Effect of dissociating cytosolic calcium and metabolic rate on intracellular P_{O_2} kinetics in single frog myocytes

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The purpose of this investigation was to utilize 2,3-butanedione monoxime (BDM; an inhibitor of contractile activation) to dissociate cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) from the putative respiratory regulators that arise from muscle contraction-induced ATP utilization in order to determine the relative contribution of $[Ca^{2+}]_c$ on intracellular P_{O_2} (P_{iO_2}) kinetics during the transition from rest to contractions in single skeletal myocytes isolated from *Xenopus laevis* lumbrical muscle. Myocytes were subjected to electrically induced isometric tetanic contractions (0.25 Hz; 2-min bouts) while peak tension and either $[Ca^{2+}]_c$ ($n = 7$; ratiometric fluorescence microscopy) or P_{iO_2} ($n = 7$; phosphorescence microscopy) was measured continuously. Cells were studied under both control and 3 mM BDM conditions in randomized order. Initial (control, 100 ± 0%; BDM, 72.6 ± 4.6%), midpoint (control, 86.7 ± 1.8%; BDM, 61.6 ± 4.1%) and end (control, 85.0 ± 2.8%; BDM, 57.5 ± 5.0%) peak tensions (normalized to initial control values) were significantly reduced ($P < 0.05$) with BDM compared with control ($n = 14$). Despite the reduced peak tension, peak $[Ca^{2+}]_c$ was not altered ($P > 0.05$) between control and BDM trials. Thus, the peak tension-to-peak $[Ca^{2+}]_c$ ratio was reduced with BDM compared with control. The absolute fall in P_{iO_2} with contractions, which is proportional to the rise in $\dot{V}_{O_2}$, was significantly reduced with BDM (13.2 ± 1.3 mmHg) compared with control (22.0 ± 2.0 mmHg). However, P_{iO_2} onset kinetics (i.e. mean response time (MRT)) was not altered between BDM (66.8 ± 8.0 s) and control (64.9 ± 6.3 s) trials. Therefore, the initial rate of change (defined as the fall in P_{iO_2} /MRT) was significantly slower in BDM fibres compared with control. These data demonstrate in these isolated single skeletal muscle fibres that unchanged peak $[Ca^{2+}]_c$ in the face of reduced metabolic feedback from the contractile sites evoked with BDM did not alter P_{iO_2} onset kinetics in isolated single frog myocytes, suggesting that metabolic signals arising from the contractile sites play a more substantial role than $[Ca^{2+}]_c$ in the signalling pathway to oxidative phosphorylation during the transition from rest to repeated tetanic contractions.

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At the transition to an elevated muscle metabolic demand, ATP demand increases in immediate, square-wave fashion whereas the ATP contribution from oxidative phosphorylation is less rapid. The rapidity with which the mitochondria respond to an elevated metabolic stress will be inversely related to the reliance placed upon glycolysis and substrate-level phosphorylation to meet energetic demands. This is important as slowed oxygen uptake ($\dot{V}_{O_2}$) onset kinetics, which results in an increased O_2 deficit, culminates ultimately in a more rapid onset of fatigue. It has been suggested that the rate-limiting step to the rise in $\dot{V}_{O_2}$ at exercise onset is located within the myocyte and is not contingent upon residing O_2 availability (for review see Tschakovsky & Hughson, 1999; Grassi, 2000). However, O_2 may become limiting under extreme stresses such as very heavy, maximal exercise (Grassi, 2000) and also in disease states such as chronic heart failure where O_2 delivery is slowed (Meakins & Long, 1927; Hepple *et al.* 1999).

Currently, the exact site(s) of 'metabolic inertia' within the muscle remains obscure. However, muscle oxidative capacity is considered an integral determinant of $\dot{V}_{O_2}$ kinetics as it is well accepted that exercise training-induced mitogenesis results in faster $\dot{V}_{O_2}$ kinetics (Hagberg *et al.* 1980). Within the mitochondria, specific putative sites of limitation include cytochrome c oxidase (Kindig *et al.* 2001, 2002; Jones *et al.* 2003), and more controversially, pyruvate dehydrogenase (PDH; Timmons *et al.* 1998;

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Howlett *et al.* 1999; Grassi *et al.* 2002; Rossiter *et al.* 2003; Howlett & Hogan, 2003). Important roles for the NADH/NAD^+ ratio, phosphorylation potential, creatine (Cr) and phosphocreatine (PCr), ADP and inorganic phosphate (P_i) in a cytosolic signalling pathway between sites of ATP hydrolysis and production have been postulated (for review see Mahler, 1985; Balaban, 1990; Meyer & Foley, 1996). Mahler (1985) suggested that the mitochondrial creatine kinase (CK) reaction may be a rate-limiting step in this process. Recently, our laboratory demonstrated that the CK-catalysed breakdown of PCr at exercise onset does play an important role in regulating $\dot{V}_{O_2}$ kinetics (Kindig *et al.* 2004). In addition, Ca^{2+} released from the sarcoplasmic reticulum (SR) during contractile activation has been implicated as a rapid modulator of mitochondrial ATP production and thus has been postulated to play a role in setting the $\dot{V}_{O_2}$ in response to increased energy demand (for review see Hansford, 1994; Balaban, 2002). Indeed, Ca^{2+} has been shown to activate several calcium-sensitive dehyrogenases (Hansford, 1994) including PDH (Randle *et al.* 1974) which will act to maximize the NADH/NAD^+ ratio. In mitochondria isolated from skeletal muscle, elevating $[Ca^{2+}]$ within the physiological range stimulated mitochondrial respiratory rate directly, regardless of the residing substrate (Kavanagh *et al.* 2000). Recently, Territo *et al.* (2001) demonstrated, in cardiac mitochondria, that raising extramitochondrial Ca^{2+} levels resulted in elevated mitochondrial $[Ca^{2+}]$ and ATP production concomitantly in < 200 ms

In the current investigation, we utilized BDM in order to reduce peak tension (and ATP utilization) yet maintain peak cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$). In doing so, $[Ca^{2+}]_c$ was dissociated from the metabolites ([ADP], $[P_i]$, etc.) arising from the ATP utilization at the contractile sites such that $[Ca^{2+}]_c$ will be increased relative to the rise in the other cytosolic signalling pathway components. The purpose of this investigation was to study this effect on intracellular P_{O_2} (P_{iO_2}) kinetics at the onset of moderate intensity contractions in intact single myocytes isolated from frog muscle. We tested the hypothesis that if $[Ca^{2+}]_c$ is the primary determinant for increased oxidative phosphorylation at the transition from rest to contractions in frog skeletal muscle, then a similar initial rate of change of oxidative phosphorylation in the face of reduced metabolic demand with BDM would result in overall faster P_{iO_2} kinetics of individual fibres (mean response time (MRT)) compared with a matched control trial. Alternatively, if the oxidative phosphorylation activation rate is proportional to the metabolic signals arising directly from contractile site ATP utilization, then the initial rate of change of oxidative phosphorylation would be decreased with BDM, resulting in unchanged MRT between BDM and control trials.

Methods

Female adult *Xenopus laevis* were used in this investigation. All procedures were approved by the University of California–San Diego animal use and care committee and conform to National Institutes of Health standards.

Myocyte preparation

Single muscle cells ($n = 14$) were isolated and prepared as previously described (Hogan, 1999). Briefly, frogs were submerged in chilled water, then stunned, decapitated and pithed, and the lumbrical muscles (II–IV) were removed from the hind feet. Single myocytes were dissected out with tendons intact in a chamber of physiological Ringer solution at a pH = 7.0. Cells were injected via micropipette pressure injection (PV830 pneumatic pico-pump, World Precision Instruments, Sarasota, FL, USA) with either a solution consisting of 0.5 mM Pd-*meso*-tetra (4-carboxyphenyl) porphine bound to bovine serum albumin (for phosphorescence quenching) and the Ca^{2+} indicator dye fura 2 (10 mM; Molecular Probes, Eugene, OR, USA) or fura 2 alone (for fluorescence microscopy).

Experimental protocol

Platinum clips were attached to the tendons of each myocyte to facilitate fibre positioning within the Ringer solution-filled chamber. One tendon was fixed, whereas the contralateral was attached to an adjustable force transducer (model 400A, Aurora Scientific, Aurora, Ontario, Canada), allowing the muscle to be set at optimum muscle length. The analog signal from the force transducer was recorded via a data acquisition system (AcqKnowledge, Biopac Systems, Santa Barbara, CA, USA) for subsequent analysis. Fibres were perfused throughout the experiment with Ringer solution equilibrated with 5% CO_2 and 4% O_2 in N_2 balance. Constant perfusion was maintained throughout the protocol to maintain the extracellular P_{O_2} at ~30 Torr and to reduce the occurrence of an appreciable unstirred layer surrounding the cell. Tetanic contractions were elicited using direct (8–10 V) stimulation of the muscle (model S48, Grass Instruments, Warwick, RI, USA). The stimulation protocol consisted of ~250 ms trains of 70 Hz impulses of 1 ms duration. Myocytes were subjected to trials of ~120 s at a ~0.25 Hz stimulation frequency with a 15 min recovery period between trials.

Separate experiments were performed to obtain either the $[Ca^{2+}]_c$ ($n = 7$) or P_{O_2} ($n = 7$) response to contractions under control and matched 3 mM BDM conditions. The effects of BDM on force production in skeletal muscle are fully reversible (Sun *et al.* 2001), so all experiments were randomized. One trial per treatment per cell was obtained for the $[Ca^{2+}]_c$ data whereas two trials per treatment per cell were acquired for all P_{O_2} data. In the latter, the

two control and two BDM trials were averaged for data analysis.

Cytosolic [Ca^{2+}] measurement

$[Ca^{2+}]_c$ was measured using an epifluorescent microscope system that consisted of a Nikon inverted microscope with a × 40 fluor objective and a DeltaScan illumination and detection system (Photon Technology International, South Brunswick, NJ, USA) as previously described (Stary & Hogan, 2000). Fibres injected with fura 2 were illuminated sequentially (20 Hz) with two excitation wavelengths of 340 and 380 nm, and the resulting fluorescence emission was measured at 510 nm. The ratio of 340/380 nm fluorescence was used to obtain the Ca^{2+}-dependent signal (Grynkiewicz *et al.* 1985).

Assessment of P_{iO_2}

Each myocyte was observed with a Nikon × 40 fluor objective (0.70 numerical aperture). The phosphorescence quenching of the porphyrin compound within the myocyte was measured via a system consisting of a flash lamp (Oxygen Enterprises, Philadelphia, PA, USA), a 425 nm band-pass excitation filter, a 630 nm cut-on emission filter, and a photomultiplier tube for collection of the phosphorescence signal. To calculate phosphorescence lifetimes from the intracellular O_2 probe, the phosphorescent decay curves from a series of 10 flashes (15 Hz) were averaged, and a mono-exponential function was fitted to the subsequent best-fit decay curve (analysis software from Medical Systems, Greenvale, NY, USA). The O_2 dependence of phosphorescence quenching is described by the Stern-Volmer equation where:

$$\tau_o/\tau = 1 + k_q \tau_o P_{O_2},$$

thus

$$P_{O_2} = (\tau_o/\tau - 1)/(k_q \tau_o),$$

where τo and τ are the phosphorescence lifetimes at anoxia and a given P_{O_2}, respectively, and k_q, the quenching constant (in Torr s^{-1}), is a second-order rate constant that is related to the frequency of collisions between O_2 and the excited triplet state of the porphyrin and the probability of energy transfer when collisions occur. The constants k_q and τ_o were set at 690 mmHg s^{-1} and 100 μs for Pd-*meso*-tetra (4-carboxyphenyl) porphine bound to albumin in solution for this preparation as established previously (Hogan, 1999). Phosphorescent decay curves were recorded every 4 s from each cell throughout the experimental period.

Data and statistical analysis

Following experimental procedures, the mean response time (MRT) was calculated as the time to 63% of the fall in P_{iO_2} with contractions. Both peak tension and $[Ca^{2+}]_c$ data were normalized to the initial control point. Data are presented as mean ± S.E.M. Differences between trials in regard to the P_{iO_2} fall and MRT were tested via a paired *t* test. Changes in peak tension and $[Ca^{2+}]_c$ were tested via a repeated measures 2-way ANOVA. When significant *F* values were present, the Bonferroni *post hoc* test was employed for determination of between-group differences. Statistical significance was accepted at $P < 0.05$.

Results

In Fig. 1, tension and P_{iO_2} data for one representative cell over four contractions bouts (2 control and 2 BDM) are shown. BDM induced a significant reduction in peak tension ($P < 0.05$) compared with control

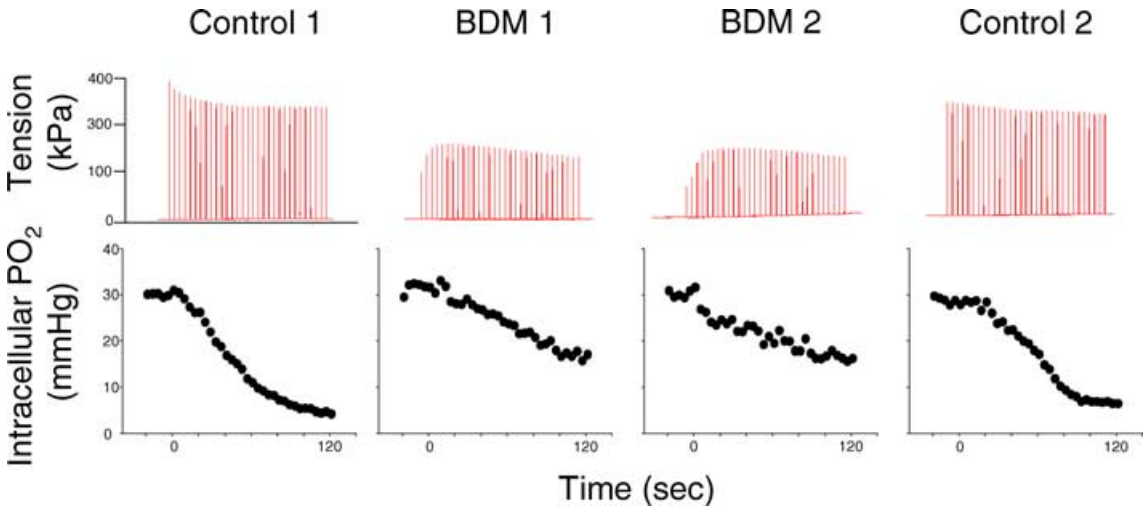

Figure 1. Data for a representative myocyte
Tension (upper panel) and intracellular P_{O_2} (lower panel) for one representative single myocyte at rest and during a 2-min bout of isometric tetanic contractions under control and 3 mM BDM conditions.

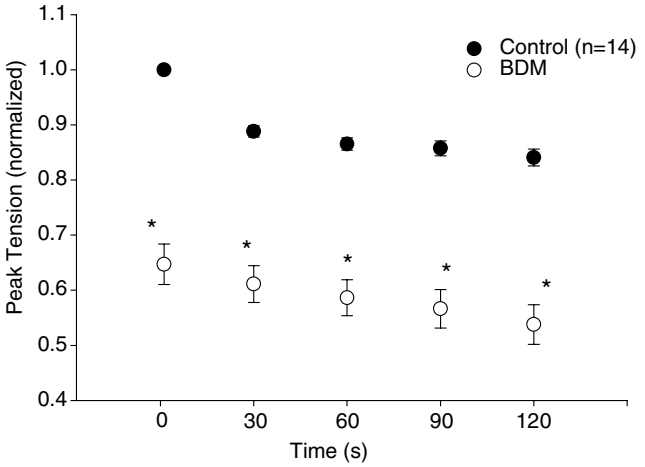

Figure 2. Mean (± S.E.M.) peak tension for control and BDM trials
Peak tension, normalized to initial control values, was significantly reduced (* $P < 0.05$) throughout duration of the isometric contractions bout in BDM compared with the control trial. Peak tension data were pooled from both the $[Ca^{2+}]_c$ ($n = 7$) and P_{iO_2} ($n = 7$) experiments.

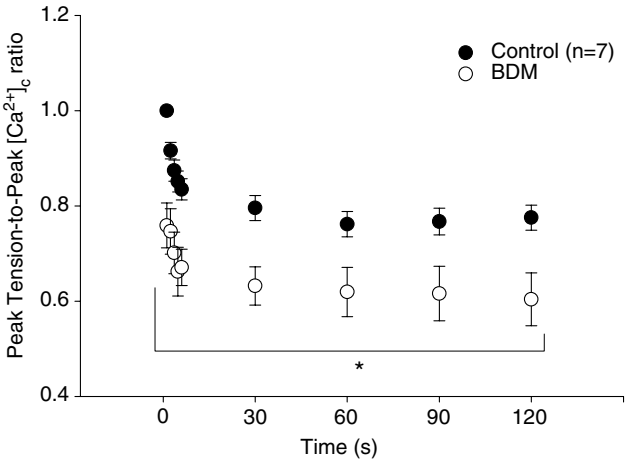

Figure 4. The peak tension-to-peak cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) ratio for control and matched BDM trials
The peak tension-to-peak $[Ca^{2+}]_c$ ratio was significantly reduced (* $P < 0.05$) across a 2-min bout of isometric tetanic contractions in BDM compared with control.

over the duration of the contractions bout ($n = 14$; Fig. 2). Specifically, initial (control, $100 \pm 0\%$; BDM, $72.6 \pm 4.6\%$), midpoint (control, $86.7 \pm 1.8\%$; BDM, $61.6 \pm 4.1\%$) and end (control, $85.0 \pm 2.8\%$; BDM, $57.5 \pm 5.0\%$) peak tensions (normalized to initial control values) were significantly reduced with BDM compared with control.

Despite the significant loss of peak tension from BDM administration, peak $[Ca^{2+}]_c$ was not altered ($P > 0.05$) between control and BDM trials ($n = 7$; Fig. 3). Accordingly, the peak tension-to-peak $[Ca^{2+}]_c$ was reduced ($n = 7$; $P < 0.05$) with BDM compared with control across the duration of the contractions bout (Fig. 4). Neither resting nor baseline $[Ca^{2+}]_c$ values during contractions differed ($P > 0.05$) between control and BDM trials.

As shown in Fig. 5 (left panel), the fall in P_{iO_2} from resting baseline to the end of contractions was significantly reduced ($P < 0.05$) with BDM (13.2 ± 1.3 mmHg) compared with control (22.0 ± 2.0 mmHg). There was a trend ($P = 0.09$) for the ratio of the fall in peak tension with BDM (0.63 ± 0.03) to be greater than the ratio of the fall in P_{iO_2} (0.52 ± 0.06), indicative of a greater aerobic cost per contractions in the BDM trial compared with control. Additionally, P_{iO_2} MRT was not altered ($P > 0.05$) between BDM (66.8 ± 8.0 s) and control (64.9 ± 6.3 s) trials (Fig. 5, right panel). A normalization of the MRT to P_{iO_2} reductions yields an initial rate of change (fall in P_{iO_2} /MRT), which was significantly faster ($P < 0.05$) in control *versus* BDM as clearly seen in Fig. 1.

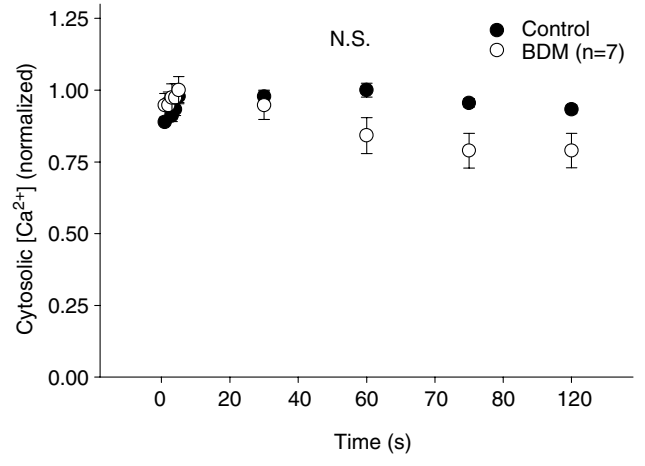

Figure 3. Peak cytosolic $[Ca^{2+}]$ for control and BDM trials
Peak cytosolic $[Ca^{2+}]$ (mean ± S.E.M.), normalized to initial contraction values, was not different (N.S.; $P > 0.05$) between BDM and matched control across a 2-min bout of isometric tetanic contractions.

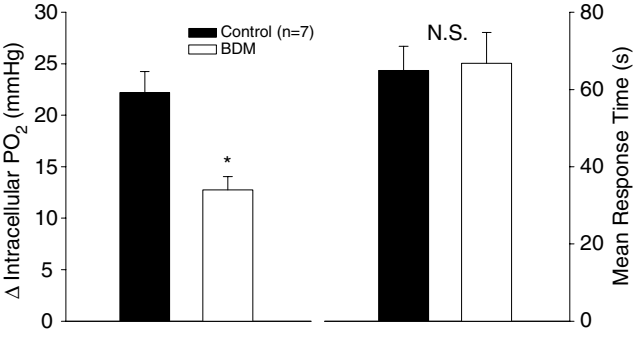

Figure 5. Intracellular P_{O_2} (P_{iO_2}) data for BDM and control trials
The fall in P_{iO_2} with contractions was significantly greater (* $P < 0.05$) in control compared with the BDM trial (left panel). The mean response time for the fall in P_{iO_2} with not different (N.S.; $P > 0.05$) between control and BDM trials (right panel).

Discussion

Cytosolic Ca^{2+} has been suggested to be a key mitochondrial signalling factor in muscle. In the current investigation, BDM was employed to reduce peak tension (and ATP utilization) yet maintain peak $[Ca^{2+}]_c$. Thus, peak $[Ca^{2+}]_c$ was dissociated from the cytosolic signalling metabolites ([ADP], [Pi], etc.) arising from contraction-induced ATP utilization such that $[Ca^{2+}]_c$ was greater relative to the rise in the other cytosolic signalling processes. The key and novel finding from the current investigation is that BDM administration did not alter the P_{iO_2} MRT compared with control. In fact, the initial rate of change (i.e. fall in P_{iO_2} /MRT) was actually faster in control compared with the BDM trial in concert with apparent first-order metabolic control of oxidative phosphorylation by metabolic signals arising from the contractile sites (Mahler, 1985; Meyer, 1988; C.A. Kindig, R.A. Howlett & M.C. Hogan, unpublished observations). These data demonstrate that $[Ca^{2+}]_c$, in itself, does not exert control over the speed of the oxidative phosphorylation increase in response to repetitive tetanic contractions and suggest that $[Ca^{2+}]_c$ may not be an integral metabolic signalling component for oxidative phosphorylation in this isolated single skeletal muscle model.

Methodological considerations

A direct relationship between $\dot{V}_{O_2}$ and P_{iO_2} for single myocytes lacking myoglobin, such as in *Xenopus* muscle, is described by Fick's law of diffusion where:

$$\dot{V}_{O_2} = \dot{D}_{O_2}(\text{extracellular}P_{O_2} - \text{intracellular}P_{O_2})$$

where $\dot{D}_{O_2}$ is the rate of diffusion of O_2 and extracellular P_{O_2} is the external partial pressure of oxygen.

For our single myocyte preparation, this relationship has been verified previously (Howlett & Hogan, 2001). In intact muscle, the capillary (more specifically, red blood cell)-to-myocyte interface determines muscle-diffusing O_2 capacity and thus $\dot{D}_{O_2}$ is a dynamic variable (Federspiel & Popel, 1986; Mathieu-Costello *et al.* 1991). In this preparation, $\dot{D}_{O_2}$ represents the muscle O_2 diffusion coefficient, which at the temperature of our experimental set-up, is $1.01 \times 10^5\ cm^2\ s^{-1}$ (Mahler *et al.* 1985) and remains unchanged across the rest–contractions transition. Thus, in our single muscle fibre preparation, the fall in P_{iO_2} reflects linearly the rise in $\dot{V}_{O_2}$. One consideration in the present study using the isolated single *Xenopus* skeletal muscle fibre preparation is that these large myoglobin-free fibres may yield slightly different $\dot{V}_{O_2}$ onset kinetics compared with mammalian skeletal muscle which have a much smaller diameter and contain myoglobin.

BDM, a drug with phosphatase-like activity, apparently affects anuran and mammalian muscle differently in terms of specific sites of action (Sellin & McArdle, 1994). In frog muscle, at concentrations greater than 2 mM, BDM has been shown to have a direct effect on the contractile apparatus where it inhibits cross-bridge cycling, whereas at concentrations greater than 5 mM BDM may impair SR Ca^{2+} release (Sun *et al.* 2001). In the current investigation, BDM was titrated at 3 mM in order to significantly reduce peak tension production (Figs 1 and 2) yet not affect SR Ca^{2+} handling (i.e. not alter peak $[Ca^{2+}]_c$ with contractions; Fig. 3).

In the current investigation, there was a trend ($P = 0.09$) toward a greater specific aerobic cost of contractions (absolute fall in P_{iO_2} /force) in BDM compared with the control trial. This would be consistent with an increased (relative to control) energetic cost of Ca^{2+}-ATPase activity with BDM in the face of reduced mechanical work which is consistent with a significant ATP cost of SR Ca^{2+} handling as has been demonstrated previously in dog muscle (Hogan *et al.* 1998).

Cytosolic Ca^{2+}

Cytosolic Ca^{2+} is believed to control the two major ATPase reactions in the myocyte, myosin-ATPase, through its interaction with troponin, and Ca^{2+}-ATPase of the SR. There are many targets for Ca^{2+}-induced activation of oxidative phosphorylation. It is well-established that Ca^{2+} activates several calcium-sensitive dehydrogenases located within the permeability barrier of the inner mitochondrial membrane including pyruvate, isocitrate and 2-oxogluturate (fro review see Hansford, 1994). Additionally, it is thought that glycerol 3-phosphate dehydrogenase, which is located on the outer surface of the inner mitochondrial membrane and thus exposed to the cytosolic environment, is also sensitive to $[Ca^{2+}]$ (Hansford & Chappell, 1967). Furthermore, it has been demonstrated that Ca^{2+} directly activates oxidative phosphorylation in mitochondria isolated from skeletal muscle (Kavanaugh *et al.* 2000). These studies have made Ca^{2+} an attractive candidate as a putative controlling signal of oxidative phosphorylation in cardiac and skeletal muscle (for review see Hansford, 1994; Balaban, 2002).

The time delay prior to a discernible rise in $\dot{V}_{O_2}$ at the transition to an elevation in metabolic demand is thought to occur within a matter of seconds, if not almost immediately (Bangsbo *et al.* 2000; Behnke *et al.* 2002; Kindig *et al.* 2003*b*). Thus, from the above, it would appear most likely, although not necessary, that for Ca^{2+} to modulate $\dot{V}_{O_2}$ onset kinetics, Ca^{2+} would have to move from the cytosol into the mitochondria in a similar time course and, also, over the brief contractile transient when cytosolic levels are elevated.

Mitochondria have been shown to take up Ca^{2+} within the time scale of physiological Ca^{2+} pulses (Sparagna *et al.* 1995; Duchen *et al.* 1998). This Ca^{2+} uptake is mediated by a uniporter and is driven by a large potential difference across the inner mitochondrial membrane (Duchen *et al.* 1998). In mitochondria isolated from cardiac muscle, Territo *et al.* (2001) demonstrated that mitochondrial $[Ca^{2+}]$ and ATP production increases to step increases in extramitochondrial $[Ca^{2+}]$ occurred within 150 ms. In muscle fibres isolated from rat soleus, Bruton *et al.* (2003) demonstrated that Ca^{2+} uptake into the mitochondria increased after a single tetanic contraction. Most pertinent to the current investigation, Lannergren *et al.* (2001) demonstrated that, in contracting single *Xenopus* myocytes, mitochondrial $[Ca^{2+}]$ had increased markedly within the first 10 tetanic contractions. However, how rapidly Ca^{2+} within the mitochondria was detected and the time course of that rise was not reported (Lannergren *et al.* 2001). Thus, as a whole, these data suggest that mitochondrial $[Ca^{2+}]$ increases in a manner such that it theoretically might play a role in modulating the rise in $\dot{V}_{O_2}$ at the transition to elevated metabolic demand.

In the present investigation, mitochondrial $[Ca^{2+}]$ was not measured. However, as BDM has no known effect on mitochondrial Ca^{2+} uptake, if it is assumed that mitochondrial $[Ca^{2+}]$ is largely contingent upon residing cytosolic values (as discussed above), then in the current investigation, both would be similar under control and BDM conditions (see Fig. 3). Despite the elevated $[Ca^{2+}]_c$-to-metabolic demand ratio with BDM application, the P_{iO_2} MRT did not differ from control values (Fig. 5). This would initially appear to be in conflict with investigations of metabolic control by Ca^{2+} in cardiac muscle (for review see Balaban, 2002). However, in heart muscle, unlike skeletal muscle (as discussed below), key regulatory feedback metabolites including [Cr], [ADP], $[P_i]$ and [NADH] remain nearly constant over a wide range of metabolic rates (for review see Balaban, 2002).

Putative controllers of oxidative phosphorylation in skeletal muscle

The data presented herein demonstrate that unchanged $[Ca^{2+}]_c$, in the face of reduced cytosolic metabolite signals commensurate with the reduced peak tension and ATP utilization, does not alter the P_{iO_2} MRT, at least in frog skeletal muscle. What singular component is responsible for the 'metabolic inertia' seen at the transition to an elevated metabolic demand is not clear. Unlike that reported in cardiac muscle, alterations in key skeletal muscle cytosolic signals such as ADP, P_i, NADH and PCr occur almost instantaneously at the onset of exercise and tend to change in linear/quasi-linear fashion with increases in exercise intensity. Thus putative mechanisms for metabolic control include: kinetic limitation by cytosolic [ADP] and/or $[P_i]$ in accordance with Michaelis-Menten kinetics (Chance & Williams, 1955), non-equilibrium thermodynamic control via the phosphorylation potential (i.e. $[ATP]/[ADP][P_i]$) and electron transport chain redox potential (i.e. [NADH]/[NAD]), and alterations in Gibbs free energy of cytoplasmic ATP hydrolysis (for review see Balaban, 1990; Meyer & Foley, 1996). Additionally, it was demonstrated recently that [PCr] and the [PCr]/[Cr] ratio may play an important role in the regulation of mitochondrial ADP-stimulated respiration (Walsh *et al.* 2001). An attractive hypothesis is that mitochondrial respiration may be controlled by local production of ADP via CK-catalysed breakdown of PCr (e.g. Mahler, 1985). Additionally, the regulatory effect of $[O_2]$ on oxidative phosphorylation during exercise is an issue that remains currently unresolved due, in part, to differences associated with work intensity and the organ/system studied (for review see Tschakovsky & Hughson, 1999; Grassi, 2000). Within isolated mitochondria, as P_{O_2} falls across a physiological range, cytochrome c is reduced, whereas $\dot{V}_{O_2}$ remains uniform to levels near 1 mmHg (Wilson *et al.* 1983; Wilson & Rumsey, 1988). In intact, exercising muscle, the microcirculatory P_{O_2} considered rate limiting is dependent upon the capillary-to-myocyte interface, fibre type and mitochondrial capacity. For the purposes of the current study, extracellular P_{O_2} was tightly controlled at 30 mmHg which is well above that considered 'rate limiting' at the onset of contractions in this preparation (Hogan, 2001; Kindig *et al.* 2003*a*).

Regardless of the exact mechanism(s) that ultimately determines the speed of the rise in oxidative phosphorylation at exercise onset, the data in the current investigation support the proposition that muscle respiration at the transition to an altered metabolic demand is under first-order control (Mahler, 1985; Meyer, 1988). First-order respiratory control requires a similar time course for $\dot{V}_{O_2}$ onset kinetics to step changes in metabolism and a time course (i.e. MRT) independent of submaximal ATP demand. Recently, our laboratory demonstrated that altering metabolic demand in single contracting frog fibres by altering the contraction duration did not affect the P_{iO_2} MRT (C.A. Kindig, R.A. Howlett & M.C. Hogan, unpublished observations). However, the initial rate of P_{iO_2} change was greater in the trial with the augmented contraction duration, demonstrating that, in accordance with a greater initial impulse (i.e. larger signal for augmented respiration) with increased contractile duration, the available mitochondria were able to respond more rapidly to the higher metabolic rate (C.A. Kindig, R.A. Howlett & M.C. Hogan, unpublished observations). Similarly (and as clearly seen in Fig. 1) the MRT was not different between control and

BDM trials in the current investigation, whereas the initial rate of change was more rapid in the trial with the greater metabolic demand (i.e. control > BDM). Although the potential for compensatory mechanisms affecting the MRT cannot be dismissed, the present study suggests that the respiratory control signals that originate from myosin-ATPase activity are a greater determinant of P_{iO_2} onset kinetics than is a relative increase in $[Ca^{2+}]_c$ in these isolated single skeletal muscle fibres.

Summary

The pharmacologic agent BDM reduced peak tension and thus ATP utilization yet did not affect peak $[Ca^{2+}]_c$ in contracting single frog myocytes. Hence, BDM peak $[Ca^{2+}]_c$ was dissociated from the cytosolic signalling metabolites (i.e. reduced [ADP], $[P_i]$, etc. commensurate with the reduced peak tension) such that $[Ca^{2+}]_c$ was greater relative to the rise in the other cytosolic signalling processes. While BDM reduced the fall in P_{iO_2} with contractions in a manner similar to the loss of peak tension, this manipulation did not alter the P_{iO_2} MRT. With invariant MRTs and different metabolic rates, the initial rate of P_{iO_2} change was faster in control compared with the BDM trial, suggesting that $\dot{V}_{O_2}$ on-kinetics in isolated frog skeletal muscle fibres are significantly more contingent upon metabolic signals from the contractile sites compared with $[Ca^{2+}]_c$.

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