Role of A_1 adenosine receptors in regulation of vascular tone


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Tawfik, Huda E., J. Schnermann, Peter J. Oldenburg, and S. Jamal Mustafa. Role of A_1 adenosine receptors in regulation of vascular tone. Am J Physiol Heart Circ Physiol 288: H1411–H1416, 2005. First published November 11, 2004; doi:10.1152/ajpheart.00684.2004.—The vascular response to adenosine and its analogs is mediated by four adenosine receptors (ARs), namely, A_1, A_2A, A_2B, and A_3. A_2A ARs and/or A_2B ARs are involved in adenosine-mediated vascular relaxation of coronary and aortic beds. However, the role of A_1 ARs in the regulation of vascular tone is less well substantiated. The aim of this study was to determine the role of A_1 ARs in adenosine-mediated regulation of vascular tone. A_1 AR-knockout [A_1AR<sup>—/—</sup>] mice and available pharmacological tools were used to elucidate the function of A_1 ARs and the impact of these receptors on the regulation of vascular tone. Isolated aortic rings from A_1AR<sup>—/—</sup> and wild-type [A_1AR<sup>+/+</sup>] mice were precontracted with phenylephrine, and concentration-response curves for adenosine and its analogs, 5'-N-ethyl-carboxamidoadenosine (NECA, nonselective), 2-chloro-N<sup>-</sup>-cyclopentyladenosine (CCPA, A_1AR selective), 2-(2-carboxyethyl)phenethylaminethyl-5'-N-ethylcarboxamido-adenosine (CGS-21680, A_2A selective), and 2-chloro-N<sup>-</sup>-3-iodobenzyladenosine 5'-N-methyluronamide (CI-IBMECA, A_3 selective) were obtained to determine relaxation. Adenosine and NECA (0.1 μM) caused small contractions of 13.9 ± 3.0 and 16.4 ± 6.4%, respectively, and CCPA at 0.1 and 1.0 μM caused contractions of 30.8 ± 4.3 and 28.1 ± 3.9%, respectively, in A_1AR<sup>—/—</sup> rings. NECA- and CCPA-induced contractions were eliminated by 100 nM of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, selective A_1AR antagonist). Adenosine, NECA, and CGS-21680 produced an increase in maximal relaxation in A_1AR<sup>—/—</sup> compared with A_1AR<sup>+/+</sup> rings, whereas CI-IBMECA did not produce contraction in either A_1AR<sup>—/—</sup> or A_1AR<sup>+/+</sup> rings. CCPA-induced contraction at 1.0 μM was eliminated by the PLC inhibitor U-73122. These data suggest that activation of A_1 ARs causes contraction of vascular smooth muscle through PLC pathways and negatively modulates the vascular relaxation mediated by other adenosine receptor subtypes.

A_1 adenosine receptor; knockout mice; smooth muscle; modulate the vascular response. This possibility is supported by the observation that A_1 receptors are coexpressed with the other adenosine receptor subtypes in both the coronary and aortic vascular beds (6, 7, 9, 10, 18–20).

Earlier evidence indirectly suggests that the adenosine A_1 receptor negatively modulates the effect of adenosine on vascular tone (9, 10). The selective A_1 receptor agonist 2S-N<sup>-</sup>-endo-norbornyl (S-ENBA) induced contraction in theophylline (a nonselective adenosine receptor blocker)-treated compared with nontreated aortic rings. This effect was explained as the consequence of an upregulation of A_1 adenosine receptors due to theophylline treatment (9). Selective activation of adenosine A_1 receptors by S-ENBA also significantly attenuated the relaxation mediated by isoproterenol and inhibited basal and isoproterenol-stimulated cAMP accumulation in porcine coronary rings (10). Treatments with the A_1 receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) or pertussis toxin antagonized this inhibitory effect (10). These findings together indirectly suggest that A_1 receptors may play a role in modulating vascular function by attenuating relaxation. However, more definitive characterization of A_1 receptor-mediated effects and elucidation of the possible signaling mechanisms are necessary for the development of potential therapies in the management of vascular disease. A_1 receptor signaling in vascular smooth muscle is less understood, although it is generally agreed that vascular contraction results from calcium mobilization subsequent to activation of the PLC pathway (2).

Characterization of the A_1 adenosine receptor-mediated vascular response was performed in this study by combining a traditional pharmacological approach with newly available gene-modified animal models. Isolated in vitro preparations of mouse aortic rings were used to study the effects of A_1 adenosine receptors on vascular smooth muscle tone. We hypothesized that adenosine A_1 receptors negatively modulate adenosine-mediated regulation of vascular tone through the PLC pathway.

MATERIALS AND METHODS

A_1 adenosine receptor-knockout [A_1AR<sup>—/—</sup>] mice and their respective wild-type [A_1AR<sup>+/+</sup>] littermates of either sex (12–14 wk old) were used in this study. A_1AR<sup>—/—</sup> mice of a mixed C57BL/6/129 genetic background were bred at East Carolina University animal facility as a subcolony of the original A_1AR strain maintained at the National Institutes of Health. The generation and initial characterization of the A_1AR<sup>—/—</sup> and A_1AR<sup>+/+</sup> mice have been described previously (21). Heterozygous [A_1AR<sup>—/+</sup>] mice were bred to obtain A_1AR<sup>—/+</sup> and A_1AR<sup>+/+</sup> mice. For PCR genotyping, genomic DNA was isolated from tail snips. DNA fragments of

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predicted lengths were detected by 1.5% agarose gel electrophoresis and ethidium bromide staining. The mice were kept in community cages on a 12:12-h light-dark cycle and were maintained on standard laboratory mouse diet with access to water ad libitum. All animal care and experimentation protocols were approved and carried out in accordance with the East Carolina University Institutional Animal Care and Use Committee and were in accordance with the principles and guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Preparation of isolated aortic rings. While mice were under deep anesthesia with pentobarbital sodium (100 mg/kg ip), a thoracotomy was performed, and the aorta was gently removed. Fat and connective tissue were removed, and the aorta was cut transversely into three or four rings of 3–4 mm in width. The rings were mounted vertically between two stainless steel wire hooks with extreme care being taken to avoid damaging the endothelium. The rings were suspended in 10-ml organ baths that contained Krebs-Henseleit solution continuously gassed with 95% O2:5% CO2 (37°C, pH 7.4). Aortic rings were allowed to equilibrate for 60 min at an initial resting tension of 1 g, and the bathing solution was changed every 15 min according to our previously described protocol (23). Composition of the Krebs-Henseleit solution was (in mM) 118 NaCl, 4.8 KCl, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, 2.5 CaCl2, and 11 glucose. Changes in the isometric tension were measured with a fixed-range precision-force transducer (model TSD 125C; BIOPAC Systems) connected to a differential amplifier (model DA 100B; BIOPAC Systems). Data were recorded on a Dell computer using an MP 100 WSW digital acquisition system (BIOPAC Systems) and were analyzed using Acqknowledge 3.5.7 software (BIOPAC Systems) (17).

Protocol for agonist dose-response curves. After rings were equilibrated, the responsiveness of each individual ring was checked by successive administration of a submaximally effective concentration of KCl (35 mM). The integrity of the vascular endothelium was tested pharmacologically by acetylcholine-induced relaxation of phenylephrine-precontracted rings. Tissues that did not elicit a reproducible and stable contraction with phenylephrine (1 μM) and relaxed <50% with 0.1 μM acetylcholine were discarded from the study. Preparations were then washed several times with Krebs-Henseleit solution and allowed to relax fully for 30 min before the experimental protocol began.

To determine the vasodilator responses to adenosine and adenosine analogs, the aortic rings were precontracted with phenylephrine at a submaximal dose of 1 μM (Emax, 10 μM). Concentration-response curves (CRCs) for aortic relaxation by agonists were obtained by cumulative addition of agonists to organ baths that contained phenylephrine-precontracted rings. The concentration in the organ bath was increased in 1-log concentration steps. In all cases, agonists were added to yield the next higher concentration only when the response to the earlier dose reached a steady state. One CRC was constructed for each ring. Agonist CRCs were performed in pairs of aortic rings from both A1AR(−/−) and A1AR(+/+) mice and studied in a parallel fashion in the same bath. The adenosine analog 2-chloro-N-6-3-iodobenzyladenosine-5′-methyloxamino-hexyl)-1H-pyrrole-2,5-dione (U-73122, 4 μM) for 30 min (2, 5). A1AR(−/−) and A1AR(+/+) groups at individual agonist concentrations were calculated by Student’s unpaired t-test. A P value of <0.05 was considered significant. The concentration required to produce a 50% response (EC50) in aortic relaxation was obtained by graphic analysis of individual curves by nonlinear regression (curve-fit) analysis.

**Chemicals.** Phenylephrine and acetylcholine were dissolved in distilled water. CCPA, 5′-N-ethyl-carboxamidoadenosine (NECA), 2-(2-carboxyethyl)phenethyl amino-5′-N-ethylcarboxamido-adenosine (CGS-21680), 2-chloro-N′-3-iodobenzyladenosine-5′-N-methyl-luronamide (CI-BIMECA), and DPCPX were dissolved in 100% DMSO as 10 mM stock solutions, which were followed by serial dilutions in distilled water. U-73112 and U-73343 were dissolved in DMSO at desired concentrations. We have shown before (23) that DMSO at the concentration used does not alter the response curve. All chemicals were purchased from Sigma Chemical (St. Louis, MO).

**RESULTS**

Vascular effects of adenosine receptor agonists in isolated aortic rings of A1AR(−/−) and A1AR(+/+) mice. To examine the possible negative modulatory effects of A1 receptors on other adenosine receptor subtypes, the nonselective adenosine receptor agonists adenosine and NECA were used. NECA is the only available agonist for A2B adenosine receptors that predominates adenosine-mediated relaxation in mouse aorta (23). Adenosine and NECA relaxed phenylephrine-precontracted A1AR(−/−) and A1AR(+/+) aortic rings in a dose-dependent manner. In A1AR(−/−) aortic rings, the CRC for both adenosine- and NECA-induced relaxation was shifted to the left, which demonstrates an increase in maximal relaxation compared with A1AR(+/+) aortic rings (Figs. 1 and 2). The EC50 values for A1AR(−/−) aortic relaxation with NECA (1.56 ± 1.7 μM) and adenosine (0.14 ± 1.17 μM) were lower than those for A1AR(+/+) with NECA (8.87 ± 3.7 μM) and adenosine (2.98 ± 3.7 μM; Figs. 1 and 2). Also, adenosine and NECA caused constriction of A1AR(+/+) aortic rings: 0.1 μM adenosine caused a 16.4 ± 6.4% contraction, whereas 0.1 μM NECA caused 13.9 ± 3.1% (Figs. 1 and 2). These contractile responses were significantly greater than those of A1AR(−/−) rings, as neither adenosine nor NECA produced any contraction (P < 0.05). In A1AR(−/−) rings, contraction induced by NECA was significantly greater compared with baseline values within the same group (P < 0.05; Fig. 2).

For the signaling experiments, rings were precontracted with KCl (50 mM) instead of phenylephrine to avoid a possible blocking effect of the PLC inhibitors on phenylephrine-induced vascular smooth muscle contraction. After equilibration for 1 h, CRCs for CCPA (0.1 nM to 100 μM) were obtained by cumulative addition to the organ bath after incubation with PLC inhibitors.

The role of the PLC pathway in A1 receptor-mediated contraction of vascular tone was tested in the presence and absence of the PLC inhibitor 1-(6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl)-1H-pyrrrole-2,5-dione (U-73112, M) for 30 min (2, 5). A1AR(−/−) and A1AR(+/+) mouse aortic rings were incubated with U-73112 before they were contracted with KCl (50 mM), and the CRCs for CCPA were conducted in the presence of the inhibitor. To rule out nonspecific effects of the PLC blocker U-73112, a separate experiment was performed on A1AR(−/−) aortic rings using an inactive analog of the PLC inhibitor. 1-(6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl)-2,5-pyrrolidine-dione (U-73343), as a control. Experiments were performed in parallel using two rings from the same aorta; one served as a control (without inhibitor) and one served as the treatment (with inhibitor).
doses (10–100 μM) and wild-type [A1AR(−/−)] mouse aortic rings. Each point represents a mean ± SE. *P < 0.05, A1AR(−/−) vs. A1AR(+/−) group; n = 4 animals/group.

To identify the contribution of A1 receptors to NECA-induced contraction in A1AR(+/+) aortic rings, a CRC was generated for CCPA (a selective A1 receptor agonist) in both A1AR(+/+) and A1AR(−/−) aortic rings. CCPA produced significant contraction in A1AR(+/+) aortic rings. At doses of 0.1 and 1.0 μM, CCPA produced contractions of 30.8 ± 4.3 and 28.1 ± 3.9%, respectively (P < 0.05), whereas there was no contractile effect in A1AR(−/−) rings (Fig. 3). CCPA at higher doses (10–100 μM) produced relaxation in both A1AR(−/−) and A1AR(+/+) aortic rings.

To test the possible inhibitory effects of A1 receptors on A2A-mediated relaxation, we examined the concentration-response relationship for CGS-21680 (a selective A2A receptor agonist) in both A1AR(−/−) and A1AR(+/+) aortic rings. CGS-21680 caused a small relaxation of 8.0 ± 4.2% at a dose of 100 μM in A1AR(+/+) aortic rings. This is because the involvement of A2A receptors in adenosine-mediated relaxation in mouse aorta is less than that of A2B receptors (23). Furthermore, CGS-21680 significantly increased the maximal relaxation in A1AR(−/−) rings to 39.5 ± 4.3% at a dose of 100 μM (P < 0.05). The EC50 value for aortic relaxation of A1AR(−/−) aortic rings with CGS-21680 was lower than that of A1AR(+/+) (0.22 ± 0.33 vs. 0.36 ± 0.44 μM, respectively; Fig. 4).

To identify the possible contribution of A3 receptors to NECA-mediated contraction of aortic rings, CRCs for CI-IBMECA (a selective A3 agonist) were performed on both A1AR(−/−) and A1AR(+/+) aortic rings. CI-IBMECA did not produce contraction in either A1AR(−/−) or A1AR(+/+) mouse aortic rings. CI-IBMECA produced relaxation at a very high dose (100 μM) with a significant increase in A1AR(+/+) mouse aortic rings (P < 0.05; Fig. 5).
Effects of A1 adenosine receptor blockade on adenosine-mediated regulation of vascular tone in mouse aortic rings.

The selective A1 receptor antagonist DPCPX was used to further characterize the effect of A1 receptors in A1AR(+/-) mice. Aortic rings from A1AR(+/-) mice were incubated for 1 h with 100 nM of DPCPX before being precontracted with phenylephrine. This concentration and incubation period of DPCPX has been shown to completely block the A1 receptor-mediated contractile effect (19). DPCPX completely inhibited NECA- and CCPA-induced contraction in A1AR(+/-) mouse aortic rings and resulted in an increase in the maximal relaxation caused by NECA (*P < 0.05; A1AR(+/-) vs. A1AR(+/+) groups; n = 7 animals/group.

Effects of PLC inhibitor on CCPA-mediated contraction in A1AR(-/-) mouse aortic rings. Incubating the tissues with the PLC inhibitor U-73122 (4 μM) eliminated CCPA (1 μM)-mediated contraction in A1AR(+/-) aortic rings (Fig. 8). U-73122 also blocked CCPA-mediated contraction in tissues precontracted with phenylephrine (data not shown). U-73122 did not affect CCPA-induced relaxation in A1AR(-/-) mouse aortic rings (data not shown).

The inactive analog of PLC, U-73433, was used as a control. U-73433 (20 μM) did not show any effect on CCPA-mediated contraction in A1AR(+/-) mouse aortic rings (Fig. 8).

DISCUSSION

The objectives of this study were to determine whether and to what extent A1 adenosine receptor activation causes negative modulation of adenosine-mediated relaxation of vascular tone. The physiological significance of the coexistence of A1 receptors with the other adenosine receptor subtypes in the vasculature is still not well understood. An A1 receptor-mediated increase of vascular tone is not self evident, because...
another adenosine receptor subtype, the A3 receptor, elicits the same negative effect on cAMP in the vascular smooth muscle but does not cause vasoconstriction (24). Nevertheless, activation of A1 receptors by N6-(R-phenylisopropyl)adenosine (R-PIA, a selective A1 agonist) caused contraction in isolated mouse aortic rings (18). Also, NECA, a nonselective adenosine receptor agonist, caused contraction in isolated carotid rings from both A2A receptor-knockout and wild-type mice (19). Both of these effects of NECA and R-PIA were eliminated by the nonselective adenosine receptor antagonist 8-(p-sulphophenyl)-theophylline and by the selective A1 receptor antagonist DPCPX (18, 19). Additional evidence supporting a negative coregulatory role of adenosine A1 receptors includes the observation that adenosine and N⁶-cyclohexyladenosine (a selective A1 receptor agonist) caused constriction in perfused afferent arterioles from mouse kidney, and this response was not observed in arterioles from A1AR−/− mice (5). These findings suggest that activation of A1 adenosine receptors negatively influences the tone of vascular smooth muscle.

However, more definitive characterization of A1 receptors is necessary to elucidate the function and the impact of these receptors on adenosine-mediated regulation of vascular tone. In the present study, we used aortic rings from mice with targeted deletion of A1 adenosine receptors in combination with the available selective pharmacological agonists and antagonists for better identification of the role of A1 receptors in the regulation of vascular smooth muscle tone. Our data demonstrated that adenosine and NECA at a dose of 0.1 μM induced small contractions in A1AR+/+ aortic rings (when added after phenylephrine). Most importantly, direct activation of A1 receptors by CCPA caused significant contraction in A1AR+/+ phenylephrine-precontracted aortic rings at doses of 0.1 and 1.0 μM. CCPA is the most potent and selective A1 adenosine receptor agonist with a Kᵢ of 0.8 compared with 2,300 for A2A, 18,800 for A2B, and 42 nM for A3 (11). The contractile responses caused by adenosine, NECA, or CCPA were not observed in A1AR−/− aortic rings. NECA- and CCPA-induced contractions were completely abolished by 100 nM DPCPX. DPCPX is a highly selective A1 receptor antagonist with a Kᵢ of 3.9 compared with 130 for A2A, 1,000 for A2B, and 4,000 nM for A3 (11). Taken together, these findings suggest that adenosine-, NECA-, and CCPA-induced contractions of aortic rings were primarily due to activation of A1 adenosine receptors.

We reported earlier that adenosine-mediated mouse aortic relaxation is predominantly mediated by A2B receptors, whereas A2A receptors were suggested to be less important in this vascular bed (23). In contrast, adenosine-mediated coronary vasodilation is mainly through the activation of A2A receptors. In human coronary arterioles, Sato et al. (20) reported the presence of adenosine A1 receptors using 8-cyclopentyl-1,3-dipropylxanthine (an A1 receptor-selective antagonist), which enhanced coronary relaxation by adenosine. In the same study, the selective A1 receptor agonist S-ENBA attenuated the highly selective A2A receptor agonist CGS-21680-mediated coronary relaxation. To examine whether A1 receptors have a modulatory effect on A2B and A2A adenosine receptor subtypes in the aortic vascular bed, we performed CRCs for NECA and CGS-21680 in A1AR−/− and A1AR+/+ mouse aortic rings. NECA is the only available A2B receptor agonist, whereas CGS-21680 is a highly selective A2A receptor agonist; Kᵢ values are 15 for A2A compared with 2,600 nM for A1 receptors (4). A1AR−/− rings demonstrated an increase in maximal relaxation to NECA and lowered the EC₅₀ value compared with A1AR+/+ rings. Also, CGS-21680 caused more relaxation in A1AR−/− compared with A1AR+/+ rings and shifted the dose-response curve to the left. More importantly, DPCPX increased the maximal relaxation mediated by NECA and CGS-21680 in A1AR+/+ aortic rings. These observations suggest that the relaxation caused by both A1AR−/− and A2B receptors was unchanged in A1AR−/− mouse aortic rings. This suggests that the NECA- and CGS-21680-mediated increases in relaxation in A1AR−/− aortic rings were most likely due to the deletion of the negative modulatory effect of A1 adenosine receptors.

In addition to A2B and A1 receptors, which are positively coupled to cAMP, A1 receptor-knockout mice also possess a receptor that is known to decrease cAMP through its coupling to Gᵢi proteins, namely, the A3 receptor. We demonstrated earlier (22) that direct activation of A3 receptors did not cause contraction; rather it inhibited or negatively modulated the A2A receptor-mediated coronary vasodilation. To examine whether A3 receptor activation contributed to the aortic contraction elicited by NECA, we generated Cl-IBMECA CRCs in A1AR−/− and A1AR+/+ aortic rings. Neither A1AR−/− nor A1AR+/+ rings showed a contractile response to Cl-IBMECA. At high doses, Cl-IBMECA caused relaxation in both A1AR−/− and A1AR+/+ aortic rings with significant increases in maximal relaxation of A1AR−/− compared with A1AR+/+ aortic rings. This relaxation may be due to the lack of selectivity of this agonist at high doses, which might affect other adenosine receptors that mediate smooth muscle relaxation (possibly A2B).

We have also previously shown that adenosine A1 receptors are involved in the upregulation of PKC in porcine coronary artery (12, 13, 16). This increase in the expression of PKC was found to occur through an upstream activation of pertussis toxin-sensitive G protein after activation of adenosine A1 receptors by S-ENBA (12, 13). Furthermore, in isolated porcine coronary smooth muscle cells, activation of adenosine A1 receptor with S-ENBA caused upregulation of PKC-α, -β1, -βII, -γ, -ε, and -ζ isoforms in a dose-dependent manner (16). Adenosine A1 receptor activation has been shown to cause production of inositol 1,4,5-trisphosphate in rabbit airway smooth muscle (2), which is the possible mechanism behind A1 receptor-mediated negative modulation of airway vascular tone.

Recent work by Hansen et al. (5) on perfused mouse kidney afferent arterioles demonstrated a contractile response to N⁶-cyclohexyladenosine (a selective A1 receptor agonist) in arterioles of A1AR+/+ but not A1AR−/− mice. Pretreatment with pertussis toxin or the PLC inhibitor U-73122 blocked this contractile effect. In our study, U-73122 eliminated CCPA-mediated contraction in A1AR+/+ mouse aortic rings and had no effect on CCPA-induced relaxation in both A1AR−/− and A1AR+/+ rings, whereas U-73343 (an inactive analog) did not affect CCPA-mediated contraction in A1AR+/+ rings. These finding suggest that the PLC pathway has a major role in A1 adenosine receptor-mediated contraction of vascular tone.

In summary, the present study provides the first direct evidence that A1 adenosine receptors play a negative modulatory role in adenosine-mediated regulation of vascular tone, and this effect is mediated through a PLC signaling pathway.
Selective activation of A1 receptors causes contraction on top of phenylephrine-induced contraction and negatively modulates the other adenosine receptor subtypes by reducing vascular smooth muscle relaxation that is mediated by A2B and A2A receptor subtypes. A more definitive characterization of A1 receptors in other vascular beds like the coronary circulation awaits further study in the isolated heart of A1AR<sup>-/-</sup> mice. Understanding the role of A1 receptors in different vascular beds and its signaling mechanisms could be important for developing better therapeutic approaches for various vascular diseases.

**GRANTS**

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**REFERENCES**


