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INFLUENCE OF STIMULATION PARAMETERS ON THE RELEASE OF ADENOSINE, LACTATE AND CO_2 FROM CONTRACTING DOG GRACILIS MUSCLE

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SUMMARY

1. The addition of adenosine, CO_2 and lactate to the venous blood draining an isolated constant-flow perfused gracilis muscle was studied in anaesthetized and artificially ventilated dogs during twitch and tetanic contractions.

2. Venous adenosine concentration increased from 154 ± 33 nM (mean ± S.E.M.) to 279 ± 121 or 280 ± 125 nM after 10 min of 1·5 or 3 Hz twitch contractions and to 240 ± 120 or 276 ± 139 nM after 10 min of 1 or 5 s tetani occurring at 0·1 Hz. Twitch contractions at 0·1 or 0·5 Hz for 10 min did not significantly elevate venous adenosine.

3. Venous lactate concentration was significantly increased after 10 min of 1·5 or 3 Hz twitches or 5 s tetani at 0·1 Hz. There was a good correlation ($r = 0{\cdot}70$; $P < 0{\cdot}001$) between venous adenosine and lactate concentrations.

4. Venous partial pressure of CO_2 (P_{CO_2}) was significantly elevated after 10 min of 1·5 or 3 Hz twitch contractions or 1 or 5 s tetani at 0·1 Hz. There was also a good correlation ($r = 0{\cdot}58$; $P < 0{\cdot}001$) between venous adenosine concentration and P_{CO_2}.

5. Venous partial pressure of O_2 (P_{O_2}) decreased during all contractions except those at 0·1 Hz, but the oxygen cost per unit of tension × time was similar during every pattern of stimulation, and the percentage of the total energy production achieved by anaerobic means during muscle contractions did not exceed that at rest, indicating that there had been no limitation to the oxygen supply. Venous P_{O_2} was poorly correlated with venous adenosine concentration ($r = 0{\cdot}28$), but quite well correlated with venous lactate concentration ($r = 0{\cdot}53$; $P < 0{\cdot}001$). If the indirect influence of P_{O_2} on venous adenosine concentration via an increase in lactate concentration was eliminated by partial correlation, then the coefficient for the relationship between venous adenosine concentration and venous P_{O_2} became 0·15.

6. There was a significant correlation between the venous adenosine concentration and the venous pH ($r = 0{\cdot}53$; $P < 0{\cdot}001$). If the influence of oxygenation on venous adenosine and pH was eliminated by partial correlation, the coefficient for the relationship between venous adenosine and pH increased to 0·95.

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7. These data support a role for pH in the control of adenosine release from skeletal muscle, and confirm that the amounts of lactate released during muscle contractions were large enough to have caused the adenosine release. Adenosine output did not appear to be directly related to muscle oxygenation, but an indirect association whereby oxygen lack stimulated lactate production, and lactate in turn stimulated adenosine production remains possible.

INTRODUCTION

Adenosine causes around half of the steady-state vasodilatation that accompanies contractions in oxidative skeletal muscles (Kille & Klabunde, 1984; Ballard, Cotterrell & Karim, 1987; Schwartz & McKenzie, 1990), but the mechanism which links the adenosine release to the muscle contractions remains uncertain. It was originally proposed that when the energy consumption increased, there was insufficient oxygen to maintain the ATP level aerobically, causing net breakdown of ATP to adenosine (Imai, Riley & Berne, 1964; Dobson, Rubio & Berne, 1971). This concept was initially well supported, since muscles did release more adenosine during contractions if their blood (and hence oxygen) supply was limited (Belloni, Phair & Sparks, 1979; Phair & Sparks, 1979). However, later studies found that elevation of the blood flow could not abolish the adenosine release (Ballard *et al.* 1989), the release lagged behind the change in oxygen tension by about 4 min (Ballard *et al.* 1987), and there was no correlation between the adenosine production and either the oxygen consumption (Bockman & McKenzie, 1983) or the venous partial pressure of O_2 (P_{O_2}) (Ballard *et al.* 1989). Therefore, the decrease in muscle pH which occurs during flow restriction was examined to determine whether it might influence the adenosine release.

Sodium acetate infusion (which would reduce intracellular pH) increased the adenosine content of dog gracilis muscle (Steffen, McKenzie, Bockman & Haddy, 1983). Lactic acid infusion also stimulated adenosine release (Ballard, 1991), and the adenosine output was negatively correlated with venous pH; points obtained during acid infusion and during muscle stimulation fell on a single line, suggesting that endogenously produced lactic acid might stimulate adenosine release during muscle contractions. Neither the P_{O_2}, the partial pressure of CO_2 (P_{CO_2}) nor the pH alone can be shown to control muscle blood flow, but a constant relationship exists between the steady-state blood flow during exercise and the venous P_{O_2} and P_{CO_2} together (Mohrman & Regal, 1988); this is consistent with the possibility that adenosine release is controlled by the overall pH change, since oxygen deficiency could depress the pH by stimulating lactic acid production, but with adequate oxygenation, pH would still decrease due to CO_2 production and the liberation of hydrogen ions during fatty acid oxidation.

The primary purpose of the present study was to determine whether the rise in lactate concentration during muscle contractions was large enough to have caused the increase in adenosine concentration. We also wished to examine the role of hypoxia in the release of both the lactate and the adenosine, since adenosine output might be indirectly dependent on muscle oxygenation if oxygen insufficiency caused lactate formation, and the increased lactate in turn stimulated adenosine formation. Finally, we wished to determine the relationship between muscle P_{CO_2} and adenosine

output, since CO_2 influences muscle pH, and an elevated P_{CO_2} has been shown to stimulate adenosine release from cardiac muscle (Mustafa & Mansour, 1984). A range of stimulation parameters were applied to an isolated gracilis muscle, in order to vary the amounts of adenosine, lactate and CO_2 added to the venous blood, and correlation analysis of these variables was performed. The experiments were performed using a constant-flow rate of 200% of resting flow, since the adenosine release during muscle contractions at this flow rate does not differ from that under free-flow conditions (Ballard *et al.* 1985).

METHODS

Twenty-three mongrel dogs, weighing 10·5–24·5 kg, were anaesthetized by intravenous injection of 30 mg kg^{-1} sodium pentobarbitone (Sagatal, RMB Animal Health Ltd, Dagenham, UK). Anaesthesia was maintained throughout the experiment by further intravenous infusions of sodium pentobarbitone when required (average dose 6·5 mg kg^{-1} h^{-1}). The neck was opened in the mid-line, and a tracheal cannula inserted. Positive pressure ventilation at a stroke rate of 18 min^{-1} and an initial stroke volume of 17 ml kg^{-1} was begun. Arterial P_{O_2} was determined using a Strathkelvin Instruments model 781 oxygen meter (S.I., Glasgow, UK), and maintained at 118·3 ± 3·1 mmHg (mean ± S.E.M., $n = 23$) by supplementing the inspired air with oxygen. Arterial P_{CO_2} was determined using an Orion model 950200 carbon dioxide electrode attached to an Orion model EA920 ion analyser (Orion Research, Boston, MA, USA), and maintained at 31·4 ± 0·9 mmHg by adjustments to the stroke volume. Arterial pH was determined using a glass capillary electrode (Radiometer, Copenhagen, Denmark; model BMS2), and maintained at 7·44 ± 0·01 by infusions of sodium bicarbonate (50–300 mM). Oesophageal temperature was maintained at 37·1 ± 0·2 °C by heating coils under the table.

A gracilis muscle was vascularly isolated as previously described (Ballard *et al.* 1987). The proximal tendon was fixed to a post on the surgical table, and the distal tendon was sectioned and attached to a transducer (Grass, model FT10) to record isometric developed tension. Around 2 cm of the branch of the obturator nerve supplying the gracilis muscle was dissected free, and placed over a bipolar stimulating electrode. The muscle was wrapped in plastic film to protect it from dehydration. Sixty minutes after all surgical procedures had been completed, the animal was given heparin sodium (500 i.u. kg^{-1}; Sigma Chemical Co., USA) and connected to the perfusion circuit as previously described (Ballard, 1991). The muscle was perfused under free-flow conditions for at least 30 min after connection of the circuits, then blood flow was measured by timed collections of the venous outflow, and constant-flow perfusion was begun at approximately double the free-flow rate. The obturator nerve was then crushed proximal to the stimulating electrode.

Systemic arterial blood pressure was recorded from a brachial artery using a Gould P23XL blood pressure transducer (Spectramed Inc., Oxnard, CA, USA), and held constant at 82·1 ± 4·2 mmHg by connection of a carotid artery to a pressure bottle. Saline was infused intravenously or added to the arterial pressure bottle whenever necessary, in order to maintain both the blood pressure and the haematocrit within physiological limits. Femoral arterial pressure was recorded from the perfusion circuit using a second Gould P23XL transducer. Signals were amplified using Gould 13-4615-50 transducer amplifiers, and recorded on a Gould RS3400 recorder.

Analytical procedure

Triplicate plasma samples (0·5 ml) were deproteinized with 3 ml ice-cold acetone, and extracted with 3 ml chloroform. Two of the samples were evaporated under nitrogen for 30 min at 0·5 °C, and their plasma adenosine concentrations were determined by high-pressure liquid chromatography (HPLC) as previously described (Ballard, 1991). Plasma lactate concentration was measured in two 50 μl aliquots of the remaining sample using a semi-micro version of the enzymatic assay described by Olson (1962): glycine-semicarbazide buffer (700 μl), NAD (40 mg ml^{-1}, 10 μl) and plasma extract (50 μl) or standard lithium lactate solution (0–10 mM, 50 μl) was placed in a semi-micro cuvette, and the absorbance at 340 nm recorded. L-lactic dehydrogenase (5 μl; Sigma Chemical Co.) was added to start the reaction, and the absorbance was measured again after a 90 min incubation at room temperature. Plasma concentrations were corrected for the recovery of 134·3 ± 7·9% ($n = 32$).

Experimental procedure

Muscle stimulation with constant-flow perfusion. Control blood samples were collected from the arterial and venous sections of the perfusion circuit for adenosine, lactate, pH and blood gas analysis. The obturator nerve was then stimulated using 6 V, 2 ms pulses (Grass, model S48) for 10 min, and blood samples were again collected for analysis. Stimulation was stopped, and the perfusion pressure was allowed to return to control before another stimulation was performed. Altogether, six different patterns of stimulation were given: twitch contractions were produced by stimulation of the obturator nerve at 0·1, 0·5, 1·5 or 3 Hz for 10 min; tetanic contractions were produced by delivering 1 or 5 s pulses of 30 Hz stimulation at 0·1 Hz (i.e. a 1 or a 5 s tetanus every 10 s for 10 min). Each muscle received between one and six of these stimulation patterns.

Muscle stimulation with free-flow perfusion. The hyperaemia response to each pattern of stimulation was determined in a separate set of experiments; the muscle was perfused under free-flow conditions at arterial blood pressure throughout the experiment. Fifteen minutes after the obturator nerve had been sectioned, the resting blood flow was again determined, and stimulation was begun at 0·5 Hz. After 5 min of contractions the steady-state flow rate was measured, then the contraction frequency was increased to 1·5 Hz. The same procedure was used to determine the free-flow rate at 1·5 and 3 Hz, then the muscle was allowed to recover for 20–30 min, before using this procedure to determine the flow rate during the 1 and 5 s tetanic stimulation procedures.

When all of the experimental procedures had been completed, the animal was killed by I.V. infusion of saturated potassium chloride, and the gracilis muscle was removed and weighed.

Statistical analysis

Values are the means ± S.E.M. of the number of tests (n) shown. Animals received between one and six different stimulation patterns, each pattern being preceded by control measurements. Thus, for stimulation data, n also equals the number of animals, but for control data, n includes one to six measurements in each animal. Arterial and venous lactate or adenosine concentrations (which were not normally distributed) were compared using Wilcoxon's signed ranks test. All other data were compared to control values in the same animal using a paired t test, or to values in another group of animals using Student's t test. Single correlation analysis was used to determine whether a significant relationship existed between two variables; where three variables were thought to interact with each other, partial correlation was used to eliminate the influence of the third variable, giving a net correlation coefficient for the relationship between the remaining two.

RESULTS

Constant-flow perfusion

The resting free flow to this group of gracilis muscles was $7{\cdot}3 \pm 1{\cdot}5$ ml min^{-1} (100 g)$^{-1}$ ($n = 16$). The muscles were perfused at a constant flow rate of $14{\cdot}1 \pm 1{\cdot}9$ ml min^{-1} (100 g)$^{-1}$. Altogether, fifty-three stimulations were performed in sixteen animals.

Force of contraction

Typical haemodynamic and force recordings are shown in Fig. 1, and the contractile force with different stimulation parameters is summarized in Table 1. No fatigue occurred during twitch contractions at 0·1 Hz; in four of the six muscles, the force gradually increased throughout the stimulation period, so that maximum force development occurred at the end of the 10 min stimulation period. Fatigue occurred at all higher frequencies of twitch contraction; the higher the frequency of contraction, the earlier the onset of the fatigue, and the greater the overall decline in force which occurred.

The degree of fatigue was also dependent on the duration of the contraction; tetanic contractions produced a greater decline in force than twitch contractions at

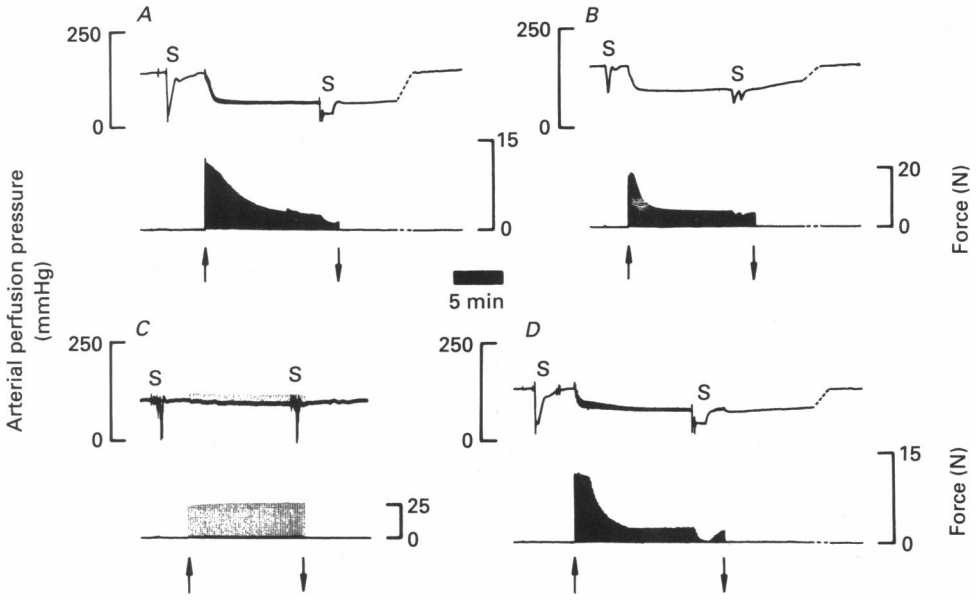

Fig. 1. Example of traces showing the femoral arterial perfusion pressure and force response to twitch contractions at 1·5 (*A*), 3 (*B*) or 0·1 (*C*) Hz, and to 5 s tetanic contractions at 0·1 Hz (*D*). Arrows indicate where stimulation was switched on and off in each case. The artifacts in the pressure tracing (S) during the control period and after 10 min contraction were caused by the blood sampling procedure.

TABLE 1. Contractile force with different stimulation parameters

	Time to peak force (min)	Peak force (N (100 g)$^{-1}$)	5 min force (N (100 g)$^{-1}$)	Decline in force (%)	10 min force (N (100 g)$^{-1}$)	Decline in force (%)
Twitch						
0·1 Hz	7·2±1·8	42·5±4·7	40·7±4·3	0	42·5±4·7	0
0·5 Hz	3·6±3·3	28·8±5·0	23·4±3·7*	17·6±4·6	19·1±10·1*	30·9±9·6
1·5 Hz	1·7±1·0	23·6±3·8	13·0±2·1*	38·2±9·1	11·5±2·4*	44·8±10·4
3·0 Hz	0·8±0·1	17·2±3·1	8·3±2·2*	53·1±6·6	6·6±1·9*	62·5±6·4
Tetanus						
1 s, 0·1 Hz	2·3±1·1	57·2±12·9	49·1±10·7*	11·9±2·6	40·7±9·1*	24·2±6·9
5 s, 0·1 Hz	0·6±0·1	48·2±11·9	12·6±3·1*	71·9±5·1	11·3±2·6*	74·8±4·9

Values are the means±S.E.M. of six to nine tests; force is expressed in newtons per hundred grams wet weight of muscle (determined at the end of the experiment). Stimulation was continued for 10 min in each case; tetani were produced by stimulation at 30 Hz and repeated at a frequency of 0·1 Hz. Asterisks indicate where the force had declined significantly from its peak value ($P < 0·05$, paired t test).

the same frequency, and a 5 s tetanus caused a greater decrease in force than a 1 s tetanus.

Haemodynamic recordings

The change in femoral arterial perfusion pressure in response to muscle stimulation is shown in Table 2. The arterial perfusion pressure decreased to a new stable value

TABLE 2. Haemodynamic response to gracilis muscle stimulation for 10 min at different frequencies

	Arterial perfusion pressure (mmHg)		Decrease in arterial perfusion pressure (%)	T_{90} for recovery (min)	n
	Control	Stimulation			
Twitch					
0·1 Hz	128·1 ± 12·1	121·4 ± 10·7	5·0 ± 2·0	1·5 ± 0·4	6
0·5 Hz	146·1 ± 12·6	116·4 ± 15·9*	21·7 ± 20·5	8·1 ± 4·3	9
1·5 Hz	158·8 ± 19·5	117·4 ± 31·5*	32·3 ± 8·1	13·5 ± 4·7	9
3·0 Hz	132·3 ± 14·6	98·9 ± 18·5*	28·4 ± 5·5	15·7 ± 3·9	8
Tetanus					
1 s, 0·1 Hz	135·5 ± 19·0	112·2 ± 23·8*	20·9 ± 7·0	10·8 ± 4·6	8
5 s, 0·1 Hz	136·8 ± 21·8	102·4 ± 24·9*	31·7 ± 7·7	15·7 ± 4·1	8

Values are the means ± S.E.M. of the number of tests shown. T_{90} is the time taken for perfusion pressure to recover to 90% of its precontrol value. Asterisks indicate where arterial perfusion pressure was significantly reduced by muscle stimulation ($P < 0·05$, paired t test).

TABLE 3. Blood gas profile of femoral venous blood at rest and during muscle contractions

	pH	n	P_{O_2} (mmHg)	n	P_{CO_2} (mmHg)	n
Control	7·33 ± 0·01	53	54·3 ± 2·2	53	44·6 ± 1·6	52
Twitch						
0·1 Hz	7·39 ± 0·01	6	50·4 ± 3·4	6	35·6 ± 1·6	6
0·5 Hz	7·33 ± 0·04	10	30·8 ± 3·1*	10	50·5 ± 5·0	10
1·5 Hz	7·25 ± 0·04*	10	26·2 ± 2·2*	10	63·4 ± 7·2*	9
3·0 Hz	7·24 ± 0·03*	9	20·7 ± 2·3*	9	58·4 ± 3·4*	8
Tetanus						
1 s, 0·1 Hz	7·23 ± 0·04*	9	23·7 ± 2·2*	9	58·1 ± 5·0*	9
5 s, 0·1 Hz	7·22 ± 0·03*	9	25·3 ± 2·3*	9	61·8 ± 4·1*	9

Values are means ± S.E.M. of the number of tests shown. Asterisks indicate those values which are significantly different from control ($P < 0·05$, Student's t test).

over the first 2–4 min of stimulation, and remained at this level until the stimulation was withdrawn, then returned to a postcontrol value not significantly different from the precontrol. Both the magnitude of the vasodilatation and the time required for recovery of vascular tone depended on the frequency and the duration of the contractions.

Blood gases and pH

The arterial pH was 7·43 ± 0·01 ($n = 106$), the P_{CO_2} was 34·1 ± 0·6 mmHg ($n = 103$) and the P_{O_2} was 112·7 ± 1·6 mmHg ($n = 106$). These values were not altered by stimulation of the muscle. The venous blood gas and pH values at rest and during contractions are shown in Table 3. Venous pH was significantly reduced, and venous P_{CO_2} was significantly elevated by 1·5 or 3 Hz twitch contractions and by either of the tetanic contractions. Venous P_{O_2} was significantly reduced by the same conditions, and additionally by twitch contractions at 0·5 Hz.

Oxygen saturation was calculated from the P_{O_2}, P_{CO_2} and pH using the seven-coefficient computer model described by Kelman (1966). Oxygen content was then calculated from the haematocrit, oxygen saturation and P_{O_2}, and oxygen consumption from the arteriovenous difference in oxygen contents and the flow rate (Table 4). All types of muscle contraction resulted in a significant increase in oxygen

TABLE 4. Oxygen consumption, oxygen cost, and contributions of aerobic and anaerobic energy production to total energy use with different types of gracilis muscle contraction

	Oxygen consumption (ml min^{-1} $(100\ g)^{-1}$)	Oxygen cost (μl N^{-1} $contraction^{-1}$)	Aerobic ATP production (μmol min^{-1} $(100\ g)^{-1}$)	Anaerobic ATP production (μmol min^{-1} $(100\ g)^{-1}$)	Anaerobic ATP production (%)	n
Control	0·40 ± 0·07	—	94·2	6·02	6·0	48
Twitch						
0·1 Hz	0·47 ± 0·09*	1·92 ± 0·44	111·0	3·02	2·6	6
0·5 Hz	1·06 ± 0·21*	4·61 ± 2·99	250·2	5·49	2·1	9
1·5 Hz	1·59 ± 0·34*	1·90 ± 0·56	375·0	11·75	3·0	9
3·0 Hz	1·87 ± 0·39*	2·41 ± 0·97	441·0	14·58	3·2	8
Tetanus						
1 s, 0·1 Hz	1·53 ± 0·32*	8·89 ± 3·01	360·6	7·82	2·1	8
5 s, 0·1 Hz	1·45 ± 0·33*	44·26 ± 19·76	342·0	12·60	3·6	8

Oxygen consumption and oxygen cost values are the means ± S.E.M. of the number of tests shown. Asterisks indicate where the oxygen consumption is significantly greater than control ($P < 0·05$, paired t test). Aerobic ATP production was calculated from the oxygen consumption, assuming a value of 6·0 micromoles ATP per micromole O_2, and anaerobic ATP production was calculated from lactate release, assuming a value of 1·5 micromoles ATP per micromole lactate.

consumption above the control level. The oxygen cost of the different types of contraction was found by dividing the oxygen consumption by the frequency of contractions and the force of contraction at the time of blood sampling, and is also shown in Table 4; stimulation frequency did not significantly alter the oxygen cost of each twitch contraction. The oxygen cost for 0·1 Hz tetani exceeded that for 0·1 Hz twitch contractions (duration approximately 0·25 s) but only in proportion to the increased duration of the contraction.

Adenosine and lactate concentrations

Under resting conditions, the arterial plasma adenosine concentration was 73 ± 10 nM and the venous plasma adenosine concentration was 154 ± 33 nM ($n = 53$; $P < 0·01$). Thus, adenosine was added to the venous blood at a rate of 162 ± 37 pmol min^{-1} under resting conditions.

The arterial and venous adenosine concentrations with different stimulation conditions are shown in Fig. 2; the venous adenosine concentration was significantly greater than arterial during 1·5 or 3 Hz twitch contractions and during either of the tetanic contractions ($P < 0·01$). Since the venous adenosine concentration was slightly higher than arterial at rest, the venous adenosine concentration during

muscle contractions was also compared to the resting venous concentration; a significant difference was again found during 1·5 ($P < 0·05$) or 3 Hz twitch contractions ($P < 0·02$) and both tetanic contractions ($P < 0·02$).

The resting arterial and venous plasma lactate concentrations were $2·24 \pm 0·25$ and $3·11 \pm 0·30$ mM respectively ($n = 46$; $P < 0·01$), which was equivalent to the addition

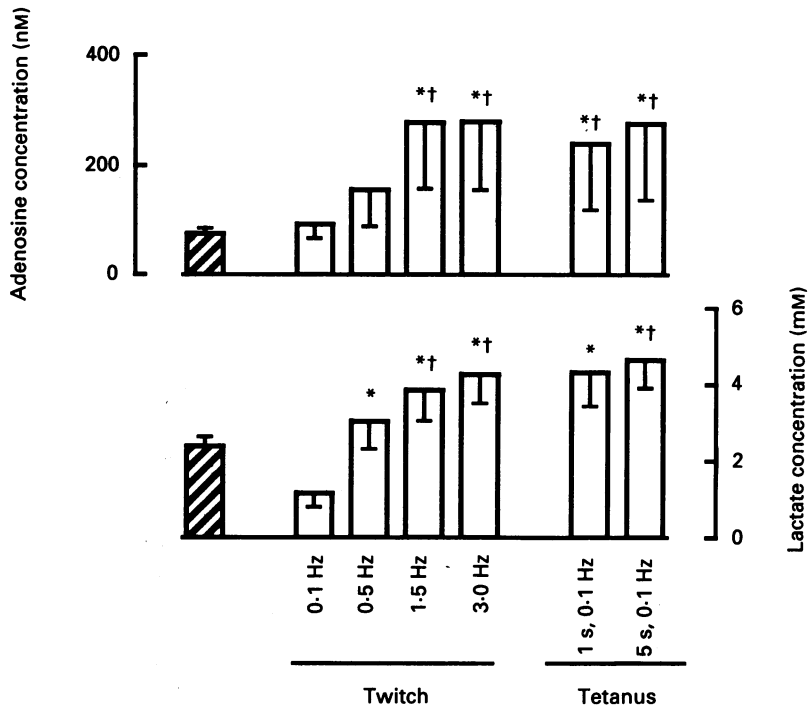

Fig. 2. The influence of stimulation parameters on venous adenosine and lactate concentrations. Values are the means ± S.E.M. of fifty-three tests for arterial adenosine, forty-six tests for arterial lactate and six to ten tests for venous concentrations. * denotes venous concentration (open bars) significantly greater than arterial (hatched bars) and † denotes venous concentration during contractions significantly greater than resting venous concentration ($P < 0·05$, Wilcoxon's signed ranks test).

of $2·35 \pm 0·31$ nmol min^{-1} of lactate to the venous blood. The arterial and venous lactate concentrations during muscle contractions are shown in Fig. 2; the venous lactate concentration was significantly greater than arterial during 0·5 ($P < 0·05$), 1·5 and 3 Hz twitch contractions ($P < 0·01$) and both tetanic contractions ($P < 0·01$). However, the venous lactate during contractions was significantly greater than the resting venous concentration during only 1·5 or 3 Hz twitch contractions and the 5 s tetanus (all $P < 0·01$).

The venous adenosine concentration was inversely related to the venous pH ($r = 0·53$, $P < 0·001$; Fig. 3*A*), and positively correlated with both the venous P_{CO_2} ($r = 0·58$, $P < 0·001$; Fig. 3*B*) and the venous lactate concentration ($r = 0·70$, $P < 0·001$; Fig. 3*C*). Venous adenosine concentration was not well correlated with either the

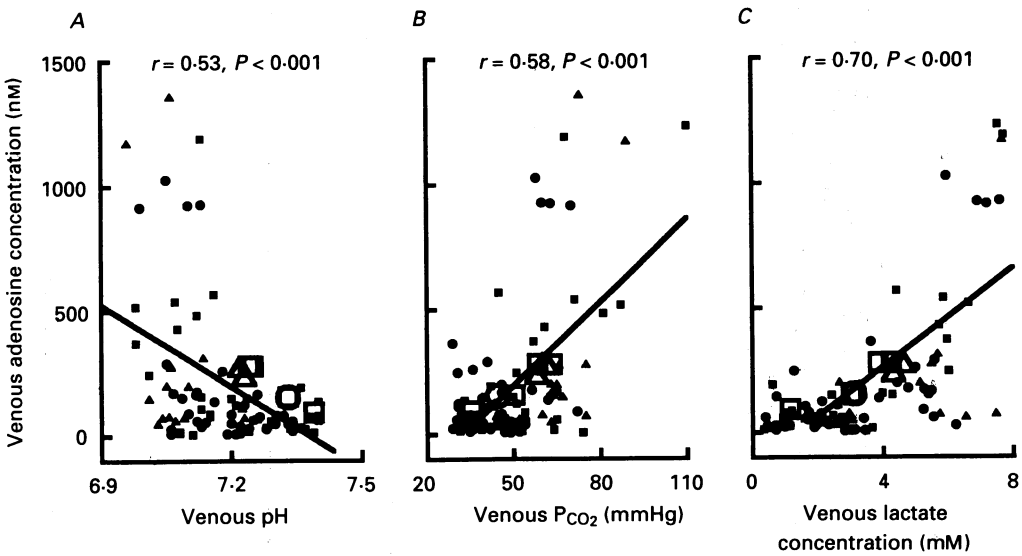

Fig. 3. Relationship between venous adenosine concentration and venous pH (*A*), venous P_{CO_2} (*B*), and venous lactate concentration (*C*) at rest (○, ●) and during twitch (□, ■) or tetanic (△, ▲) contractions. The regression lines and the correlation coefficients were obtained from the individual data points (filled symbols); the mean value for each treatment is also shown for reference (open symbols).

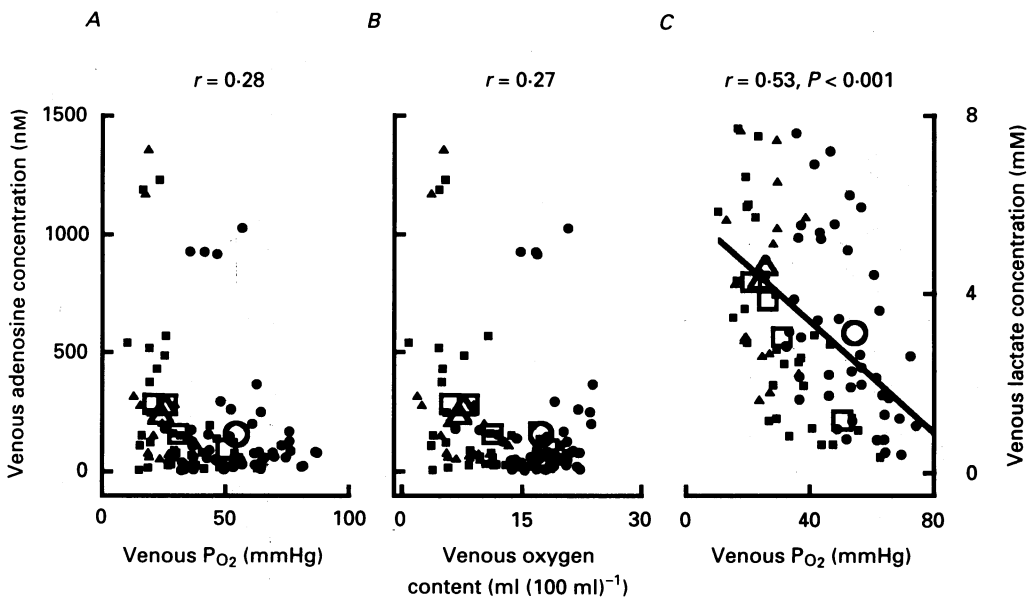

Fig. 4. Relationship between venous adenosine concentration and venous P_{O_2} (*A*) or venous oxygen content (*B*), and between venous lactate concentration and venous P_{O_2} (*C*) at rest (○, ●) and during twitch (□, ■) or tetanic (△, ▲) contractions. The regression line and the correlation coefficients were obtained from the individual data points (filled symbols); the mean value for each treatment is also shown for reference (open symbols).

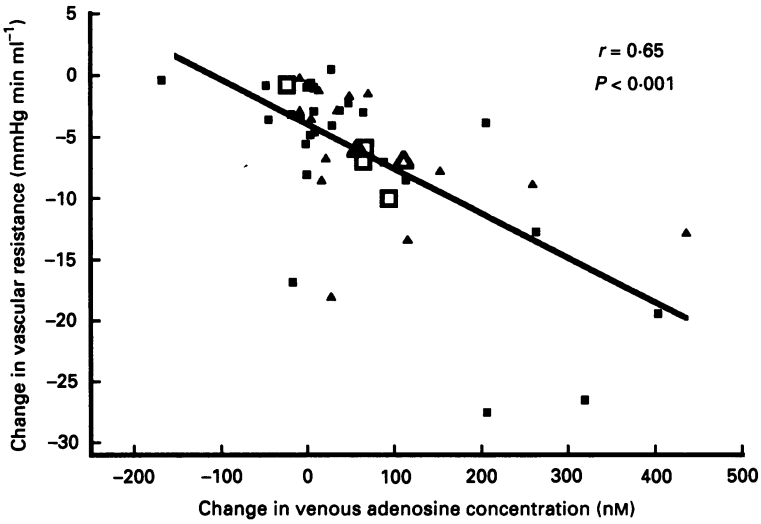

Fig. 5. Relationship between the change in vascular resistance (resistance during muscle contractions minus resting vascular resistance) and the change in venous adenosine concentration (contracting minus resting venous adenosine concentration). The regression line and the correlation coefficient were obtained from the individual data points (filled symbols); the mean value for each treatment is also shown for reference (open symbols). □, ■ twitch contractions; △, ▲ tetanic contractions.

TABLE 5. Haemodynamic and force responses to 10 min of stimulation under free-flow conditions

	Flow rate (ml min^{-1} (100 g)$^{-1}$)	Flow rate (percentage of control)	Peak force (N (100 g)$^{-1}$)	5 min force (N (100 g)$^{-1}$)	Decline in force (%)
Twitch					
0·5 Hz	14·9±4·0	146·7±22·9	35·4±7·2	34·1±7·4	5·0±2·7*
1·5 Hz	21·5±5·6	208·0±30·8	33·4±7·6	21·8±5·2	36·5±4·6
3·0 Hz	24·0±6·1	238·6±31·1	21·6±5·3	12·7±3·4	42·6±3·0
Tetanus					
1 s, 30 Hz	18·2±5·0	176·1±24·1	70·0±9·3	54·2±7·7	21·0±8·4
5 s, 30 Hz	20·2±6·2	185·9±19·6	54·3±7·8	24·3±4·9	58·7±4·7

Values are the means±S.E.M. of six tests for flow values and seven tests for force values. Asterisks indicate where force values differ significantly from those recorded under similar conditions using constant flow perfusion.

venous P_{O_2} ($r = 0{\cdot}28$; Fig. 4*A*) or the venous oxygen content ($r = 0{\cdot}27$; Fig. 4*B*), but there was a significant correlation between the venous lactate concentration and the venous P_{O_2} ($r = 0{\cdot}53$, $P < 0{\cdot}001$; Fig. 4*C*). The decrease in vascular resistance during muscle contractions correlated well with the change in venous adenosine concentration ($r = 0{\cdot}65$, $P < 0{\cdot}001$; Fig. 5).

Partial correlation of the relationship between venous adenosine, venous lactate and venous P_{O_2} showed that the net correlation between adenosine and P_{O_2} (after the

influence of lactate had been removed) had a coefficient of only 0·15; however, the correlation coefficient for the relationship between venous adenosine and venous pH increased to 0·95 if the influence of venous P_{O_2} was partialled out.

Free flow

Seven animals each received five different stimulation patterns under free flow conditions. The resting free flow to the gracilis muscles used in these tests was $9·3 \pm 3·1$ ml min^{-1} $(100\ g)^{-1}$ ($n = 7$; not significantly different from the group used for constant flow experiments). The flow rate and contractile force observed during each stimulation is shown in Table 5.

DISCUSSION

The main purpose of this study was to determine whether the increase in lactate concentration and/or decrease in pH during muscle contractions was sufficiently large to have caused the adenosine release which occurred. Highly significant correlations were found between the venous adenosine concentration and both the venous lactate concentration (Fig. 3*C*) and the venous pH (Fig. 3*A*). This is in agreement with previous reports that the muscle contents of adenosine and lactate were increased to a similar degree during contractions of the cat soleus muscle, which is also predominantly composed of red muscle fibres (Bockman & McKenzie, 1983; Schwartz & McKenzie, 1990). A highly significant correlation was found between the increase in venous adenosine concentration and the decrease in vascular resistance (Fig. 5), confirming the conclusions of other authors (Steffen *et al.* 1983; Ballard *et al.* 1987; Schwartz & McKenzie, 1990) that adenosine was at least partially responsible for the steady-state vasodilatation in red muscles during exercise.

It had previously been shown that lactic acid infusion caused a dose-dependent release of adenosine, which was well correlated with the decrease in venous pH (Ballard, 1991). The present study confirms that venous adenosine concentration and pH are negatively correlated during muscle contractions also (Fig. 3*A*). Previously, intra-arterial infusion of around 4 mM lactic acid caused a similar adenosine release to that seen with 3 Hz contractions (Ballard, 1991); presumably the concentration of lactic acid reaching the muscle cell cytoplasm during the infusion was somewhat lower than 4 mM. In the present experiments, 3 Hz contractions raised the venous lactate concentration to 4·26 mM. Given that the lactate concentration would be higher in the muscle cell than in the venous blood during contractions, it appears that the increase in lactate concentration alone should be sufficient to account for the adenosine release that took place.

The venous P_{CO_2} increased from 44·6 to 58·4 mmHg during 3 Hz contractions, which would contribute around 0·1 units to the overall decrease in pH; given the significant correlation between adenosine release and venous P_{CO_2} in this preparation (Fig. 3*B*), and also that CO_2 is known to cause adenosine release from isolated heart and cultured vascular smooth muscle (Mustafa & Mansour, 1984; Belloni, Bruttig, Rubio & Berne, 1986), it remains possible that the elevation in P_{CO_2} also contributed to the adenosine release here. This question is the subject of further investigation in our laboratory. Therefore, the present findings support the concept that muscle pH

may be the factor which controls the release of adenosine, and further report that the amount of lactic acid released during muscle contractions was large enough to have caused the observed adenosine release.

The venous adenosine concentration was used in these experiments as an index of the perivascular concentration; since arterial adenosine concentration is not altered by muscle contractions (Ballard *et al.* 1987), and vasodilatation does not appear to materially alter the surface area which is used for diffusion (Tominaga, Curnish, Belardinelli, Rubio & Berne, 1980; authors' unpublished observations), the change in the perivascular adenosine concentration is the main determinant of the amount of adenosine added to the venous blood. The plasma adenosine concentrations showed quite large interanimal variations: some dogs had a low (< 50 nM) arterial level, and released only small amounts of adenosine into the venous blood during muscle stimulation, whereas others had relatively high (around 200 nM) arterial concentrations, and released larger amounts from the muscle upon stimulation. Thus the concentration values were not normally distributed, which necessitated comparison of arterial and venous concentrations using non-parametric statistics. A wide interdog variation in the capacity of gracilis muscle to produce adenosine (ratio of 5′-nucleotidase activity to AMP deaminase activity) has previously been reported (Bockman & McKenzie, 1983), which probably accounts for much of the variability.

These data did not support a direct role for muscle oxygenation in the control of adenosine release since the venous adenosine concentration was only poorly correlated with venous P_{O_2} (Fig. 4*A*) or venous oxygen content (Fig. 4*B*). Partial correlation gave a net correlation coefficient for the relationship between venous adenosine and P_{O_2} of only 0·15 if the influence of lactate was removed, whereas the net correlation coefficient between adenosine and pH increased to 0·95 if the influence of P_{O_2} was removed; this suggests that if the oxygenation level had any influence on adenosine release, it achieved this via its influence on lactate production and hence pH. Previous suggestions that adenosine release was caused by oxygen insufficiency were based on ischaemia studies (Berne, Rubio, Dobson & Curnish, 1971; Belloni *et al.* 1979; Phair & Sparks, 1979), in which the muscle pH would also presumably have been depressed. Phair & Sparks (1979) found that muscle adenosine content increased progressively with the degree of flow restriction, but the relationship between adenosine content and the oxygen supply–demand ratio was not linear.

We cannot say for certain whether oxygen insufficiency caused the lactate formation: the correlation between venous lactate concentration and venous oxygen tension was quite good, but indirect evidence suggests that the oxygen supply to the muscle was not a limiting factor. Firstly, the muscle was releasing lactate at rest with a high blood flow; secondly, the lactate release increased very sharply for contraction frequencies above 0·5 Hz whereas the venous oxygen tension scarcely changed (Table 3); and thirdly, the percentage of the energy production achieved by anaerobic means during even the most stressful contractions did not exceed the percentage at rest (Table 4). Also, the oxygen cost per unit of tension × time was similar for all types of muscle contraction (Table 4). This finding is in broad agreement with previous studies (Stainsby & Barclay, 1972; Wilson & Stainsby, 1978). Hypoxia did not occur during 0·1 Hz twitch contractions, because the venous P_{O_2} was not even depressed. Since the oxygen cost for the most stressful stimuli (3 Hz twitches and 5 s tetani) did

not differ from that for 0·1 Hz contractions, it would be reasonable to assume that the oxygen supply was adequate during these contractions also, and hence that the increase in venous lactate concentration was not caused by oxygen deficiency.

Another explanation for the constancy of the oxygen cost of the various stimulation patterns is suggested by the degree of muscle fatigue, which increased with the contraction frequency; if the oxygen supply had been limited by the controlled flow condition, force production might have decreased to the maximum level permitted by the oxygen supply. However, the degree of fatigue did not differ significantly between constant- and free-flow perfused preparations, and the level of controlled flow was similar to the maximum free-flow rate achieved during stimulation (Table 5). For a red muscle, the energy supply is preferentially derived from fatty acid breakdown (Beatty, Peterson & Bocke, 1963; Rennie, Winder & Holloszy, 1976; Maizels, Ruderman, Goodman & Lau, 1977), which liberates hydrogen ions. Thus, the intracellular pH decreases in proportion to the rate of energy use, even in the presence of an adequate oxygen supply, which may explain the relationship between contraction frequency and fatigue (Metzger & Moss, 1987).

Dog gracilis and cat soleus muscles have previously been reported to accumulate lactate under fully aerobic conditions, in proportion to the stimulation frequency (Connett, Gayeski & Honig, 1984; Schwartz & McKenzie, 1990). Dog muscle is composed entirely of fibres with a high oxidative capacity (Bockman & McKenzie, 1983; Maxwell, Barclay, Mohrman & Faulkner, 1977) which contain the B (or H) isozyme of lactate dehydrogenase (Gollnick & Armstrong, 1976). This isozyme shows very pronounced substrate inhibition in response to pyruvate accumulation (Everse & Kaplan, 1973). Thus the oxygen level must decrease sufficiently to cause an increase in mitochondrial NADH before it could stimulate lactate production in this type of muscle (Everse & Kaplan, 1975). However, it has been determined that the P_{O_2} of individual mitochondriae in the dog gracilis muscle did not drop below 2 mmHg during 4 Hz contractions (Connett *et al.* 1984), and even at 5 Hz, measurement of NADH fluorescence indicated that it was consistently converted to NAD^+ during the contractions (Jobsis & Stainsby, 1968). Therefore it is unlikely that the oxygen level could have decreased sufficiently to cause NADH accumulation under the present experimental conditions, which may explain why lactate release did not appear to be dependent on oxygen deficiency.

The lactate content of the cat gracilis muscle, which is predominantly glycolytic, was found to be elevated in proportion to the contraction frequency (Schwartz & McKenzie, 1990). However, the adenosine content of the muscle did not correlate with its lactate content during contractions (Bockman & McKenzie, 1983). This suggests that the control of adenosine production is somewhat different in red and white muscles. Thus, even though lactate accumulation results from moderate hypoxia in glycolytic muscles, it may not stimulate adenosine release there. This difference may be relevant to the observations that white glycolytic muscles produce much less adenosine than red oxidative muscles during exercise and reactive hyperaemia (Klabunde & Mayer, 1979; Bockman & McKenzie, 1983; Schwartz & McKenzie, 1990).

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