J Physiol 587.7 (2009) pp 1565–1578

The Journal of Physiology

Elucidation in the rat of the role of adenosine and A_{2A}-receptors in the hyperaemia of twitch and tetanic contractions

Clare J. Ray and Janice M. Marshall

The Medical School, University of Birmingham, Birmingham B15 2TT, UK

Adenosine is implicated in playing a role in blood flow responses to situations where O_2 delivery (D_{O_2}) is reduced (hypoxia) or O_2 consumption ($\dot{V}_{O_2}$) is increased (exercise). Strong isometric contractions have been shown to limit vasodilatation, potentially leading to a greater mismatch between D_{O_2} and $\dot{V}_{O_2}$ than during twitch contractions. Thus, we hypothesized that adenosine makes a greater contribution to the hyperaemia associated with isometric tetanic than isometric twitch contractions and aimed to elucidate the adenosine-receptor subtypes involved in the response. In four groups of anaesthetized rats, arterial blood pressure (ABP), femoral blood flow (FBF) and tension in the extensor digitorum longus muscle were recorded; isometric twitch and tetanic contractions were evoked by stimulation of the sciatic nerve for 5 min at 4 Hz and 40 Hz, respectively. Groups 1 (twitch) and 3 (tetanic) were time controls for Groups 2 and 4, which received the selective A_{2A}-receptor antagonist ZM241385 before the third and 8-sulphophenyltheophylline (8-SPT; a non-selective adenosine receptor antagonist) before the fourth contraction. Time controls showed consistent tension and hyperaemic responses: twitch and tetanic contractions were associated with a 3-fold and 2.5-fold increase in femoral vascular conductance (FVC, FBF/ABP) from baseline, respectively. ZM241385 reduced these responses by 14% and as much as 25%, respectively; 8-SPT had no further effect. We propose that, while twitch contractions produce a larger hyperaemia, adenosine acting via A_{2A}-receptors plays a greater role in the hyperaemia associated with tetanic contraction. These results are considered in relation to the A_1-receptor-mediated muscle dilatation evoked by systemic hypoxia.

(Received 19 September 2008; accepted after revision 3 February 2009; first published online 9 February 2009)
Corresponding author C. J. Ray: The Medical School, University of Birmingham, Birmingham B15 2TT, UK.
Email: c.j.ray@bham.ac.uk

Matching blood flow to metabolic activity is particularly important in skeletal muscle during and after muscle contraction when metabolism must increase to meet increased energy requirements. Elevation of blood flow is also essential to restore normal cellular metabolite levels. The increase in blood flow that accompanies muscle contraction is known as exercise hyperaemia. Various substances, including those released in association with contraction and increased metabolic activity such as K^+ ions, lactate, H^+ ions, adenosine and the adenine nucleotide ATP, and other mediators of vascular tone released from skeletal muscle fibres, vascular smooth muscle and the endothelium, including nitric oxide (NO), prostanoids and endothelial derived hyperpolarizing factor (EDHF), have been implicated in mediating exercise hyperaemia (Clifford & Hellsten, 2004).

Adenosine has long been implicated more generally in vasodilatation in situations in which O_2 supply is diminished (hypoxia) or O_2 demand is increased (exercise), when it is considered to increase blood flow to match metabolic requirements (Berne *et al.* 1983). Indeed, in dogs, skeletal muscle vasodilatation evoked by systemic hypoxia was accompanied by release of adenosine into the venous efflux (Mo & Ballard, 1997). The adenosine receptor antagonist aminophylline attenuated the increase in forearm blood flow evoked by acute systemic hypoxia in humans (Leuenberger *et al.* 1999). Further, our own experiments, using receptor-specific adenosine receptor antagonists, demonstrated that adenosine acting at the A_1-receptors on vascular endothelium mediates approximately 50% of the muscle vasodilator response to systemic hypoxia in the rat, but that stimulation of A_{2A}-receptors plays no role in this response, even though the muscle vasodilatation induced by infused adenosine was attributable to A_{2A}- and A_1-receptors (Bryan & Marshall, 1999; Ray *et al.* 2002).

DOI: 10.1113/jphysiol.2008.163683

Early studies on exercise hyperaemia, prior to the development of specific adenosine receptor antagonists, investigated the role of adenosine by measuring its release. Adenosine was detected only when the muscle was made ischaemic or contracted under ischaemic or constant flow conditions (Dobson *et al.* 1971; Bockman *et al.* 1975, 1976; Belloni *et al.* 1979). This may be explained by the avid uptake and metabolism of adenosine (see Ray *et al.* 2002), for, with the development of more sensitive techniques for its detection, adenosine was measured in the venous efflux of contracting dog skeletal muscle at constant high flow rates (Ballard *et al.* 1987) and in free flow conditions (Fuchs *et al.* 1986) and in the interstitial space of contracting muscles (Hellsten *et al.* 1998). Moreover, since the development of adenosine transport and deaminase inhibitors and antagonists of adenosine receptors, studies in a number of species have demonstrated that exercise hyperaemia is reduced by as much as 40% by adenosine receptor antagonists (see Marshall, 2007).

It is generally held that strong isometric contraction limits the vasodilatation that accompanies muscle contraction by physically restricting the blood flow (Barcroft & Millen, 1939; Bonde-Petersen *et al.* 1975; Sadamoto *et al.* 1983). Such physical limitation of O_2 delivery (D_{O_2}) to muscle might be expected to lead to a greater mismatch between D_{O_2} and O_2 consumption ($\dot{V}_{O_2}$) than during twitch contractions when blood flow is able to increase during relaxation periods. It is therefore reasonable to hypothesize that adenosine makes a greater contribution to the hyperaemia that accompanies isometric tetanic contraction than to isometric twitch contractions.

Thus, the aim of the present study was to investigate this hypothesis by testing the effect of adenosine receptor antagonists on the hyperaemia evoked by isometric twitch contractions and by tetanic contraction. This was of particular interest because in the majority of previous studies, isometric twitch contractions were used to investigate the role of adenosine (e.g. Ballard *et al.* 1987; Lo *et al.* 2001; Poucher *et al.* 1990). Indeed, the highly selective A_{2A}-receptor antagonist ZM241385 substantially reduced the hyperaemic response to isometric *twitch* contractions of cat hindlimb and the same effect was observed with the adenosine receptor antagonist 8-phenyltheophylline (8-PT), which is non-selective between receptor subtypes. Thus, it was concluded that stimulation of A_{2A}-receptors, but not other adenosine receptors, are responsible for up to 30% of exercise hyperaemia (Poucher, 1996). As mentioned above, 50% of the hindlimb dilatation evoked in the rat by systemic hypoxia, is mediated by adenosine acting on endothelial adenosine A_1- but not A_{2A}-receptors (Bryan & Marshall, 1999; Marshall, 2000). This raises the possibility that there is either a fundamental difference in the functional roles of adenosine receptors in rat and cat hindlimb, or in the mechanisms by which adenosine increases blood flow in exercise and systemic hypoxia. Thus, a secondary aim of this study was to elucidate the adenosine receptor subtypes involved in the hyperaemic responses to isometric twitch and tetanic contractions in the rat and to compare the results with the role of A_1- and A_{2A}-receptors in the response to hypoxia in the rat and the results of Poucher (1996) on exercise hyperaemia in the cat.

Methods

Surgical preparation

Experiments were performed in accordance with UK legislation (Home Office Animals (Scientific Procedures) Act 1986) on 34 male Wistar rats (265 ± 3 g). Anaesthesia was induced with halothane (3.5% in O_2) and was judged to be at a surgical level when the pedal withdrawal reflex was absent. Anaesthesia was maintained with Saffan (7–12 mg kg^{-1} h^{-1} I.V.; Schering-Plough Animal Health, UK) infused via a cannula in the jugular vein and was judged to be adequate by the absence of withdrawal reflexes and stability of arterial blood pressure (ABP; Coney & Marshall, 2007). All animals were killed by anaesthetic overdose at the end of the experiments.

Animals were prepared surgically using techniques previously described (Ray & Marshall, 2005). Briefly, the trachea was cannulated to maintain a patent airway and ABP was monitored via a cannula in the brachial artery. The left femoral artery and vein were cannulated to allow the collection of arterial blood samples for analysis of blood gases, and the administration of pharmacological antagonists (see below). A transonic, perivascular flow probe (0.5V; Transonic Systems Inc., NY, USA) connected to a flow meter (T106, small animal flow meter; Transonic Systems Inc.) was placed around the right femoral artery to allow continuous measurement of femoral blood flow (FBF).

The extensor digitorum longus (EDL) muscle was chosen as a representative muscle of the rat hindlimb for the present study; it is a mixed muscle containing approximately 42% glycolytic and 56% oxidative fibres and receives a similar blood flow, per gram of tissue, at rest and during exercise, as the whole hindlimb (Armstrong & Laughlin, 1983). The right EDL tendon and sciatic nerve were isolated and sectioned at their distal and proximal ends, respectively. The ankle was immobilized in a clamp and the tendon was attached to an isometric force transducer (TRI-201 Letica Scientific, Spain) by means of a length of inextensible suture to allow isometric tension to be developed and measured. The sciatic nerve was attached to a hook electrode to allow stimulation of the muscle as described below. Baseline tension was set at 5 g.

Data were collected via a PowerLab/8SP (ADInstruments, UK) onto an Apple PowerPC G5

computer. Femoral vascular conductance (FVC) was derived on-line by dividing FBF by ABP. Thirty to sixty minutes were allowed for the stabilization of all cardiovascular variables before experimental protocols were begun.

Experimental protocols

Group 1. Isometric twitch contractions: time controls. Maximum twitch of the EDL was established by stimulating the sciatic nerve with increasing voltage (1–9 V). Contractions were then evoked with a supra-maximal voltage (generally 2–4 V) at 4 Hz (0.1 ms pulse duration) for 5 min (1200 pulses). This stimulation was repeated a further four times at 30 min intervals ($n = 7$) giving a total of five contractions. The data for the fifth period of contractions were not used in the present study but served as part of the time control for the experiments described in our companion paper (Ray & Marshall, 2009).

Group 2. Involvement of adenosine in the response to isometric twitch contractions. Two 5 min periods of isometric twitch contractions, as described above, were performed at 30 min intervals. The adenosine A_{2A}-receptor antagonist ZM241385 (0.05 mg kg^{-1}; Bryan & Marshall, 1999) was then administered and 30 min later, a third period of contractions was evoked. ZM241285 is a competitive antagonist with a high affinity in the nanomolar range at the A_{2A}-receptor subtype and has ∼100-fold selectivity for A_{2A}-receptors over A_{2B}-receptors (Poucher *et al.* 1995). The antagonist 8-sulphophenyltheophylline (8-SPT; 10 mg kg^{-1}), which is non-selective between adenosine receptor subtypes (Evoniuk *et al.* 1987), was then administered and 30 min later a final, fourth period of contractions was evoked ($n = 10$).

Group 3. Isometric tetanic contraction: time controls. Maximum twitch of the EDL was established as above and isometric tetanic contraction was evoked with a supra-maximal voltage (generally 2–4 V) at 40 Hz for 5 min. This stimulation was repeated a further three times ($n = 7$).

Group 4. Involvement of adenosine in the response to isometric tetanic contraction. The protocol used for Group 2 was performed, except that the stimulation parameters were chosen to produce isometric tetanic contraction as in Group 3 ($n = 10$).

Analysis

It was observed in Group 1 that the responses evoked by the first period of muscle contractions were significantly different from those evoked by all subsequent periods of contractions (see Results, Groups 1 and 3). Therefore, in all experiments of Groups 2 and 4, the second contraction was considered as the control to which all subsequent contractions were compared. Responses evoked by time control stimulations 1–4 only are shown for Groups 1 and 3 (see Figs 2 and 5) for ease of comparison with Group 2 and Group 4 data.

All variables were analysed to give mean (± S.E.M.) tension, FBF, ABP and FVC for 1 min periods, immediately preceding stimulation (baseline), for the 5 min during stimulation (S1–5) and for the 7 min of recovery after stimulation (R1–7). The change in integrated tension (ΔInt tension) or tension time index (TTI) for the stimulation period was calculated by subtracting the integrated tension for the 5 min preceding muscle contraction from the integrated tension for the 5 min of contraction. The change in integrated FVC (ΔInt FVC) was calculated by subtracting the integrated FVC for the 12 min preceding muscle contraction from the integrated FVC for the 5 min of contraction and the first 7 min of recovery and is expressed in conductance units (c.u.).

Differences within groups were determined with ANOVA for repeated measures and differences between groups with factorial ANOVA, with Scheffé's *post hoc* test when appropriate. $P < 0.05$ was considered significant. For clarity, symbols on figures show significant differences for time points indicated by *post hoc* tests (see specific figure legends).

Results

Group 1. Isometric twitch contractions: time controls

Pattern of response. Stimulation of the sciatic nerve at 4 Hz with a supra-maximal voltage produced rhythmic twitching of the hindlimb muscles. As can be seen from the original traces shown in Fig. 1, tension of the EDL peaked during the first minute of stimulation, and then fatigued to a level that was maintained for the remainder of the stimulation period (Fig. 1*B*). Mean femoral vascular conductance (FVC) increased from baseline at the start of stimulation and reached a peak during the second minute where it was maintained until the first 30 sec to 1 min of recovery before declining steadily back towards baseline levels (Fig. 1*D*). Arterial blood pressure (ABP) was maintained throughout (Fig. 1*A*) and, therefore, mean femoral blood flow (FBF) followed the pattern of FVC (Fig. 1*C*).

Considering the grouped data, the mean tension achieved by the EDL was greater during time control 1 than in the subsequent three periods of contraction (Fig. 2*A*). Further, mean FBF (Fig. 2*B*) and FVC (Fig. 2*D*) were greater during the recovery period and mean ABP was significantly greater at S5, R1 and R2 (Fig. 2*C*), of

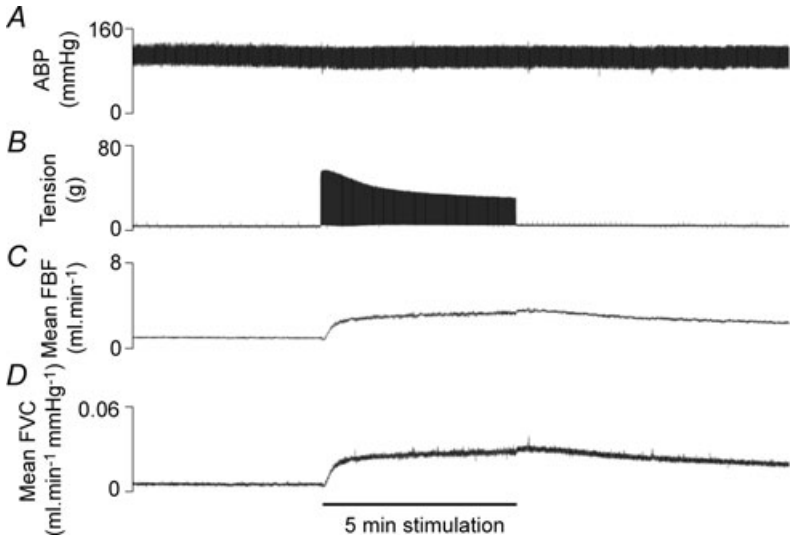

Figure 1. Isometric twitch contractions
Representative original trace showing ABP (*A*), EDL tension (*B*), mean FBF (*C*) and mean FVC (*D*), before, during and after 5 min isometric twitch contractions.

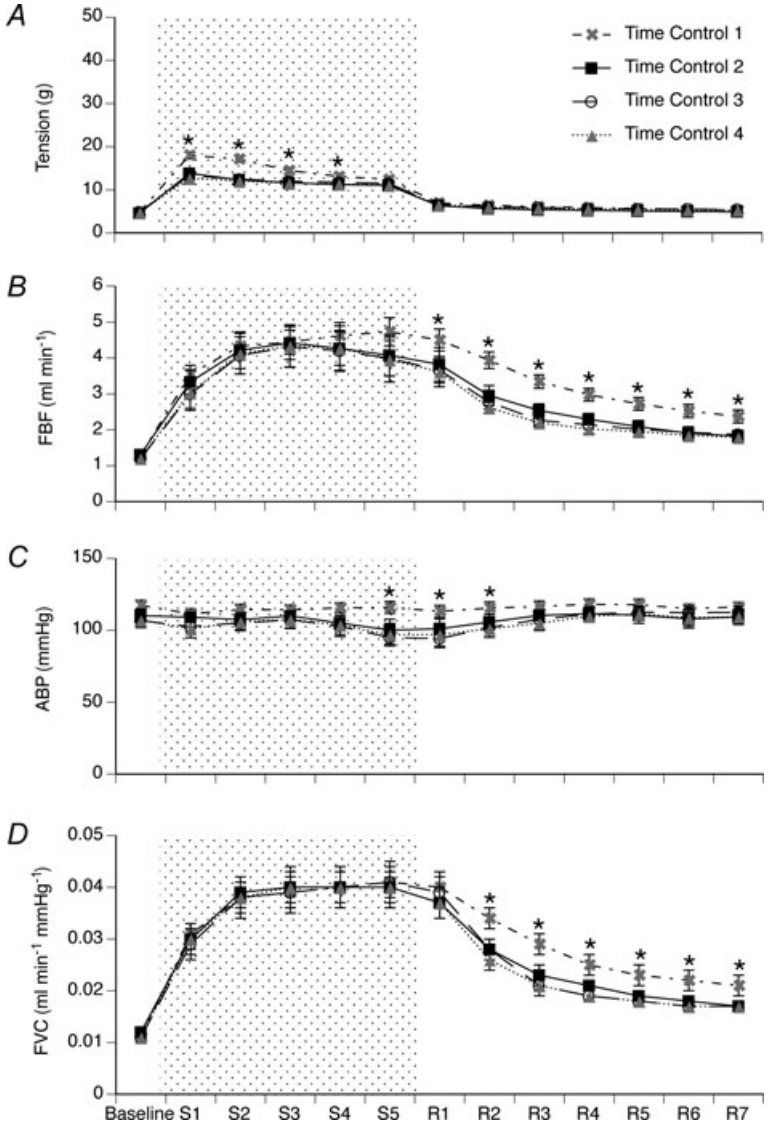

Figure 2. Isometric twitch contractions: time controls
A–D show mean (± S.E.M.) EDL tension, FBF, ABP and FVC for time control stimulation 1 (×, dashed and dotted lines), 2 (■, continuous lines), 3 (○, dashed lines) and 4 (▲, dotted lines) in the 1 min before (baseline), 5 min of (S1–5) and 7 min after (R1–7) sciatic nerve stimulation (4 Hz). *$P < 0.05$ time control 1 S1–5 and/or R1–7, *vs* time control 2–4. There was no significant difference in tension, FBF, ABP and FVC at any time point for time controls 2–4. $n = 7$.

time control 1 than time controls 2–4. Importantly, the changes in mean tension, FBF and FVC (Fig. 2) and the ΔInt tension and ΔInt FVC (Fig. 7*A* and *C*) evoked in time control 2–4 were fully comparable. Thus, in Group 2, Contraction 2 acted as the control for Contractions 3 and 4.

Group 2. Involvement of adenosine in the response to isometric twitch contractions

The pattern of response evoked by stimulation at 4 Hz was as described above for Group 1 (see above; Fig. 3*A*). Administration of the adenosine A_{2A}-receptor antagonist ZM241385 had no effect on mean or integrated tension achieved by EDL and the subsequent administration of 8-SPT, an adenosine receptor antagonist which is non-selective between receptor subtypes, also had no effect (Figs 3*A* and 7*E*).

Mean FVC during contraction was also unchanged by ZM241385. However, during recovery FVC was significantly lower after ZM241385 (Fig. 3*D*). Subsequent administration of 8-SPT had no further effect on FVC during stimulation or recovery (Fig. 3*D*). As mean ABP was unchanged by either antagonist (Fig. 3*C*), mean FBF followed the pattern of FVC and was significantly lower during recovery after ZM241385 and was not further altered by 8-SPT (Fig. 3*B*).

The effect of ZM241385 on the entire hyperaemic response (S1–R7) can be seen in Fig. 7*G*: in all animals ZM241385 tended ($P = 0.07$ *vs* control 2) to reduce the ΔInt FVC evoked by isometric twitch contraction (∼14% reduction from the ΔInt FVC evoked by control 2), while 8-SPT had no further effect.

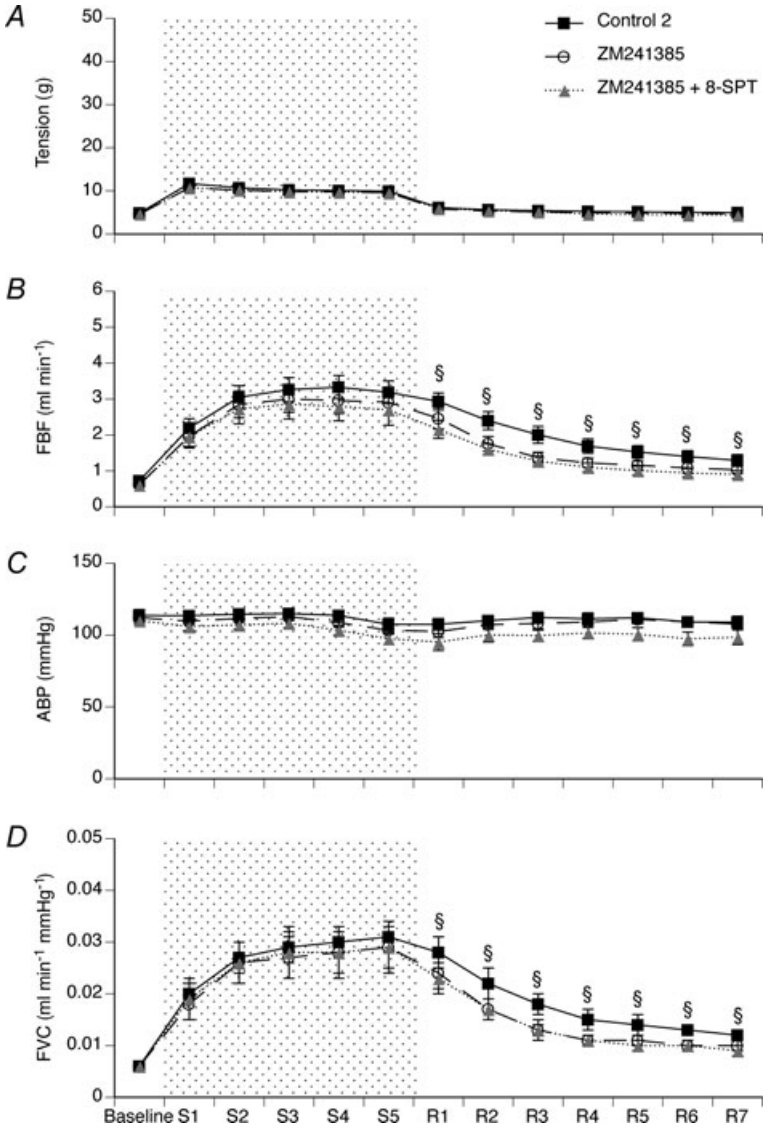

Figure 3. Involvement of adenosine in the response to isometric twitch contractions
A–D show mean (± S.E.M.) EDL tension, FBF, ABP and FVC for control stimulation 2 (■, continuous lines), stimulation after ZM241385 (○, dashed lines) and stimulation after ZM241385 + 8-SPT (▲, dotted lines) in the 1 min before (baseline), 5 min of (S1–5) and 7 min after (R1–7) sciatic nerve stimulation (4 Hz). §$P < 0.05$ control 2 S1–5 and/or R1–7, *vs* stimulation after ZM241385 and stimulation after ZM241385 + 8-SPT. There was no significant difference in tension, FBF, ABP and FVC at any time point between stimulation after ZM241385 and stimulation after ZM241385 + 8-SPT. $n = 10$.

Group 3. Isometric tetanic contraction: time controls

Stimulation of the sciatic nerve with a supra-maximal voltage at 40 Hz produced a tetanic contraction of the hindlimb muscles. As shown in the original traces, tension of the EDL peaked during the first minute of stimulation, then fatigued steadily over the remaining stimulation period (Fig. 4*C*). Concomitantly, in each experiment, mean FVC decreased transiently for the first 20 s of the stimulation period, coinciding with the peak tension developed by the EDL. Mean FVC then increased steadily, reaching a peak during the second minute of contraction where it was maintained. On cessation of muscle contraction, in each experiment, FVC showed a further transient increase for ∼20 s before steadily decreasing during the recovery period (Fig. 4*D*). ABP was maintained (Fig. 4*A*) and therefore FBF followed the pattern of FVC (Fig. 4*B*).

Considering the grouped data, there was no significant difference in the mean tension achieved by the EDL during time controls 1–4 (Fig. 5*A*). The transient increases and decreases in FVC and FBF at the beginning and end of contraction are not apparent in Fig. 5 because each value represents the mean of the data collected over a 1 min period (see Analysis in Methods). However, the increase in mean FVC and FBF evoked in time control 1 was significantly less during stimulation and greater during recovery than for all other contractions (Fig. 5*B* and *D*). There was no difference between the ABP values for time controls 1–4 (Fig. 5*C*). Despite there being no difference in mean tensions in time controls 1–4, the integrated tension of time control 4 was smaller than that of time control 2 (Fig. 7*B*). However, importantly, the ΔInt FVC for the entire hyperaemic response for time controls 2–4 were fully comparable (Fig. 7*D*). Therefore, as for the twitch contractions of Groups 1 and 2, the second tetanic contraction acted as the control for Contractions 3 and 4 in Group 4.

Group 4. Involvement of adenosine in the response to isometric tetanic contraction

The pattern of response during Contraction 2 was as described for Group 3 (see above; Fig. 6). Administration of ZM241385 had no effect on mean tension achieved by the EDL. Subsequent administration of 8-SPT also had no effect (Fig. 6*A*). In contrast to Group 3 in which the integrated tension of time control 4 was less than time control 2, there was no change in integrated tension between Contraction 2 and Contractions 3 and 4 in the presence of ZM241385 and 8-SPT (Fig. 7*F*).

FVC during the first 3 min of contraction (S1–S3) was unchanged by ZM241385. However, in the last 2 min of contraction (S4–S5) and during recovery, FVC was significantly lower after ZM241385 than before (Fig. 6*D*). Administration of 8-SPT had no further effect on FVC (Fig. 6*D*). As mean ABP was unchanged by either drug (Fig. 6*C*), mean FBF followed the pattern of FVC and was significantly lower in S4 and S5 and during recovery after ZM241385 (Fig. 6*B*). The effect of ZM241385 on the entire hyperaemic response (S1–R7) can be seen in Fig. 7*H*: ZM241385 significantly reduced the ΔInt FVC evoked by isometric tetanic contraction (∼25% reduction from the ΔInt FVC evoked by control 2), while 8-SPT had no further effect. These results are in contrast to Group 3 time controls in which there was a decrease in ΔInt tension with repetition, but the hyperaemic response (ΔInt FVC) was consistent.

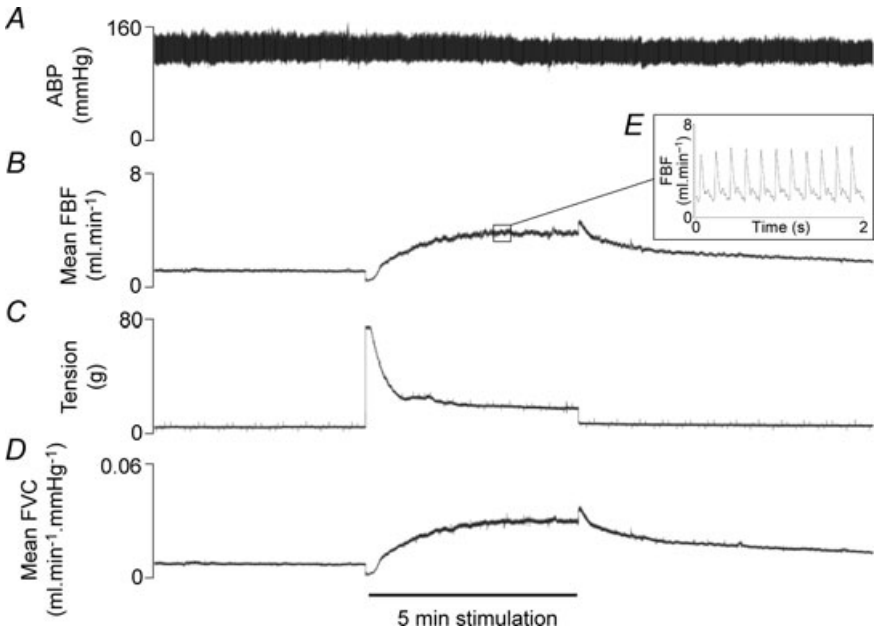

Figure 4. Isometric tetanic contraction
Representative original trace showing ABP (*A*), mean FBF (*B*), EDL tension (*C*) and mean FVC (*D*), before, during and after a 5 min isometric tetanic contraction. There is a fall in mean FBF in the first 20 s of sciatic nerve stimulation corresponding with the peak tension developed by the EDL and an increase in mean FBF for 10 s immediately after the cessation of muscle contraction. *E* shows a 2 s trace of FBF from the plateau phase of isometric tetanic contraction, illustrating pulsatile blood flow.

Arterial blood gases and pH. P_{aO_2}, P_{aCO_2} and arterial pH values in the minute before and in the fifth minute of muscle contraction are shown in Table 1. There was no change in P_{aO_2}, P_{aCO_2} or arterial pH during the period of contraction, or with time, in Groups 1 and 3. In Groups 2 and 4, there was no change in P_{aCO_2} or arterial pH during contraction, or with drug treatments. However in Group 2, P_{aO_2} in the minute before Contractions 3 and 4 was lower after ZM241385 and 8-SPT administration than at the beginning of the protocol before Contraction 2 and in the fifth minute of Contractions 3 and 4 P_{aO_2} was significantly higher than before contraction and comparable to that measured in Contraction 2. A similar trend was observed in Group 4.

Discussion

The present study was designed to compare the contribution of adenosine to exercise hyperaemia evoked by isometric twitch and tetanic contractions. The results indicate that adenosine makes a contribution to the exercise hyperaemia that accompanies both isometric twitch and tetanic contractions in hindlimb muscle of the rat. More specifically, it is likely that adenosine acting via A_{2A}-receptors, contributes ∼14% of the hyperaemia evoked by 5 min of isometric *twitch* contractions (at 4 Hz) and ∼25% of the hyperaemia evoked by isometric *tetanic* contraction (40 Hz). The discussion that follows considers these findings in more detail and the insight they provide into the mechanisms by which adenosine is released and acts to increase blood flow in response to contraction and by implication to systemic hypoxia.

Blood flow and twitch *vs* tetanic contraction

Firstly, any changes in arterial blood gases and pH that occurred during the protocols were very small and are unlikely to have affected the vascular responses in the

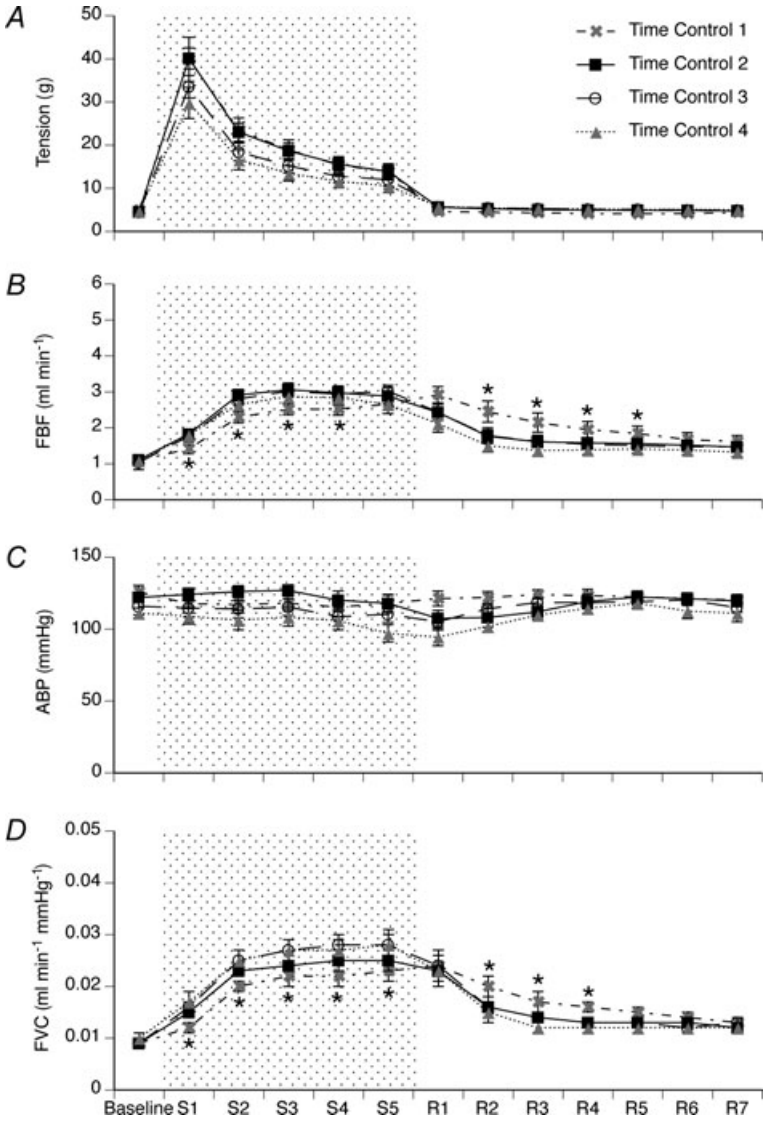

Figure 5. Isometric tetanic contraction: time controls
A–D show mean (± S.E.M.) EDL tension, FBF, ABP and FVC for time control stimulation 1 (✖, dashed and dotted lines), 2 (■, continuous lines), 3 (○, dashed lines) and 4 (▲, dotted lines) in the 1 min before (baseline), 5 min of (S1–5) and 7 min after (R1–7) sciatic nerve stimulation (40 Hz). $^*P < 0.05$ time control 1 S1–5 and/or R1–7, *vs* time control 2–4. There was no significant difference in tension, FBF, ABP and FVC at any time point for time controls 2–4. $n = 7$.

hindlimb. P_{aCO_2} tended to be slightly lower than normal throughout the protocols, particularly in Groups 1 and 3. However, changes in P_{aCO_2} have very little influence on the cardiovascular responses of the rat (Walker & Brizzee, 1990). Considering the pattern of muscle contraction evoked by the two different stimulation protocols used in the present study, stimulation of the sciatic nerve at 4 Hz produced rhythmic twitching of the hindlimb muscles, tension peaking in EDL during the first minute of stimulation and fatiguing to a level that was maintained for the remaining 4 min. This pattern of response is similar to that evoked by low-frequency stimulation in rat soleus and EDL muscles (2 Hz for 15 min; Lo *et al.* 2001), cat EDL and tibialis anterior muscles (3 Hz, 20 min; Poucher, 1996) and in gracilis muscle of the cat (1 Hz, 20 min; Poucher *et al.* 1990) and dog (4 Hz, 20 min; Ballard *et al.* 1987). By contrast, stimulation of the sciatic nerve at 40 Hz produced tetanic contraction of the hindlimb muscles, tension in EDL peaking during the first minute of stimulation and fatiguing substantially during the remaining 4 min. This is also very similar to the pattern of contraction evoked by stimulation of motor nerves at 40 Hz in hindlimb muscles of the rat (Smith *et al.* 2001) and cat (Daniels *et al.* 2000).

Interestingly, for Group 1 time controls the mean tension achieved by the EDL was greater during time control 1 than in the subsequent three periods of contraction. This phenomenon has been noted before in a similar study to our own. Thus, in the cat Poucher (1996) reported that peak tension in the EDL, evoked by sciatic nerve stimulation (3 Hz) fell significantly from the first to the second control contraction, but did not attempt to explain the mechanisms underlying it. Clearly, we can only speculate on the basis of our own findings. There are several potential explanations for this interesting observation including changes occurring in the sciatic nerve and at the neuromuscular junction after an initial period of electrical stimulation and differences in the removal of metabolites and restoration of cellular energy sources, from an initial bout of exercise. There may also be rearrangement of the balance between

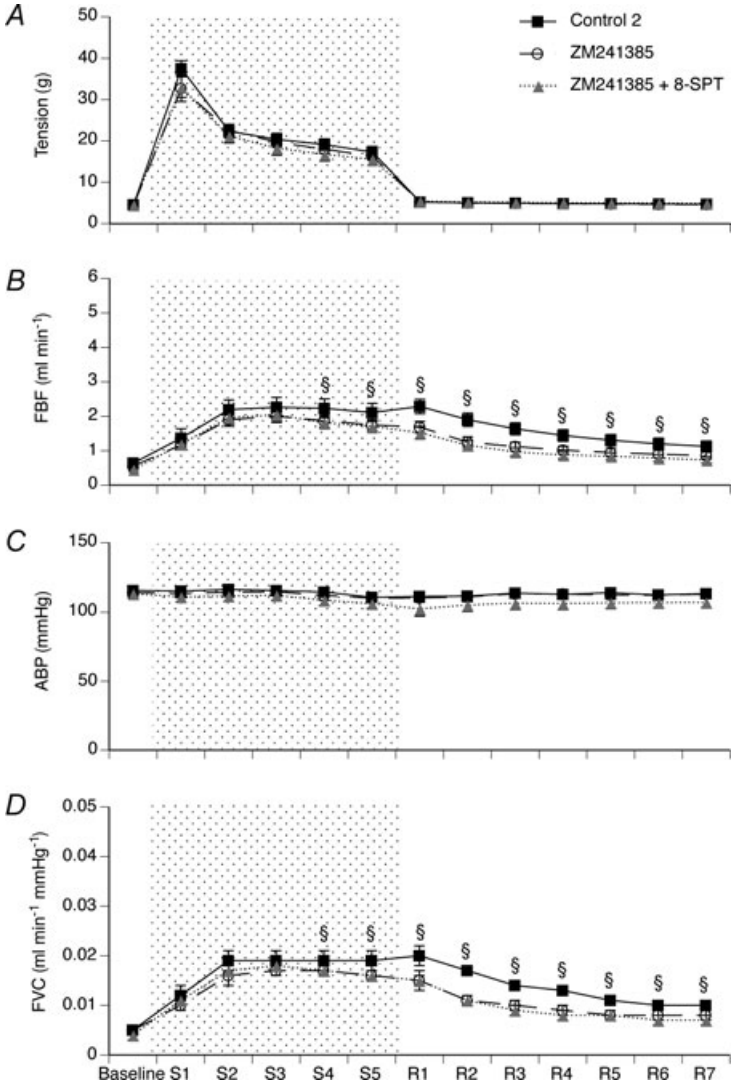

Figure 6. Involvement of adenosine in the response to isometric tetanic contraction
A–D show mean (± S.E.M.) EDL tension, FBF, ABP and FVC for control stimulation 2 (■, continuous lines), stimulation after ZM241385 (○, dashed lines) and stimulation after ZM241385 + 8-SPT (▲, dotted lines) in the 1 min before (baseline), 5 min of (S1–5) and 7 min after (R1–7) sciatic nerve stimulation (40 Hz). §$P < 0.05$ time control 2 S1–5 and/or R1–7, *vs* stimulation after ZM241385 and stimulation after ZM241385 + 8-SPT. There is no significant difference in tension, FBF, ABP and FVC at any time point between stimulation after ZM241385 and stimulation after ZM241385. $n = 10$.

the elastic-connective elements of the muscle and its contractile components in the first contraction, such that subsequent contractions are consistent (Orizio *et al.* 2003). The present observation could have significant implications for the design of experiments involving repeated bouts of exercise and therefore warrants further investigation. Importantly, the changes in tension evoked in time control 2–4 for Group 1 were fully comparable so in all groups Contraction 2 acted as the control for Contractions 3 and 4.

During isometric twitch contractions, FBF and FVC, representing gross blood flow and vascular conductance

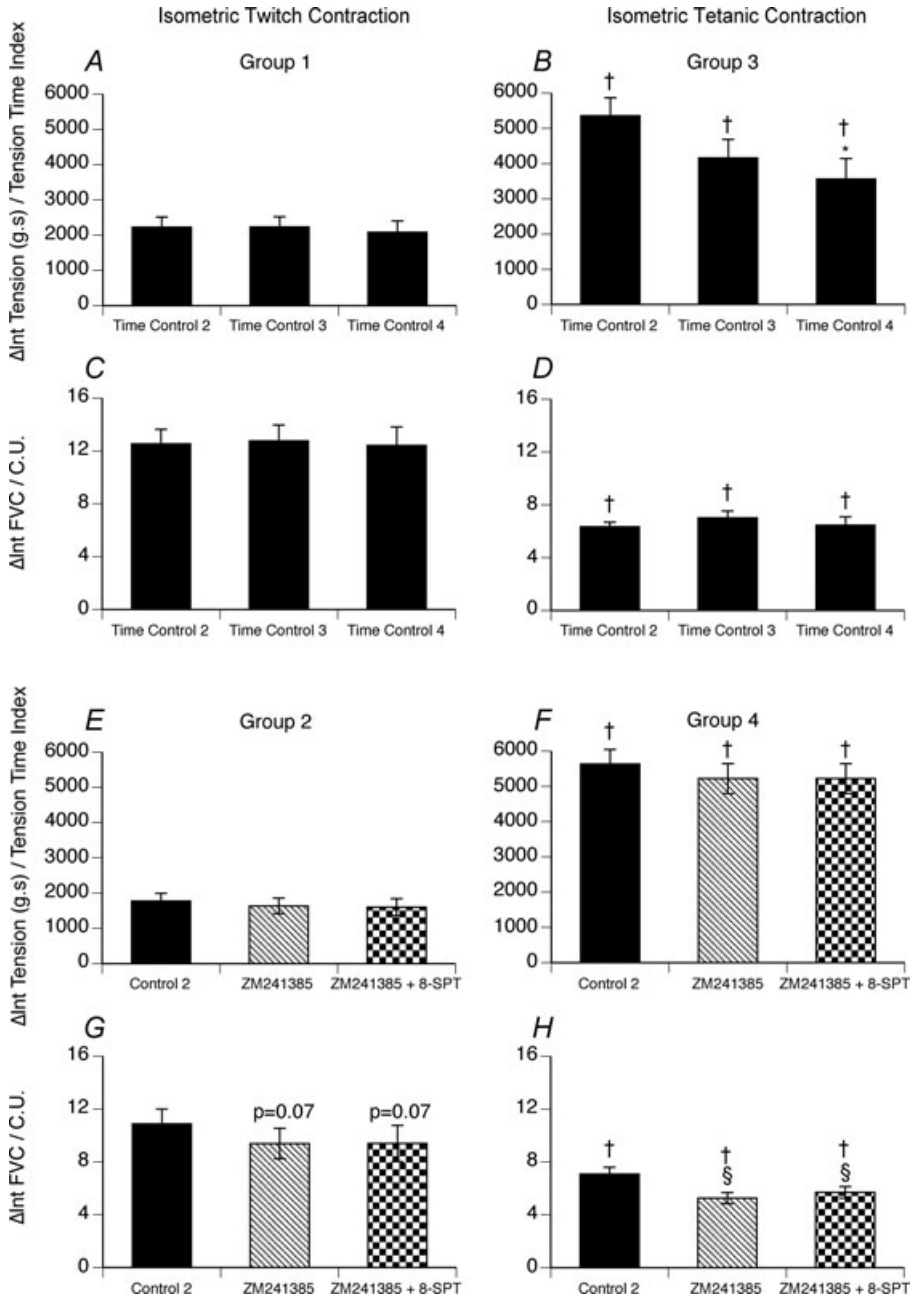

Figure 7. Comparison of change in integrated tension (ΔInt tension; tension time index (TTI)) and the change in integrated FVC (ΔInt FVC) for isometric twitch and tetanic contractions
A and *B* show mean (± S.E.M.) ΔInt tension/TTI (S1–5), while *C* and *D* show mean (± S.E.M.) ΔInt FVC (S1–5 + R1–7) for time control isometric twitch (Group 1) and tetanic (Group 3) contractions (*$P < 0.05$ *vs* time control 2). *E* and *F* show ΔInt tension/TTI (S1–5) while *G* and *H* show ΔInt FVC (S1–5 + R1–7) for 5 min isometric twitch (Group 2) and tetanic (Group 4) contraction before and after ZM241385 and after ZM241385 + 8-SPT. *P* values shown in *G* are *vs* control 2, §$P < 0.05$ *vs* control 2. There is no significant difference between ΔInt FVC for ZM241385 and ZM241385 + 8-SPT in *G* or *H*. †$P < 0.05$ isometric twitch *vs* tetanic for equivalent periods of contraction.

Table 1. Arterial blood gases and pH

	1 min before	5th min	1 min before	5th min	1 min before	5th min
Group 1						
	Time control 2		Time control 3		Time control 4	
P_{aO_2}	83.7 ± 3.44	85.8 ± 2.13	84.4 ± 1.39	88.8 ± 1.79	86.0 ± 3.22	86.7 ± 2.25
P_{aCO_2}	33.0 ± 1.51	32.6 ± 1.66	33.2 ± 1.94	29.00 ± 4.95	29.0 ± 3.35	33.6 ± 1.67
pH	7.48 ± 0.01	7.47 ± 0.01	7.47 ± 0.01	7.47 ± 0.01	7.48 ± 0.01	7.46 ± 0.01
Group 2						
	Control 2		ZM241385		ZM241385 + 8-SPT	
P_{aO_2}	85.6 ± 1.73	85.7 ± 2.00	78.7 ± 1.97†	82.8 ± 2.26*	80.7 ± 1.93†	86.2 ± 1.66*
P_{aCO_2}	41.3 ± 1.34	38.7 ± 0.84	41.4 ± 1.00	39.6 ± 1.03	37.5 ± 1.63	39.0 ± 1.55
pH	7.44 ± 0.01	7.44 ± 0.01	7.45 ± 0.01	7.43 ± 0.01	7.45 ± 0.02	7.45 ± 0.01
Group 3						
	Time control 2		Time control 3		Time control 4	
P_{aO_2}	86.9 ± 2.38	88.4 ± 2.07	85.2 ± 2.85	84.7 ± 2.72	95.9 ± 9.87	88.9 ± 2.41
P_{aCO_2}	31.0 ± 1.36	32.4 ± 1.62	25.9 ± 3.33	26.2 ± 1.38	25.9 ± 3.33	26.2 ± 1.38
pH	7.50 ± 0.01	7.48 ± 0.01	7.52 ± 0.01	7.51 ± 0.01	7.53 ± 0.01	7.52 ± 0.01
Group 4						
	Control 2		ZM241385		ZM241385 + 8-SPT	
P_{aO_2}	86.1 ± 3.74	86.4 ± 7.30	82.8 ± 5.14	85.6 ± 7.62	83.2 ± 5.14	85.6 ± 7.62
P_{aCO_2}	36.9 ± 3.57	35.2 ± 3.13	32.1 ± 2.88	35.2 ± 2.93	37.6 ± 3.42	37.7 ± 4.31
pH	7.43 ± 0.01	7.42 ± 0.01	7.45 ± 0.01	7.41 ± 0.01	7.42 ± 0.02	7.42 ± 0.01

The table shows P_{aO_2}, P_{aCO_2} and pH in the minute before and in the 5th minute of isometric twitch (Groups 1 and 2) and isometric tetanic (Groups 3 and 4) contraction performed under control conditions (time controls,Groups 1 and 3) and after ZM241385 and after ZM241385 + 8-SPT (Groups 2 and 4). *$P < 0.05$ *vs* 1 min before contraction; †$P < 0.05$ *vs* control 2.

of hindlimb muscles, increased ∼3-fold, both variables returning to baseline over the 7 min recovery period. This pattern of response is comparable to that reported in cat and dog (Ballard *et al.* 1987; Poucher *et al.* 1990; Poucher, 1996). Our use of a transonic flow probe for measuring blood flow enabled us to show that FBF and FVC also increased *during* isometric tetanic contraction, indicating substantial vasodilatation in the hindlimb muscles. This is an important finding given the accepted view that tetanic contraction impairs muscle vasodilatation (see introduction) and given that most of the established methods available for recording blood flow in humans and animals (e.g. venous occlusion plethysmography, venous drop counters, electro-magnetic flow transducers), cannot be used effectively *during* sustained muscle contraction. Indeed, even recent recordings made with ultrasonic flow probes which can give continuous recordings, also showed no increase in human forearm blood flow during sustained isometric contractions of up to 70% of maximum (Kagaya & Homma, 1997).

In the present study, there was certainly a transient decrease in FBF during the first 20 s of tetanic contraction when the force achieved by EDL was at its maximum, but thereafter, pulsatile blood flow was recorded throughout contraction (Fig. 4*E*) and FBF and FVC were increased by ∼2.5-fold from baseline. A transient further increase in FBF occurred for 20 s immediately after contraction (Fig. 4*B*), but this barely distorted the gradual recovery of FBF and FVC, and both variables returned to baseline during the 7 min recovery period, suggesting little mechanical impairment of blood flow. It seems reasonable to assume that the mechanical responses of the EDL are representative of rat hindlimb muscles in general, given they have similar metabolic profiles (see Armstrong & Laughlin, 1983). Thus, the present results indicate that in the rat hindlimb, isometric tetanic contraction causes relatively little impairment of the associated hyperaemia and increase in FVC, at least in part because the muscles show substantial fatigue during the period of contraction. Any mismatch between D_{O_2} and $\dot{V}_{O_2}$ during contraction is apparently made good by the hyperaemia, leaving little accumulation of vasodilator metabolites to cause further vasodilatation when contraction ceases.

In previous studies, tension time index (TTI, the equivalent of ΔInt tension; see Analysis) was used to compare force produced by muscle during dynamic and static exercise (Daniels *et al.* 2000). In the present study, the TTI was at least 2-fold greater for isometric tetanic contraction than for twitch contraction and yet, the total exercise hyperaemia (S1–5 + R1–7) was 2-fold greater

for the twitch contractions than for tetanic contraction (Fig. 7*A* and *B*). This finding might seem to contrast with the accepted view that skeletal muscle blood flow increases in proportion to the metabolic demands of the muscle (Clifford & Hellsten, 2004; Clifford, 2007) and that blood flow increases linearly with muscle work load (Andersen & Saltin, 1985). However, in experiments on humans, Newman *et al.* (1995) showed that when muscle shortens and performs work, or twitches isometrically (cf Groups 1 and 2 of the present study), it fatigues more rapidly than muscles that exert sustained contraction for an equivalent length of time under isometric conditions (cf Groups 3 and 4 of the present study). As a consequence, the isometric twitch contractions had an energy requirement (measured as ATP turnover), that was almost 2-fold greater than the sustained isometric contraction (Newman *et al.* 1995). Further, in a study on human subjects in which the TTIs for dynamic and static exercise were matched, the blood flow response to dynamic exercise was greater than for static exercise (Laaksonen *et al.* 2003). If these findings can be extrapolated to rat hindlimb muscles, then it is reasonable to propose that the 5 min period of isometric twitch contractions had a higher metabolic cost and therefore produced more vasodilator metabolites than tetanic contraction. Thus, the magnitude of exercise hyperaemia was greater for isometric twitch contractions than for tetanic contraction of the same duration.

Contributions of adenosine

The time control experiments of Group 1 and 3 showed that generally the first period of stimulation (time control 1) produced contractions and cardiovascular changes that were greater than those evoked by subsequent stimulations (time control 2–4). This phenomenon was also noted in the cat when EDL and tibialis anterior muscle group was stimulated at 3 Hz for 20 min (Poucher, 1996). Importantly, time control stimulations 2–4 produced contractions and cardiovascular changes that were fully comparable. Thus, it was reasonable to use the second period of stimulation as the control when testing whether the adenosine receptor antagonists affected the evoked responses. In Groups 2 and 4, ZM241385 was administered after Contraction 2. ZM241385 is a highly selective adenosine A_{2A}-receptor antagonist with a high affinity in the nanomolar range at the A_{2A}-receptor subtype and has ∼100-fold selectivity for A_{2A}- over A_{2B}-receptors (Poucher *et al.* 1995). Moreover, at the dose used in the present study, ZM241385 reversed the vasodilator effect of CGS 21680, a highly selective A_{2A}-receptor agonist, and blocked ∼50% of the vasodilator effect of adenosine (which is ∼50% mediated by A_1- and 50% mediated by A_{2A}-receptors). This study therefore demonstrated that A_{2A}-receptors are responsible for the A_2-mediated component of rat hindlimb dilatation and that ZM241385 used at 0.05 mg kg^{-1} is an effective A_{2A}-receptor antagonist (Bryan & Marshall, 1999).

As indicated in the introduction, Poucher (1996) showed in the cat, that ZM241385 reduced by ∼27% the increase in hindlimb vascular conductance evoked *during* twitch contractions, with no effect on the tension generated. Further, 8-SPT, which is non-selective between the adenosine receptor subtypes, had comparable effects indicating the likelihood that the only adenosine receptors involved were the A_{2A}-receptors. In the present study, ZM241385 similarly had no effect on the tension generated by the EDL during twitch contractions, but it also had no effect on the increase in FBF or FVC that occurred *during* the 5 min period of isometric twitch contractions and reduced the increase in FBF and FVC of the post-contraction recovery period by ∼28%. This meant that overall, ZM241385 tended to reduce the entire hyperaemic response (S1–5 + R1–7; $P = 0.07$) by ∼14%. Nevertheless, since 8-SPT had no further effects on the tension or hyperaemic responses, this again suggested the likelihood that only the A_{2A}-receptors were involved.

It seems likely that the apparent disparity between the present study and that of Poucher (1996) in the timing and relative contribution of adenosine acting on A_{2A}-receptors to the hyperaemia of twitch contractions, can be attributed to the difference in the duration of exercise; 5 min *vs* 20 min, respectively. In rat EDL muscle contracting at 2 Hz for 15 min, interstitial adenosine concentration as measured by microdialysis, was not significantly elevated in the first 5 min of contractions, but was substantially increased in the next 10 min of contractions and the first 10 min of recovery (Lo *et al.* 2001). Similarly, during a 20 min period of twitch contractions of dog gracilis muscle, adenosine concentrations in venous efflux rose slowly to reach a maximum at 10 min that was maintained until the contractions ceased, returning to baseline in the first 5 min of recovery (Ballard *et al.* 1987).

Turning to the isometric tetanic contractions, although in the time control experiments of Group 3, the TTI was smaller in Contraction 3 than 2, this was not the case in Group 4 when ZM241385 was given after Contraction 2. This suggests that A_{2A}-receptor blockade ameliorated fatigue. Concomitantly, A_{2A}-receptor blockade had no effect on the increase in FBF and FVC that occurred during the first 3 min of isometric tetanic contraction, but it reduced the increase in FBF and FVC during the fourth and fifth minute of contraction and during recovery, this amounting to ∼25% reduction in the entire exercise hyperaemia.

Given that the increase in FBF evoked by tetanic contraction was reduced by ZM241385, the lessening of fatigue is not obviously attributable to an improved supply of O_2 (in fact D_{O_2} during contraction was actually decreased after ZM241385, and $\dot{V}_{O_2}$ was maintained by an increased O_2 extraction ratio; data not shown)

or nutrients, or to increased wash-out of factors that contribute to fatigue. Rather, the present results suggest that stimulation of A_{2A}-receptors contributes to fatigue. One possibility is that the action of adenosine on A_{2A}-receptors on the skeletal muscle fibres that are coupled to ATP-sensitive K^+ (K_{ATP}) channels facilitates the efflux of K^+, which has been implicated in fatigue and in recovery from fatigue (see Paterson *et al.* 1992; Renaud *et al.* 1996). The very fact that TTI was better maintained during tetanic contraction after ZM241385, is consistent with the idea that the concomitant attenuation of the exercise hyperaemia was due to blockade of A_{2A}-receptors on the blood vessel walls. The alternative possibility that it reflects a reduction in the dilator action of K^+ released from muscle fibres via A_{2A}-coupled K_{ATP} channels is less attractive given that inhibition of K_{ATP} channels had little effect on functional hyperaemia (Paterson *et al.* 1992). Since 8-SPT, which is non-selective between adenosine receptor subtypes, had no further effect on TTI or the exercise hyperaemia, we can conclude that only the A_{2A} subtype of adenosine receptors is implicated in the hyperaemic response to tetanic contraction. As far as we are aware, this is a novel finding.

To check the efficacy of 8-SPT as a general adenosine receptor antagonist, in a further group of experiments in which isometric tetanic contractions were performed as in Groups 3 and 4, ($n = 4$) 8-SPT and ZM241385 were administered after the second and third contractions, respectively: 8-SPT reduced the ΔInt FVC from 9.29 ± 0.80 CU to 7.46 ± 0.66 CU and the subsequent administration of ZM241385 had no further effect (7.99 ± 1.18 CU; data not shown). Given that the effects of ZM241385 (which blocks A_{2A}-receptors) and 8-SPT (which blocks all adenosine receptors) administered before the 3rd contraction were fully comparable, this provides further evidence that only the adenosine A_{2A}-receptors are involved in the adenosine component of the skeletal muscle vasodilator response to exercise.

We argued above, from published evidence, that isometric tetanic contraction has higher TTI but a lower metabolic cost and is associated with less ATP turnover than isometric twitch contractions when performed for the same period of time, and that this explains why exercise hyperaemia is larger for twitch than tetanic contractions. We also argued from our own results that, at least in the rat, sustained tetanic contraction causes little impairment of blood flow through the hindlimb muscles. However, the effects of ZM243185 indicate that adenosine acting most likely via A_{2A}-receptors makes a much larger contribution in percentage terms to the exercise hyperaemia of tetanic contraction than twitch contractions. Further, adenosine apparently contributed earlier to the hyperaemia associated with tetanic contraction than to that associated with twitch contractions (see above). Taken together, these findings suggest that the metabolic consequences of tetanic contraction more readily lead to adenosine accumulation than twitch contractions. Alternatively, although gross hindlimb blood flow was minimally impaired by tetanic contraction, the distribution of blood flow through the capillary network may have been more heterogenous during tetanic than twitch contraction, such that regions of the muscle became relatively hypoxic. This would be consistent with the accepted view that adenosine accumulates when O_2 supply does not meet O_2 demand (Berne *et al.* 1983).

It is of course possible that the many other substances, including potassium, nitric oxide, prostanoids, EDHF and ATP, that have been proposed as potential mediators of exercise hyperaemia make different contributions to the exercise hyperaemia of twitch and tetanic contractions. For example, during isometric twitch contraction, the smaller role for adenosine might be due to an enhanced role for one or more of the other substances. Moreover, it has been suggested that 'redundancy' exists between the factors involved in exercise hyperaemia and that when the action of one vasodilator is blocked others increase their contribution in compensation and so maintain the increase in blood flow (for further discussion see Clifford & Hellsten, 2004). If this occurs to any extent when the effect of adenosine is removed, then the exact contribution of adenosine to isometric twitch or tetanic contraction is impossible to judge from the effects of adenosine receptor blockade. All we can say is that adenosine makes a functional contribution to the hyperaemia of both twitch and tetanic contractions.

The origin of adenosine

The origin of the adenosine that mediates exercise hyperaemia has been discussed in a number of reviews (Rådegran & Hellsten, 2000; Clifford & Hellsten, 2004; Marshall, 2007). There are several potential sources including the skeletal muscle fibres, vascular endothelial, nerve and red blood cells. It seems most likely that adenosine is formed extracellularly by the action of ecto 5′-nucleotidase (Hellsten & Frandsen, 1997), the activity of which is increased during muscle contraction (Hellsten, 1999), from adenine nucleotides (AMP, ADP or ATP) that are predominantly released from skeletal muscle fibres (Hellsten *et al.* 1998; Mo & Ballard, 2001; Lo *et al.* 2001). It should be noted that during systemic hypoxia there is no change in the interstitial concentrations of adenosine or the adenine nucleotides but there is a significant increase in venous adenosine suggesting formation by the vascular tissue (Mo & Ballard, 2001). Adenosine generated from ATP released from sympathetic or other nerve fibres is unlikely to significantly raise the concentration measured in the interstitial space. Moreover, adenine nucleotides or adenosine originating from red blood cells or

endothelium during contraction seem unlikely to reach the interstitium in physiologically significant amounts, as the endothelium is a very effective metabolic barrier (see Lo *et al.* 2001; Marshall, 2007). If adenosine did originate from the endothelium during muscle contraction, then we would expect it to produce exercise hyperaemia via A_1- not A_{2A}-receptors (see Bryan & Marshall, 1999), particularly as A_1-receptors have a higher affinity for adenosine than A_{2A}-receptors (Olssson & Pearson, 1990). Thus, we propose that the origin and site of action of the adenosine that contributes to exercise hyperaemia and to the dilatation of systemic hypoxia are functionally distinct; the adenosine that contributes to exercise hyperaemia originates mainly from muscle fibres and acts on extraluminal A_{2A}-receptors, whereas the adenosine that contributes to the dilatation of systemic hypoxia is likely to originate mainly from endothelial cells and acts on endothelial A_1-receptors (Bryan & Marshall, 1999; Ray *et al.* 2002; Marshall, 2007).

In summary, we propose that adenosine that originates from muscle fibres, accumulates in the interstitium during both twitch and tetanic muscle contraction and acts directly on A_{2A}-receptors on the vascular smooth muscle to make a contribution to exercise hyperaemia that is greater for tetanic than twitch contractions. Given A_{2A}-receptors have already been implicated as the adenosine receptor involved in the hyperaemic response to twitch contractions in the cat (Poucher, 1996), there is no reason to suggest a species difference between rats and cats (see introduction). Despite the fact that Martin *et al.* (2006*a*) divided human subjects into 'responders' and 'non-responders' based on the presence or absence of vasodilatation to intra-arterial infusion of adenosine in the forearm, they demonstrated that the non-selective adenosine receptor antagonist aminophylline significantly inhibited exercise hyperaemia by ∼10% at high workloads in both groups (Martin *et al.* 2006*b*), leading them to suggest that adenosine plays a limited role in exercise hyperaemia in humans. Moreover, Rådegran & Calbet (2001) demonstrated in humans that endogenous adenosine controls at least ∼20% of the hyperaemic response to submaximal knee exercise. Many studies have also demonstrated significant increases in interstitial adenosine concentrations during exercise in humans (e.g. Costa *et al.* 2000; Hellsten *et al.* 1998; Langberg *et al.* 2002; Lott *et al.* 2001). Given this evidence, we now propose that A_{2A}-receptors generally play an important functional role in mediating the adenosine component of exercise hyperaemia, including in humans. Testing this proposal clearly depends on the availability of selective adenosine receptor antagonists that can be used in humans, but there is already immunohistochemical evidence that A_1-, A_{2A}- and A_{2B}-receptors are present on vascular smooth muscle and endothelium of skeletal muscle in humans (Lynge & Hellsten, 2000).

References

Andersen P & Saltin B (1985). Maximal perfusion of skeletal muscle in man. *J Physiol* **366**, 233–249.

Armstrong RB & Laughlin MH (1983). Blood flows within and among rat muscles as a function of time during high speed treadmill exercise. *J Physiol* **344**, 189–208.

Ballard HJ, Cotterrell D & Karim F (1987). Appearance of adenosine in venous blood from the contracting gracilis muscle and its role in vasodilatation in the dog. *J Physiol* **387**, 401–413.

Barcroft H & Millen JLE (1939). The blood flow through muscle during sustained contraction. *J Physiol* **97**, 17–31.

Belloni FL, Phair RD & Sparks HV (1979). The role of adenosine in prolonged vasodilation following flow-restricted exercise of canine skeletal muscle. *Circ Res* **44**, 759–766.

Berne RM, Knabb RM, Ely SW & Rubio R (1983). Adenosine in the local regulation of blood flow: a brief overview. *Fed Proc* **42**, 3136–3142.

Bockman EL, Berne RM & Rubio R (1975). Release of adenosine and lack of release of ATP from contracting skeletal muscle. *Pflugers Arch* **355**, 229–241.

Bockman EL, Berne RM & Rubio R (1976). Adenosine and active hyperemia in dog skeletal muscle. *Am J Physiol* **230**, 1531–1537.

Bonde-Petersen F, Mork A & Nielsen E (1975). Local muscle blood flow and sustained contractions of human arm and back muscles. *Eur J Appl Physiol* **34**, 43–50.

Bryan PT & Marshall JM (1999). Adenosine receptor subtypes and vasodilatation in rat skeletal muscle during systemic hypoxia: a role for A_1 receptors. *J Physiol* **514**, 151–162.

Clifford PS (2007). Skeletal muscle vasodilatation at the onset of exercise. *J Physiol* **583**, 825–833.

Clifford PS & Hellsten Y (2004). Vadodilatory mechanisms in contracting skeletal muscle. *J Appl Physiol* **97**, 393–403.

Coney AM & Marshall JM (2007). Contribution of α_2-adrenoceptors and Y_1 neuropeptide Y receptors to the blunting of sympathetic vasoconstriction induced by systemic hypoxia in the rat. *J Physiol* **582**, 1349–1359.

Costa F, Heusinkveld J, Ballog R, Davis S & Biaggionic I (2000). Estimation of skeletal muscle interstitial adenosine during forearm dynamic exercise in humans. *Hypertension* **35**, 1124–1128.

Daniels JM, Stebbins CL & Longhurst JC (2000). Hemodynamic responses to static and dynamic muscle contractions at equivalent workloads. *Am J Physiol Regul Integr Comp Physiol* **279**, R1849–R1855.

Dobson JG, Rubio R & Berne RM (1971). Role of adenine nucleotides, adenosine, and inorganic phosphate in the regulation of skeletal muscle blood flow. *Circ Res* **29**, 375–384.

Evoniuk G, von Borstel RW & Wurtman RJ (1987). Antagonism of the cardiovascular effects of adenosine by caffeine or 8-(p-sulphophenyl)-theophylline. *J Pharmacol Exp Ther* **240**, 428–432.

Fuchs BD, Gorman MW & Sparks HV (1986). Adenosine release into venous plasma during free flow exercise. *Proc Soc Exp Bio Med* **181**, 364–370.

Hellsten Y (1999). The effect of muscle contraction on the regulation of adenosine formation in rat skeletal muscle cells. *J Physiol* **518**, 761–768.

Hellsten Y & Frandsen U (1997). Adenosine formation in contracting primary rat skeletal muscle cells and endothelial cells in culture. *J Physiol* **504**, 695–704.

Hellsten Y, Maclean D, Rådegran G, Saltin B & Bangsbo J (1998). Adenosine concentrations in the interstitium of resting and contracting human skeletal muscle. *Circulation* **98**, 6–8.

Kagaya A & Homma S (1997). Brachial arterial blood flow during static handgrip exercise of short duration at varying intensities studied by a Doppler ultrasound method. *Acta Physiol Scand* **160**, 257–265.

Laaksonen MS, Kalliokoski KK, Kyrolainen H, Kemppainen J, Teras M, Sipila H, Nuutila P & Knuuti J (2003). Skeletal muscle blood flow and flow heterogeneity during dynamic and isometric exercise in humans. *Am J Physiol Heart Circ Physiol* **284**, H979–H986.

Langberg H, Bjorn C, Boushel R, Hellsten Y & Kjær (2002). Exercise-induced increase in interstitial bradykinin and adenosine concentrations in skeletal muscle and peritendinous tissue in humans. *J Physiol* **542**, 977–983.

Leuenberger UA, Gray K & Herr MD (1999). Adenosine contributes to hypoxia-induced forearm vasodilation in humans. *J Appl Physiol* **87**, 2218–2224.

Lo SM, Mo FM & Ballard HJ (2001). Interstitial adenosine concentration in rat red or white skeletal muscle during systemic hypoxia or contraction. *Exp Physiol* **86**, 593–598.

Lott ME, Hogeman CS, Vickery L, Kunselman AR, Sinoway LI & MacLean DA (2001). Effects of dynamic exercise on mean blood velocity and muscle interstitial metabolite responses in humans. *Am J Physiol Heart Circ Physiol* **281**, H1734–H1741.

Lynge J & Hellsten Y (2000). Distribution of adenosine A1, A2A and A2B receptors in human skeletal muscle. *Acta Physiol Scand* **169**, 283–290.

Marshall JM (2000). Adenosine and muscle vasodilatation in acute systemic hypoxia. *Acta Physiol Scand* **168**, 561–573.

Marshall JM (2007). The roles of adenosine and related substances in exercise hyperaemia. *J Physiol* **583**, 835–845.

Martin EA, Nicholson WT, Eisenach JH, Charkoudian N & Joyner MJ (2006*a*). Bimodal distribution of vasodilator responsiveness to adenosine due to difference in nitric oxide contribution: implications for exercise hyperaemia. *J Appl Physiol* **101**, 492–499.

Martin EA, Nicholson WT, Eisenach JH, Charkoudian N & Joyner MJ (2006*b*). Influences of adenosine receptor antagonism on vasodilator responses to adenosine and exercise in adenosine responders and nonresponders. *J Appl Physiol* **101**, 1678–1684.

Mo FM & Ballard HJ (1997). Intracellular lactate controls adenosine output from dog gracilis muscle during moderate systemic hypoxia. *Am J Physiol Heart Circ Physiol* **272**, H318–H324.

Mo FM & Ballard HJ (2001). The effect of systemic hypoxia on interstitial and blood adenosine, AMP, ADP and ATP in dog skeletal muscle. *J Physiol* **536**, 593–603.

Newman DJ, Jones DA, Turner DL & McIntyre D (1995). The metabolic cost of different types of contractile activity of the human adductor pollicis muscle. *J Physiol* **488**, 815–819.

Olsson, RA & Pearson JD (1990). Cardiovascular purinoceptors. *Physiol Rev* **70**, 761–845.

Orizio C, Gobbo M, Veicsteinas A, Baratta RV, Zhou BH & Solomonow M (2003). Transients of the force and surface mechanomyogram during cat gastrocnemius tetanic stimulation. *Eur J Appl Physiol* **88**, 691–606.

Paterson DJ, Vejlstrup N, Willford D & Hogan MC (1992). Effect of a sulphonylurea on dog skeletal muscle performance during fatiguing work. *Acta Physiol Scand* **144**, 399–400.

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

Poucher SM, Keddie JR, Singh P, Stoggall SM, Caulkett PWR, Jones G & Collis MG (1995). The *in vitro* pharmacology of ZM241385, a potent, non-xanthine, A2a selective adenosine receptor antagonist. *Br J Pharmacol* **115**, 1096–1102.

Poucher SM, Nowell CG & Collis MG (1990). The role of adenosine in exercise hyperaemia of the gracilis muscle in anaesthetized cats. *J Physiol* **427**, 19–29.

Rådegran G & Calbet JAL (2001). Role of adenosine in exercise-induced human skeletal muscle vasodilatation. *Acta Physiol Scand* **171**, 177–185.

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

Ray CJ, Abbas MR, Coney AM & Marshall JM (2002). Interactions of adenosine, prostaglandins and nitric oxide in hypoxia-induced vasodilatation: *in vivo* and *in vitro* studies. *J Physiol* **544**, 195–209.

Ray CJ & Marshall JM (2005). Measurement of nitric oxide release evoked by systemic hypoxia and adenosine from rat skeletal muscle in vivo. *J Physiol* **568**, 967–978.

Ray CJ & Marshall JM (2009). Nitric oxide (NO) does not contribute to the generation or action of adenosine during exercise hyperaemia in rat hindlimb. *J Physiol* **587**, 1579–1591.

Renaud J-M, Gramolini A, Light P & Comtois A (1996). Modulation of muscle contractility during fatigue and recovery by ATP-sensitive potassium channel. *Acta Physiol Scand* **156**, 203–212.

Sadamoto T, Bonde-Petersen F & Suzuki Y (1983). Skeletal muscle tension, flow, pressure and EMG during sustained isometric contractions in hymans. *Eur J Appl PhysiolOccup Physiol* **51**, 395–408.

Smith SA, Mitchell JH & Garry MG (2001). Electrically induced static exercise elicits a pressor response in the decerebrate rat. *J Physiol* **537**, 961–970.

Walker BR & Brizzee BL (1990). Cardiovascular responses to hypoxia and hypercapnia in barodenervated rats. *J Appl Physiol* **68**, 678–686.

Acknowledgements

This work was supported by the British Heart Foundation.