidoadenosine (CGS21680; 0.1 nM to 1 μ M), the nonselective A_{2B} agonist *N*-ethylcarboxamidoadenosine (NECA; 0.1 nM to 1 μ M), and the A_3 agonist N^6 -(3-iodobenzyl)adenosine-5'-*N*-methyluronamide (IB-MECA; 1 nM to 1 μ M) were constructed. Because there are no selective A_{2B} agonists currently available, we used the reported most potent A_{2B} agonist NECA (Alexander et al., 1996; Müller and Stein, 1996; Zocchi et al., 1996; Cooper et al., 1997; Feoktistov and Biaggioni, 1998) to study the role of A_{2B} in vasodilation. After completing the control vascular response, the vasodilations elicited by A_1 , A_{2A} , A_{2B} , and A_3 receptor agonists were reexamined after a 30-min incubation of the vessels with the respective adenosine receptor antagonists 8-cyclopentyl-1,3-dipropylxanthine (CPX; 0.1 μ M; Müller and Stein, 1996), 4-(2-[7-amino-2-(2-furyl)[1,2,4]-triazolo[2,3-*a*][1,3,5]triazin-5-yl amino]ethyl)phenol (ZM241385; 10, 30, and 50 nM and 1 μ M; Tocris Cookson Incorporated, Ballwin, MO; Poucher et al., 1995), 5-amino-9-chloro-2-(2-furyl)1,2,4-triazolo[1,5-*c*]quinazoline (CGS15943; 0.1 μ M; Alexander et al., 1996; Cooper et al., 1997), and 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(\pm)-dihydropyridine-3,5-dicarboxylate (MRS1191; 1 μ M; Research Biochemicals Inc., Natick, MA; Dunwiddie et al., 1997). In these experiments, usually one or two agonists and antagonists were studied in each microvessel. The sequence of agonist administration was alternated in each experiment. After completing the concentration-diameter curve, the vessels were washed at least three times, and the other run of the drug study was performed after equilibration of

the vessels with PSS-albumin for at least 1 h. The reproducibility of the response was confirmed in our pilot studies.

To elucidate the possible cellular mechanisms involved in the coronary arteriolar dilation to adenosine and its receptor agonists, the following series of experiments were performed. The role of endothelium in vasodilation was evaluated after endothelial denudation. The technique for endothelial removal has been described in detail in our previous study (Ishizaka and Kuo, 1996). Briefly, a nonionic detergent, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (0.4%), was intraluminally perfused into the vessel for 1 to 2 min to remove endothelial cells. Only vessels that exhibited normal basal tone showed no vasodilation to endothelium-dependent vasodilator bradykinin (1 nM; Ishizaka and Kuo, 1996) and showed unaltered vasodilation to sodium nitroprusside (1 nM to 0.1 mM) after endothelial removal were accepted for data analysis. The involvement of NO and prostaglandins in agonist-induced dilation was examined before and after treatment of isolated arterioles with the specific inhibitors *N*^G-nitro-L-arginine methyl ester (L-NAME; 10 μ M, 30 min) and indomethacin (10 μ M, 30 min), respectively. The involvement of smooth muscle K_{ATP} channels in vasodilation to adenosine and its agonists was examined before and after the incubation of denuded vessels with a specific inhibitor glibenclamide (5 μ M, 30 min). Because more than 99% of glibenclamide binds to albumin (Olsen et al., 1995), this set of experiments was performed in PSS without albumin.

Chemicals. Adenosine receptor agonists were purchased from Research Biochemicals Inc, and other drugs and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), except as specifically stated. Adenosine, bradykinin, L-NAME, and sodium nitroprusside were dissolved in PSS. Indomethacin, pinacidil, IB-MECA, and CPX were dissolved in ethanol, and NECA, glibenclamide, CGS21680, CGS15943, ENBA, MRS1191, and ZM241385 were dissolved in dimethyl sulfoxide (DMSO) as stock solutions (10 mM). Subsequent concentrations of these drugs were diluted in PSS. The final concentrations of ethanol and DMSO in the vessel bath were 0.1 and 0.03%, respectively. Vehicle control studies indicated that these final concentrations of ethanol and DMSO had no effect on the arteriolar function.

Data Analysis. At the end of each experiment, the vessel was relaxed with 100 μ M sodium nitroprusside to obtain its maximal diameter at 60 cm H₂O intraluminal pressure. We have previously shown that this concentration of sodium nitroprusside produced maximal relaxation of isolated vessels because their diameters were not further increased by a calcium-free solution containing EDTA (1 mM; Hein and Kuo, 1998). Therefore, all diameter changes in response to agonists were normalized to the vasodilation in response to 100 μ M sodium nitroprusside and expressed as a percentage of maximal dilation. All data are presented as mean \pm S.E. In each set of interventions, the vessels have their own control, with each vessel being from a different heart. The control vessels were pooled when comparing a series of experiments examining the effect of antagonists on vasodilation. Statistical comparisons of vasomotor responses under various treatments were performed using two-way ANOVA with repeated measures and tested with Fisher's protected least significant difference multiple range test. Differences in resting diameter before and after pharmacological interventions were compared by paired Student's *t* tests. The EC₅₀ values (concentrations of adenosine agonists that cause 50% of maximal dilation) were averaged from individual concentration-response curves. Mean values of EC₅₀ were calculated as the antilogarithm of mean values of pEC₅₀. The potency of ZM241385 to antagonize adenosine-induced vasodilation was estimated using Schild analysis (Arunlakshana and Schild, 1959). Dose-response ratios using EC₅₀ values for adenosine in the absence and presence of three different concentrations of ZM241385 (10, 30, and 50 nM) were calculated and used to construct a Schild plot from which the K_B and pA₂ ($-\log_{10}K_B$) values were determined. Significance was accepted at *P* < .05.

Results

Vasodilation of Coronary Arterioles to Adenosine and Adenosine Receptor Agonists. In this study, all vessels developed a similar level of basal tone (constricted to $68 \pm 1\%$ of their maximal diameter) at 36–37°C bath temperature with 60 cm H₂O intraluminal pressure. The average resting and maximal diameters of the vessel were 88 ± 3 and 129 ± 4 μ m, respectively. To examine the putative adenosine receptors involved in the dilation of coronary arterioles to adenosine, concentration-diameter relationships were established for adenosine, A₁ agonist ENBA, A_{2A} agonist CGS21680, nonselective agonist NECA, and A₃ agonist IB-MECA (Fig. 1). All agonists produced vasodilation in a concentration-dependent manner. The highest concentration of adenosine, CGS21680, and NECA produced about 90% of maximal dilation, with pEC₅₀ values of 6.62 ± 0.07 , 7.50 ± 0.11 , and 7.12 ± 0.13 , respectively. Thus, the relative order of potency for these agonists was CGS21680 > NECA > adenosine, suggesting that A_{2A} is the major adenosine receptor subtype for coronary arteriolar vasodilation. The pEC₅₀ values for ENBA and IB-MECA cannot be determined because the maximal concentration used in this study produced only 46 and 70% dilation, respectively. Any agonist concentration used higher than that presented in the present study causes nonspecific dilation of the microvessel and loss of basal tone due to the toxic effect of solvent (e.g., ethanol, >0.1%; DMSO, >0.03%).

Effect of Adenosine Receptor Blockade on Coronary Arteriolar Dilation to Adenosine and Adenosine Receptor Agonists. To evaluate the relative contribution of specific adenosine receptors in coronary arteriolar dilation to adenosine, concentration-dependent dilation to adenosine was examined in the absence and presence of competitive adenosine receptor antagonists. Blockade of A₁ and A₃ receptors by CPX (0.1 μ M) and MRS1191 (1 μ M), respectively, had no effect on vasodilation to adenosine (Fig. 2). The nonselective adenosine receptor antagonist CGS15943 slightly inhibited vasodilation elicited by higher concentrations (1 and 10 μ M) of adenosine. In contrast, the A_{2A} receptor antagonist ZM241385 (1 μ M) abolished vasodilation induced by the

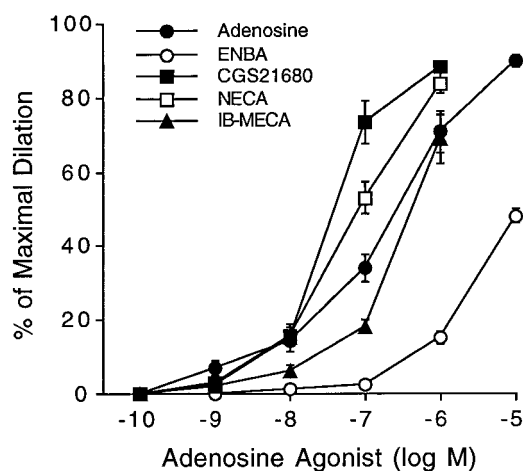


Fig. 1. Vasodilatory response of coronary arterioles to adenosine and adenosine receptor agonists. Adenosine (*n* = 5), A₁ agonist ENBA (*n* = 5), A_{2A} agonist CGS21680 (*n* = 5), nonselective agonist NECA (*n* = 5), and A₃ agonist IB-MECA (*n* = 5) elicited concentration-dependent dilation of isolated coronary arterioles. (*n* = number of vessels.)

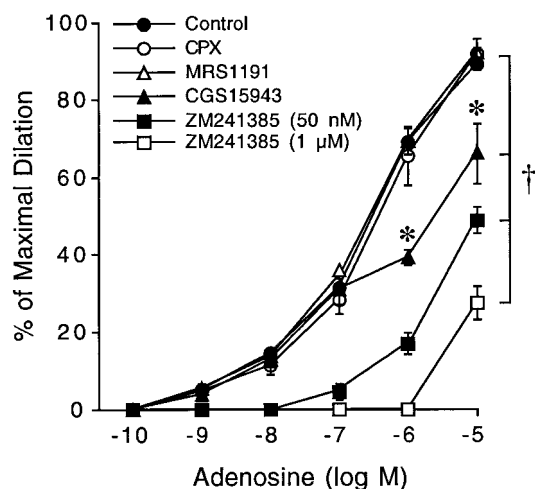


Fig. 2. Contribution of adenosine receptor subtypes to coronary arteriolar dilation to adenosine. Adenosine produced concentration-dependent dilation of isolated coronary arterioles (resting diameter = $83 \pm 5 \mu\text{m}$; maximal diameter = $128 \pm 6 \mu\text{m}$; $n = 14$). Vasodilation to adenosine was not altered by A_1 and A_3 antagonists CPX ($0.1 \mu\text{M}$, $n = 3$) and MRS1191 ($1 \mu\text{M}$, $n = 3$), respectively. The vasodilatory response to higher concentrations of adenosine was inhibited by nonselective antagonist CGS15943 ($0.1 \mu\text{M}$, $n = 3$). Adenosine-induced dilation was almost completely blocked by selective A_{2A} antagonist ZM241385 ($1 \mu\text{M}$, $n = 5$), suggesting that porcine coronary arteriolar dilation to adenosine is mediated by A_{2A} receptors. * $P < .05$, CGS15943 versus Control (ANOVA followed by Fisher's test). † $P < .05$, all interventions versus Control (ANOVA followed by Fisher's test). ($n =$ number of vessels.)

lower concentrations of adenosine ($\leq 1 \mu\text{M}$) and reduced the response to the highest concentration of adenosine ($10 \mu\text{M}$) from 93% (control) to 30% (Fig. 2). To study the nature of the antagonism by ZM241385, vasodilation to adenosine in the absence and presence of three different concentrations of ZM241385 (10, 30, and 50 nM) was examined. The adenosine-induced dilation was shifted to the right by ZM241385 in a concentration-dependent manner (Fig. 3A). The slope of the Schild plot was 1.25 (Fig. 3B), and pA_2 and K_B values for ZM241385 were 8.45 ± 0.04 and 3.54 nM , respectively. These results indicate that coronary arteriolar dilation to adenosine is primarily mediated by the activation of A_{2A} receptors.

The dilation of arterioles to A_1 agonist ENBA was not altered by CPX ($0.1 \mu\text{M}$, Fig. 4A) but was significantly inhibited by ZM241385 ($1 \mu\text{M}$, Fig. 4A). Coronary arteriolar dilation to A_{2A} agonist CGS21680 was inhibited by ZM241385 in a concentration-dependent manner (Fig. 4B). Vasodilations in response to the nonselective adenosine receptor agonist NECA, only at the higher concentrations, were partially inhibited ($\sim 50\%$) by CGS15943 ($0.1 \mu\text{M}$; Fig. 5). This concentration of CGS15943 has been shown to inhibit the A_{2B} receptor-mediated function in human embryonic kidney cells (Cooper et al., 1997). ZM241385 ($1 \mu\text{M}$) abolished the vasodilation to the lower concentrations of NECA ($\leq 0.1 \mu\text{M}$) and significantly reduced the dilation from 85 to 35% at the highest concentration of NECA ($1 \mu\text{M}$). The remaining dilation to NECA was abolished by adding CGS15943 ($0.1 \mu\text{M}$) to the ZM241385-treated vessels (Fig. 5). These results suggest that ENBA and CGS21680 cause dilation by activating A_{2A} receptors, whereas NECA activates both A_{2A} and A_{2B} receptors. IB-MECA-induced vasodilation was not affected by A_3 antagonist MRS1191 ($1 \mu\text{M}$); in contrast, ZM241385 ($1 \mu\text{M}$) effectively abolished the vasodilation to IB-MECA (Fig. 6), suggesting that the dilation elicited by IB-MECA is also

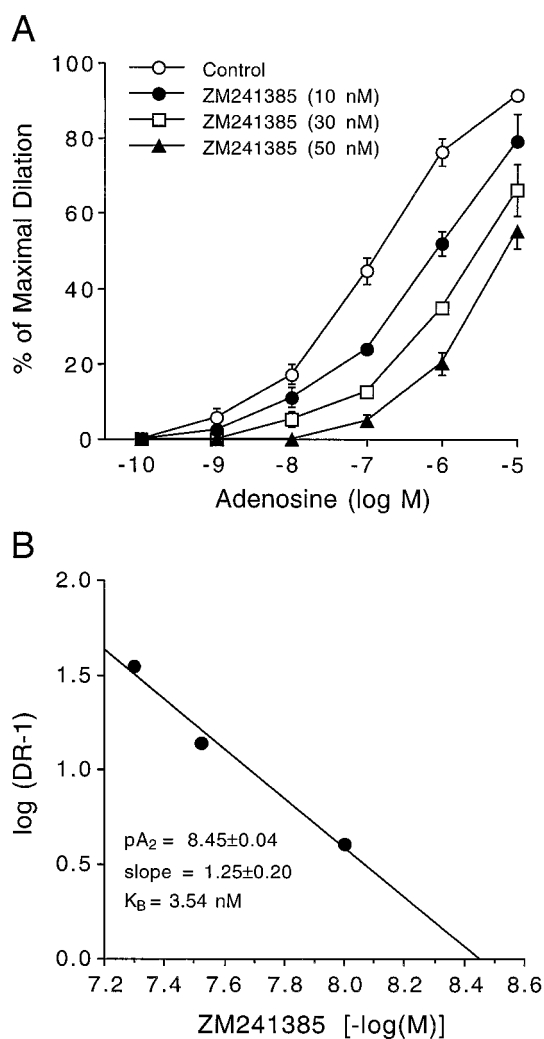


Fig. 3. Effects of ZM241385 on coronary arteriolar dilation to adenosine. A, adenosine produced concentration-dependent dilation of isolated coronary arterioles (resting diameter = $93 \pm 6 \mu\text{m}$; maximal diameter = $137 \pm 9 \mu\text{m}$; $n = 6$). This vasodilatory response was inhibited in a concentration-dependent manner by ZM241385 (10, 30, and 50 nM). B, Schild plot of results shown in A. ($n =$ number of vessels.)

mediated by A_{2A} receptors. Because A_{2A} and A_{2B} receptors appear to be the major adenosine receptor subtypes that mediate the dilations of these coronary arterioles (Figs. 1–5), it is likely that adenosine-induced dilation is also mediated by the activation of the same receptor subtypes. This interpretation is further supported by the results depicted in Fig. 7: adenosine-induced dilation, except at the highest concentration ($10 \mu\text{M}$), was abolished by the A_{2A} antagonist ZM241385 ($1 \mu\text{M}$). The residual dilation at the highest concentration of adenosine was blocked by adding the antagonist CGS15943 ($0.1 \mu\text{M}$) to the ZM241385-treated vessels (Fig. 7). It is important to note that ZM241385 and CGS15943 did not alter resting vascular diameter and did not alter coronary arteriolar function through a nonspecific effect because endothelium-independent vasodilation in response to sodium nitroprusside was not affected by these antagonists (data not shown).

Contribution of Endothelium-Derived NO to Vasodilations Elicited by Adenosine and CGS21680. The contribution of NO to adenosine- and CGS21680-induced vaso-

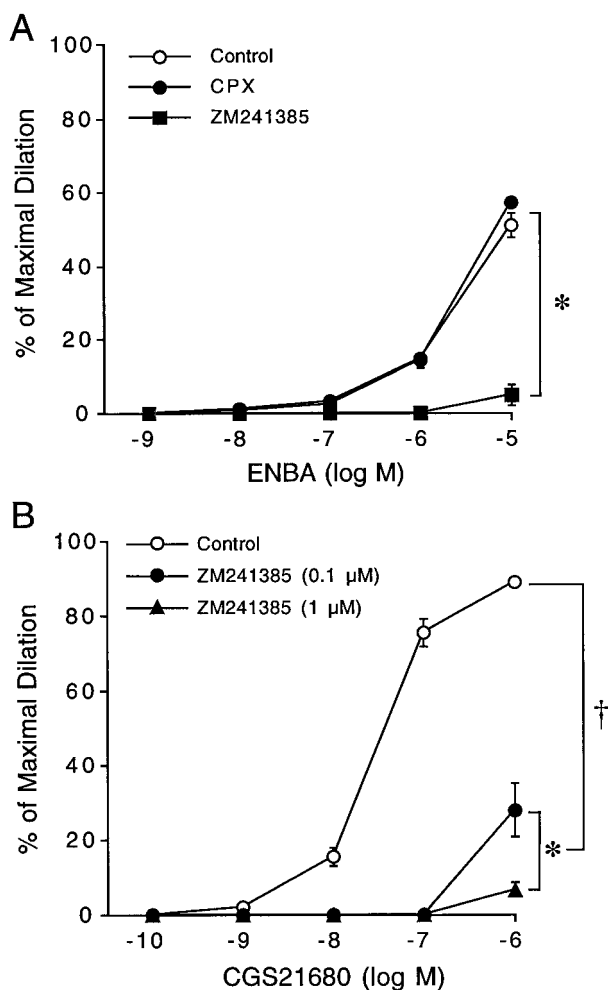


Fig. 4. Contribution of adenosine receptors to coronary arteriolar dilations to ENBA and CGS21680. A, A_1 agonist ENBA produced concentration-dependent dilation of isolated coronary arterioles (resting diameter = $86 \pm 6 \mu\text{m}$; maximal diameter = $128 \pm 7 \mu\text{m}$; $n = 8$). Vasodilation to ENBA was not altered by A_1 antagonist CPX ($0.1 \mu\text{M}$, $n = 4$). The A_{2A} antagonist ZM241385 ($1 \mu\text{M}$, $n = 4$) significantly inhibited dilation to ENBA, suggesting that porcine coronary arteriolar dilation to this adenosine receptor agonist is mediated by A_{2A} receptors. $*P < .05$, ZM241385 versus Control or CPX. B, A_{2A} agonist CGS21680 produced concentration-dependent dilation of isolated coronary arterioles (resting diameter = $79 \pm 4 \mu\text{m}$; maximal diameter = $122 \pm 6 \mu\text{m}$; $n = 10$). Vasodilation to CGS21680, except at the highest concentration ($1 \mu\text{M}$), was abolished by $0.1 \mu\text{M}$ ZM241385 ($n = 6$). The remaining dilation at $1 \mu\text{M}$ CGS21680 was abolished by a higher concentration of ZM241385 ($1 \mu\text{M}$, $n = 4$), suggesting that porcine coronary arteriolar dilation to CGS21680 is mediated by A_{2A} receptors. $*P < .05$, ZM241385 $0.1 \mu\text{M}$ versus $1 \mu\text{M}$ (ANOVA followed by Fisher's test). $^\dagger P < .05$, ZM241385 (0.1 and $1 \mu\text{M}$) versus Control (ANOVA followed by Fisher's test). ($n =$ number of vessels.)

dilations was examined and compared in the absence and presence of L-NAME ($10 \mu\text{M}$). The resting vessel diameter was slightly decreased by L-NAME but not in a significant manner. L-NAME significantly shifted the vasodilatory response curves of adenosine (Fig. 8A) and CGS21680 (Fig. 8B) to the right. These data suggest that NO contributes in part to the coronary arteriolar dilations to adenosine and CGS21680. It is important to note that the cyclooxygenase inhibitor indomethacin ($10 \mu\text{M}$) did not alter vasodilations to adenosine or CGS21680 (data not shown), indicating that prostaglandins are not involved in these dilations.

Contribution of Vascular Smooth Muscle K_{ATP} Channels to Arteriolar Dilations Elicited by Adenosine and

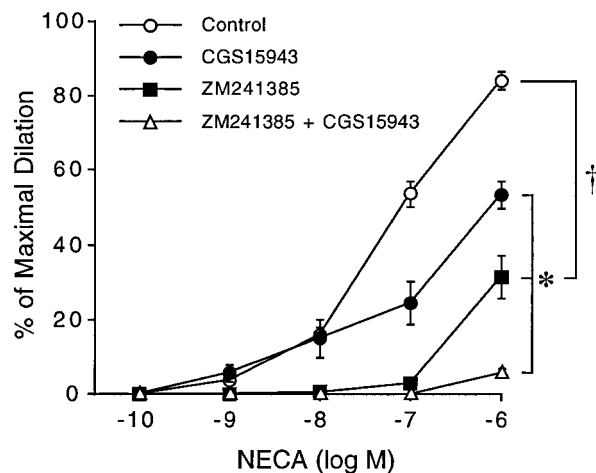


Fig. 5. Contribution of adenosine receptors to coronary arteriolar dilation to NECA. Nonselective adenosine receptor agonist NECA produced concentration-dependent dilation of isolated coronary arterioles (resting diameter = $83 \pm 5 \mu\text{m}$; maximal diameter = $125 \pm 6 \mu\text{m}$; $n = 8$). Vasodilation to NECA was inhibited by nonselective antagonist CGS15943 ($0.1 \mu\text{M}$, $n = 3$) and by selective A_{2A} antagonist ZM241385 ($1 \mu\text{M}$, $n = 5$). The addition of CGS15943 to the ZM241385-treated vessels almost completely blocked the vasodilatory response ($n = 4$), suggesting that porcine coronary arteriolar dilation to NECA is mediated by A_{2A} and A_{2B} receptors. $*P < .05$, ZM241385 plus CGS15943 versus CGS15943 or ZM241385 (ANOVA followed by Fisher's test). $^\dagger P < .05$, all interventions versus Control (ANOVA followed by Fisher's test). ($n =$ number of vessels.)

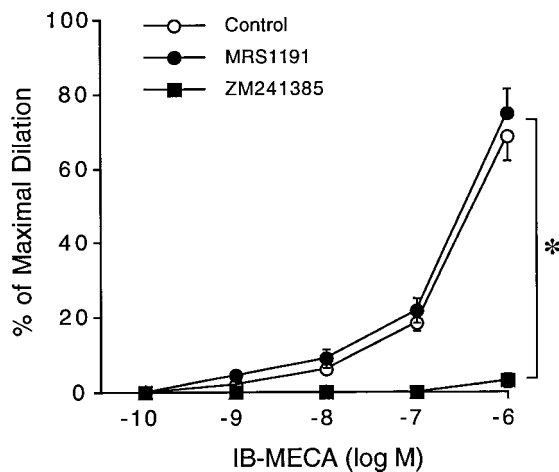


Fig. 6. Contribution of adenosine receptors to coronary arteriolar dilation to IB-MECA. A_3 agonist IB-MECA produced concentration-dependent dilation of isolated coronary arterioles (resting diameter = $98 \pm 8 \mu\text{m}$; maximal diameter = $138 \pm 8 \mu\text{m}$; $n = 7$). Vasodilation to IB-MECA was not altered by A_3 antagonist MRS1191 ($1 \mu\text{M}$, $n = 3$). The A_{2A} antagonist ZM241385 ($1 \mu\text{M}$) abolished dilation to IB-MECA ($n = 4$), suggesting that porcine coronary arteriolar dilation to this adenosine receptor agonist is mediated by A_{2A} receptors. $*P < .05$, ZM241385 versus Control or MRS1191 (ANOVA followed by Fisher's test). ($n =$ number of vessels.)

CGS21680. The results of the experiments designed to determine the contribution of smooth muscle K_{ATP} channels to vasodilations elicited by adenosine and CGS21680 are illustrated in Fig. 8. In this series of studies, the endothelium was initially removed to eliminate its contribution to vasodilation. Disruption of endothelium did not affect basal tone but attenuated both adenosine- and CGS21680-induced vasodilations. It is worth noting that the inhibition of vasodilation by the disruption of endothelium was identical to that produced by L-NAME (Fig. 8). Glibenclamide ($5 \mu\text{M}$) did not alter the basal tone of denuded vessels but produced a near-

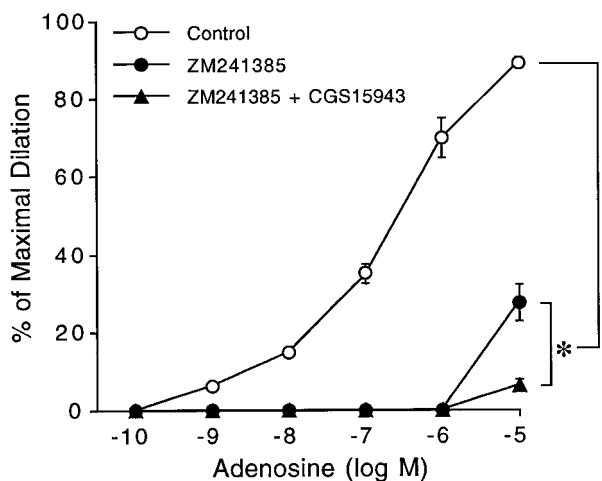


Fig. 7. Contribution of A_{2A} and A_{2B} receptors to coronary arteriolar dilation to adenosine. Adenosine produced concentration-dependent dilation of isolated coronary arterioles (resting diameter = $85 \pm 8 \mu\text{m}$; maximal diameter = $129 \pm 10 \mu\text{m}$; $n = 5$). Vasodilation to adenosine, except at the highest concentration, was abolished by A_{2A} antagonist ZM241385 ($1 \mu\text{M}$, $n = 5$). The addition of the nonselective antagonist CGS15943 ($0.1 \mu\text{M}$) to the ZM241385-treated vessels abolished the residual vasodilation, suggesting that porcine coronary arteriolar dilation to the highest concentration of adenosine is mediated by A_{2A} and A_{2B} receptors. * $P < .05$, ZM241385 plus CGS15943 versus ZM241385 (ANOVA followed by Fisher's test). † $P < .05$, all interventions versus Control (ANOVA followed by Fisher's test). ($n =$ number of vessels.)

complete inhibition of vasodilations in response to adenosine (Fig. 8A) and CGS21680 (Fig. 8B). These results suggest that activation of smooth muscle K_{ATP} channels contributes to adenosine- and CGS21680-induced dilations of coronary arterioles.

Discussion

In the present study, we provide the first direct evidence that coronary microvascular dilation to adenosine is mediated predominantly by A_{2A} receptors. This conclusion is based on the findings that CGS21680, the rather selective A_{2A} receptor agonist, is the most potent vasodilator in these microvessels and that the adenosine-induced dilation is effectively attenuated only by the selective A_{2A} antagonist ZM241385. In addition, CGS21680 mechanistically mimics the vasodilatory effect of adenosine. The A_{2A} receptors appear to be on both the microvascular endothelial and smooth muscle cells. Activation of these receptors elicits vasodilation by endothelial release of NO and by the smooth muscle opening of K_{ATP} channels. To provide a perspective on our observations and conclusions, the results from the present study are discussed below in reference to methodological considerations and to previous findings regarding adenosine receptors, endothelial dependence, and potassium channels in mediating adenosine-induced coronary dilation.

Methodological Considerations. Although it has previously been shown in isolated guinea pig hearts that A_{2A} receptors mediated coronary vasodilation (Belardinelli et al., 1998; Shryock et al., 1998), another study used isolated rabbit hearts and suggested that A_1 receptors were involved in coronary vasodilation to adenosine (Nakhostine and Lamontagne, 1993). These discrepancies may result from the species differences and/or uncontrolled factors associated with perfused heart study. Regardless, the implication from these

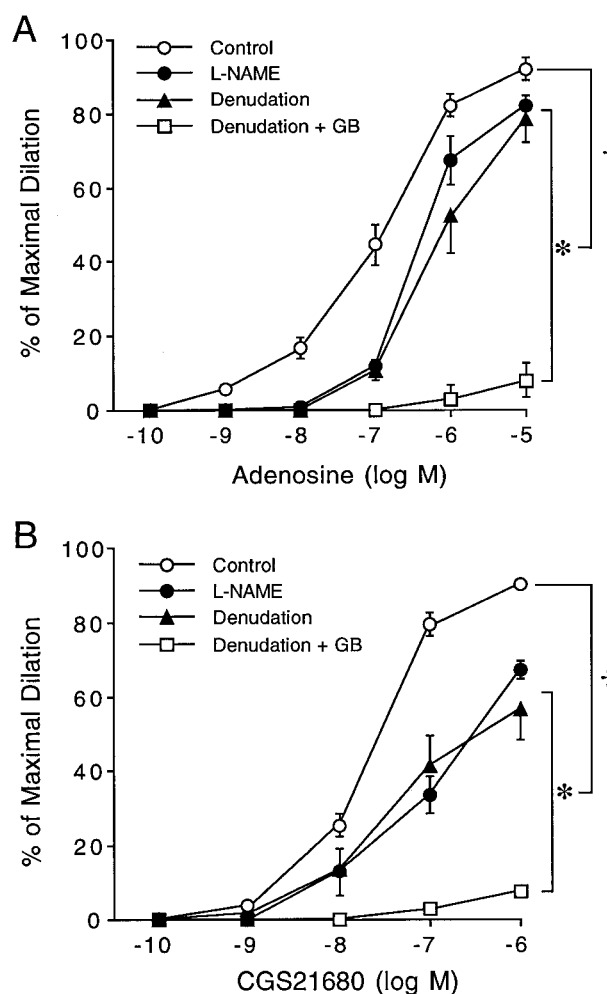


Fig. 8. Contribution of NO, endothelium, and K_{ATP} channel in coronary arteriolar dilations to adenosine and CGS21680. Adenosine (A; resting diameter = $89 \pm 5 \mu\text{m}$; maximal diameter = $129 \pm 7 \mu\text{m}$; $n = 9$) and CGS21680 (B; resting diameter = $91 \pm 6 \mu\text{m}$; maximal diameter = $129 \pm 7 \mu\text{m}$; $n = 8$) produced concentration-dependent dilation of isolated coronary arterioles. The vasodilatory responses were significantly attenuated in a similar manner by NO synthase inhibitor L-NAME ($10 \mu\text{M}$, $n = 5$ for adenosine and $n = 4$ for CGS21680) and by endothelial denudation ($n = 4$ for adenosine and $n = 4$ for CGS21680). Subsequent administration of the K_{ATP} channel inhibitor glibenclamide (GB, $5 \mu\text{M}$) nearly abolished dilation of denuded vessels to adenosine ($n = 4$) and CGS21680 ($n = 4$). * $P < .05$, L-NAME or Denudation versus Denudation plus GB (ANOVA followed by Fisher's test). † $P < .05$, all interventions versus Control (ANOVA followed by Fisher's test). ($n =$ number of vessels.)

studies that specific adenosine receptors are being activated in the coronary microvessels can only be suggested because we (Kuo et al., 1991; Kuo and Chancellor, 1995; Liao and Kuo, 1997) and other investigators (Pohl et al., 1994; Jones et al., 1995b) have shown that the in vivo and in situ studies of vasodilators are potentially confounded by local changes of hemodynamic factors such as pressure and flow. For example, vasodilation associated with increased flow can be potentiated by a threshold concentration of adenosine (Kuo and Chancellor, 1995), and the vasodilation to adenosine can be enhanced by a reduction of luminal pressure (T.W.H. and L.K., unpublished observations). In this regard, the vasodilation (e.g., in the form of increased flow or reduced pressure) observed in the intact heart study can be an integrated response from the direct action of adenosine agonist and the indirect activation of flow-induced dilation and myogenic di-

lation. Therefore, the results from *in vivo/in situ* studies are difficult to interpret, and the adenosine receptor subtypes involved in the dilation of coronary microvessels to adenosine remain unknown. Although the *in vitro* study can directly assess the role of receptor subtypes in vasodilation, these studies also yield inconsistent results (White and Angus, 1987; King et al., 1990; Abebe et al., 1994, 1995; Makujina et al., 1994; Niiya et al., 1994). It is worth noting that these *in vitro* studies were performed in the large conduit coronary arteries in the presence of pharmacological constrictors. We and other investigators have shown that different pre-constrictors elicit different signal transduction pathways and confound the vasomotor function (Nakajima and Ueda, 1990; Rosenblum and Nelson, 1990; Balwierczak, 1991; Saito et al., 1994; Falloon et al., 1995; Kuo and Chancellor, 1995). For example, the vessels precontracted with KCl could mask the potassium channel for vasodilation to adenosine (Herlihy et al., 1976; Jiang et al., 1991; Kuo and Chancellor, 1995), and the increased smooth muscle calcium by precontractors could possibly alter the vasodilatory response of adenosine and its agonists. Furthermore, it is well recognized that many receptors in the coronary circulation are not equally distributed in large versus small blood vessels (Foulkes et al., 1991; Jones et al., 1993), and thus the results obtained from large conduit vessels cannot be readily applied to the microcirculation. Here, an advantage of our isolated vessel approach is to directly identify specific adenosine receptor subtypes and to elucidate the mechanisms involved in the dilation of coronary microvessels to adenosine without the potentially confounding factors associated with intact heart preparations. These isolated microvessels developed basal tone, which precludes the use of pharmacological precontractors.

However, the isolated microvessel preparation used in the present study is not amenable to detailed quantification of the pharmacology that will be necessary to determine agonist and antagonist potency or affinity values. The basal tone developed by these isolated coronary arterioles is sensitive (i.e., loss of basal tone) to repeated stimulation with pharmacological agonists and to concentrations of agonists that elicit maximal dilation. For example, the concentration of 100 μM adenosine produces maximal dilation, but this high concentration is harmful to the vessels and often disables them from regaining basal tone. Therefore, we avoided using this high concentration of adenosine in the present study. Nevertheless, 10 μM adenosine, 1 μM NECA, and 1 μM CGS21680 produced near-maximal dilation in the present study. In contrast, the maximal dilation for ENBA and IB-MECA cannot be obtained due to the toxic effect of solvents (e.g., ethanol, >0.1%; DMSO, >0.03%) at higher concentrations. Consequently, we used only one or two adenosine agonists at the range of submaximal response in each vessel, which provided us with reliable experiments and semiquantitative data.

Coronary Dilation Caused by Adenosine Analogs. The adenosine receptor subtypes that have been implicated in mediating dilation to adenosine in various vascular beds include A_1 , A_{2A} , A_{2B} , and A_3 (Fredholm et al., 1994). The characterization of these adenosine receptors in conduit arteries has been performed by using potent and selective adenosine receptor agonists. In the present study, we used the A_1 agonist ENBA, A_{2A} agonist CGS21680, and A_3 agonist IB-MECA to determine receptor subtypes involved in the

vasodilation to adenosine. Although selective A_{2B} agonists are not available, we used the nonselective adenosine receptor agonist NECA, which has been reported to be among the most potent A_{2B} agonists (Cooper et al., 1997; Mutafova-Yambolieva and Keef, 1997). Based on binding affinities, ENBA is 15-fold selective for A_1 compared with A_2 and A_3 receptors (Müller and Stein, 1996), CGS21680 is 170-fold selective for A_{2A} over other adenosine receptors (Müller and Stein, 1996), and IB-MECA is reported as having 50-fold selectivity for A_3 receptors (Gallo-Rodriguez et al., 1994). Among these adenosine receptor agonists, CGS21680 was the most potent vasodilator, indicating that A_{2A} receptors represent the major receptor subtype for coronary arteriolar dilation. Our results not only showed that coronary arterioles can dilate concentration-dependently to all of these agonists but also indicated that these vasodilatory responses are mediated primarily by A_{2A} receptors. More specifically, the vasodilations caused by ENBA (A_1) and IB-MECA (A_3) were not affected by their corresponding selective antagonist, CPX and MRS1191, but were significantly attenuated by the A_{2A} receptor antagonist ZM241385. The concentration of CPX (0.1 μM) used in the present study is 200-fold more than the K_i value (0.5 nM; Jacobson and van Rhee, 1997b) for the A_1 receptor. Importantly, previous studies in isolated heart preparations demonstrated that this concentration of CPX selectively antagonized A_1 -mediated (negative dromotropic effect) rather than A_{2A} -mediated (increase in coronary conductance) responses to A_1 -selective agonist 2-chloro- N^6 -cyclopentyladenosine (CCPA; Belardinelli et al., 1998). On the other hand, the increase in coronary conductance by CCPA was inhibited by selective A_{2A} antagonists ZM241385 and SCH58261 (Belardinelli et al., 1998; Shryock et al., 1998). Likewise, in accord with these findings, the inhibition of ENBA-induced dilation by ZM241385 but not by CPX in our study suggested that A_{2A} rather than A_1 receptors play a major role in coronary arteriolar dilation.

The concentration of MRS1191 (1 μM) used in the present study is 300-fold more than the K_i value (31 nM) for the A_3 receptor. This concentration has been shown to block A_3 receptor activation by IB-MECA in Chinese hamster ovary cells (Jacobson et al., 1997a). As mentioned above, IB-MECA is only 50-fold selective for A_3 over A_1 and A_{2A} receptors. It is likely that IB-MECA activates A_{2A} receptors to produce vasodilation in our preparation. The possible activation of A_{2A} receptors by A_3 agonists was suggested by a recent study showing that [^{125}I]AB-MECA, an A_3 -agonist ligand, bound to the A_{2A} receptors in the brain striatum (Shearman and Weaver, 1997). In our study, dilation of coronary arterioles to IB-MECA was not affected by MRS1191 (1 μM) but was abolished by ZM241385, suggesting that IB-MECA is mediating vasodilation via activation of A_{2A} receptors rather than A_3 receptors.

To determine the role of A_{2B} receptors in vasodilation, we used nonselective adenosine receptor antagonist CGS15943 because it has been shown in recent studies to potently block A_{2B} receptor function (Alexander et al., 1996; Cooper et al., 1997; Klotz et al., 1998). The K_i value of CGS15943 for A_{2B} receptors has been reported to be 0.1 μM (Müller and Stein, 1996). At the concentration of 0.1 μM , CGS15943 potently inhibited A_{2B} receptor activation in human embryonic kidney cells (Cooper et al., 1997). Using the same concentration of CGS15943, we found that the vasodilation at the lower con-

centrations of NECA was not affected but that the dilation at the higher concentrations of NECA was attenuated. It is important to note that A_{2B} is a low-affinity receptor, whereas A_{2A} is a high-affinity receptor (Bruns et al., 1986). It is likely that higher concentrations of NECA are required to activate the A_{2B} receptor for a functional response. As a result, the reported potent A_{2B} antagonist CGS15943 attenuated vasodilations to higher concentrations of NECA but not to lower concentrations. In contrast to CGS15943, ZM241385 (1 μ M) abolished dilation induced by the lower concentrations of NECA, and the dilation at the higher concentrations was only attenuated. These results suggested that NECA might activate A_{2A} receptors at the lower concentrations and activate both A_{2A} and A_{2B} receptors at the higher concentrations. This contention is supported by the present finding that CGS15943 abolished the NECA-induced dilation in the presence of A_{2A} blocker ZM241385.

Interestingly, recent studies demonstrated that although A_1 -selective agonists such as CCPA and ENBA and the A_3 -selective agonist IB-MECA have submicromolar affinities for A_{2A} receptors (Müller and Stein, 1996), functional responses that are efficiently coupled to activation of A_{2A} receptors, such as vasodilation, can be elicited by concentrations that are lower than those needed to cause 50% occupancy of A_{2A} receptors (Belardinelli et al., 1998; Shryock et al., 1998). This high A_{2A} receptor reserve for the coronary vasodilator action of adenosine and adenosine analogs might explain our present findings that non- A_{2A} receptor agonists (e.g., A_1 and A_3 receptor agonists) are capable of causing coronary dilation via A_{2A} receptor activation. Nevertheless, the present pharmacological data provide strong evidence that the A_{2A} receptor is the major adenosine receptor subtype that mediates the coronary arteriolar dilation to adenosine analogs.

Adenosine-Induced Dilation and A_{2A} Receptors. The strongest evidence in support of A_{2A} receptor-mediated dilation of coronary arterioles to adenosine is that the selective and specific A_{2A} antagonist ZM241385 inhibited the adenosine response in a concentration-dependent manner. This recently developed nonxanthine compound is the most potent and selective A_{2A} antagonist available (Poucher et al., 1995). In the present study, ZM241385 potently ($pA_2 = 8.45$) and competitively antagonized the vasodilation to adenosine. This pA_2 value was similar to that reported previously for ZM241385 in attenuating the vasorelaxation to CGS21680 in the guinea pig isolated heart preparation ($pA_2 = 9.01$; Poucher et al., 1995). In addition, recent ligand binding studies have demonstrated that ZM241385 has 6,700-fold selectivity for A_{2A} over A_1 binding sites and 500,000-fold selectivity for A_{2A} over A_3 binding sites (Poucher et al., 1995). In our preparation, ZM241385 (1 μ M) almost abolished the vasodilation elicited by CGS21680 without affecting the vasomotor response to sodium nitroprusside, indicating that ZM241385 is a selective and specific antagonist for the A_{2A} receptor in coronary arterioles.

Although ZM241385 (1 μ M) abolished the vasodilation elicited by the lower concentrations of adenosine (≤ 1 μ M), the dilation at the highest concentration (10 μ M) was only attenuated. It is likely that adenosine activates other receptor subtypes at high concentrations. Because our findings suggest that A_{2B} receptors might be involved in the dilation elicited by a high concentration of NECA, it is possible that activation of A_{2B} receptors is responsible for the observed

residual dilation. Indeed, adding CGS15943 (0.1 μ M) to the ZM241385-treated vessels (i.e., to fully occupy all A_1 and A_{2A} receptors) resulted in a near-complete inhibition of the adenosine response (i.e., only 5% of dilation was observed). As mentioned earlier, the A_{2B} receptor has been previously characterized as a low-affinity receptor compared with the high-affinity A_{2A} receptor for adenosine (Fredholm et al., 1994), and hence at the highest concentration of adenosine (10 μ M), A_{2B} receptors, in addition to A_{2A} receptors, are likely to have been activated. This might explain the effectiveness of blocking adenosine-induced dilation by the combined administration of ZM241385 and CGS15943 in the present study. Collectively, these results provide evidence that the A_{2A} receptor is the main receptor subtype responsible for coronary arteriolar dilation to adenosine, whereas the A_{2B} receptor plays a lesser role in this dilation.

Location of Adenosine Receptors and Role of NO and K_{ATP} Channels. The location of the A_{2A} receptor mediating vasodilation to adenosine is generally considered to reside in the vascular smooth muscle cells (Abebe et al., 1994; Marala and Mustafa, 1998). However, coronary vasodilation to adenosine also has been shown to be at least in part dependent on the endothelium (Rubanyi and Vanhoutte, 1985; Vials and Burnstock, 1993; Abebe et al., 1994; Kuo and Chancellor, 1995). In the present study, the dilation of coronary arterioles to adenosine as well as CGS21680 was attenuated by endothelial removal, suggesting that endothelial A_{2A} receptors contribute to the vasodilation to adenosine. This is consistent with the finding in large conduit coronary arteries that CGS21680-induced relaxation was reduced after endothelial disruption, although the vascular response to adenosine was not examined in these large vessels (Abebe et al., 1994).

The activation of endothelial A_{2A} receptors by adenosine and CGS21680 may stimulate the production and release of NO because incubation of cultured carotid artery endothelial cells with adenosine (Li et al., 1995) or of intact coronary arterial rings (Abebe et al., 1995) with NECA or CGS21680 produced an increase in NO degradation products (e.g., nitrite and nitrate) in the medium. In our previous (Kuo and Chancellor, 1995) and present studies, coronary arteriolar dilation to adenosine was attenuated in a similar manner by removal of the endothelium and by the NO synthase inhibitor L-NAME, suggesting a role for endothelial NO in coronary arteriolar dilation. Interestingly, the response of these microvessels to CGS21680 was also inhibited, in an identical manner, by denudation and L-NAME, suggesting that the activation of endothelial A_{2A} receptors mediates the release of NO and subsequent vasodilation. However, a considerable amount of dilation to adenosine and CGS21680 still remains after denudation or inhibition of the NO pathway, evidence that vasodilation to adenosine A_{2A} receptor activation is not solely NO dependent.

The remaining dilations in response to adenosine and CGS21680 after denudation may be a result of activating smooth muscle K_{ATP} channels because the increased coronary flow by adenosine in the intact heart can be attenuated by glibenclamide (Nakhostine and Lamontagne, 1993; Randall, 1995). Recent patch-clamp (Dart and Standen, 1993) and intact heart (Nakhostine et al., 1993) studies suggest that A_1 receptor activation is responsible for the opening of K_{ATP} channels by adenosine. However, these results are in-

consistent with the present and previous reports that adenosine A_{2A} receptors are the major receptor subtypes responsible for the coronary vasodilation (Nekooeian and Tabrizchi, 1996; Belardinelli et al., 1998). Therefore, the linkage of K_{ATP} channels to the vascular adenosine receptor subtypes remains unclear. In the present study, after removal of the endothelium, glibenclamide nearly abolished vasodilation in response to adenosine, suggesting that opening of smooth muscle K_{ATP} channels is essential for vasodilation to adenosine. Likewise, glibenclamide also nearly abolished vasodilation to CGS21680, indicating that opening of smooth muscle K_{ATP} channels is mediated by A_{2A} receptors. In contrast to resistance vessels, the dilation of large coronary arteries to adenosine and CGS21680 was not affected by glibenclamide (Makujina et al., 1994). This apparent discrepancy may be attributed to the difference in vessel size. In fact, heterogeneous vasodilation to adenosine has been documented between large and small vessels (Habazettl et al., 1992). In addition, the possible differences in the vasodilatory mechanism between small resistance and large conduit vessels should be considered. Moreover, the use of precontractors (i.e., KCl or prostaglandin $F_{2\alpha}$) in the large-vessel study may also contribute to this discrepancy because K_{ATP} channel activity and vascular dilation to adenosine are likely to be influenced by pharmacological precontractors (Herlihy et al., 1976; Jiang et al., 1991). Regardless of the issues on vessel size and experimental approach, our data support the contention that adenosine elicits coronary arteriolar dilation by the activation of A_{2A} receptors that are coupled to the opening of K_{ATP} channels in the vascular smooth muscle.

In conclusion, the results of the present study provide direct functional evidence that coronary arteriolar dilation in response to adenosine is mediated predominantly by A_{2A} receptors. The A_{2A} receptor appears to be on both the microvascular endothelial and smooth muscle cells. Activation of these receptors elicits vasodilation by endothelial release of NO and by the smooth muscle opening of K_{ATP} channels. We also provide indirect evidence for a possible role of A_{2B} receptors in the vasodilation caused by high concentrations of adenosine and adenosine receptor agonists.

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Send reprint requests to: Lih Kuo, Ph.D., Department of Medical Physiology, Cardiovascular Research Institute, Texas A&M University System Health Science Center, 336 Reynolds Medical Building, College Station, TX 77843-1114. E-mail: lkuo@tamu.edu
