Potassium initiates vasodilatation induced by a single skeletal muscle contraction in hamster cremaster muscle

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The rapid onset of vasodilatation within seconds of a single contraction suggests that the vasodilators involved may be products of skeletal muscle activation, such as potassium (K^+). To test the hypothesis that K^+ was in part responsible for the rapid dilatation produced by muscle contraction we stimulated four to five skeletal muscle fibres in the anaesthetized hamster cremaster preparation *in situ* and measured the change in diameter of arterioles at a site of overlap with the stimulated muscle fibres before and after a single contraction stimulated over a range of stimulus frequencies (4, 10, 20, 30, 40, 60 and 80 Hz; 250 ms train duration). Muscle fibres were stimulated in the absence and presence of an inhibitor of a source of K^+, the voltage dependent K^+ channel inhibitor 3,4-diaminopyridine (DAP, 3×10^{-4} M) and inhibitors of the K^+ dilatory signal transduction pathway, either a Na^+ K^+-ATPase inhibitor (ouabain; 10^{-4} M) or an inward rectifying K^+ channel inhibitor (barium chloride, $BaCl_2$; 5×10^{-5} M). We observed significant inhibitions of the rapid dilatation at all stimulus frequencies with each inhibitor. The dilatory event at 4 s was significantly inhibited at all stimulus frequencies by an average of $65.7 \pm 3.6\%$, $58.8 \pm 6.1\%$ and $64.4 \pm 2.1\%$ in the presence DAP, ouabain and $BaCl_2$, respectively. These levels of inhibition did not correlate with non-specific changes in force generation by skeletal muscle measured *in vitro*. Therefore, our data support that K^+ is involved in the rapid dilatation in response to a single muscle contraction across a wide range of stimulus frequencies.

Blood flow to active skeletal muscle increases rapidly at the onset of exercise. Rapid vasodilatation has been implicated as a component of this process (Tschakovsky *et al.* 1996; Shoemaker *et al.* 1998; Naik *et al.* 1999; Hamann *et al.* 2004; Saunders & Tschakovsky, 2004; Tschakovsky & Sheriff, 2004). Microvascular studies support this by showing that vasodilatation occurs within seconds in response to skeletal muscle contraction (Marshall & Tandon, 1984; Dodd & Johnson, 1991; Mihok & Murrant, 2004; VanTeeffelen & Segal, 2006). The identity of the vasodilator(s) responsible for the rapid dilatation remain unclear. Mihok & Murrant (2004) show that there may be multiple dilators responsible for the dilatation resulting from a single contraction and that dilator production is dependent on the stimulation parameters used to induce the skeletal muscle contraction. They observed that low stimulus frequencies (4 Hz) resulted in a single rapid dilatation peaking within 3–4 s of the contraction before returning to baseline by 10 s; higher stimulus frequencies (20, 30 and 40 Hz) resulted in a biphasic dilatation with two peaks, one peak at approximately 4 s and another at approximately 20 s. Even higher stimulus frequencies

(60 and 80 Hz) resulted in a single larger dilatation peaking at approximately 4 s but lasting much longer than the dilatation at low frequencies. Although aspects of the pattern of dilatation differ depending on stimulus frequency, what remains constant is the presence of a very rapid dilatation at approximately 4 s. The rapid nature of the dilatation indicates that the vasodilators may be a product of skeletal muscle activation, ones that are produced even before the skeletal muscle cells themselves contract. Both acetylcholine (ACh), used for transmission of the action potential from the α motor neuron to the skeletal muscle cell at the neuromuscular junction, and potassium (K^+), released from skeletal muscle cell as the action potential develops along the fibres, are vasodilatory products that fit this description. VanTeeffelen & Segal, 2006) have shown that the early component of the dilatation in response to a single contraction of the whole muscle was, in part, dependent on ACh. Whether K^+ plays a role in this early dilatation is unknown.

K^+ has been implicated in the vasodilatations that produce the changes in blood flow in response to exercise (Tominaga *et al.* 1973; Mohrman & Sparks, 1974; Radawski

et al. 1975; Stowe *et al.* 1975; Hnik *et al.* 1976; Hirche *et al.* 1980; Wilkerson *et al.* 1982; Vyskocil *et al.* 1983; Kiens *et al.* 1989; Lott *et al.* 2001) although some studies question its significance (Mohrman, 1982; Lash & Bohlen, 1987; Wilson *et al.* 1994). K^+ levels in both the plasma (Tominaga *et al.* 1973; Radawski *et al.* 1975; Stowe *et al.* 1975; Bockman *et al.* 1976; Wilkerson *et al.* 1982; Juel *et al.* 1990; Wilson *et al.* 1994) and the interstitial space (Hirche *et al.* 1980; Juel *et al.* 2000; Lott *et al.* 2001) increase in response to muscle contraction and in some cases the release has been shown to occur immediately upon the initiation of contraction (Scott *et al.* 1970; Mohrman & Sparks, 1974; Vyskocil *et al.* 1983; Kiens *et al.* 1989) and in response to a single contraction (Mohrman & Sparks, 1974; Hnik *et al.* 1976). A primary source of K^+ resulting from skeletal muscle cell activation is the slow voltage dependent K^+ (K_v) channels associated with the action potential being propagated down nerves and skeletal muscle cell membranes and thus K^+ has the potential to be released directly into the interstitial space surrounding any vasculature overlapping the active skeletal muscle fibres. K^+ is vasodilatory at lower concentrations, in the range of 1–15 mM (Dawes, 1941; Emanuel *et al.* 1959; Chen *et al.* 1972; Duling, 1975; Kiens *et al.* 1989; Wilson *et al.* 1994; Burns *et al.* 2004) and interstitial K^+ levels produced by muscle contraction are at levels that promote vasodilatation (Vyskocil *et al.* 1983; Juel *et al.* 2000; Lott *et al.* 2001). The vasodilatatory response to K^+ has been attributed to a transient hyperpolarization of vascular smooth muscle cells by stimulation of inward rectifying K^+ (K_{IR}) channels (Loeb *et al.* 2000; Burns *et al.* 2004) and the sodium–potassium pump (Na^+ K^+-ATPase) (Haddy, 1983; Burns *et al.* 2004). Hyperpolarization will close voltage gated Ca^{2+} channels, decrease intracellular Ca^{2+} and produce vasodilatation. Therefore, there is evidence that K^+ may be involved in the events initiating the rapid dilatation in response to skeletal muscle contraction.

The goal of this study was to determine whether K^+ is involved in producing the rapid dilatation in response to a single contraction. We tested the hypothesis that K^+ was, in part, responsible for producing the initial, rapid vasodilatory peak observed at all stimulus frequencies. We used an anaesthetized hamster cremaster muscle preparation *in situ* to directly stimulate a small bundle of skeletal muscle fibres (4–5) for a single contraction (train duration 250 ms) over a range of stimulus frequencies (4, 10, 20, 30, 40, 60 and 80 Hz) and monitored the diameter of arterioles overlying the active skeletal muscle cells. The diameter of the overlying arterioles were measured in the absence and the presence of a blocker of source of K^+, 3,4-diaminopyridine (DAP, 3×10^{-4} M) to block K_v channels, or blockers of key components in the signalling pathway to K^+, barium chloride ($BaCl_2$, 5×10^{-5} M) to block K_{IR} channels or ouabain (10^{-4} M) to block the Na^+ K^+-ATPase.

Methods

All experimental procedures were approved by the institutional animal care and use committee and were conducted in accordance with the guidelines of the Canadian Council on Animal Care as set out in the *Guide to the Care and Use of Experimental Animals*.

In situ protocol

Adult male Golden hamsters (100–130 g) were anaesthetized with sodium pentobarbital (70 mg kg^{-1} I.P.) and tracheotomized. Polyethelyne catheters (outer tip diameter approximately 0.5 mm) were placed in the left femoral artery and vein to monitor mean arterial pressure and for supplemental pentobarbital administration, respectively. Supplemental pentobarbital was given as needed during surgery and constantly infused throughout the experimental protocol (10 mg ml^{-1} saline, 0.56 ml h^{-1}). Hamster esophageal temperature was maintained at 37°C via convective heat from a coiled water-filled glass tube (42.0°C) secured under the hamster. The right cremaster was prepared for *in situ* microscopy as previously described (Baez, 1973; Murrant, 2005). Briefly, the cremaster was isolated, cut longitudinally, separated from the testis and epididymis and gently spread over a semicircular lucite platform. The edges of the tissue were secured with insect pins to maintain tension but not stretch the muscle. Once exposed, the cremaster muscle was constantly superfused with a bicarbonate-buffered salt solution containing (mmol l^{-1}): NaCl 131.9, KCl 4.7, $CaCl_2$ 2.0, $MgSO_4$ 1.2, $NaHCO_3$ 30, and 0.3 mg l^{-1} tubarine (curare) equilibrated with gas containing 5% CO_2–95% N_2 (pH 7.35–7.45). Cremaster muscle temperature was maintained at 34.0°C. Following the surgery, all preparations were allowed to equilibrate for 45–60 min prior to data collection. The cremaster microvasculature was visualized by transillumination with a tungsten lamp and with an Olympus BX51WI microscope using a ×20 long working distance water immersion objective (n.a. 0.50) and a ×1.6 magnification changer. The microscope image was displayed via a video camera (Hitachi VK-C370) on a monitor and recorded on a videotape recorder (Sony, SVO-9600 MD). Final magnification of the site was ×2000. Diameter measurements were reproducible to within ± 0.3 μm.

Transverse arterioles of approximately 40 μm maximum diameter were observed. Transverse arterioles were identified as previously described (Mihok & Murrant, 2004). Our only selection criterion required that muscle fibres associated with the transverse arteriole run approximately perpendicular to the vessel. This architecture is common and can be found in all areas of the tissue preparation.

Muscle fibre bundles (3–5 fibres) were stimulated directly using a platinum wire microelectrode (tip

diameter approximately 25 μm), which was placed in close proximity to, but not touching, muscle fibres running approximately perpendicular to the arteriole (observation site). The microelectrode was positioned at least 1000 μm away from the chosen site of the arteriole–stimulated muscle fibre intersection. Curare was added to the superfusate to ensure direct electrode stimulation of the skeletal muscle cells, but not nervous stimulation of the muscle fibres. Blocking nicotinic cholinergic membrane receptors on the skeletal muscle ensured that if a motor nerve was stimulated by the electrode, then the resulting acetylcholine release would not stimulate skeletal muscle cell contraction. Therefore the addition of curare isolated any muscle contraction to the control of the electrode only. The ground electrode was placed in the superfusate around the outer rim of the tissue support pedestal. Each stimulus was a square wave pulse of 0.4 ms duration and 8–15 V (Grass S48 stimulator, Quincy, MA, USA). The voltage was chosen to maximally stimulate four to five muscle fibres and then kept constant throughout the duration of the experiment.

After the equilibration period, arteriolar diameter at the observation site was continuously recorded for 1 min before muscle stimulation, and during and for 2 min after each single contraction. Muscle fibres were stimulated with a range of stimulus frequencies of 4, 10, 20, 30, 40, 60 and 80 Hz with a train duration of 250 ms. For each experiment, the order of stimulus frequencies was randomized and a 4–5 min rest period was allowed between each contraction. To test for a role of K^+ in the dilatations to a single muscle contraction, the cremaster muscle was superfused with either 3×10^{-4} M DAP ($n = 12$) for 10 min or 10^{-4} M ouabain ($n = 14$) for 20 min or 5×10^{-5} M $BaCl_2$ ($n = 14$) for 30 min, and the stimulation protocol was repeated. Following each experiment, arteriolar diameters were recorded after 2 min of superfusion of the preparation with 10^{-4} M sodium nitroprusside (considered to produce maximal dilatation).

Experiments showed that $BaCl_2$ induced a significant decrease in baseline diameter (Table 1). In order to determine the effects of this change in baseline diameter on arteriolar vasodilatation in response to muscle contraction we contracted the muscle cells over the same range of stimulus frequencies used *in situ* in the absence and the presence of 10^{-8} M phenylpherine (PHE), added to decrease the baseline diameter to approximately the same levels as with $BaCl_2$.

Time controls for the above experiments involving a drug treatment that followed an incubation time were conducted by stimulating the skeletal muscle cells over a range of stimulus frequencies and then waiting 30 min, with no treatment, and repeating the stimulation protocol. This was done in order to ensure that there was no change in responsiveness of the preparation over the longest drug incubation time used in this study.

Table 1. Baseline and maximal arteriolar diameters for *in situ* experiments

	Baseline Diameter* (μm)		Maximum diameter (μm)	n
	Control	Experimental		
DAP	22.0 ± 2.4	19.9 ± 2.7	41.8 ± 3.1	12
$BaCl_2$	21.7 ± 1.8	15.3 ± 1.5†	40.1 ± 2.3	14
Ouabain	20.8 ± 1.8	17.2 ± 1.5	39.3 ± 2.3‡	14
Phenylephrine	22.5 ± 2.4	15.7 ± 1.3†	46.5 ± 3.3	8

*Baseline diameters were not significantly different across stimulus frequencies within each experiment therefore baseline diameters for 4 Hz were used as representative. †Significant difference from control values. ‡Significant difference from phenylephrine maximum diameter.

In a separate set of experiments the effectiveness of the concentration and exposure duration for both ouabain and $BaCl_2$ at blocking the dilatory response to K^+ were tested by recording the arteriolar dilatory response to 15 mM KCl (equimolar NaCl removed to preserve the osmolarity of the superfusate) for 5 min in the absence of blockers and then again following exposure to 10^{-4} M ouabain ($n = 4$) for 20 min or 5×10^{-5} M $BaCl_2$ ($n = 4$) for 30 min.

Following each experiment animals were killed with an overdose of sodium pentobarbital (70 mg kg^{-1} I.V.).

In vitro protocol

In the *in situ* protocol, skeletal muscle cells were exposed to DAP, $BaCl_2$ and ouabain each of which could alter skeletal muscle function, which may in turn result in an altered dilatory response to muscle contraction. To determine if there were systematic effects of the blockers on the force of contraction of the cremaster muscle fibres, following the *in situ* experiments the left cremaster muscle was exteriorized and cut into two strips, each of which was tied at the ends with 5.0-gauge suture silk and hung in a tissue chamber containing Krebs–Henseleit solution consisting of (mmol l^{-1}): NaCl, 118; KCl, 4.75; $CaCl_2$, 2.54; KH_2PO_4, 1.18; $MgSO_4$, 1.18; $NaHCO_3$, 24.8; glucose, 10. The solution contained 0.3 mg l^{-1} tubocurarine and 10 U insulin l^{-1} and was aerated continuously with 95% O_2–5% CO_2 at 27°C (pH 7.4). The cremaster strips were attached to a fixed point at one end and a force transducer on the other (either model FT03, Grass Medical Instruments, Quincy, MA, USA, or model 60-2995, Harvard Apparatus, Southmatic, MA, USA). Stimulating electrodes were fixed at each side of the muscle. The length of each muscle was adjusted to be optimal for force development. After an hour of equilibration, force of contraction was measured as muscles were stimulated over a range of stimulus frequencies (4, 10, 20, 30, 40, 60, 80 Hz), at a constant train duration of 250 ms with

a supramaximal voltage (100 V). For each experiment, the order of stimulus frequencies was randomized. One of the pair of muscle strips (experimental) was then incubated with either 3×10^{-4} M DAP $(n = 6)$, 5×10^{-5} M BaCl $(n = 6)$, or 10^{-4} M ouabain $(n = 6)$ for 10, 20 and 30 min, respectively, the other of the pair was incubated in Krebs–Henseleit solution for the incubation time that corresponded with the experimental trial to act as an animal- and time-matched control. Following the incubation period the muscles were stimulated again over the above range of stimulus frequencies. Data were collected and analysed using the MP100WSW data acquisition system and Acqknowledge III software (Biopac Systems Inc., Goleta, CA, USA) on an IBM computer. Following each experiment muscle length and weight were measured.

Data analysis and statistics

All data are reported as means ± standard error of the mean (s.e.m.). Baseline diameter was defined as the diameter just prior to muscle stimulation. Only one arteriole per preparation was used to collect data, and n indicates the number of arterioles observed. All *in situ* experiments were videotaped and analysed off line. Images were digitized and arteriolar diameters measured via ImagePro Plus software. Arteriolar diameter was measured immediately following stimulation each second for 10 s, then every 5 s until 60 s and then at 90 and 120 s. Group means were compared with an ANOVA or a repeated measures ANOVA where data was analysed over time. When the ANOVAs identified significant differences, a protected LSD was used *post hoc* to determine if diameter changes were significantly different (Snedecor & Cochran, 1989). Differences were considered significant when $P < 0.05$.

Results

In situ protocol

Baseline and maximal diameters are shown in Table 1. Within each experiment the baseline diameters did not differ between stimulus frequencies so the base-line diameter prior to 4 Hz is shown as representative. Control baseline diameters did not significantly differ from experimental baseline diameters except following BaCl$_2$ exposure. BaCl$_2$ significantly decreased baseline diameter. PHE also significantly decreased baseline diameter (by design), to a level which was not significantly different from the baseline effects of BaCl$_2$. The maximal diameters did not differ between groups except for maximum diameters between PHE and ouabain. Significant differences in baseline diameters may lead to an altered responsiveness of the blood vessels; significant differences in baseline

diameter and maximal diameter may alter the dilatory potential (maximal diameter-baseline diameter) of the blood vessels. We found very low correlations between peak change in diameter and baseline diameter (average r^2 value 0.2 ± 0.03) as well as very low correlations between the peak change in diameter and dilatory potential (average r^2 value 0.1 ± 0.03) indicating that differences in baseline diameter and maximal diameter did not have a significant influence on the ability of the blood vessels to dilate.

Figure 1 shows the three patterns of dilatation typically observed for a single contraction over a range of stimulus frequencies. A single impulse (4 Hz for 250 ms), which produces a twitch contraction, produced a single, rapid dilatation that peaked at approximately 3 s and returned to baseline within 10 s. Stimulus frequencies of 20, 30 and 40 Hz produced a biphasic dilatation characterized by a single rapid dilatation peaking within 4 s followed by a secondary, longer lasting dilatation peaking at approximately 20 s before returning to baseline approximately 60 s later; 60 and 80 Hz produced a single, larger dilatation that peaked within 10 s and, in some cases, required over 120 s to recover. Contractions at each stimulus frequency produced significant dilatations within 1 s of the contraction (the first time period measured). Characteristic of all stimulus frequencies was an initial dilatory event that peaked at approximately 4 s. A second dilatory event occurred at approximately 20 s at stimulus frequencies of 20, 30 and 40 Hz.

Figure 2 shows the effect of 3×10^{-4} M DAP on the magnitude of the dilatory event at 4 s and the magnitude of the dilatation at 20 s across all stimulus frequencies. DAP significantly inhibited the initial dilatory event at all stimulus frequencies but did not consistently inhibit the magnitude of the dilatation at 20 s. The dilatory event at 20 s was inhibited significantly by DAP at frequencies where there is a pronounced dilatory peak at 20 s, 20 and 40 Hz, but not consistently as there was no inhibition of the second dilatory 30 Hz. The dilatation at 20 s was not inhibited at higher stimulus frequencies, 60 and 80 Hz, but there is no pronounced dilatory peak at 20 s at these frequencies.

Ouabain significantly inhibited the initial dilatory event at 4 s across all stimulus frequencies as well as dilatory events at 20 s at most stimulus frequencies where a distinct second prolonged peak occurs (20–80 Hz) (Fig. 3). BaCl$_2$ produced a significant decrease in baseline diameter. To test whether this change in baseline diameter influenced the ability of the blood vessel to respond to muscle contraction, we added 10^{-8} M PHE in order to decrease baseline diameter similarly (Table 1). Figure 4 compares the change in diameter from control changes (control – experimental) induced by the range of stimulus frequencies in the presence of BaCl$_2$ and PHE. We observed that BaCl$_2$ significantly inhibited the initial dilatory event at 4 s across most stimulus frequencies

(except 4 and 40 Hz) but did not consistently inhibit dilatations at 20 s when compared to PHE. Experiments where both ouabain and $BaCl_2$ were added simultaneously, in order to determine if the initial dilatory response could be eliminated completely, resulted in spontaneous skeletal muscle activity (where the muscle appeared to be twitching), and thus these experiments were not continued.

Given that all experiments involved a 10–30 min blocker incubation period between when control and experimental data were taken, we undertook time control experiments to ensure that the reactivity of our preparation did not change over time. Figure 5 shows that the initial dilatory response to muscle contraction across all stimulus frequencies at 4 and 20 s did not systematically differ significantly from the dilatory response following 30 min, which encompasses our longest incubation time. Thus, the vasculature was equally reactive to skeletal muscle stimulation over time.

In a separate set of experiments we tested the efficacy of 5×10^{-5} M $BaCl_2$ and 10^{-4} M ouabain in their ability to inhibit the dilatory effects of K^+. We found that the dilatation in response to 15 mM K^+ was transient, peaking within 2 s before returning to baseline diameter by 7 s. $BaCl_2$ significantly decreased the $15.0 \pm 2.6 \mu m$ peak change in diameter induced by K^+ to $7.9 \pm 1.8 \mu m$ and ouabain significantly decreased the $10.5 \pm 2.0 \mu m$ change in diameter induced by K^+ to $3.4 \pm 1.4 \mu m$.

In vitro protocol

We tested the effects of DAP, $BaCl_2$ and ouabain on force generation of cremaster strips *in vitro* to assess any systematic changes in skeletal muscle force generation in the presence of each of the blockers (Table 2). We found that $BaCl_2$ exposure decreased force significantly at all tetanic stimulus frequencies by up to $54.8 \pm 5.8\%$ when compared to time matched controls and ouabain decreased force significantly at all tetanic stimulus frequencies by up to $46.7 \pm 10.6\%$. Twitch contraction force development (4 Hz at 250 ms train duration) was not significantly inhibited by either $BaCl_2$ or ouabain but there was a trend to decrease twitch force development. Conversely, DAP did not significantly inhibit force at any stimulus frequency; in fact there was a trend to amplify developed force in the presence of DAP and a significant increase in developed force at 4 and 10 Hz (Table 2).

Discussion

The purpose of this study was to determine the role of K^+ in the rapid vasodilatations in response to a single muscle contraction. We found that inhibiting either a source of K^+, by blocking the K_V channel on skeletal muscle cells, or
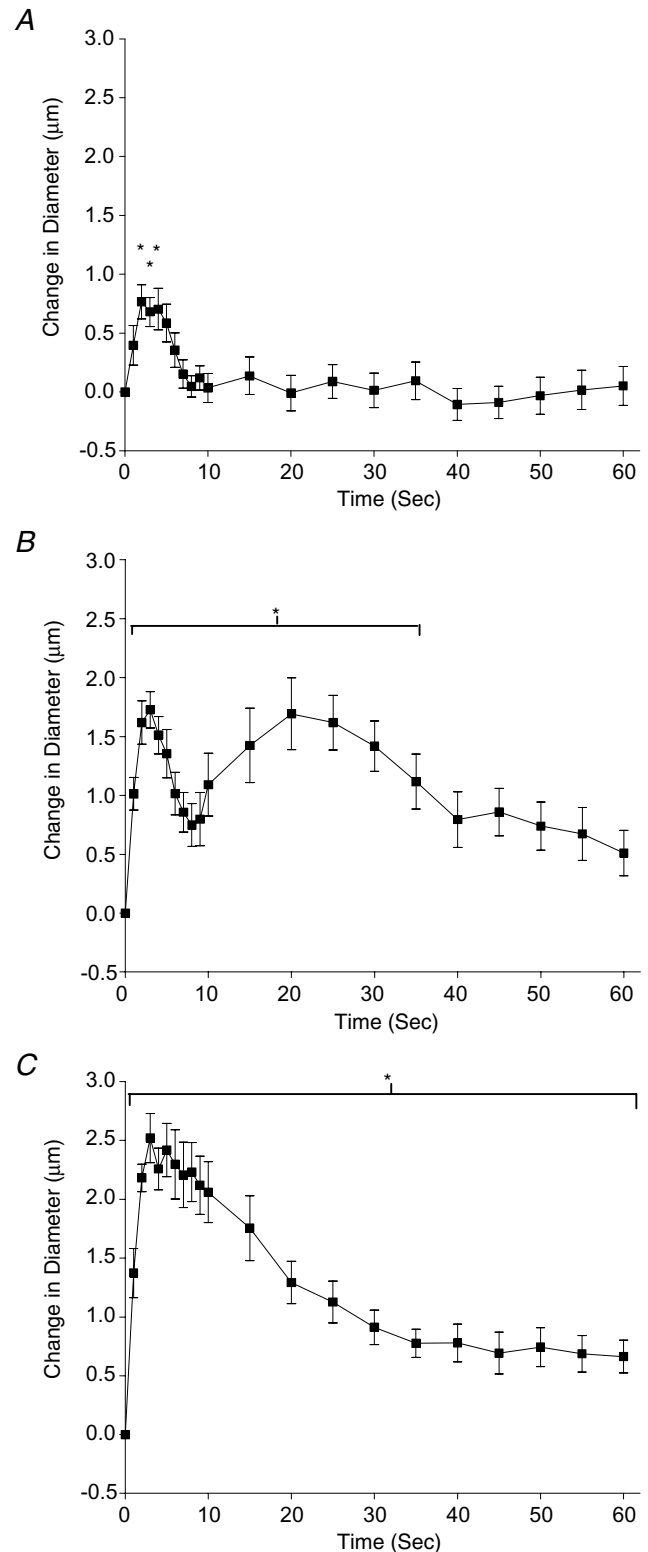

Figure 1. The change in diameter over time in response to a single contraction at 4 Hz (*A*) 20 Hz (*B*) and 60 Hz (*C*)
Representative data (control data from the $BaCl_2$ experiment) showing the three typical patterns of dilatation observed over a range of stimulus frequencies. *Data or the range of data significantly different from 0.
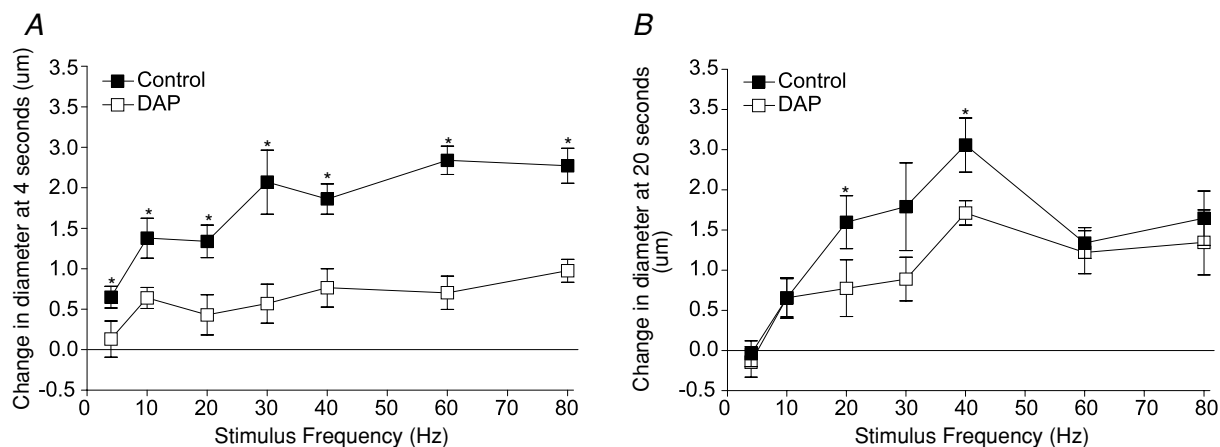
A

B

Figure 2. The effect of 3×10^{-4} M DAP on the change in diameter at 4 s (*A*) and 20 s (*B*) across all stimulus frequencies tested
*Control change in diameter significantly different from diameter in the presence of DAP. At 4 s the *P* values for main and interactive effects were: drug < 0.0001, frequency < 0.0001, and drug–frequency interaction $= 0.13$, and at 20 s these were 0.0077, < 0.0001 and 0.53, respectively.

by inhibiting mechanisms in the K^+ dilatory signal transduction pathway, the K_{IR} channel and Na^+ K^+-ATPase on cells of the vasculature (smooth muscle and/or endothelial cells), was successful in attenuating the rapid dilatation produced by a single muscle contraction at all stimulus frequencies. Therefore, there is a dilatory role for K^+ in response to muscle contraction at all stimulus frequencies.

Experimental considerations

The hamster cremaster model was used to evaluate the dynamics between contracting skeletal muscle and the associated vasculature and processes initiating vaso-

dilatation. The hamster cremaster muscle is a very thin muscle, approximately five to six skeletal muscle layers thick, consisting of mostly fast twitch fibres (~13–20% type IIa and 60–75% type IIb, 15% type I; Sarelius *et al.* 1983), similar to extensor digitorum longus (EDL) of the hamster (Phipps & Elbrink, 1983) as well as other species (Crow & Kushmerick, 1982; Beaune *et al.* 1994; Faucher *et al.* 2005). The cremaster muscle has also been shown to be biochemically similar to EDL in terms of the relative amounts of eNOS and nNOS (Lau *et al.* 2000). Hamster cremaster muscle has contractile properties (Murrant, 2005) that are typical of other mixed muscles (Murrant & Barclay, 1995; McGuire *et al.* 2003; Singh *et al.* 2004).
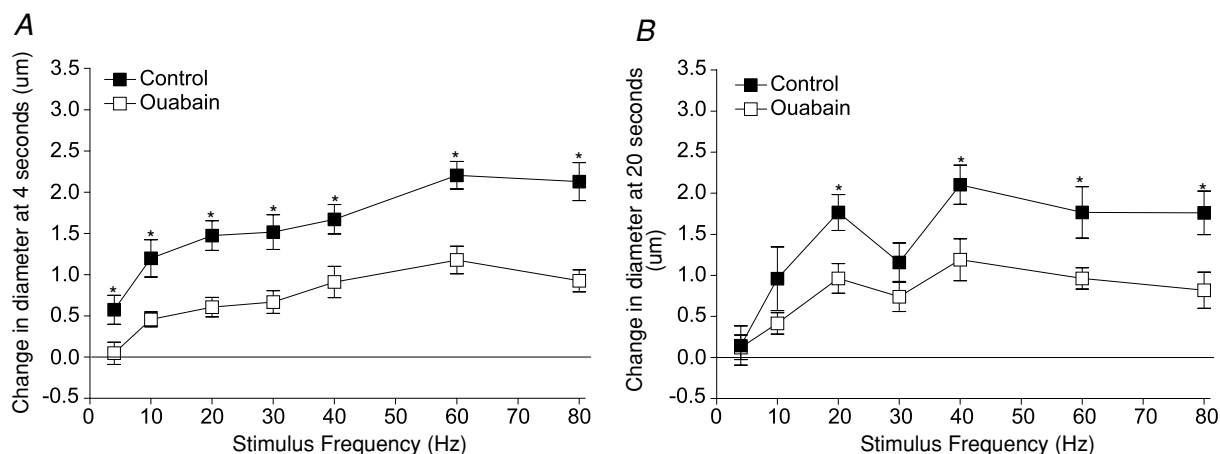
A

B

Figure 3. The effect of 10^{-4} M ouabain on the change in diameter at 4 s (*A*) and 20 s (*B*) across all stimulus frequencies tested
*Control change in diameter is significantly different from diameter in the presence of ouabain. At 4 s the *P* values for main and interactive effects were: drug < 0.0001, frequency < 0.0001, and drug–frequency interaction $= 0.58$, and at 20 s these were < 0.0001, < 0.0001, and 0.41, respectively.
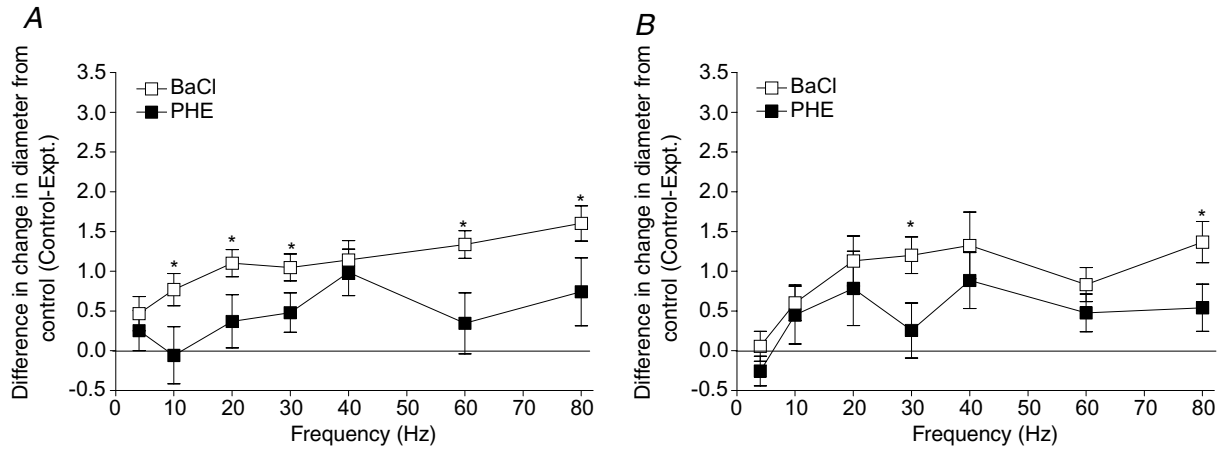
Figure 4. The effect of 5×10^{-5} M $BaCl_2$ (□) and 10^{-8} M PHE (■) on the change in diameter at 4 s (A) and 20 s (B) across all stimulus frequencies tested

The change in diameter is represented by the change in diameter under control conditions minus the change in diameter under experimental conditions (Control–Expt.). *Control change in diameter significantly different from diameter in the presence of $BaCl_2$. At 4 s the P values for main and interactive effects were: drug < 0.0001, frequency $= 0.0095$, and drug–frequency interaction $= 0.58$, and at 20 s these were 0.0044, 0.0045, 0.86, respectively.

The cremaster muscle has a vasculature architecture that differs from other more three dimensional muscles, but the control of capillary recruitment (Sarelius, 1986; Sweeney & Sarelius, 1989) is similar to other muscles whose architecture and capillary recruitment mechanisms have been studied (tibialis anterior: Lund *et al.* 1987; Delashaw & Duling, 1988; and gastrocnemius: Hilton *et al.* 1978). Therefore, the fact that the cremaster is composed of muscle fibres similar to other fast twitch muscles (such as EDL) and that it has capillary recruitment control mechanisms similar to other skeletal muscles make this a good model for the study of active hyperaemia and blood flow control in skeletal muscle.

The advantages of using this preparation to study the events initiating the rapid dilatation in response to skeletal muscle contraction are (1) that the cremaster is thin and ideal for visualization of all levels of the microvasculature via microscopy and small fibre bundles associated with any level of the vasculature can be targeted; (2) unlike thicker preparations, a few muscle fibres, anywhere in the preparation, can be stimulated to contract such that any level of the vasculature can be studied; and (3) only a very
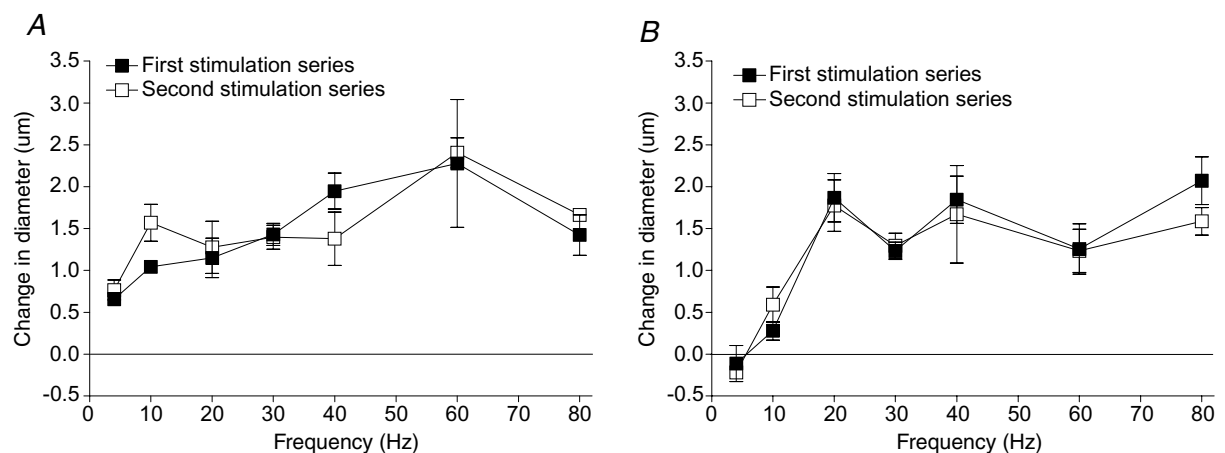

Figure 5. Time controls for the data in Figs 2–4

Skeletal muscle cells were stimulated over a range of stimulus frequencies (first stimulation series, ■) and then again following 30 min (second stimulation series, □). A, effect of stimulating the preparation twice with no treatment on the change in diameter at 4 s. B, effect of stimulating the preparation twice with no treatment on the change in diameter at 20 s. At 4 s the P values for main and interactive effects were: time $= 0.6143$, frequency $= < 0.0001$ and time–frequency interaction $= 0.6410$, and at 20 s these were 0.6143, < 0.0001, and 0.8720, respectively.

Table 2. *In vitro* force data (in grams) under control conditions and after BaCl$_2$, ouabain or DAP exposure at each stimulus frequency tested

Stimulus freq. (Hz)	DAP control‡	DAP	BaCl$_2$ control‡	BaCl$_2$	Ouabain control‡	Ouabain
4	0.8 ± 0.1	1.2 ± 0.3*	2.0 ± 0.2	1.5 ± 0.3	1.4 ± 0.3	0.9 ± 0.1
10	1.2 ± 0.2	1.9 ± 0.5*	3.4 ± 0.4	2.0 ± 0.6*	2.6 ± 0. 6	1.6 ± 0.3*
20	2.8 ± 0.4	4.0 ± 1.2	5.9 ± 0.5	3.1 ± 0.8*	3.9 ± 0.7	2.2 ± 0.4*
30	3.6 ± 0.5	4.6 ± 1.3	6.6 ± 0.5	3.5 ± 1. 0*	4.9 ± 0.9	2.6 ± 0.4*
40	4.2 ± 0.5	4.9 ± 1.3	7.3 ± 0.4	4.5 ± 1.3*	5.4 ± 1.0	3.3 ± 0.6*
60	4.7 ± 0.6	5.2 ± 1.3	7.9 ± 0.6	4.7 ± 1.3*	5.5 ± 1.0	2.9 ± 0.4*
80	4.6 ± 0.7	4.9 ± 1.3	7.7 ± 0.5	4.8 ± 1.3*	5.5 ± 1.1	3.4 ± 0.6*

‡Time and animal matched controls. *Significant difference from the time and animal matched controls.

small, localized region of a blood vessel can be affected, and therefore local dilatory events can be monitored in the absence of vascular compression and in the absence of interference of conducted dilatory responses from contracting muscles in other regions of the tissue (Berg *et al.* 1997; Murrant & Sarelius, 2000; Emerson & Segal, 2001) both of which can occur when contracting the whole muscle. This preparation is therefore ideal for studying the very early events of vasodilatation at the onset of muscle contraction and is ideal for identifying the initiators of active hyperaemia.

Significant differences were observed in the baseline diameter following BaCl$_2$ application as well as differences in maximal diameter between the PHE and ouabain groups. Differences in baseline diameter could result in changes in smooth muscle reactivity as a result of differences in the smooth muscle cell length. Because of the length–tension relationship in smooth muscle, the length of the smooth muscle can influence its ability to constrict or dilate to vasoactive substances (Davis & Gore, 1989; Seow, 2000). Differences in baseline diameter, along with differences in the maximal diameter, also changes the dilatory potential of the vessel (dilatory potential = maximal diameter − baseline diameter). We could not identify that either differences in baseline diameter or maximal diameter affected the behaviour of the blood vessel response to muscle contraction. We could not demonstrate strong correlations between the peak change in diameter and the baseline diameters under control condition or correlations between peak change in diameter and dilatory potential in this study as well as others (Mihok & Murrant, 2004; Murrant, 2005). Thus, the difference in baseline diameter and maximal diameter does not significantly impact the response of the vessels to muscle contraction or our interpretation of the data.

Effects of developed force *in vitro*

Force could not be measured directly in the *in situ* preparation. The *in vitro* experiments using hamster cremaster strips allow us to obtain a force response relative to that *in situ* for correct data interpretation. The *in vitro* developed force is expressed in Table 2 in grams, not normalized to muscle mass or normalized to cross-sectional area as is usual for *in vitro* skeletal muscle preparations. These normalizations create a large variability in the data as both normalizations use the weight of the muscle. The cremaster muscle consists of five to six layers of muscle cells in multiple orientations, only one of which will be aligned parallel to the ties on the end of the muscle and contributing to force generation. Therefore only about one-sixth of the weight will be contributing to force generation. Muscle strip weight is also variable as the ends of the muscle strip are tied first then the muscle strip is cut out leaving tissue at the sides of each preparation that is not included in the ties, and therefore not contributing to force but contributing to weight. Therefore, normalizations were not used in the expression of the data in Table 2 as they are misleading. If we were to normalize force to mN mm^{-2} (based on muscle length at optimal length in mm, weight in mg, and density of 1.06 g cm^{-3}) for BaCl$_2$ controls at 80 Hz the result is 27.1 ± 5.2 mN m^{-2} (average length 11.2 ± 0.03 mm, average weight 53.3 ± 24.2 mg). This force represents only-one sixth of the force that could be generated by a muscle of this weight, and therefore the predicted force generated would be 6 times this or 162.6 mN mm^{-2}, which is comparable to other *in vitro* skeletal muscle preparations (Brooks & Faulkner, 1988; Murrant & Barclay, 1995; McGuire *et al.* 2001; Mishima *et al.* 2005).

The dilatory responses did not correlate with force generated by the skeletal muscle cells themselves. We observed that both BaCl$_2$ and ouabain significantly inhibited force generation while DAP had a tendency to amplify force. BaCl$_2$ has been shown to inhibit K$^+$ influx and efflux via K$_{IR}$ channels on skeletal muscle cells with a pronounced decrease in contractile force and decrease in M-wave (compound action potential) area (Clausen & Overgaard, 2000). Ouabain has been shown to increase the rate of force decline within seconds of exposure (Nielsen & Clausen, 1996; Harrison *et al.* 1997) and also decreases the M-wave area (Ferguson *et al.* 2001).

The interference with excitability by both blockers is thought to be the cause of the decrease in force generation. DAP significantly increased force at 4 and 10 Hz and there was a non-significant trend to increase force at all other stimulus frequencies. DAP has been observed to increase contractile force (Van Lunteren & Moyer, 1996) possibly by prolonging the action potential. Both the delayed repolarization and a reduced accumulation of K^+ in the t-tubules are thought to be the mechanisms by which DAP may amplify force generation. Regardless of the specifics of the direct effects of the blockers on skeletal muscle we show that both $BaCl_2$ and ouabain significantly inhibited force generation, and therefore the reduced vascular reactivity in the presence of these blockers could be due to the reduced force production. Although we do demonstrate that $BaCl_2$ and ouabain significantly decreased force production we also show that DAP did not have an effect on force generation where as all three blockers significantly inhibited the vaso-dilatations induced by muscle contraction. Thus, there was no systematic change in diameter that correlated with the effects of the inhibitors on force generation and the changes in vasodilatation in the presence of the blockers may not be attributable to changes in force generation.

Inhibition of a source of K^+

We observed that the initial rapid dilatation at 4 s was inhibited across all stimulus frequencies with DAP and that the 20 s dilatation was not consistently inhibited. DAP was used to target inhibition of the skeletal muscle cells' K_v channels but both vascular smooth muscle cells and endothelial cells were exposed to DAP as well. At the vasculature level, DAP has been shown to cause depolarization and vasoconstriction of cerebral arterioles (Knot & Nelson, 1995), possibly through K_v, on smooth muscle themselves (Cheong *et al.* 2001*a*,*b*; Kerr *et al.* 2001; Thorneloe *et al.* 2001; Amberg *et al.* 2003). K_v channels have been identified on microvascular endothelial cells (Fan & Walsh, 1999; Cheong *et al.* 2001*a*,*b*; Hogg *et al.* 2002), but their physiological relevance has not been yet been determined. We did not observe a significant decrease in baseline diameter in the presence of DAP, and therefore the potential depolarization produced in our vessels with our concentration of DAP did not significantly depolarize our vessels. Thus the effect of depolarization with DAP via its effects on vascular smooth muscle or endothelial cells was considered minimal and the decrease in vasodilatation after DAP incubation was primarily attributed to blocking K_v channels on skeletal muscle cells.

Inhibition of the sites of action of K^+

We observed that both $BaCl_2$ and ouabain decreased contraction-induced vasodilatation at 4 s at all stimulus frequencies except 4 Hz and 40 Hz with $BaCl_2$. Ouabain also decreased the amount of dilatation at 20 s where a significant second dilatory event occurs at stimulus frequencies of 20, 30 and 40 Hz and above. The presence of a delayed second dilatation that is longer lasting implies that there is a second dilator present, acting on a different time scale. Our data indicate that K^+ may not be the primary dilator involved in the second dilatation, as K_V and K_{IR} channel blocking did not systematically inhibit the second dilatation. The dilator causing the 20 s dilatation may also be dependent on Na^+ K^+-ATPase as their inhibition systematically decreased the amount of dilatation as there may be overlap of signalling pathways when multiple dilators are involved. Determining the identity of the other dilators involved will be a necessary first step in determining the role of Na^+ K^+-ATPase in the second dilatation.

The primary target for $BaCl_2$ and ouabain were the vascular cells, but which cell type, endothelial cells or vascular smooth muscles cells, was the primary site of inhibition is unknown. K_{IR} channels and Na^+K^+-ATPase have been identified on both cell types (for review see Jackson, 2005) and both microvascular smooth muscle cells and endothelial cells are connected via gap junctions (Little *et al.* 1995); therefore changes in the membrane potential of one cell type can be reflected in changes in the other cell type (Beach *et al.* 1998; Emerson & Segal, 2000). Under these circumstances it is difficult to discern in which cell type the signals are initiated. Skeletal muscle cells were also exposed to the $BaCl_2$ and ouabain *in situ*, and we observed *in vitro* a significant decrease in force generated by skeletal muscle cells exposed to both of these inhibitors, probably as a result of an interference with excitability as discussed previously. Decreasing the excitability of the skeletal muscle cells may decrease the amount of K^+ efflux per action potential and therefore may contribute to the decreased dilatory response that we observed in the presence of these blockers. The effects of $BaCl_2$ and ouabain are similar to DAP at 4 s but not at 20 s. Regardless, the potential decrease in K^+ efflux from skeletal muscle as a result of ouabain and $BaCl_2$ incubation would still implicate K^+ in the dilatory response to muscle contraction.

Blocking K_{IR} channels with $BaCl_2$ produced a significant baseline vasoconstriction, probably the result of depolarization of smooth muscle cells (Edwards & Hirst, 1988; Hirst & Edwards, 1989). To ensure that the decrease in vasodilatory response was not strictly due to depolarization events, we used PHE to depolarize and constrict the vascular smooth muscle (Xia & Duling, 1995; Xia *et al.* 1995; Welsh & Segal, 1998) and mimic the depolarization and baseline constriction seen with $BaCl_2$. The relationship between membrane potential and diameter change for PHE is similar to that of other constrictors (high KCl) (Welsh & Segal, 1998). Although

there was a tendency for less dilatation to occur in the presence of PHE, there was greater inhibition with $BaCl_2$. Therefore, although depolarization may explain some of the effects of $BaCl_2$ it does not explain them all, and the totality of the data, from all blockers used in this study, support a role for K^+ in initiation of the rapid dilatation in response to skeletal muscle contraction.

The role of K^+ in active hyperaemia

Our data indicate that K^+ is one of the dilators involved in the rapid dilatation observed at the onset of contraction and that a single contraction, at any stimulus frequency, may yield enough K^+ to produce a vasodilatory response. We have identified a role for K^+ in the initiation of the rapid dilatation induced by a single muscle contraction, but its precise role in active hyperaemia, especially beyond a single contraction, will need to be further defined. There are indications that its dilatory role may be limited and confined to very early events of initiation. (1) K^+ has the ability promote a transient dilatation, and therefore depending on how long the contraction bout is the role for K^+ may diminish over time. (2) The contribution of K^+ to the overall amount of dilatation differs depending on the stimulus frequency used to stimulate the skeletal muscle cells. We have shown that the amount of overall vasodilatation attributable to K^+ is dependent on the stimulus frequency. At low stimulus frequencies (4 Hz), where the dilatation in response to muscle contraction is composed of only a single rapid peak, the contribution of K^+ will be much greater than the contribution of K^+ at higher stimulus frequencies where there appear to be multiple dilators (60 Hz) and K^+ plays less of a role in the total dilatation. Therefore, given the very transient ability of K^+ to dilate, and the variable contribution of its affects depending on stimulus frequency, the physiological role for K^+ in active hyperaemia, especially over time, is unknown. The literature does support a role for K^+ in the initiation of vasodilatory processes and our data support this role, but, given the very transient nature of the effects of K^+, its role may be confined to only the very early events of initiation.

We have shown that we could partially block the contraction induced dilation with the concentrations of DAP, $BaCl_2$ and ouabain used in this study. We also show that the concentrations of ouabain and $BaCl_2$ used in this study were effective at blocking approximately 50% of the dilatory response to 15 mM K^+. Although a 100% block is not necessary to demonstrate a role for K^+ in skeletal muscle contraction induced dilations, we do not know the full extent of the role of K^+. Our inability to fully inhibit the rapid dilatation to muscle contraction may be due to the concentrations of the drugs used and it is possible that higher concentrations of the blockers would have yielded full inhibition. Our inability to inhibit the

full response could also indicate that there are other vaso-dilators involved in the rapid dilatation induced by muscle contraction. Further studies are required to determine if more than one dilator is responsible for the rapid dilatation induced by skeletal muscle contraction.

In conclusion, we have shown that there is a role for K^+ in the rapid response to a single contraction in cremaster muscle. The role is detectable within 4 s of the contraction at all stimulus frequencies, but less defined in a secondary dilatation, by 20 s. Our data indicate that there are potentially multiple dilators involved in response to a single contraction and that K^+ is at least partially responsible for the very rapid component of this dilatation. What is yet to be determined is the role for K^+ over time and the physiological relevance of such a dilator whose dilatory ability is transient.

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