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Functional characterization of coronary vascular adenosine receptors in the mouse

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1 Coronary responses to adenosine agonists were assessed in perfused mouse and rat hearts. The roles of nitric oxide (NO) and ATP-dependent K^+ channels (K_{ATP}) were studied in the mouse.

2 Resting coronary resistance was lower in mouse *vs* rat, as was minimal resistance (2.2 ± 0.1 *vs* 3.8 ± 0.2 mmHg ml^{-1} min^{-1} g^{-1}). Peak hyperaemic flow after 20–60 s occlusion was greater in mouse.

3 Adenosine agonists induced coronary dilation in mouse, with pEC_{50}s of 9.4 ± 0.1 for 2-[p-(2-carboxyethyl)phenethylamino]-5′-N-ethyl carboxamidoadenosine (CGS21680, A_{2A}-selective agonist), 9.3 ± 0.1 for 5′-N-ethylcarboxamidoadenosine (NECA, A_1/A_2 agonist), 8.4 ± 0.1 for 2-chloroadenosine (A_1/A_2 agonist), 7.7 ± 0.1 for N^6-(R)-(phenylisopropyl)adenosine (R-PIA, A_1/A_{2B} selective), and 6.8 ± 0.2 for adenosine. The potency order (CGS21680 = NECA > 2-chloroadenosine > R-PIA > adenosine) supports A_{2A} adenosine receptor-mediated dilation in mouse coronary vessels. 0.2–2 μM of the A_{2B}-selective antagonist alloxazine failed to alter CGS21680 or 2-chloroadenosine responses.

4 pEC_{50}s in rat were 6.7 ± 0.2 for CGS21680, 7.3 ± 0.1 for NECA, 7.6 ± 0.1 for 2-chloroadenosine, 7.2 ± 0.1 for R-PIA, and 6.2 ± 0.1 for adenosine (2-chloroadenosine > NECA = R-PIA > CGS21680 > adenosine), supporting an A_{2B} adenosine receptor response.

5 NO-synthase antagonism with 50 μM N^G-nitro L-arginine (L-NOARG) increased resistance by ~25%, and inhibited responses to CGS21680 ($pEC_{50} = 9.0 \pm 0.1$), 2-chloroadenosine ($pEC_{50} = 7.3 \pm 0.2$) and endothelial-dependent ADP, but not sodium nitroprusside (SNP). K_{ATP} channel blockade with 5 μM glibenclamide increased resistance by ~80% and inhibited responses to CGS21680 in control ($pEC_{50} = 8.3 \pm 0.1$) and L-NOARG-treated hearts ($pEC_{50} = 7.3 \pm 0.1$), and to 2-chloroadenosine in control ($pEC_{50} = 6.7 \pm 0.1$) and L-NOARG-treated hearts ($pEC_{50} = 5.9 \pm 0.2$).

6 In summary, mouse coronary vessels are more sensitive to adenosine than rat vessels. A_{2A} adenosine receptors mediate dilation in mouse coronary vessels *vs* A_{2B} receptors in rat. Responses in the mouse involve a sensitive NO-dependent response and K_{ATP}-dependent dilation.

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Abbreviations: CGS21680, 2-[p-(2-carboxyethyl)phenethylamino]-5′-N-ethyl carboxamidoadenosine; K_{ATP}, ATP-dependent K^+ channel; L-NOARG, N^G-nitro L-arginine; NECA, 5′-N-ethylcarboxamidoadenosine; NO, nitric oxide; R-PIA, N^6-(R)-(phenylisopropyl) adenosine; SNP, sodium nitroprusside

Introduction

Adenosine receptor sub-types mediating coronary vasodilation appear to differ between species, and controversy exists regarding mechanisms contributing to adenosine-mediated dilation in different (and also within) vascular beds. For example, while there is evidence of NO-dependent components to adenosine responses in coronary vessels (Newmann *et al*., 1988; Leipert *et al*., 1992; Vials & Burnstock, 1993; Zanzinger & Bassenge, 1993; Abebe *et al*., 1994; Kuo & Chancellor, 1995; Hein *et al*., 1999; 2000), this is not universally observed (Sabouni *et al*., 1990; Kirkeboen *et al*., 1994; Lewis & Hourani, 1997; Kemp & Cocks, 1999; Lew & Kao, 1999). There is only preliminary data available regarding the receptor sub-type mediating coronary dilation in the mouse (Morrison *et al*., 2001), and there are no data regarding mechanisms involved. Therefore, the primary goal of the present study was to functionally characterize the adenosine receptor sub-type mediating coronary dilation in mouse heart. To identify the receptor, coronary responses to adenosine (the endogenous and non-selective agonist), CGS 21680 (A_{2A} selective agonist), NECA (A_1/A_2 agonist), 2-chloroadenosine (A_1/A_2 agonist), and R-PIA (A_1/A_{2B} agonist) were examined, together with the effects of A_{2B}-selective antagonism with alloxazine. The potential contributions of NO and K_{ATP} channel activation to adenosine receptor responses were assessed *via* competitive inhibition of NO-synthase with L-NOARG and inhibition of K_{ATP} channels with glibenclamide. Coronary vascular function in mouse was also compared to that in the more thoroughly characterized rat heart.

Methods

Langendorff perfused heart model

Hearts were isolated from mice and rats as described by us in detail previously (Headrick, 1996; Headrick *et al*., 2000).

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Specifically, adult male C57/B16 mice (7–12 weeks old, 26.3±0.3 g body weight, 118±8 mg blotted heart weight) or male Wistar rats (12–16 weeks old, 337±15 g body weight, 1.22±0.19 g blotted heart weight) were anaesthetized with 50 mg kg^{-1} sodium pentobarbitone, a thoracotomy performed and hearts rapidly excised into ice-cold perfusion fluid. The aorta was cannulated and hearts initially perfused in a retrograde fashion at a hydrostatic pressure of 100 mmHg with modified Krebs bicarbonate buffer containing (in mM): NaCl, 120; $NaHCO_3$, 25; KCl, 4.7; KH_2PO_4, 1.2; $CaCl_2$, 2.5; Mg_2SO_4 1.2; glucose, 15; and EDTA, 0.6. Perfusate was equilibrated with 95% O_2, 5% CO_2 at 37°C, giving a pH of 7.4. Perfusate temperature was maintained at 37°C and hearts were constantly bathed in perfusate within a small water jacketed chamber maintained at 37°C. The left ventricle was vented with a polyethylene drain to prevent accumulation of Thebesian drainage. Coronary perfusion was constantly monitored *via* a cannulating ultrasonic flow-probe (Transonic Systems Inc., Ithaca, NY, U.S.A.) in the aortic perfusion line and aortic perfusion pressure was monitored using a P23XL pressure transducer (Viggo-Spectramed, Oxnard, CA, U.S.A.) connected to a MacLab data acquisition unit (ADInstruments, Castle Hill, Australia). Aortic pressure was held constant at 100 mmHg. After an initial 20 min of stabilization at intrinsic heart rate all hearts were electrically paced *via* silver electrodes using a Grass stimulator (Grass S9 stimulator, Quincy, MA, U.S.A.). Mouse hearts were paced at a rate of 400 beats min^{-1} while rat hearts were paced at 300 beats min^{-1} (0.5 ms square pulses 20% above threshold, typically 1–4 V). Hearts were allowed to stabilize for an additional 10 min before experimentation. Hearts were then switched to constant flow perfusion at a rate giving an aortic pressure of 100 mmHg prior to drug infusion (13.2±0.7 ml min^{-1} g^{-1} in mice, 10.6±0.8 ml min^{-1} g^{-1} in rats). This permitted analysis of coronary dilation without the complication of changes in coronary flow rate and drug delivery. Flow was delivered by a Gilson MiniPuls 2 peristaltic pump (Gilson Inc., Middleton WI, U.S.A.).

Experimental protocol

Stabilized hearts were treated with a dilatory agonist (2-chloroadenosine, R-PIA, CGS21680, NECA, adenosine, SNP, or ADP) infused in incremental concentrations. Agonists were infused at each concentration for 1–3 min period during which vascular responses stabilized. Only one concentration-response curve was acquired per heart. Changes in aortic pressure were measured, and coronary flow was constantly monitored to verify a constant flow rate.

To examine the effects of A_{2B} antagonism in mouse hearts, responses to CGS21680 and 2-chloroadenosine were acquired in the presence of 200 nM alloxazine ($n=7$ for both groups). Responses to 2-chloroadenosine were also acquired in a subset of hearts treated with a higher 2 μm concentration of alloxazine ($n=4$). Alloxazine infusion was initiated 10 min prior to acquisition of agonist concentration-response curves.

For NO-synthase inhibition in mouse hearts, infusion of 50 μM L-NAME (final concentration) was initiated after stabilization. After a further 10 min period concentration-response curves for CGS21680 ($n=10$), 2-chloroadenosine ($n=8$), ADP ($n=6$), or SNP ($n=7$) were acquired, as described above. To further examine mechanisms of adenosine-mediated dilation, K_{ATP} channel inhibition was studied in mouse hearts. Infusion of 5 μM glibenclamide was initiated alone or in conjunction with 50 μM L-NAME. After a further 10 min period, concentration-response curves for CGS21680 and 2-chloroadenosine were acquired ($n=6$ in all groups). In preliminary experiments 5 μM glibenclamide was shown to fully block dilatory responses to 0.1–3.0 μM of the K_{ATP} opener minoxidil (data not shown).

Reactive hyperaemic responses were assessed in a separate group of mouse ($n=7$) and rat hearts ($n=8$) which were perfused at a constant aortic pressure of 100 mmHg after pacing was initiated. After a further 10 min stabilization hearts were subjected to 20 s of total coronary occlusion followed by reperfusion for 15 min, before studying the response to 60 s of coronary occlusion and reperfusion. Flow responses were monitored and the peak hyperaemic response determined (in absolute units and as per cent of baseline resistance), together with per cent flow-debt repayment. Flow-debt repayment was calculated as:

$$100\% \times \text{excess flow during hyperaemia (ml g}^{-1}\text{)/flow-debt (ml g}^{-1}\text{)},$$

where flow-debt is the product of baseline flow (ml min^{-1} g^{-1}) × period of occlusion (min), and excess flow during hyperaemia was calculated as the volume of flow during the period of the hyperaemic response minus the baseline flow. The period of the hyperaemic response was defined as the time from onset of reperfusion to the point where flow fell to the pre-occlusion level.

Data analysis

Responses to agonists were compared between treatment groups using a multi-way analysis of variance for repeated measures, followed by Tukeys HSD *post-hoc* test for multiple comparisons when differences were detected. pEC_{50} values were obtained from concentration-response data (expressed as absolute units) by fitting the following four-parameter logistic equation to data for individual experiments:

$$\text{Response} = A + B - \frac{B}{1 + ([\text{agonist}]/EC_{50})^{\text{slope factor}}}$$

where A is the response at zero dose (i.e. the pre-infusion value), and B is the response at infinite dose. The equation was fit to data using the Statistica program (Statsoft, Tulsa, OK, U.S.A.), and individual pEC_{50} values derived from each fit. pEC_{50}s were compared between treatment groups by one-way analysis of variance with Tukeys HSD *post-hoc* test. In all statistical tests $P<0.05$ was considered indicative of statistical significance. Dilatory responses to agonists were calculated as a per cent of resting resistance, or were scaled (as a per cent) to the maximal dilatory response observed. All values are reported as mean±s.e.mean.

Chemicals

2-Chloroadenosine, adenosine, CGS21680, R-PIA, NECA, ADP, SNP, alloxazine, L-NOARG, glibenclamide, and minoxidil were all purchased from Sigma/RBI (Sigma

Chemical, Castle Hill, Australia). All other chemicals used were of analytical grade or better.

Results

Coronary vascular function in mouse vs *rat heart*

Baseline coronary tone, peak reactive hyperaemic responses, and minimal vascular tone observed during infusion of the relatively non-selective A_2 adenosine receptor agonist 2-chloroadenosine are shown in Table 1. Resting coronary resistance was lower in mouse *vs* rat hearts ($\sim$7.5 *vs* 9.5 mmHg ml^{-1} min^{-1} g^{-1}). The minimal resistance achieved during dilation with 2-chloroadenosine was also lower in mouse (2.2 mmHg ml^{-1} min^{-1} g^{-1}) *vs* rat heart (3.8 ml^{-1} min^{-1} g^{-1}) (Table 1). The peak reactive hyperaemic response and extent of flow repayment following 60 s occlusion was significantly greater in mouse *vs* rat (Table 1). Similarly, the peak response following a shorter 20 s occlusion was greater in mouse (35.8 ± 2.3 ml^{-1} min^{-1} g^{-1} at $\sim$5 s of reperfusion) *vs* rat (24.3 ± 1.2 ml^{-1} min^{-1} g^{-1} at $\sim$15 s), as was repayment (136 ± 9% in mouse *vs* 98 ± 6% in rat). Collectively, data show that resting and minimum coronary resistances are lower in mouse. However, the dynamic range over which coronary vessels dilate is similar in both species ($\sim$5.5 mmHg ml^{-1} min^{-1} g^{-1}).

Effects of adenosine receptor agonists in mouse vs *rat heart*

All adenosine receptor agonists concentration-dependently dilated the coronary circulation in mouse and rat hearts (Figure 1). Response magnitude, expressed as per cent change in resistance, was similar in both species. CGS21680 was the most potent agonist in mouse, whereas it was one of the less potent in rat. The rank order of potencies was CGS21680 = NECA > 2-chloroadenosine > R-PIA > adenosine for mouse. Statistical analysis of pEC_{50}s yielded *P* values of 0.879 for NECA *vs* CGS21680, and less than 0.001 for 2-chloroadenosine *vs* NECA and CGS21680, R-PIA *vs* 2-chloroadenosine, and adenosine *vs* R-PIA. This rank order of potencies supports A_{2A}-mediated dilation, and contrasted with that in the rat: 2-chloroadenosine > NECA = R-PIA > CGS21680 > adenosine (Table 2). Statistical analysis of pEC_{50}s yielded *P* values of 0.001 for NECA *vs* 2-chloroadenosine, 0.835 for R-PIA *vs* NECA, and less than 0.005 for CGS21680 *vs* R-PIA and adenosine *vs* CGS21680. This rank order of potencies supports an A_{2B}-mediated response in rat. In addition, sensitivity to all adenosine agonists was higher in mouse *vs* rat. Furthermore, as shown in Table 2 (and apparent in curves depicted in Figure 1), the steepness or slope of concentration-response curves is greater in mouse *vs* rat. Slope factors for all adenosine agonists were significantly higher in mouse (Table 2).

A_{2B}-selective antagonist alloxazine, when infused at 200 nM concentration, failed to alter responses to either A_{2A}-selective CGS21680 ($pEC_{50} = 9.2\pm0.1$) or non-selective 2-chloroadenosine ($pEC_{50} = 8.2\pm0.1$) (Figure 2). A 10 fold higher concentration of alloxazine also failed to alter responses to 2-chloroadenosine in mouse heart (Figure 2B).

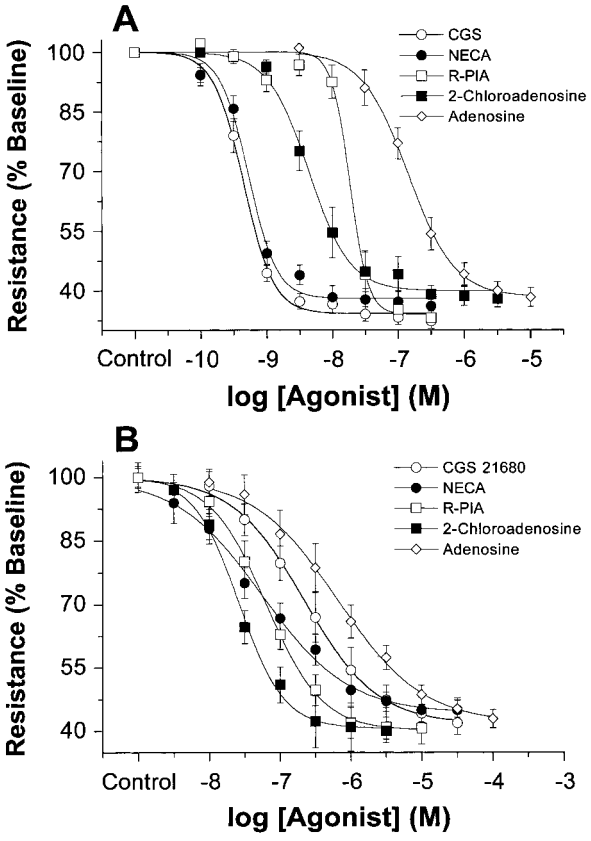

Figure 1 Concentration-response curves for adenosine agonist mediated vasodilation in (A) mouse, and (B) rat hearts. Responses to adenosine ($n=8$ for mouse, $n=7$ for rat), 2-chloroadenosine ($n=7$ for mouse, $n=7$ for rat), R-PIA ($n=6$ for mouse, $n=8$ for rat), NECA ($n=7$ for mouse, $n=7$ for rat), and CGS 21680 ($n=8$ for mouse, $n=7$ for rat) were studied. Responses are shown as per cent of baseline coronary resistance. All values are means$\pm$s.e.mean.

Table 1 Coronary vascular functional parameters in mouse and rat hearts

	Resting resistance (mmHg ml^{1} min^{1} g^{1})	*Min. resistance* (mmHg ml^{1} min^{1} g^{1})	*Peak resistance* (ml min^{-1} g^{-1})	*% Repayment*
Mouse	7.6 ± 0.4	2.2 ± 0.1	38.6 ± 1.8	137 ± 9
Rat	9.4 ± 0.7*	3.8 ± 0.2	25.4 ± 1.1	105 ± 7*

Resting resistance was determined at the end of stabilization immediately prior to adenosine agonist infusions. The minimum resistance was the resistance achieved during maximal stimulation with 2-chloroadenosine. Peak hyperaemia was the peak reactive hyperaemic conductance achieved following 60 s of coronary occlusion, and the per cent repayment is the flow-debt repayment during this hyperaemic response. Values are means$\pm$s.e.mean of individual experiments ($n\geqslant6$). *$P<0.05$ *vs* values in mouse hearts.

Table 2 Concentration-response data for adenosine agonists in mouse and rat hearts

	pEC$_{50}$	*Response* (mmHg ml^{-1} min^{-1})	*Response* (% Resistance)	*Slope*
Mouse				
CGS21680	9.4±0.1	5.2±0.4	68±2	2.4±0.4
CGS21680 + L-NOARG	9.0±0.1†	6.1±0.8	64±2	2.2±0.5
CGS21680 + Glibenclamide	8.3±0.1†‡	5.9±0.3	64±6	1.5±0.3
CGS21680 + L-NOARG + Glib	7.3±0.1†‡	7.2±0.7	56±3	3.2±0.5
2-Chloroadenosine	8.4±0.1	4.0±0.4	62±2	2.1±0.4
2-Chloro + L-NOARG	7.3±0.2†	5.4±0.8	68±2	2.6±0.5
2-Chloro + Glibenclamide	6.7±0.1†‡	6.1±0.7	63±3	1.2±0.3
2-Chloro + L-NOARG + Glib	5.9±0.2†‡	8.2±0.7†	68±2	3.3±1.1
NECA	9.3±0.1	4.5±0.6	63±3	3.4±1.6
R-PIA	7.7±0.1	6.1±0.7	67±2	3.9±0.7
Adenosine	6.8±0.1	5.1±0.4	63±2	2.2±0.4
SNP	7.1±0.3	5.7±0.5	64±1	1.2±0.2
SNP + L-NOARG	7.2±0.2	7.4±0.4†	72±1†	1.1±0.2
ADP	7.4±0.1	5.0±0.8	64±3	4.8±1.1
ADP + L-NOARG	6.7±0.1†	7.0±0.9	69±2	7.8±1.6
Rat				
CGS21680	6.7±0.2*	5.5±0.3	59±2	0.8±0.2*
2-Chloroadenosine	7.6±0.1*	5.6±0.5	61±4	1.4±0.2*
NECA	7.3±0.1*	5.2±0.6	57±4	0.7±0.3*
R-PIA	7.2±0.1*	5.4±0.4	60±3	1.1±0.3*
Adenosine	6.2±0.1*	5.3±0.3	58±3	0.7±0.2*

The negative log of half-maximal effective concentrations (pEC_{50}) was calculated from individual EC_{50} values obtained for each agonist using the logistic equation:

$$\text{Response} = A \pm B - \frac{B}{1 + ([\text{agonist}]/EC_{50})^{\text{slope factor}}}$$

Response amplitude (*B*) was calculated in absolute resistance units and per cent of resistance. Slope is the slope factor in this equation. Responses were acquired in the absence and presence of 50 μM L-NOARG, 5 μM glibenclamide or 50 μM L-NOARG + 5 μM glibenclamide (L-NOARG + Glib). Values are means ± s.e.mean of individual experiments ($n \geq 6$). *$P<0.05$ rat *vs* mouse; †$P<0.05$ *vs* untreated hearts; ‡$P<0.05$ *vs* L-NOARG treated hearts.

Effects of NO-synthase inhibition in mouse heart

Treatment of mouse hearts with 50 μM L-NOARG significantly increased baseline coronary resistance by ~25% to 9.5±0.6 mmHg ml^{-1} min^{-1} g^{-1} ($P<0.05$). Responses to the adenosine receptor agonists CGS21680 and 2-chloroadenosine were significantly reduced by L-NOARG (Figures 3 and 4). Inhibitory effects were present whether responses were expressed relative to resting resistance (Figure 3) or scaled to maximal dilation (data not shown). Effects of L-NOARG were primarily observed at low to moderate agonist concentrations, with no depression of responses to high agonist levels (i.e. above 10^{-8} M CGS21680 and 10^{-7} M 2-chloroadenosine) (Figure 3). Coronary sensitivities to both CGS21680 and 2-chloroadenosine (reflected by pEC_{50}s) were significantly depressed by L-NOARG treatment, with a greater shift in 2-chloroadenosine sensitivity (13 fold) *vs* CGS21680 sensitivity (2.5 fold) (Table 2). Representative traces from hearts treated with 2-chloroadenosine and CGS21680 ± L-NOARG, are shown in Figure 4.

L-NOARG failed to reduce responses or alter sensitivity to the endothelial-independent dilator SNP (Figure 3C, Table 2). There were no significant inhibitory effects when responses were expressed either as per cent of resting coronary resistance (Figure 3C), or scaled to the maximal dilation observed (data not shown). Indeed, due to slightly increased resting resistance with L-NOARG, there was an insignificant trend towards slightly enhanced responses to higher levels of nitroprusside (when expressed relative to the initial resting resistance). Dilatory responses to the endothelial-dependent dilator ADP were significantly inhibited by L-NOARG (Figure 3D, Table 2).

Curiously, a significant number of hearts displayed phasic oscillations in coronary tone during treatment with L-NOARG, rendering it difficult to acquire concentration-response data in those hearts. Representative traces from various experiments, including the latter hearts displaying oscillations in tone, are shown in Figure 4.

Effects of K_{ATP} channel inhibition in mouse heart

Treatment of mouse hearts with 5 μM glibenclamide significantly increased baseline coronary resistance by ~35% to 9.8±0.5 mmHg ml^{-1} min^{-1} g^{-1} ($P<0.05$). Treatment with 5 μM glibenclamide + 50 μM L-NOARG further increased resting resistance to 12.6±0.7 mmHg ml^{-1} min^{-1} g^{-1} ($P<0.05$). Responses to CGS21680 and 2-chloroadenosine were significantly inhibited by glibenclamide, and were further inhibited by co-treatment with glibenclamide + L-NOARG (Figure 5). The degree of inhibition observed with glibenclamide was considerably greater than that with L-NOARG alone. The pEC_{50}s for CGS21680 and 2-chloroadenosine were shifted to higher concentrations by an order of magnitude in the presence of glibenclamide (Table 2). The substantial inhibitory effects of glibenclamide were additive to

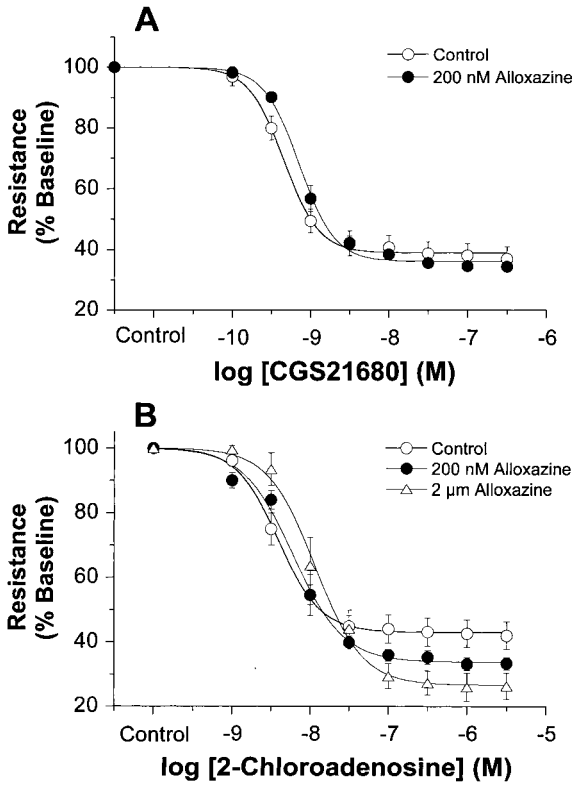

Figure 2 Concentration-response curves for (A) CGS21680 and (B) 2-chloroadenosine in mouse hearts in the presence and absence of alloxazine. Responses were obtained for CGS21680 alone ($n=8$) or in the presence of 200 nM alloxazine ($n=7$), and for 2-chloroadenosine alone ($n=7$) or in the presence of 200 nM ($n=7$) or 2 μm ($n=4$) alloxazine. Control data for CGS21680 and 2-chloroadenosine are taken from Figure 1. Responses are shown as per cent coronary resistance. All values are means ± s.e.mean. $^*P<0.05$ *vs* values in untreated hearts ($P<0.05$).

those for L-NOARG. The pEC_{50}s for CGS21680 and 2-chloroadenosine were further shifted by at least an order of magnitude in hearts treated with glibenclamide + L-NOARG together, relative to pEC_{50}s for hearts treated with either glibenclamide or L-NOARG alone (Table 2).

Discussion

Little information exists regarding adenosine receptor sub-types mediating coronary dilation in the mouse, or mechanisms of adenosine-mediated coronary dilation in this species. Our data indicate that mouse heart is more sensitive to adenosine agonists than rat heart, and show that different receptor sub-types mediate dilation in these species. Adenosine-mediated coronary dilation in the mouse involves NO-dependent and K_{ATP}-dependent components.

Coronary vascular function in mouse vs *rat heart*

Baseline and maximally dilated coronary resistances were lower in mouse *vs* rat heart (Table 1). Additionally, reactive hyperaemic responses were higher in the mouse (Table 1). The mouse circulation therefore appears to operate at lower resting and dilated resistances than in the rat, likely reflecting correlations between vascular density, mitochondrial capacity, metabolic rate and body mass (Pietschmann *et al*., 1982; Rakusan & Tomanek, 1986). Owing to greater metabolic rate in mouse (i.e. mouse heart rate is 550–600 beats min^{-1} *vs* ~350 beats min^{-1} in the rat), the murine heart requires a much greater O_2 delivery. This can be accomplished by limited adaptations in O_2 carrying capacity of blood and O_2 transport from blood to mitochondria, and/or it can be met by increased coronary vascularization and coronary flow, as shown here. Higher hyperaemic responses and lower coronary resistance in mouse heart permits greater resting and maximal O_2 delivery.

The adenosine receptor mediating coronary dilation in mouse and rat hearts

Endogenous adenosine may play an important role in modulating coronary vascular resistance during and following pathophysiological stimuli (Berne, 1980; Olsson & Pearson, 1990), although its role in regulation of coronary tone under more physiological conditions *in vivo* is questionable (Tune *et al*., 2001). We show that murine coronary vessels are highly sensitive to adenosine agonists. Sensitivities to all agonists, including adenosine, are higher in mouse than in rat (Figure 1, Table 2). Importantly, the rank order of agonist potencies differs between mouse and rat (Table 2). As noted initially by Gurden *et al*. (1993), two differing orders of agonist potencies are observed for A_2 receptor mediated vascular responses. They observed an order of potency of CGS21680 ≥ NECA > R-PIA in canine coronary vessels, reflecting an A_{2A} receptor-mediated response, and NECA > R-PIA > CGS21680 in guinea-pig aorta, reflecting an A_{2B} receptor-mediated response. Kull *et al*. (1999) documented a functional potency profile of NECA ≥ CGS21680 > R-PIA ≥ 2-chloroadenosine > adenosine for rat and human A_{2A} receptors. We obtained an order of CGS21680 = NECA > 2-chloroadenosine > R-PIA > adenosine in the mouse which is comparable to that for canine (Gurden *et al*., 1993), human and rat A_{2A} receptors (Kull *et al*., 1999). In contrast we obtained a profile of 2-chloroadenosine > NECA = R-PIA > CGS21680 > adenosine in rat, supporting a primarily A_{2B} response.

To test for A_{2B} receptors we also infused the A_{2B} selective antagonist alloxazine (Figure 2). While there is a lack of potent and highly selective A_{2B} antagonists, alloxazine exhibits ~10 fold selectivity for murine A_{2B} receptors *vs* A_{2A} receptors (Brackett & Daly, 1994) and completely inhibits A_{2B} receptors at 2 μM. We chose a lower concentration in order to minimize antagonism at the A_{2A} receptors which appear to be present. At 200 nM alloxazine failed to alter responses to A_{2A} selective CGS21680 and non-selective 2-chloroadenosine (Figure 2). Hearts receiving the maximally effective 2 μM concentration also displayed unaltered responses to 2-chloroadenosine (Figure 2B). These data verify A_{2A} mediated responses to both receptor agonists, and indicate an absence of functional A_{2B} receptor in mouse coronary vessels.

Previous studies indicate that A_{2A} receptors mediate coronary dilation in dog (Glover *et al*., 1996), pig (Lew & Kao, 1999; Hein *et al*., 1999), and guinea-pig (Belardinelli *et al*., 1998). Functional identification of A_{2A} receptors in the pig (Lew & Kao, 1999; Hein *et al*., 1999) has recently been

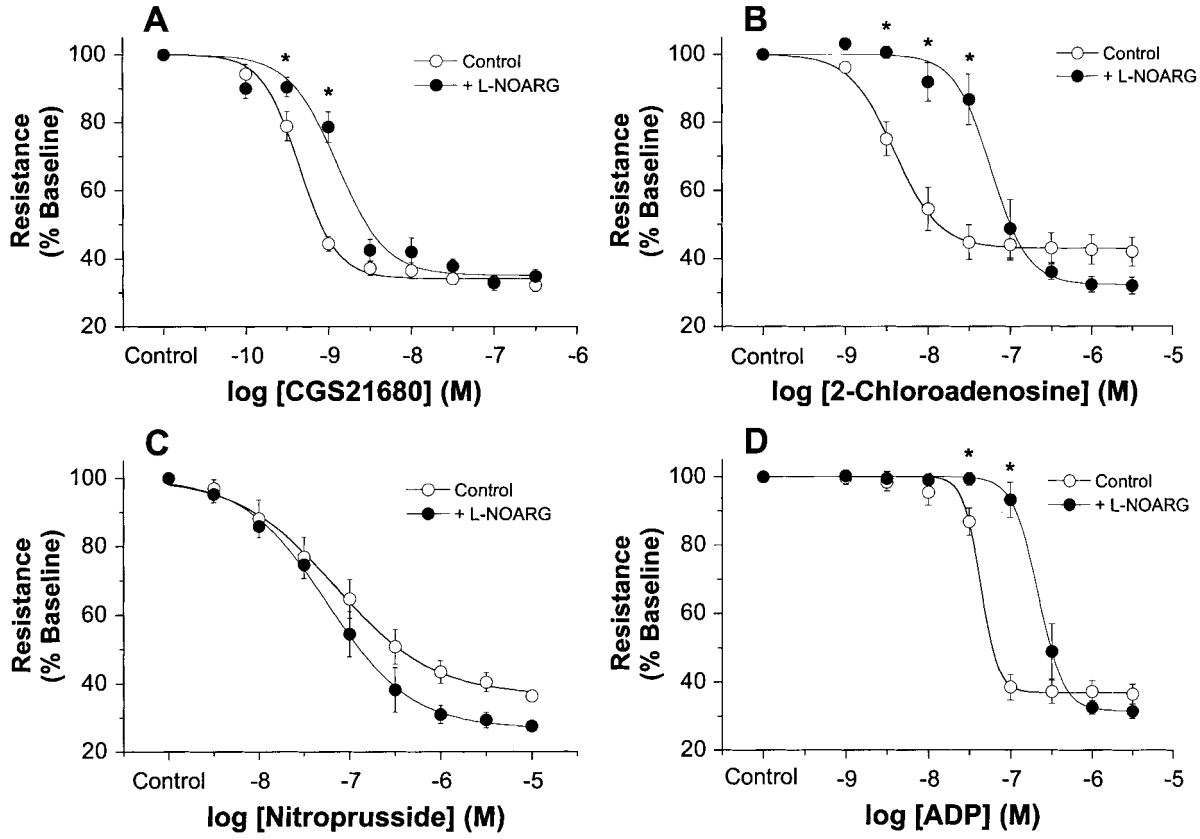

Figure 3 Concentration-response curves from mouse hearts in the presence and absence of 50 μM L-NOARG. Responses were obtained for (A) CGS21680 ($n=10$), (B) 2-chloroadenosine ($n=8$), (C) SNP ($n=6$ in both groups), and (D) ADP ($n=7$ in both groups). Control responses for CGS21680 and 2-chloroadenosine are taken from the data shown in Figure 1. Responses are shown as per cent of baseline coronary resistance. All values are means$\pm$s.e.mean. * $P<0.05$ *vs* values in untreated hearts ($P<0.05$).

supported by molecular analysis (Hein *et al.*, 2001). In contrast, A_{2B} receptors are of primary importance in mediating coronary dilation in humans (Kemp & Cocks, 1999) and in the rat (Lewis & Hourani, 1997; Rose'meyer *et al.*, 1999). A preliminary study by Morrison *et al.* (2001) indicates that gene deletion of the A_{2A} receptor eliminates CGS21680-mediated coronary dilation in the mouse. While findings from genetically modified knock-out animals are not directly applicable to wild-type tissues, and although we have only assessed receptor identity in a single strain of mice, and rodents strains can show variability in cardiovascular responses, our data together with the findings of Morrison *et al.* (2001) support A_{2A}-mediated coronary dilation in the murine heart.

It is worth noting that the slope of concentration-response curves is greater for all agonists in rat *vs* mouse (Table 2). This suggests that the efficacy of adenosine agonists at murine A_{2A} receptors may be greater than at rat A_{2B} receptors, and/or supports heterogeneity in adenosine responses in rat. Lewis & Hourani (1997) provide evidence that both A_{2A} and A_{2B} receptors may mediate dilation in rat heart, and there is support for an unidentified, potentially intracellular receptor in rat vessels (Prentice & Hourani, 1996; Prentice *et al.*, 1997) in addition to mouse aorta (Prentice *et al.*, 2001). This multiplicity of effector mechanisms may contribute to a broader concentration range over which agonists act in rat. The narrow range and steep slope in mouse is consistent with a single coronary receptor sub-type.

In assessing the identity of the adenosine receptor we chose to assess the rank order of agonist potencies, and study effects of a selective antagonist. This conventional pharmacological approach remains a key method for identification of adenosine receptors (Belardinelli *et al.*, 1998; Brackett & Daly, 1994; Gurden *et al.*, 1993; Hein *et al.*, 1999; Kemp & Cocks, 1999; Kull *et al.*, 1999; Lew & Kao, 1999; Prentice *et al.*, 1996; 1997; Rose'meyer *et al.*, 1999), and was adopted despite alternate methodologies, including epigenetic approaches (targeted gene knock-out and anti-sense oligonucleotides) (Nyce, 1999). Despite selectivity of epigenetic techniques, these methods possess limitations. Anti-sense studies in intact organs are largely restricted to brain and lungs due to difficulties in selectively introducing oligonucleotides, and these studies are limited by poor penetration into cells and nuclei. With respect to gene knock-out, an A_{2A} knockout mouse has been assessed in terms of neurophysiological responses (Ledent *et al.*, 1997), and there are preliminary data regarding adenosine and CGS21680 mediated coronary dilation in this model (Morrison *et al.*, 2001). While these findings support an important A_{2A} response in murine vessels, interpretation of gene knock-out data are limited since they assess effects of a genes long-term absence rather than the normal role of the gene product

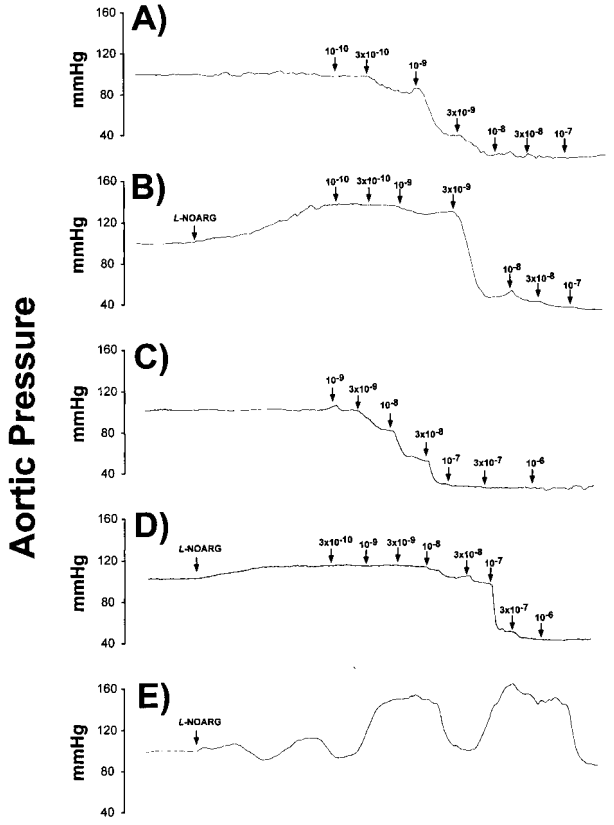

Figure 4 Representative tracings from mouse hearts treated with (A) CGS21680, (B) CGS21680 + 50 μM L-NOARG, (C) 2-chloroadenosine, and (D) 2-chloroadenosine + 50 μM L-NOARG. The trace shown in (E) is from a heart displaying phasic oscillations in coronary tone during infusion of 50 μm L-NOARG.

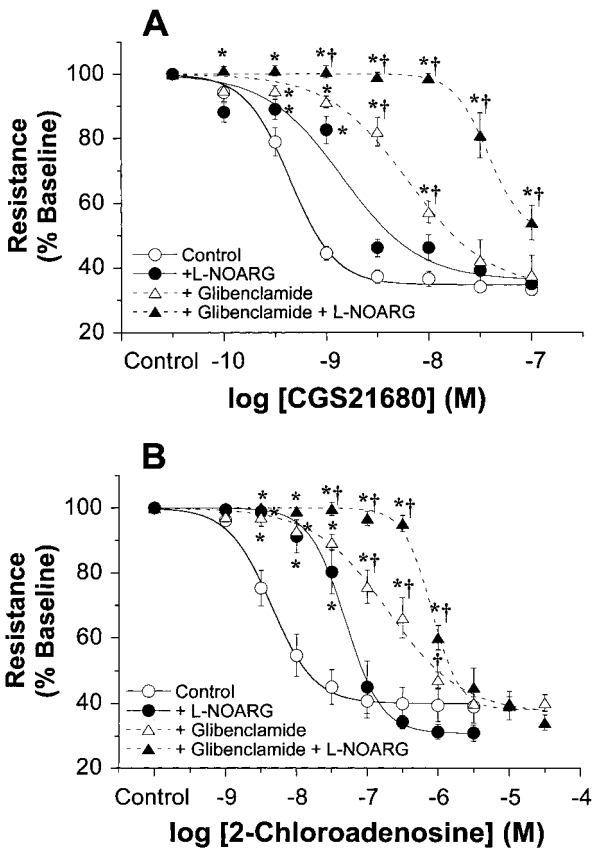

Figure 5 Concentration-response curves from mouse hearts in the presence and absence of 5 μM glibenclamide or 5 μM glibenclamide + 50 μM L-NOARG. Responses were obtained for (A) CGS21680 ($n=10$), and (B) 2-chloroadenosine ($n=8$). Control responses and responses in the presence of L-NOARG alone are also shown (from Figure 3). Responses are shown as per cent of baseline coronary resistance. All values are means ± s.e.mean. *$P<0.05$ *vs* values in untreated hearts ($P<0.05$). †$P<0.05$ *vs* values in L-NOARG treated hearts ($P<0.05$).

itself. Absence of the gene may lead to unknown developmental, morphological and functional changes, together with unpredicted compensatory responses. Receptor identification remains best achieved *via* complimentary pharmacological and molecular approaches.

Role of NO in adenosine receptor-mediated coronary dilation in mouse

There is evidence that adenosine is a mixed endothelial-dependent/independent dilator, activating both endothelial and smooth muscle receptors within the same vessel (Headrick & Berne, 1990; Moritoki *et al*., 1990; Rose'meyer & Hope, 1990; Headrick *et al*., 1992; Vials & Burnstock, 1993; Hein *et al*., 1999). To test the potential involvement of NO in adenosine responses, we studied effects of NO-synthase inhibition with L-NOARG. L-NOARG significantly increased baseline resistance and inhibited responses to endothelial-dependent ADP but not SNP, verifying selective inhibition of NO-dependent responses, and supporting a role for endogenous NO in control of resting tone in murine coronary vessels (Table 1, Figure 3). Curiously, a number of hearts displayed phasic oscillations in coronary resistance during L-NOARG treatment (Figure 4). Though the mechanism of these oscillations is unclear, it may reflect competition between constriction due to reduced NO release and relaxation due to locally released dilators. Importantly, while L-NOARG did not alter responses to SNP, it significantly attenuated responses to 2-chloroadenosine and CGS 21680, substantially increasing EC_{50}s and threshold concentrations at which agonists induced dilation (Figure 3, Table 2).

While our data agree with studies supporting partial NO- or endothelial-dependent coronary responses to adenosine in guinea-pig (Newmann *et al*., 1988; Leipert *et al*., 1992; Vials & Burnstock, 1993), dog (Rubanyi & Vanhoutte, 1985; Zanzinger & Bassenge, 1993), and pig (Abebe *et al*., 1994; Kuo & Chancellor, 1995; Hein *et al*., 1999; 2000), they contrast with studies in human and porcine coronary vessels (Sabouni *et al*., 1990; Kirkebeon *et al*., 1994; Kemp & Cocks, 1999; Lew & Kao, 1999), and in rat heart (Lewis & Hourani, 1997). Varying observations from different species supports pronounced species differences in the mechanisms of adenosine-mediated coronary dilation. Contradictory observations made within a single species (e.g. Abebe *et al*., 1994; Kirkebeon *et al*., 1994; Kuo & Chancellor, 1995; Hein *et al*., 1999; Lew & Kao, 1999) demonstrates a need for further research. In this respect, both A_{2A} and A_{2B} receptors may mediate coronary dilation in the same species and there appear to be differences in transduction mechanisms for these

sub-types. There is a greater weight of unequivocal data supporting NO-dependence of adenosine A_{2A} responses (Newmann *et al*., 1988; Leipert *et al*., 1992; Vials & Burnstock, 1993) whereas there is controversy regarding NO-dependence of coronary A_{2B} responses (Kirkebeon *et al*., 1994; Kemp & Cocks, 1999; Lewis & Hourani, 1997; Lew & Kao, 1999).

Functional antagonism vs *NO-synthase inhibition*

There may be two effects of L-NOARG in coronary vessels – enhanced functional antagonism increasing the resistance over which dilatory agonists can induce relaxation, and direct antagonism of NO-synthase dependent responses. Lewis & Hourani (1997) recently concluded that apparent antagonism of adenosine *via* NO-synthase inhibitors may reflect functional antagonism (i.e. vasoconstriction). They found that L-NOARG reduced adenosine responses in rat, but also reduced responses to NO-synthase independent SNP. However, responses to SNP were quite low in their study and L-NOARG only modestly reduced these in contrast with 2–10 fold differences in adenosine-response magnitude (Lewis & Hourani, 1997). We show that L-NOARG increases resting coronary resistance (Table 1), and this should alter dilatory response amplitude. If 'pre-constrictors' do not directly inhibit mechanisms of action of a dilator, enhanced functional antagonism and resting tone simply increases the amplitude (but not sensitivity) of dilatory responses, as noted by Lew (1995). Functional antagonism is useful in increasing response amplitude for dilators, removing constraints imposed by degree of pre-constriction, and permitting analysis of agonist efficacies (Broadley & Nicholson, 1979; Lew, 1995). These factors are exemplified by responses to SNP: L-NOARG tends to enhance response amplitude to high levels of SNP when responses are expressed relative to the higher initial tone (Figure 3C). It is necessary to normalize such responses to make meaningful comparisons, and to remove differences which are not relevant (Lew, 1995). These minor differences are absent when SNP responses are scaled to maximal dilation (data not shown). Importantly, in direct contrast to SNP, responses to low levels of adenosine agonists are substantially reduced by L-NOARG while maximal responses are unaltered (Figure 3), the threshold effective concentration of 2-chloroadenosine is increased $\sim$10 fold (Figure 3), and coronary sensitivity to 2-chloroadenosine and CGS21680 is significantly reduced (Table 2). Selective effects of L-NOARG on A_2-mediated responses (and on ADP responses) supports significant NO-dependence of adenosine-mediated dilation in mouse heart.

Assuming that responses to low levels of 2-chloroadenosine in the presence of L-NOARG can be considered largely NO-synthase independent, we can estimate NO-synthase dependent dilation by subtraction of NO-synthase independent responses from control (mixed) responses (Headrick & Berne, 1990; Headrick *et al*., 1992). As shown in Figure 6 the NO-synthase dependent L-NOARG sensitive response is important at low levels of A_2 receptor activation. Dilation with 1–30 nM 2-chloroadenosine is almost entirely NO-dependent (Figure 6). At higher levels of activation NO-synthase independent dilation predominates. This is consistent with previous observations indicating that NO-dependent adenosine responses are sensitive yet small in amplitude (Headrick & Berne, 1990; Moritoki *et al*., 1990; Rose'meyer & Hope, 1990; Headrick *et al*., 1992; Vials & Burnstock, 1993). While small amplitude might be considered evidence for a minimal functional role (Vials & Burnstock, 1993), high sensitivity (Figure 6) ensures that it is quantitatively important at low, physiologically relevant levels of receptor activation.

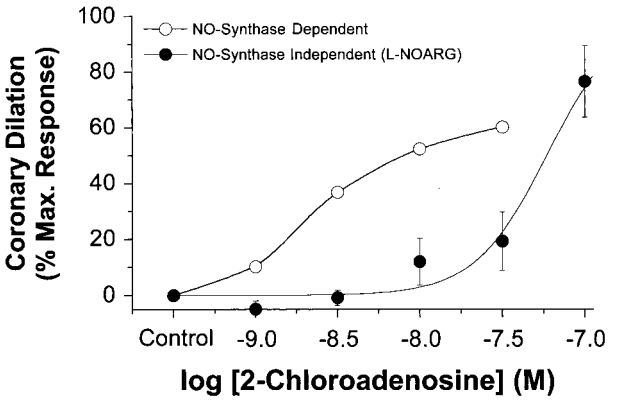

Figure 6 NO-synthase dependent and NO-synthase independent responses to 2-chloroadenosine in perfused mouse heart. The raw data is derived from the results shown in Figure 3. The NO-synthase independent response is represented by the dilatory response in the presence of 50 μM L-NOARG. The NO-synthase dependent response is calculated as the difference between the control response (in the absence of L-NOARG) and the NO-synthase independent response (in the presence of L-NOARG). Responses are scaled to per cent of maximal dilation observed.

Role of K_{ATP} channels in adenosine receptor-mediated coronary dilation in mouse

In addition to release of NO, activation of K_{ATP} channels is implicated in A_{2A} adenosine receptor-mediated coronary dilation (Kuo & Chancellor, 1995; Randall, 1995; Hein *et al*., 1999; 2001). In contrast, A_{2B} receptors may mediate coronary dilation in human vessels *via* NO and K_{ATP}-independent mechanisms (Kemp & Cocks, 1999). Our data reveal that K_{ATP} blockade with glibenclamide, at a level which fully antagonizes responses to minoxidil, markedly inhibits responses to CGS21680 and 2-chloroadenosine (Figure 5). Importantly, effects of K_{ATP} channel inhibition are additive to (and greater than) those for NO-synthase inhibition *via* L-NOARG. Considering the 120–300 fold reduction in sensitivity to CGS21680 and 2-chloroadenosine with combined NO-synthase and K_{ATP} inhibition, these two pathways appear to be the primary mechanisms contributing to A_{2A}-mediated dilation. The more pronounced inhibitory effects of K_{ATP} channel blockade and additivity with the effects of L-NOARG suggest that A_{2A} receptors trigger NO-mediated dilation *via* mechanisms distinct from activation of smooth muscle and/or endothelial K_{ATP} channels.

Concluding remarks

In conclusion, the present study demonstrates that peak coronary flows, reactive hyperaemia and adenosine sensitivity are all substantially greater in mouse *vs* rat heart. Adenosine agonist potency profiles and effects of alloxazine support A_{2A} adenosine receptor-mediated coronary dilation in mouse *vs*

A_{2B} adenosine receptor-mediated dilation in rat. Our data demonstrate a significant NO-dependent and K_{ATP} channel-dependent components to adenosine mediated coronary dilation in the mouse. A_{2A}-mediated dilation appears more strongly dependent on K_{ATP} channels than NO formation. However, although NO-independent dilation predominates over NO-dependent dilation at moderate to high agonist levels, the high-sensitivity NO-dependent response may play an important role under physiological conditions when adenosine concentrations and the level of A_{2A} receptor activation are relatively low.

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