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Oxygen delivery and oxygen consumption in rat hindlimb during systemic hypoxia: role of adenosine

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1. In anaesthetised rats, the increase in femoral vascular conductance (FVC) evoked by moderate systemic hypoxia is mediated by adenosine acting on A_1 receptors. It is also nitric oxide (NO) dependent: it is attenuated by NO synthase (NOS) inhibition, but restored when baseline FVC is restored by sodium nitroprusside (SNP), a NO donor. However, under these conditions there was in increase in the critical O_2 delivery ($D_{O_2,crit}$) at which hindlimb O_2 consumption ($\dot{V}_{O_2}$) becomes directly dependent upon O_2 delivery (D_{O_2}), indicating that $\dot{V}_{O_2}$ is regulated by newly synthesised NO.
2. In the present study, after NOS inhibition, when baseline FVC was restored with SNP infusion, the increases in FVC evoked by breathing 12 and 8% O_2 were reduced by the A_1 receptor antagonist DPCPX, by 60 and 40%, respectively ($n = 8$). The A_{2A} receptor antagonist ZM241385 reduced the FVC increase evoked by 12% O_2 (by 45%, $n = 8$), but did not alter that evoked by 8% O_2.
3. DPCPX also reduced the increases in FVC evoked by graded systemic hypoxia, breathing 14–6% O_2 and increased $D_{O_2,crit}$, from 0.64 ± 0.06 to 0.95 ± 0.07 ml O_2 min^{-1} kg^{-1} (control *vs.* DPCPX). However, ZM241385 ($n = 8$) had no effect on the FVC increases or on $D_{O_2,crit}$ (0.70 ± 0.02 ml O_2 min^{-1} kg^{-1}, $n = 8$).
4. Thus, the increases in FVC evoked by mild to severe systemic hypoxia are mediated by A_1 receptors. These responses, which are attributable to proximal arteriolar dilatation, help maintain D_{O_2}. Even after NOS inhibition, adenosine still increases FVC via A_{2A} (moderate hypoxia only) and A_1 receptors, providing baseline levels of NO are present. Furthermore, adenosine, acting via A_1 receptors, is important in determining $D_{O_2,crit}$ and therefore in maintaining $\dot{V}_{O_2}$. We propose that this is achieved by A_1-evoked dilatation of terminal arterioles and is mediated by increased synthesis of NO.

It is well established that the skeletal muscle of larger mammals such as the dog can maintain O_2 consumption ($\dot{V}_{O_2}$) at a steady rate over a wide range of O_2 delivery (D_{O_2}) values, but that below a critical D_{O_2} level ($D_{O_2,crit}$), $\dot{V}_{O_2}$ declines linearly with D_{O_2} (Duran & Renkin, 1974; Granger *et al.* 1976; Cain & Chapler, 1979; Samsel & Schumacker, 1988; Curtis *et al.* 1995). Therefore, the relationship between $\dot{V}_{O_2}$ and D_{O_2} is described by two distinct phases; a delivery-independent phase and a delivery-dependent phase. The delivery-independent phase is thought to rely on the co-ordinated vasodilatation of the terminal arterioles within the microvascular bed of muscle, leading to a more homogeneous distribution of the available O_2 and allowing $\dot{V}_{O_2}$ to be maintained (Granger *et al.* 1976; Harrison *et al.* 1990). Accordingly, $\dot{V}_{O_2}$ becomes delivery dependent when the terminal arterioles are maximally dilated and can no longer contribute to the regulation of D_{O_2}.

We recently demonstrated that this biphasic relationship between $\dot{V}_{O_2}$ and D_{O_2} also exists in the hindlimb muscles of the rat when D_{O_2} is reduced by graded systemic hypoxia (Edmunds & Marshall, 2001). Since the muscle vasodilatation evoked by systemic hypoxia, as reflected by the increase in femoral vascular conductance (FVC), was attenuated by nitro-L-arginine methyl ester (L-NAME)-mediated inhibition of nitric oxide synthase (NOS; Skinner & Marshall, 1996; Bryan & Marshall, 1999*b*), we investigated the effect of L-NAME on the D_{O_2}–$\dot{V}_{O_2}$ relationship (Edmunds & Marshall, 2001). In fact, L-NAME caused such a large decrease in FVC and femoral blood flow (FBF) that D_{O_2} was reduced too much for $D_{O_2,crit}$ to be calculated. However, when FBF, and therefore D_{O_2}, were subsequently restored by the infusion of a NO donor, then $D_{O_2,crit}$ was markedly increased. This suggests that the dilatation of the terminal arterioles, which

determines $D_{O_2,crit}$, is normally mediated by NO (Edmunds & Marshall, 2001). However, when a basal level of NO was restored after L-NAME by the infusion of a NO donor, the changes in FVC evoked by graded levels of hypoxia were also fully restored. We therefore proposed that the dilatation of the proximal arterioles, which makes the major contribution to changes in FVC (Froneck & Zweifach, 1975; Hebert & Marshall, 1988), required a basal presence of NO (i.e. it was dependent upon NO) rather than being mediated by NO. The mechanism underlying this NO-dependent dilatation of proximal arterioles remains unclear.

Previous work from our laboratory involving intravital microscopy of skeletal muscle showed that both exogenous adenosine and adenosine released during systemic hypoxia dilate the proximal and terminal arterioles but have preferential effects on terminal arterioles (Mian & Marshall, 1991). We have shown subsequently that infused adenosine can evoke an increase in FVC by stimulating A_1 or A_{2A} receptors, but that the increase in FVC evoked by systemic hypoxia was attributable only to A_1 receptor stimulation: it was attenuated by a selective A_1 receptor antagonist but not an A_{2A} antagonist (Bryan & Marshall, 1999*a*). Indeed, stimulation of either A_1 or A_{2A} receptors can stimulate NOS in human umbilical vein endothelial cells (HUVEC) and in the endothelium of freshly excised rat aorta (Sobrevia *et al.* 1997; Ray & Marshall, 2000; Rolleston & Marshall, 2000). However, it is also known that adenosine can cause dilatation by stimulating A_1 receptors on vascular smooth muscle and opening ATP-sensitive K^+ (K_{ATP}) channels (Dart & Standen, 1993), as well as by the long-accepted mechanism of stimulating A_{2A} receptors and increasing cAMP (Olsson & Pearson, 1990).

Thus, the aims of the present study were (1) to test whether the action of adenosine on A_1 receptors is responsible for the increase in FVC that persists in response to systemic hypoxia when NOS is inhibited but basal NO is restored with an NO donor, and (2) to test whether adenosine, acting via A_1 or A_{2A} receptors, is responsible for the NO-mediated dilatation of terminal arterioles that we deduced maintains $\dot{V}_{O_2}$ in the face of reduced D_{O_2}. The hypothesis that stimulation of A_{2A} receptors might contribute to the maintenance of $\dot{V}_{O_2}$ even though they play no role in the increase in FVC evoked by systemic hypoxia (Bryan & Marshall, 1999*a*) is reasonable because changes in terminal arteriolar tone can occur without significant effects on gross muscle vascular conductance (Hebert & Marshall, 1988).

Some of the results presented in this paper have already been reported in brief (Edmunds & Marshall, 2000).

METHODS

Experiments were performed on male Wistar rats (200–250 g) in which anaesthesia was induced with an oxygen–halothane mixture (3.5% halothane) and maintained with Saffan (Schering-Plough Animal Health, Welwyn Garden City, UK) delivered at 7–12 mg kg^{-1} h^{-1} I.V. during surgery and at 4–8 mg kg^{-1} h^{-1} I.V. during the experimental period (Bryan & Marshall, 1999*a*). The surgery required to record physiological variables was similar to that described previously (Marshall & Davis, 1999; Edmunds & Marshall, 2001). Briefly, arterial blood pressure (ABP) was recorded from the left brachial artery and FBF was recorded from the right femoral artery via a transonic flow probe (0.7 V) connected to a flow meter T106 (Transonic Systems, Ithaca, NY, USA). FVC was computed online as FBF divided by ABP. The trachea was cannulated so that inspired O_2 could be altered by changing the mixture of N_2 and O_2 delivered across the sidearm of the cannula. Samples of arterial blood were taken from a cannula in the right femoral artery, while samples of venous blood from the right hindlimb were taken from a cannula placed in the left femoral vein, and advanced so that the tip lay at the bifurcation of the inferior vena cava. Arterial and venous blood samples (65 μl) were analysed for O_2 content using a co-oximeter (IL-682 CO-Oximeter, Instrumentation Laboratory, Lexington, MA, USA). These measurements, together with the measurement of FBF, allowed the calculation of hindlimb D_{O_2} and $\dot{V}_{O_2}$; these parameters are expressed per unit body weight (Edmunds & Marshall, 2001). At the end of each experiment animals were killed by anaesthetic overdose followed by cervical dislocation.

All variables were recorded on an Apple Power Mac computer (4400/160) using MacLab 8/s (AD Instruments, Hastings, West Sussex, UK).

Protocols

Series 1: interactions between adenosine and NO. In group 1 ($n = 8$ animals), after a 25 min stabilisation period following surgery when animals breathed room air, the inspirate was changed to 12% or 8% O_2 for 5 min periods. The order was varied between animals and 15 min was allowed for recovery between the hypoxic challenges. The NOS inhibitor L-NAME (10 mg kg^{-1}, I.V.) was then given and 20 min later the hypoxic challenges were repeated. Following this, baseline FVC was restored by continuous infusion of sodium nitroprusside (SNP; 10 μg kg^{-1} min^{-1} I.A.), and the hypoxic challenges were repeated. The selective adenosine A_1 receptor agonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) was then administered (0.1 mg kg^{-1} I.V.) whilst SNP infusion was continued, and 20 min later the hypoxic stimuli were repeated. In group 2 ($n = 8$), the same protocol was repeated except that the selective adenosine A_{2A} receptor antagonist 4-(2-[7-amino-2-(2-furyl)(triazolo{2,3-*a*}-[1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385) was administered (0.05 mg kg^{-1} I.V.; Bryan & Marshall, 1999*a*) instead of DPCPX.

Series 2: determination of $D_{O_2,crit}$. In control rats ($n = 8$), cardiovascular variables were measured continuously while the animals breathed 21% O_2 spontaneously. After a 25 min stabilisation period, the inspirate was changed, for 5 min periods, to a range of different hypoxic mixtures: 14, 12, 10, 9, 8, 7, 6 and sometimes 5% O_2 in N_2 (Edmunds & Marshall, 2001). The order of the hypoxic mixtures was randomised and at least 15 min breathing 21% O_2 was allowed between successive periods of hypoxia. During the 5th minute of each hypoxic challenge, blood samples were taken from the femoral vein and artery cannulae, so that hindlimb D_{O_2} and $\dot{V}_{O_2}$ could be calculated at each level of hypoxia. The $D_{O_2,crit}$ was calculated as described previously (Samsel & Schumacker, 1988; Edmunds & Marshall, 2001).

In a second group of animals ($n = 8$), after an initial stabilisation period, DPCPX was administered (0.1 mg kg^{-1} I.V.; Bryan & Marshall, 1999*a*), and 20 min later the protocol described above was performed. Since preliminary experiments suggested that the value of $D_{O_2,crit}$ was increased (data not shown), a slightly different series of hypoxic challenges was used to allow $D_{O_2,crit}$ to be calculated accurately: 18, 16, 14, 12, 10, 9, 8, 7 and 6% inspired O_2 in N_2. The

DPCPX was dissolved in 10% DMSO/0.1 M NaOH (50/50 v/v) then diluted in saline. Therefore, a further series of experiments ($n = 8$) was conducted to control for this vehicle.

In a fourth group of animals ($n = 8$), after an initial stabilisation period ZM241385 was administered (0.05 mg kg^{-1}, I.V.), and after 20 min the initial protocol described was performed. *In vivo,* the effective half-life for ZM241385 is relatively short (Keddie *et al.* 1996). Therefore, supplementary doses of ZM241385 (0.05 mg kg^{-1} I.V.) were given every hour after the initial dose.

DPCPX was purchased from Research Biochemicals; ZM241385 was a kind gift from Zeneca Pharmaceuticals, and was dissolved in 3% polyethylene glycol 400/0.1 M NaOH (50/50 v/v), and then diluted in saline (see Bryan & Marshall, 1999*a*).

Statistical analysis of data

All data are expressed as means ± S.E.M. Changes in FVC were computed as the integrated FVC in conductance units, for the 5 min period during the hypoxic stimulus minus the integrated baseline FVC measured for 5 min before hypoxia (see Edmunds & Marshall, 2001). In series 1 experiments, differences in the FVC integral were determined with repeated-measures ANOVA. In all cases, the level of statistical significance was set at $P < 0.05$. In series 2 experiments, the effects of hypoxia on baseline levels of mean arterial pressure (MAP) and FBF within groups were analysed using Student's paired *t* test. Changes in MAP and FVC were compared at each level of hypoxia between different groups of animals by using one-way ANOVA for multiple comparisons, and Tukey's *post hoc* test for differences at particular levels of hypoxia. The $D_{O_2,crit}$ values were compared using Student's unpaired *t* test. Data from control animals in series 2 have been published before (Edmunds & Marshall, 2001).

RESULTS

Series 1: interactions between adenosine and NO

In group 1, in the absence of any drug, each level of systemic hypoxia (12 and 8% inspired O_2) evoked a pronounced increase in FVC, indicating hindlimb vasodilatation (Fig. 1), accompanied by a marked decrease in MAP (data not shown). After L-NAME, baseline MAP increased and FVC decreased (Table 1), which is indicative of peripheral and hindlimb vasoconstriction, respectively. Infusion of SNP after L-NAME restored baseline FVC to values similar to those seen prior to the infusion of L-NAME, although MAP fell disproportionately (Table 1).

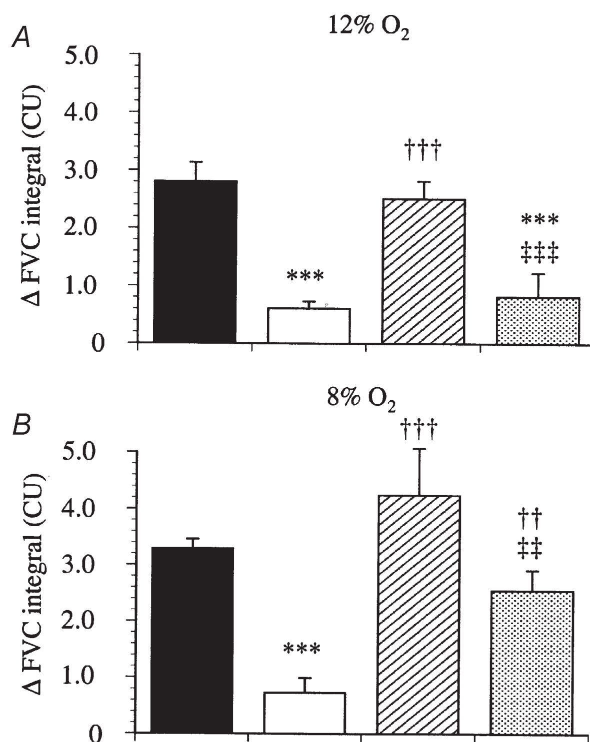

Figure 1. Effects of L-NAME, L-NAME with SNP and L-NAME with SNP and DPCPX on the changes in FVC evoked by hypoxia

Each column represents the mean ± S.E.M. of the change in integrated FVC (in conductance units, CU) after 12% inspired O_2 (*A*) and 8% inspired O_2 (*B*), for control responses (■), after L-NAME (10 mg kg^{-1}, □), after L-NAME and SNP (10 μg kg^{-1} min^{-1}, ▨) and after L-NAME, SNP and DPCPX (0.1 mg kg^{-1}, ▩). *** $P < 0.001$ *vs.* control; †† $P < 0.01$, ††† $P < 0.001$ *vs.* L-NAME; ‡‡ $P < 0.01$, ‡‡‡ $P < 0.001$ *vs.* L-NAME + SNP.

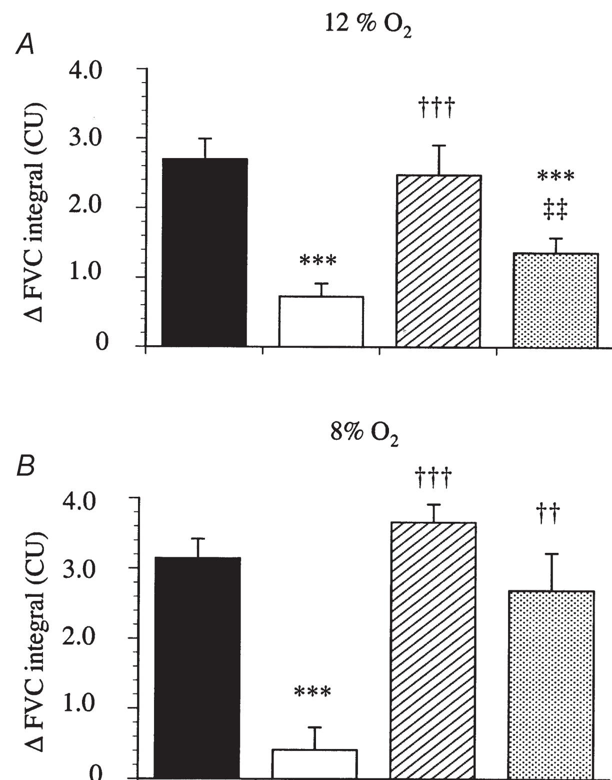

Figure 2. Effects of L-NAME, L-NAME with SNP and L-NAME with SNP and ZM243185 on the changes in FVC evoked by hypoxia

Each column represents the mean ± S.E.M. of the change in integrated FVC after 12% inspired O_2 (*A*) and 8% inspired O_2 (*B*), for control responses (■), after L-NAME (10 mg kg^{-1}, □), after L-NAME and SNP (10 μg kg^{-1} min^{-1}, ▨) and after L-NAME, SNP and ZM243185 (0.05 mg kg^{-1}, ▩). *** $P < 0.001$ *vs.* control; †† $P < 0.01$, ††† $P < 0.001$ *vs.* L-NAME; ‡‡ $P < 0.01$ *vs.* L-NAME + SNP.

Table 1. Baseline cardiovascular variables in series 1 experiments after L-NAME (10 mg kg^{-1}), L-NAME with SNP (10 μg kg^{-1} min^{-1}) and L-NAME with SNP and either DPCPX (0.1 mg kg^{-1}, group 1) or ZM241385 (0.05 mg kg^{-1}, group 2)

	MAP (mmHg)	FBF (ml min^{-1})	FVC (ml min^{-1} $mmHg^{-1}$)
Group 1			
Control	120 ± 3	2.0 ± 0.5	0.0162 ± 0.0014
L-NAME	143 ± 2***	1.1 ± 0.1**	0.0077 ± 0.0007***
L-NAME + SNP	104 ± 4*** †††	1.5 ± 0.1	0.0139 ± 0.0010††
L-NAME + SNP + DPCPX	120 ± 2††† ‡‡‡	1.7 ± 0.2†	0.0137 ± 0.0018††
Group 2			
Control	119 ± 5	2.1 ± 0.2	0.0175 ± 0.0021
L-NAME	151 ± 3***	1.2 ± 0.1*	0.0078 ± 0.0007***
L-NAME + SNP	111 ± 3* †††	1.5 ± 0.1	0.0152 ± 0.0012†
L-NAME + SNP + ZM241385	121 ± 6†††	1.8 ± 0.2	0.0149 ± 0.0020†

Values are means ± S.E.M. MAP, mean arterial pressure; FBF, femoral blood flow; FVC, femoral vascular conductance. * Significantly different *vs.* control, † significantly different *vs.* L-NAME, ‡ significantly different *vs.* L-NAME with SNP, where 1, 2 and 3 symbols indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

Table 2. MAP and FBF during air breathing and at the 5th minute of each hypoxic challenge for control, DPCPX (0.1 mg kg^{-1}) and ZM241385 (0.05 mg kg^{-1}) groups

		Control	DPCPX	ZM241385
MAP (mmHg)	Before drug	—	118 ± 4	114 ± 3
	21 % O_2	119 ± 2	129 ± 3†	118 ± 3
	14 % O_2	77 ± 4	89 ± 10	78 ± 3
	12 % O_2	71 ± 4	83 ± 6	73 ± 3
	10 % O_2	67 ± 3	71 ± 7	72 ± 3
	9 % O_2	59 ± 4	68 ± 6	72 ± 2
	8 % O_2	62 ± 2	76 ± 5	70 ± 3
	7 % O_2	50 ± 4	72 ± 7	72 ± 4
	6 % O_2	60 ± 4	78 ± 4*	80 ± 4*
FBF (ml min^{-1})	Before drug	—	2.1 ± 0.2	1.9 ± 0.1
	21 % O_2	1.8 ± 0.2	2.1 ± 0.1	2.0 ± 0.2
	14 % O_2	1.9 ± 0.1	1.8 ± 0.1	1.9 ± 0.1
	12 % O_2	1.7 ± 0.2	1.8 ± 0.2	2.0 ± 0.1
	10 % O_2	2.0 ± 0.2	1.7 ± 0.2	2.3 ± 0.1
	9 % O_2	2.0 ± 0.2	1.8 ± 0.2	2.2 ± 0.1
	8 % O_2	1.7 ± 0.2	2.1 ± 0.2	1.9 ± 0.2
	7 % O_2	1.8 ± 0.2	1.5 ± 0.1	1.8 ± 0.2
	6 % O_2	1.0 ± 0.2	1.0 ± 0.1	1.5 ± 0.2

Values are means ± S.E.M. * $P < 0.05$ *vs.* control, † $P < 0.05$ for before DPCPX *vs.* after DPCPX.

Nevertheless, as we described recently (Edmunds & Marshall, 2001), infusion of SNP completely restored the hypoxia-evoked increases in FVC, despite continued NOS blockade (Fig. 1). Under these conditions, DPCPX attenuated the hypoxia-evoked increases in FVC by about 60 and 40 % during 12 % O_2 and 8 % O_2, respectively (Fig. 3). The baseline cardiovascular variables for group 2 are also shown in Table 1; they followed a similar pattern to those in group 1. Similarly, FVC increased in response to each level of hypoxia (Fig. 2). This effect was attenuated by L-NAME, and restored by infusion of SNP (Fig. 2). Interestingly, subsequent infusion of ZM241385 partially attenuated the increase in FVC evoked by 12 % inspired O_2, but not that evoked by 8 % inspired O_2.

Series 2: determination of $D_{O_2,crit}$

Control animals. The changes in cardiovascular parameters induced by graded hypoxia have been described in detail previously (Marshall & Metcalfe, 1988; Edmunds & Marshall, 2001), and are shown in Fig. 3. As expected, each level of hypoxia caused a decrease in MAP ($P < 0.05$; Fig. 3) and an increase in FVC ($P < 0.01$; Fig. 3). In spite of a decrease in MAP during hypoxia, FBF was generally well maintained due to the increase in FVC (Table 2). However, when breathing 6 % O_2 the increase in FVC was not sufficient to sustain FBF (21 % O_2 *vs.* 6 % O_2: 1.8 ± 0.2 *vs.* 1.0 ± 0.2 ml min^{-1}, $P < 0.05$; Table 2). As described previously (Edmunds & Marshall, 2001), D_{O_2} decreased progressively with the severity of the hypoxic challenge. $\dot{V}_{O_2}$

was maintained during moderate levels of hypoxia, and was thus delivery independent (Fig. 4A). However, during more severe hypoxic challenges, when D_{O_2} was reduced to lower values, $\dot{V}_{O_2}$ declined and thus became delivery dependent. Calculation of $D_{O_2,crit}$ from each individual animal gave a value of 0.64 ± 0.06 ml O_2 min^{-1} kg^{-1} (Fig. 4A; Edmunds & Marshall, 2001).

Effects of DPCPX. Administration of the selective adenosine A_1 receptor antagonist DPCPX resulted in a small, but significant increase in baseline MAP (Table 2), indicating that under resting conditions MAP is under the tonic influence of adenosine. Baseline FVC was not altered by DPCPX (0.0171 ± 0.0012 *vs.* 0.0159 ± 0.0011 ml min^{-1} $mmHg^{-1}$, before *vs.* after DPCPX, $P > 0.05$), indicating that the increase in MAP seen with DPCPX was not the result of skeletal muscle vasoconstriction. In consequence, FBF was slightly elevated when compared with that of control animals, although this difference did not achieve statistical significance (Table 2). DPCPX blunted the hypoxia-evoked increase in FVC by approximately 50 % ($P < 0.001$ control group *vs.* DPCPX group; Fig. 3), in agreement with Bryan & Marshall (1999*a*). The vehicle for DPCPX did not alter the FVC response to hypoxia (data not shown; see Bryan & Marshall, 1999*a*). The changes in MAP evoked by hypoxia were slightly smaller after DPCPX than in control animals ($P < 0.01$, control group *vs.* DPCPX group). This effect was most apparent during the most severe hypoxic challenge (Fig. 3).

During air breathing after DPCPX, hindlimb $\dot{V}_{O_2}$ appeared to be slightly higher than in control animals, although this difference did not achieve statistical significance (0.46 ± 0.03 *vs.* 0.55 ± 0.02 ml O_2 min^{-1} kg^{-1}, $P = 0.41$; Fig. 4A *vs.* B). As indicated in Methods, two milder levels

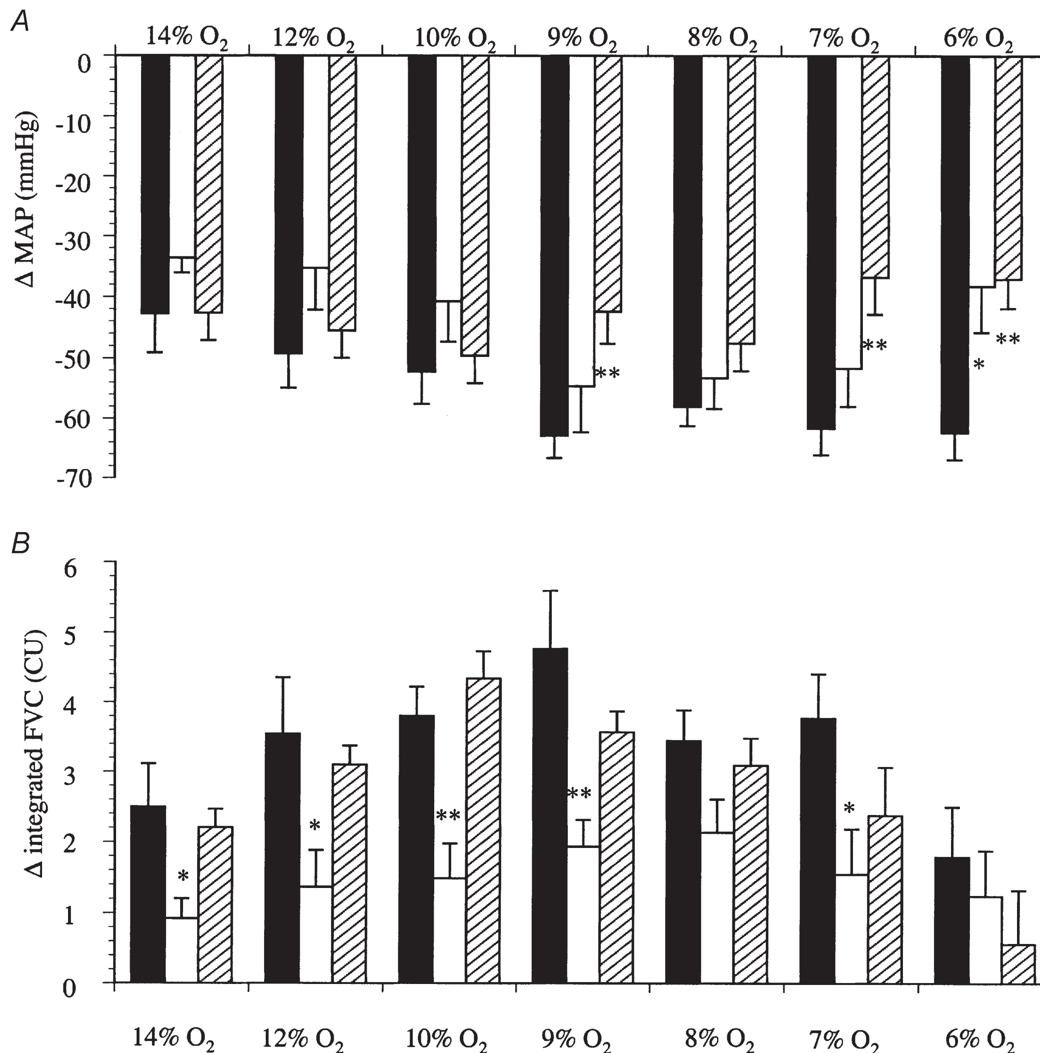

Figure 3. Changes in MAP and FVC evoked by graded systemic hypoxia

Each column represents the mean ± S.E.M. of the change in MAP (*A*) or integrated FVC (*B*) recorded over 5 min periods of hypoxia; the percentage O_2 in the inspirate is indicated for each column. Control animals, ■; after DPCPX (0.1 mg kg^{-1}, I.V.), □; and after ZM243186 (0.05 mg kg^{-1}, I.V.), ▨. * $P < 0.05$ and ** $P < 0.01$, when compared with control.

of hypoxia were included in the DPCPX group; this allowed the accurate calculation of the delivery-independent portion of the D_{O_2}–$\dot{V}_{O_2}$ relationship. Thus, after administration of DPCPX each level of systemic hypoxia resulted in decreased hindlimb D_{O_2}, and delivery-dependent and delivery-independent phases in the D_{O_2}–$\dot{V}_{O_2}$ relationship were observed. $D_{O_2,crit}$ was calculated to be 0.95 ± 0.07 ml O_2 min^{-1} kg^{-1} (Fig. 4*B*). This was significantly greater than the $D_{O_2,crit}$ calculated for control animals (0.64 ± 0.06 ml O_2 min^{-1} kg^{-1}, $P < 0.01$ *vs.* DPCPX). In animals given the vehicle for DPCPX, again two distinct phases were observed in the relationship between D_{O_2} and $\dot{V}_{O_2}$ (Fig. 4*C*); $D_{O_2,crit}$ in these animals was calculated to be 0.70 ± 0.05 ml O_2 min^{-1} kg^{-1}. This was not significantly different from that calculated in control animals, but was different from the $D_{O_2,crit}$ calculated after the infusion of DPCPX ($P < 0.01$).

Effects of ZM241385. The effects of ZM241385 on baseline values are shown in Table 1; ZM241385 did not alter MAP, FBF or FVC (0.0164 ± 0.0013 *vs.* 0.0165 ± 0.0011 ml min^{-1} mmHg^{-1}, before *vs.* after ZM241385). As the severity of the hypoxic challenges increased, ZM241385 progressively attenuated the evoked decrease in MAP (Fig. 3). However, ZM241385 did not alter the increase in FVC evoked by hypoxia (Fig. 3; see Bryan & Marshall, 1999*a*). Again, the biphasic relationship between D_{O_2} and $\dot{V}_{O_2}$ was observed (Fig. 4*D*); $D_{O_2,crit}$ was calculated to be 0.73 ± 0.02 ml O_2 min^{-1} kg^{-1}, a value that was not different from the $D_{O_2,crit}$ measured in control animals.

DISCUSSION

Changes in femoral vascular conductance

Adenosine has been proposed to be a mediator of the hypoxia-evoked vasodilatation in skeletal muscle (Mian & Marshall, 1991; Skinner & Marshall, 1996; Bryan & Marshall, 1999*b*), in the mesenteric circulation (Mian & Marshall, 1995), within the coronary circulation (Deussen *et al.* 1986; Nakhostine & Lamontagne, 1993, 1994) as well as in the cerebral circulation (Armstead, 1997; Coney

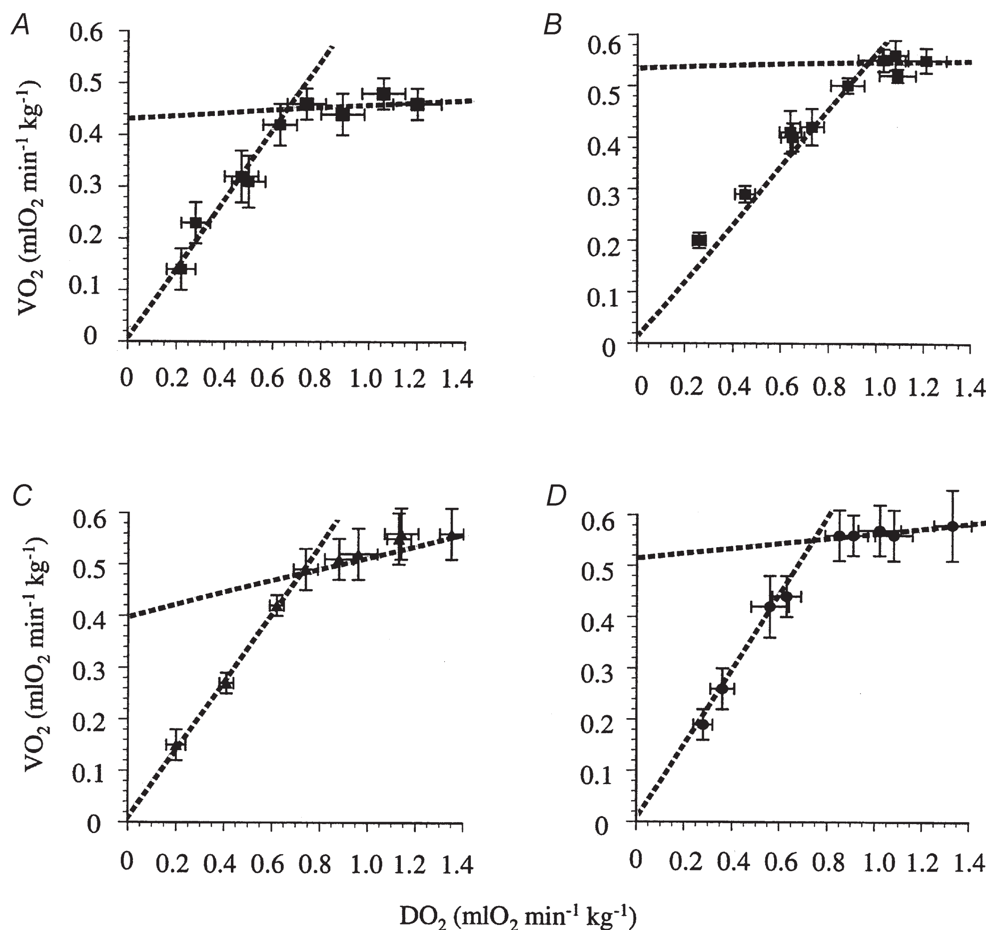

Figure 4. Effects of graded systemic hypoxia on D_{O_2} and $\dot{V}_{O_2}$ of the rat hindlimb in control animals and after administration of adenosine antagonists

Each value represents the mean ± S.E.M. for $\dot{V}_{O_2}$ and D_{O_2} during air breathing or after 5 min periods of hypoxia. *A*, control animals. *B*, animals treated with DPCPX (0.1 mg kg^{-1}). *C*, animals treated with the vehicle for DPCPX. *D*, animals treated with ZM243186 (0.05 mg kg^{-1}).

& Marshall, 1998). So, a general role for adenosine in hypoxia-evoked vasodilatation is widely accepted. Within hindlimb muscle we have shown previously that the adenosine component of the change in FVC evoked by one level of moderate systemic hypoxia (8% inspired O_2) is mediated by adenosine A_1 receptors (see Results, and Bryan & Marshall, 1999*a*). The present study confirms and extends these observations, demonstrating a role for adenosine A_1 receptors in the hypoxia-evoked increase in FVC over a wide range of hypoxic challenges (14–6% O_2). We have also extended our evidence that the increase in FVC is not altered by the selective adenosine A_{2A} receptor antagonist ZM241385 (Bryan & Marshall, 1999*a*) over the same wide range of hypoxic challenges. Nevertheless, ZM241385 did attenuate partially the decrease in MAP evoked by some of the more severe levels of hypoxia, suggesting that the adenosine released by systemic hypoxia acts on A_{2A} receptors in tissues other than muscle.

In addition, we have confirmed that the hypoxia-evoked increase in FVC is severely attenuated by inhibition of NOS (Skinner & Marshall, 1996; Bryan & Marshall, 1999*b*) and that the increase in FVC (proximal arteriolar vasodilatation) can be restored by the infusion of a NO donor (Edmunds & Marshall, 2001). A major new finding of the present study is that this restored increase in FVC was attenuated by the A_1 receptor antagonist DPCPX. Interestingly, although ZM241385 did not alter the restored increase in FVC evoked by 8% inspired O_2, it did attenuate the restored increase in FVC evoked by 12% inspired O_2. This finding, coupled with the observation that the increase in FVC evoked by adenosine infusion was almost abolished by NOS inhibition (Skinner & Marshall, 1996; Bryan & Marshall, 1999*b*), suggests that the tonic endothelial release of NO resulting mainly from shear stress (Buga *et al.* 1991) somehow sensitises the underlying smooth muscle to the vasodilator influence of adenosine.

It should be noted that NO, acting via cGMP, has been shown to sensitise smooth muscle to the actions of the vasodilators that cause an increase in cAMP (De Wit *et al.* 1994). Both A_1 and A_{2A} receptors are present on the vascular smooth muscle of skeletal muscle tissue (Rådegran & Hellsten, 2000). This observation could therefore explain our results with respect to ZM241385, because A_{2A} receptors would be expected to cause vasodilatation by a direct action on vascular smooth muscle via an increase in cAMP. However, this is an unlikely explanation of the results concerning DPCPX, since A_1 receptor stimulation would be expected to reduce rather than increase vascular smooth muscle cAMP levels. An alternative possibility is that adenosine acts directly on the vascular smooth muscle A_1 receptors that are coupled to vasodilator K_{ATP} channels (Dart & Standen, 1993). This is because cGMP, a well-known effector of NO, facilitates the opening of K_{ATP} channels (Kubo *et al.* 1994). Therefore, a synergy between smooth muscle adenosine A_1 receptors and shear-activated endothelial NO release is possible. Taken together, these findings lead to the obvious conclusion that the A_1-mediated dilatation of proximal arterioles, which is responsible for the increase in FVC evoked by systemic hypoxia, is dependent upon the existence of a basal level of NO, rather than being mediated by an increased synthesis of NO (as we originally proposed; Bryan & Marshall, 1999*b*).

However, the alternative possibility is that our original proposal was at least partially correct, but that when adenosine A_1 receptor-stimulated synthesis of NO is prevented, the action of adenosine on vascular smooth muscle A_1 receptors can predominate providing that some NO is present. This alternative proposal is attractive because adenosine A_1 receptors are present on skeletal muscle vascular endothelium (Rådegran & Hellsten, 2000) and when stimulated can induce the release of NO from endothelial cells by acting on A_1 receptors (Ray & Marshall, 2000; Rolleston & Marshall, 2000). This proposal would, of course, suggest that redundancy exists in the vasodilator signals evoked by hypoxia. This would not be surprising given the importance of maintaining the O_2 supply to tissue cells. Interestingly, as mentioned above, adenosine A_{2A} receptors are also able to stimulate the release of NO from endothelial cells (Sobrevia *et al.* 1997; Ray & Marshall, 2000; Rolleston & Marshall, 2000). During systemic hypoxia, A_{2A}-mediated NO release is not an important component of the adenosine-evoked vasodilatation of either the proximal arterioles or the terminal arterioles (see below).

Effects on D_{O_2} and $\dot{V}_{O_2}$

The second major novel finding of the present study is that DPCPX caused a substantial increase in the $D_{O_2,crit}$, calculated during progressive systemic hypoxia, from 0.64 ± 0.06 ml O_2 min^{-1} kg^{-1} in control animals to 0.95 ± 0.07 ml O_2 min^{-1} kg^{-1}. This represents a severe disruption in the ability of the hindlimb vasculature to maintain its $\dot{V}_{O_2}$ during decreases in D_{O_2} produced by graded levels of acute systemic hypoxia. The maintenance of $\dot{V}_{O_2}$ during periods of reduced D_{O_2} has been attributed to dilatation of the terminal arterioles (Granger *et al.* 1976), and the adenosine receptor antagonist 8-phenyltheophylline, which is non-selective between A_1 and A_2 receptors, reduced the responses of the terminal arterioles that dilated during hypoxia, without altering the responses of terminal arterioles that constricted (Main & Marshall, 1991). Therefore, the present results suggest that adenosine acts on A_1 receptors on terminal arterioles as an essential component in the defence of $\dot{V}_{O_2}$ when D_{O_2} is decreased by systemic hypoxia. The heterogeneous mixture of constriction and A_1 receptor-mediated dilatation of terminal arterioles allows a redistribution of blood flow and a more homogeneous distribution of O_2 within muscle (Harrison *et al.* 1990; Mian & Marshall, 1991; Marshall, 1995).

In our previous study (Edmunds & Marshall, 2001), when a basal level of NO was maintained by infusion of a NO donor under conditions of NOS blockade, $D_{O_2,crit}$ was significantly greater than that measured under control conditions. This indicates that dilatation of the terminal arterioles that maintain $\dot{V}_{O_2}$ requires an increase in the synthesis of NO. The $D_{O_2,crit}$ calculated after DPCPX in the present study compares well with that calculated after NOS inhibition (0.95 ± 0.07 ml O_2 min^{-1} kg^{-1} after DPCPX and 0.96 ± 0.07 ml O_2 min^{-1} kg^{-1} during NOS inhibition; Edmunds & Marshall, 2001). Thus, it is now reasonable to propose that this terminal arteriolar vasodilatation is, at least in part, mediated by adenosine acting on A_1 receptors and stimulating NOS. The evidence that adenosine A_1 receptors are present on skeletal muscle vascular endothelium (Rådegran & Hellsten, 2000) and cause HUVECs and rat aorta endothelial cells to release NO when stimulated (Ray & Marshall, 2000; Rolleston & Marshall, 2000) is consistent with this proposal.

The present study has not addressed the issue of whether either A_1 or A_{2A} receptors still contribute to setting $D_{O_2,crit}$ during NOS inhibition when a background level of NO is replaced by infusion of SNP (Edmunds & Marshall, 2001). The fact that these receptors contribute to the hypoxia-induced increase in FVC under these conditions (see above) raises this possibility. Technical problems, including the use of multiple drugs and increased duration of the experiments, make such studies very difficult to undertake successfully. However, we can say that if there is redundancy in the vasodilator signal at the level of the terminal arterioles, as we have proposed for the proximal arterioles (see above), then it is unable to restore $D_{O_2,crit}$ to values observed in control animals.

The action of adenosine on A_{2A} receptors was implicated in the muscle vasodilatation-associated muscle contraction in anaesthetised cats (Poucher, 1996) and in the cerebral vasodilatation effected by acute systemic hypoxia in rats (Coney & Marshall, 1998). Exogenous adenosine can stimulate A_{2A} receptors to evoke an increase in FVC (Bryan & Marshall, 1999*a*) and A_{2A} receptor stimulation can increase the synthesis of NO by HUVECs or rat aorta endothelium (Sobrevia *et al.* 1997; Ray & Marshall, 2000; Rolleston & Marshall, 2000). However, the present results not only confirm that there is no apparent role for adenosine A_{2A} receptors in the hypoxia-evoked increase in FVC (see above and Bryan & Marshall, 1999*a*), but also, given the lack of effect of ZM241385 on $D_{O_2,crit}$, demonstrate that there is no functional role for these receptors in the dilatation of the terminal arterioles during systemic hypoxia.

In summary, we have shown an integrated physiological consequence of adenosine release in the hindlimb during systemic hypoxia. Graded systemic hypoxia results in a reduction in muscle D_{O_2}, which is partially compensated for by an adenosine-evoked increase in muscle vascular conductance that is mediated via adenosine A_1 receptors. This acts to maintain muscle blood flow, and therefore limits the fall in D_{O_2}, in the face of a large decrease in MAP. This adenosine-mediated vasodilatation of proximal arterioles probably requires a basal presence of NO, but not necessarily an increase in the release of NO; it can be mediated by A_{2A} (moderate hypoxia) or A_1 receptors when NOS is blocked. Our results also indicate that adenosine, acting via A_1 receptors but not A_{2A} receptors, plays an important role in the co-ordinated terminal arteriolar response to hypoxia that is required for $\dot{V}_{O_2}$ to be maintained in the face of a reduced D_{O_2}. We suggest that this action of A_1 receptors is likely to be mediated by stimulation of NOS and release of NO.

Armstead, W. M. (1997). Role of nitric oxide, cyclic nucleotides, and the activation of ATP-sensitive K^+ channels in the contribution of adenosine to hypoxia-induced pial artery dilation. *Journal of Cerebral Blood Flow and Metabolism* **17**, 100–108.

Bryan, P. T. & Marshall, J. M. (1999*a*). Adenosine receptor subtypes and vasodilatation in rat skeletal muscle during systemic hypoxia: a role for A_1 receptors. *Journal of Physiology* **514**, 151–162.

Bryan, P. T. & Marshall, J. M. (1999*b*). Cellular mechanisms by which adenosine induces vasodilatation in rat skeletal muscle: significance for systemic hypoxia. *Journal of Physiology* **514**, 163–175.

Buga, G. M., Gold, M. E., Fukuto, J. M. & Ignarro, L. J. (1991). Shear stress-induced release of nitric oxide from endothelial cells grown on beads. *Hypertension* **17**, 187–193.

Cain, S. M. & Chapler, C. K. (1979). Oxygen extraction by canine hindlimb during hypoxic hypoxia. *Journal of Applied Physiology* **46**, 1023–1028.

Coney, A. M. & Marshall, J. M. (1998). Role of adenosine and its receptors in the vasodilatation induced in the cerebral cortex of the rat by systemic hypoxia. *Journal of Physiology* **509**, 507–518.

Curtis, S. E., Vallet, B., Winn, M. J., Caufield, J. B., King, C. E., Chapler, C. K. & Cain, S. M. (1995). Role of the vascular endothelium in O_2 extraction during progressive ischemia in canine skeletal muscle. *Journal of Applied Physiology* **79**, 1351–1360.

Dart, C. & Standen, N. B. (1993). Adenosine-activated potassium current in smooth muscle cells isolated from the pig coronary artery. *Journal of Physiology* **471**, 767–786.

Deussen, A., Moser, G. & Schrader, J. (1986). Contribution of coronary endothelial cells to cardiac adenosine production. *Pflügers Archiv* **406**, 608–614.

De Wit, C., Bismarck, P. & Pohl, U. (1994). Synergistic action of vasodilators that increase cGMP and cAMP in the hamster cremaster microcirculation. *Cardiovascular Research* **28**, 1513–1518.

Duran, W. N. & Renkin, E. M. (1974). Oxygen consumption and blood flow in resting mammalian skeletal muscle. *American Journal of Physiology* **226**, 173–177.

Edmunds, N. J. & Marshall, J. M. (2000). A role for adenosine A_1 receptors in the regulation of hindlimb oxygen consumption during graded systemic hypoxia in the rat. *Journal of Physiology* **525.P**, 15–16*P*.

Edmunds, N. J. & Marshall, J. M. (2001). Vasodilatation, oxygen delivery and oxygen consumption in rat hindlimb during systemic hypoxia: roles of nitric oxide. *Journal of Physiology* **532**, 251–259.

Froneck, K. & Zweifach, B. W. (1975). Microvascular pressure distribution in skeletal muscle and the effect of vasodilatation. *American Journal Physiology* **228**, 791–796.

Granger, H. J., Goodman, A. H. & Granger, N. D. (1976). Role of resistance and exchange vessels in local microvascular control of skeletal muscle oxygenation in the dog. *Circulation Research* **38**, 379–385.

Harrison, D. K., Kessler, M. & Knauff, S. K. (1990). Regulation of capillary blood flow and oxygen supply in skeletal muscle in dogs during hypoxemia. *Journal of Physiology* **420**, 431–446.

Hebert, M. T. & Marshall, J. M. (1988). Direct observations of the effects of baroreceptor stimulation on skeletal muscle circulation of the rat. *Journal of Physiology* **400**, 45–59.

Keddie, J. R., Poucher, S. M., Shaw, G. R., Brooks, R. & Collis, M. G. (1996). In vivo characterisation of ZM 241385, a selective adenosine A_{2A} receptor antagonist. *European Journal of Pharmacology* **301**, 107–113.

Kubo, M., Nakaya, Y., Matsuoka, S., Saito, K., Kuroda, Y. (1994). Atrial natriuretic factor and isosorbide dinitrate modulate the gating of ATP-sensitive K^+ channels in cultured vascular smooth muscle cells. *Circulation Research* **74**, 471–476.

Marshall, J. M. (1995). Skeletal muscle vasculature and systemic hypoxia. *News in Physiological Sciences* **1**,163–167.

Marshall, J. M. & Davis, W. R. (1999). The effects of acute and chronic systemic hypoxia on muscle oxygen supply and oxygen consumption in the rat. *Experimental Physiology* **84**, 57–68.

Marshall, J. M. & Metcalfe, J. D. (1988). Analysis of the cardiovascular changes induced in the rat by graded levels of systemic hypoxia. *Journal of Physiology* **407**, 385–403.

Mian, R. & Marshall, J. M. (1991). The role of adenosine in dilator responses induced in arterioles and venules of rat skeletal-muscle by systemic hypoxia. *Journal of Physiology* **443**, 499–511.

Mian, R. & Marshall, J. M. (1995). The role of adenosine in mediating vasodilatation in mesenteric circulation of the rat in acute and chronic hypoxia. *Journal of Physiology* **489**, 225–234.

Nakhostine, N. & Lamontagne, D. (1993). Adenosine contributes to hypoxia-induced vasodilatation through ATP-sensitive K^+ channel activation. *American Journal of Physiology* **265**, H1289–1293.

Nakhostine, N. & Lamontange, D. (1994). Contribution of prostaglandins in hypoxia-induced vasodilatation in isolated rabbit hearts. Relation to adenosine and K_{ATP} channels. *Pflügers Archiv* **428**, 526–532.

Olsson, R. A. & Pearson, J. D. (1990). Cardiovascular purinoceptors. *Physiological Reviews* **70**, 761–845.

Poucher, S. M. (1996). The role of the A_{2A} adenosine receptor subtype in functional hyperaemia in the hindlimb of anaesthetized cats. *Journal of Physiology* **492**, 495–503.

Rådegran, G. & Hellsten, Y. (2000). Adenosine and nitric oxide in exercise-induced human skeletal muscle vasodilatation. *Acta Physiologica Scandinavica* **168**, 575–591.

Ray, C. & Marshall, J. M. (2000). Interactions of adenosine, nitric oxide and the cyclo-oxygenase pathway in freshly excised rat aorta. *Journal of Physiology* **528.P**, 111*P*.

Rolleston, S. & Marshall, J. M. (2000). The ability of adenosine to generate nitric oxide in human umbilical vein endothelial cells. *Journal of Physiology* **523.P**, 151*P*.

Samsel, R. W. & Schumacker, P. T. (1988). Determination of the critical O_2 delivery from experimental data: sensitivity to error. *Journal of Applied Physiology* **64**, 2074–2082.

Skinner, M. R. & Marshall, J. M. (1996). Studies on the roles of ATP, adenosine and nitric oxide in mediating muscle vasodilatation induced in the rat by acute systemic hypoxia. *Journal of Physiology* **495**, 553–560.

Sobrevia, L., Yudilevich, D. L. & Mann, G. E. (1997). Activation of A_{2A}-purinoceptors by adenosine stimulates L-arginine transport (system y^+) and nitric oxide synthesis in human fetal endothelial cells. *Journal of Physiology* **499**, 135–140.

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