Trimetazidine reduces basal cytosolic Ca\(^{2+}\) concentration during hypoxia in single *Xenopus* skeletal myocytes

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(Manuscript received 16 October 2002; accepted 26 February 2003)

We tested the hypotheses that: (1) Ca\(^{2+}\) handling and force production would be irreversibly altered in skeletal muscle during steady-state contractions when subjected to severe, prolonged hypoxia and subsequent reoxygenation; and (2) application of the cardio-protective drug trimetazidine would attenuate these alterations. Single, living skeletal muscle fibres from *Xenopus laevis* were injected with the Ca\(^{2+}\) indicator fura 2, and incubated for 1 h prior to stimulation in 100 μM TMZ–Ringer solution (TMZ; \(n = 6\)) or standard Ringer solution (CON; \(n = 6\)). Force and relative free cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_c\)) were measured during continuous tetanic contractions produced every 5 s as fibres were sequentially perfused in the following manner: 3 min high extracellular \(P_O2\) (159 mmHg), 15 min hypoxic perfusion (3–5 mmHg) then 3 min high \(P_O2\). Hypoxia caused a decrease in force and peak [Ca\(^{2+}\)], in both the TMZ and CON fibres, with no significant (\(P < 0.05\)) difference between groups. However, basal [Ca\(^{2+}\)], was significantly lower during hypoxia in the TMZ group vs. the CON group. While reoxygenation generated only modest recovery of relative force and peak [Ca\(^{2+}\)], in both groups, basal [Ca\(^{2+}\)], remained significantly less in the TMZ group. These results demonstrated that in contracting, single skeletal muscle fibres, TMZ prevented increases in basal [Ca\(^{2+}\)], generated during a severe hypoxic insult and subsequent reoxygenation, yet failed to protect the cell from the deleterious effects of prolonged hypoxia followed by reoxygenation. *Experimental Physiology* (2003) 88.3, 415–421.

It has been demonstrated in cardiac (Opie, 1976) and whole skeletal muscle (Hogan et al. 1992, 1996) that decreases in O\(_2\) availability during steady-state contractions can compromise intracellular homeostasis and impair contractility. However, the intracellular factors that modulate such hypoxia-associated reductions in muscle contractility remain largely undetermined. Studies (for review see Westerblad et al. 1991) investigating high-intensity fatigue in isolated, single skeletal muscle fibre preparations, in which the frequency of contractions is increased until fatigue is induced, have provided evidence that impairment of contractility is primarily associated with changes in intracellular Ca\(^{2+}\) handling. These changes include decreases in Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) and decreased SR Ca\(^{2+}\) reuptake (Westerblad et al. 1991). Recently we demonstrated in contracting single skeletal muscle fibres that the earlier onset of fatigue induced by conditions of low O\(_2\) availability was associated with alterations in Ca\(^{2+}\) handling that were comparable to those seen during fatigue when O\(_2\) availability was greater (Stary & Hogan, 2000a). Similar alterations in Ca\(^{2+}\) handling during hypoxia followed by reoxygenation (H/R) have been demonstrated in cardiac muscle (Opie, 1976; Park & Lucchesi, 1999).

It has been shown that trimetazidine (TMZ), or 1-(2,3,4-trimethoxybenzyl)-piperazine, protects against cellular injury and improves the rate of survivability during periods of imposed H/R in cardiac muscle (Toda et al. 1979; Fitoussi et al. 1985; Lavanchy et al. 1987; Allibardi et al. 1998). It has recently been demonstrated that TMZ directly affects metabolic substrate utilization (Kantor et al. 2000), and it has subsequently been suggested that TMZ exerts its protective effects exclusively through this mechanism (Lewandowskii, 2000). However, previous evidence that TMZ modulates free cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_c\)) during hypoxia (Renaud, 1988), and recent reports that TMZ exhibits specific mitochondrial (Morin et al. 1998) and cytosolic binding properties (Morin et al. 2000) suggest that the pharmacological properties of TMZ may be more varied.
The intact, single skeletal muscle fibre preparation used in the present study is well suited for investigating discrete intracellular events and the specific effects of pharmacological compounds in skeletal muscle. Problems associated with blood flow and heterogeneity of fibre type in isolated whole organ and whole muscle preparations, which make an accurate quantification of both drug delivery and O2 availability at the cellular level difficult, are eliminated with the single fibre preparation. Additionally, by utilizing fluorescence microscopy, direct, non-invasive intracellular measurements can be made simultaneously with measurements of cellular function. Therefore, the purpose of the present study was: (1) to determine whether a 15 min period of severe hypoxia and subsequent reoxygenation alters steady-state contractility and intracellular Ca2+ handling in contracting single skeletal muscle fibres; and (2) to ascertain whether exposure to TMZ attenuates these alterations.

METHODS

Experimental preparation
Adult female *Xenopus laevis* were killed by double pithing and decapitation according to national ethical guidelines. Lumbrical muscles II–IV were removed, and single living muscle fibres of type 2 or 3 (fatigue resistant) were micro-dissected from the muscle according to size and appearance under dark-field illumination (van der Laarse & Diegenbach, 1991). Dissections and experiments were performed in standard Ringer solution containing (mM): NaCl 112, KCl 1.87, CaCl2 0.82, NaHCO3 2.38, NaH2PO4 0.07, EGTA 0.1 and glucose 5; pH 7.0 at 20°C. Following dissection, platinum clips were attached to the tendons, and the fibres were mounted in a glass chamber and placed on the stage of an inverted microscope configured for epillumination. Fibres were then pressure injected with the fluorescent Ca2+ indicator fura 2 (Molecular Probes). Following injection, fibres were incubated in either Ringer solution containing 100 μM TMZ (TMZ; n = 6) or control Ringer solution (CON; n = 6) for 1 h. All solutions in TMZ experiments contained 100 μM TMZ. Low P02 Ringer superfusate was generated by aeration with N2 until the desired P02 (< 5 mmHg) was achieved. The P02 of the Ringer solution in the chamber was monitored with a Clark-style electrode (Diamond General, Model 733, Ann Arbor, MI, USA) placed adjacent to the working fibre.

Experimental protocol
Tetanic contractions were induced by direct stimulation (50 impulses s⁻¹ of 1 ms duration at 9 V, with a train duration of 200 ms) with platinum conducting electrodes on either side of the fibre, using a Grass S48 stimulator (Quincy, MA, USA). Fibres were stimulated to produce tetani every 5 s for the duration of the contractile period, and constant perfusion was maintained during each contractile period to maintain the experimental P02 and to reduce the possible occurrence of unstirred layers surrounding the cell. Following the onset of contractions, all fibres were stimulated for a 3 min (equilibration) period at high P02. After 3 min, the superfusate was rapidly (within 15 s) switched to low P02 (hypoxia; 3–5 mmHg) for a 15 min period, followed by a rapid switch back to high P02 (reoxygenation). Reoxygenation was maintained until force ceased to recover further.

Measurements
Force and intracellular Ca2+ concentration measurements were obtained for the entire duration of the contractile period. Force development was measured with a force transducer system (Aurora Scientific, Model 400A, Aurora, Ontario, Canada). A Biopac Systems MP100WSW (Santa Barbara, CA, USA) A–D converter was used to transform the analog force signal, and the digital data were collected and analysed with AcqKnowledge III v3.5 software (Biopac Systems). All waveform analyses were performed using AcqKnowledge III software. Individual peak tensions were compared to the highest peak tension within the total experimental time period.

Relative [Ca2+], was obtained continuously using fluorescence spectroscopy. Dual wavelength ratiometric determinations were employed to eliminate signal artifacts due to variations in emitted light intensity, such as movement. Fibres were illuminated with two rapidly alternating (20 Hz) excitation wavelengths of 340 nm and 380 nm, and the resulting fluorescence emissions at 510 nm were divided (340 nm/380 nm) to obtain the Ca2+-dependent ratiometric signal (Grynkiewicz et al. 1985). Fluorescence was measured with a Photon Technology International illumination and detection system (Deltascan model, Brunswick, NJ, USA), integrated with a Nikon inverted microscope with a ×40 Fluor objective. Relative [Ca2+]i measurements were standardized in a fashion similar to force. For data analysis, five individual 340 nm/380 nm excitation peak ratios (peak [Ca2+]i) were averaged at each measurement time point and standardized to the average of peak ratios at the pre-hypoxia time point (3 min after the onset of contractions). Relative basal [Ca2+]i measurements were averaged in a similar manner and standardized to the lowest resting levels within the total experimental time period.

Adjunct experiment
In order to determine whether the reduction in force was indeed due to hypoxia, and not fatigue per se, an adjunct experiment was performed in which force and Ca2+ measurements were examined in separate, but similar, single fibres (n = 3) stimulated every 5 s for 21 min at ambient P02 perfusion.

Statistics
A two-way analysis of variance was performed for the statistical analysis in all comparisons. In all analyses, the 0.05 level of significance was used. Results are reported as mean ± S.E.M.

RESULTS

Force
During the adjunct experiment, tetanic force production was maintained (92 ± 2% of initial maximal contractions) as tetani were produced every 5 s for 21 min (the duration of the H/R protocol), indicating that the fall in force during the H/R experimental protocol was due to the low P02 treatment. Figure 1 illustrates representative force and [Ca2+]i recordings during the CON H/R protocol. Following the initial high P02 period (3 min after the onset of contractions), generated force remained near maximal initial values at 97 ± 3% in the CON group, and 89 ± 8% in the TMZ group, with no significant difference between groups (Fig. 2). Following 15 min of hypoxic perfusion, a substantial decrease in force production was observed in both the CON (to 35 ± 5% of initial maximum values)
and TMZ groups (to 20 ± 8%), with no significant difference between groups (Fig. 2). Following reoxygenation, force recovery was observed in all fibres by 3 min to 53 ± 11% (of pre-hypoxic values) in the CON group and 27 ± 10% in the TMZ group. In all cases, the maximal level of force recovery was achieved by 3 min of reoxygenation, which was then followed by a gradual loss of contractility that advanced to complete contractile failure within minutes. Although the mean force recovery of CON fibres was greater than TMZ, this was not significant.

**Figure 1**
Representative recordings of force (A) and cytosolic Ca$^{2+}$ fluorescence (B) during the control hypoxia/reoxygenation protocol. Note the increase in resting [Ca$^{2+}$], during the low $P_O$, infusion and subsequent decrease during the high $P_O$, reoxygenation period.

**Figure 2**
Relative force during the hypoxia/reoxygenation protocol for both the CON group (control; $n = 6$, mean ± S.E.M.) and the TMZ group ($n = 6$). No significant difference was observed between groups. Note the incomplete recovery of force to pre-hypoxic levels in both groups following reoxygenation.
Peak $[\text{Ca}^{2+}]_c$

Three minutes after the onset of contractions, relative peak $[\text{Ca}^{2+}]_c$ increased to 110 ± 2% in the CON group and 104 ± 2% in the TMZ group to reach steady-state maximal values (Fig. 3). Following 15 min of hypoxia, a decrease in relative peak $[\text{Ca}^{2+}]_c$ was observed in both the CON fibres (66 ± 7% of pre-hypoxia values) and TMZ fibres (41 ± 10%), with no significant difference between groups. Reoxygenation generated very minor increases in relative peak $[\text{Ca}^{2+}]_c$ in both the CON (to 74 ± 8% of initial values) and TMZ groups (to 58 ± 9%), with no significant difference between groups.

Figure 4 illustrates the ratio of relative force to relative peak $[\text{Ca}^{2+}]_c$ (force/Ca2+), an index of Ca2+ sensitivity. Following 15 min of hypoxia, force/Ca2+ had decreased significantly in both the CON (55 ± 5% of pre-hypoxic values) and TMZ groups (43 ± 10%). The 3 min period of reoxygenation generated increases in force/Ca2+ in both the CON (72 ± 6% of pre-hypoxic values) and TMZ (48 ± 13%) groups. No significant differences between treatments were observed at any time point during the pre-hypoxia, hypoxia or reoxygenation periods.

Basal $[\text{Ca}^{2+}]_c$

Three minutes after the onset of contractions, the lowest relative $[\text{Ca}^{2+}]_c$ between successive tetani (‘relative basal $[\text{Ca}^{2+}]_c$’) increased in the CON group to 138 ± 2% (of initial resting values), and to 141 ± 6.1% in fibres exposed to TMZ, with no significant difference between groups (Fig. 5). Following the 15 min period of hypoxia, relative basal $[\text{Ca}^{2+}]_c$ significantly increased in the CON group to 197 ± 6% (of initial resting values) followed by a decrease during reoxygenation to near pre-hypoxic levels (169 ± 4% of initial resting values). Exposure to TMZ resulted in a significant ($P < 0.05$) reduction of the rise in relative basal $[\text{Ca}^{2+}]_c$ observed in the CON group following hypoxia. Mean relative basal $[\text{Ca}^{2+}]_c$ decreased from pre-hypoxic levels to 126 ± 7% of initial resting values, and then remained stable following reoxygenation at 123 ± 6%.

**DISCUSSION**

The results of this study demonstrated in single skeletal muscle fibres during steady-state contractions that: (1) during 15 min of hypoxia, force and peak $[\text{Ca}^{2+}]_c$ in control fibres decreased, while basal $[\text{Ca}^{2+}]_c$ increased substantially;
(2) exposure to 100 μM TMZ abolished the increase in basal [Ca^{2+}], observed in control fibres, yet had no effect on the decrease in force or peak [Ca^{2+}]c observed during hypoxia; and (3) a 3 min period of reoxygenation failed to restore force or peak [Ca^{2+}]c to pre-hypoxic levels in both the CON and TMZ groups.

Steady-state
In the present study, fibres with slow fatiguing properties (types 2, 3) were selected in order to reduce the occurrence of high-intensity fatigue, and to maintain 'steady-state' contractions for the duration of the protocol. The results of the adjunct experiment demonstrate that these fibres were indeed capable of close to maximal (92 ± 2% of initial contractions) force production for the 21 min duration of the H/R protocol. As it has been shown (Elzinga & van der Laarse, 1988) that these fibre types have a higher oxidative capacity than fast fatiguing type 1 Xenopus fibres, maintenance of contractions is probably dependent on energy supplied predominantly through oxidative phosphorylation. It has been demonstrated that O₂ consumption does not reach maximal values until ~1–1.5 min following the onset of contractions (Elzinga & van der Laarse, 1988; Stary & Hogan, 2000b) in these single fibres, and therefore, an initial 3 min period of contractions at high Pₒ was necessary to establish a steady-state contractile period (Fig. 1). Relative peak [Ca^{2+}]c and resting [Ca^{2+}]c increased initially following the onset of contractions, then stabilized along with force for the remainder of the 3 min equilibration period in both the CON and TMZ groups (Figs 1, 2 and 3), as previously described (Westerblad et al. 1991). In addition, the results of the adjunct experiment demonstrate that any photo-bleaching effects were insignificant, as the peak fura 2 ratio following 21 min of continuous contractions was not significantly different from initial values.

Force and [Ca^{2+}]c during hypoxia
Following 15 min of hypoxic perfusion, force and relative peak [Ca^{2+}]c had substantially decreased in both CON and TMZ groups (Figs 1, 2 and 3). Studies investigating high-intensity fatigue in similar isolated, single skeletal muscle fibres have demonstrated that failure of excitation–contraction (E–C) coupling, which includes reductions in Ca^{2+} release from the SR, and decreases in myofilament Ca^{2+} sensitivity, is partially responsible for the decrease in force production (Westerblad et al. 1991). Although the loss in developed tension in both the TMZ and CON groups during hypoxia in the present study was not due to high-intensity fatigue, it is likely that a similar mechanism involving failure of E–C coupling, including a reduction in SR Ca^{2+} release, was responsible for the decreased tension development, as indicated by the simultaneous fall in peak [Ca^{2+}]c (Fig. 3).

In addition to decreases in SR Ca^{2+} release, decreases in myofilament Ca^{2+} sensitivity were observed during the present experiment following hypoxia in both the TMZ and CON groups (Fig. 4), most likely due to increases in intracellular metabolic intermediates (i.e. H⁺, inorganic phosphate and ADP) (Godt & Nosek, 1989). It has been shown that TMZ decreases intracellular acidosis associated with hypoxia (Lavanchy et al. 1987), and increases energy sparing by preserving the ATP pool (Lavanchy et al. 1987; Allibardi et al. 1998). However, despite the beneficial effects of TMZ reported in cardiac muscle during hypoxia, no differences in force reduction, peak [Ca^{2+}]c or myofilament Ca^{2+} sensitivity were observed during hypoxia between the TMZ and CON groups in the single skeletal muscle fibres used in the present study.

Although TMZ had no significant effect on force or peak [Ca^{2+}]c during hypoxia, TMZ did significantly alter the relative [Ca^{2+}]c during the period between successive tetani (‘relative basal [Ca^{2+}]c’) during hypoxia. Following 15 min of hypoxia, relative basal [Ca^{2+}]c in CON fibres had increased substantially (Figs 1 and 5) suggesting that the activity of the SR Ca^{2+} re-uptake ATPase was impaired. However, exposure to TMZ completely abolished any measurable increase in relative basal [Ca^{2+}]c in all fibres (Fig. 5). These findings are somewhat similar to previous findings in cardiac muscle in which TMZ was shown to inhibit increases in [Ca^{2+}]c under conditions similar to
those during ischaemia (Renaud, 1988). In the present study, the abolition of any increase in relative basal \([\text{Ca}^{2+}]_c\) suggests that either TMZ preserved the activity of the SR \(\text{Ca}^{2+}\) re-uptake ATPase, or cytosolic \(\text{Ca}^{2+}\) was sequestered or buffered via an alternative mechanism. One such possibility is that \(\text{Ca}^{2+}\) uptake into the mitochondria increased. Increased mitochondrial \(\text{Ca}^{2+}\) uptake has been attributed to the development of the mitochondrial permeability transition (MPT) pore, which may affect cell function and cause necrosis through abolition of the mitochondrial membrane potential (Lemasters et al. 1997). Although it has previously been demonstrated that TMZ increases mitochondrial \(\text{Ca}^{2+}\) uptake during conditions of high extracellular \(P_O\) in isolated mitochondria (Guarnieri et al. 1997), it has more recently been demonstrated that it reduces the opening of the MPT pore by competing with increases in cytosolic \(\text{Ca}^{2+}\) concentration (Morin et al. 1998, 2000), as occurs during hypoxia. This suggests that mitochondrial uptake of \(\text{Ca}^{2+}\) during hypoxia was unlikely and that increased \(\text{Ca}^{2+}\) uptake into the SR may be a possible mechanism responsible for the decrease in basal \([\text{Ca}^{2+}]_c\).

**Force and \([\text{Ca}^{2+}]_c\) during reoxygenation**

We have recently demonstrated in single fibres similar to those used in the present study (Kohin et al. 2001) that following a much briefer (5 min) period of hypoxia than that employed in the present study, force and peak \([\text{Ca}^{2+}]_c\) recovered substantially during reoxygenation to near initial levels within 3 min. However in the present study, following a substantially longer (15 min) period of hypoxia, the same period of reoxygenation resulted in only minor increases in both force production and peak \([\text{Ca}^{2+}]_c\) in fibres during the CON protocol (Figs 2 and 3). This suggests that the duration of hypoxia is an important element in the development of H/R injury in the single skeletal muscle fibres used in the present study, and that a 15 min period of hypoxia exceeds the capacity of these cells to withstand severe changes in intracellular homeostasis.

Although it has been previously shown in cardiac muscle that TMZ improves contractility during reoxygenation (Toda et al. 1979; Allibardi et al. 1998), in the contracting skeletal muscle fibres used in the present study no significant differences between the TMZ and CON groups were observed in force or relative peak \([\text{Ca}^{2+}]_c\), following 3 min of reoxygenation (Figs 2 and 3). The contrast between the previously reported protective effects of TMZ during H/R in cardiac muscle and the results of the present study in contracting, single skeletal muscle fibres may be a result of differences in \(\text{Ca}^{2+}\) handling kinetics between the two systems (Hamilton et al. 2000). Skeletal and cardiac muscle have different isoforms of the dihydropyridine receptor and the ryanodine receptor, the primary proteins involved in the release of \(\text{Ca}^{2+}\) from the SR. In cardiac muscle, it has been suggested that individual SR \(\text{Ca}^{2+}\) release units are tightly controlled by local \(\text{Ca}^{2+}\) levels through \(\text{Ca}^{2+}\)-induced \(\text{Ca}^{2+}\) release (CICR). Although it has been reported that CICR contributes somewhat to SR \(\text{Ca}^{2+}\) release in amphibian skeletal muscle, it may be critical only following the initial voltage-gated depolarization (Hamilton et al. 2000). Therefore, it is possible that the modulation of resting \(\text{Ca}^{2+}\) by TMZ observed during H/R in cardiac muscle in previous studies was sufficient to alter E–C coupling, but insufficient to successfully maintain \(\text{Ca}^{2+}\) handling and contractility during H/R in the amphibian skeletal muscle fibres used in the present study. However, in the skeletal muscle fibres used in the present study, despite the absence of complete recovery in force or peak \([\text{Ca}^{2+}]_c\), in either the CON or TMZ groups, a significantly lower basal \([\text{Ca}^{2+}]_c\) persisted during reoxyge nation in the TMZ group (Fig. 5), suggesting that the effect of TMZ observed during hypoxia remained active during reoxygenation.

**Conclusions**

In the present study, measurements of relative peak \([\text{Ca}^{2+}]_c\) and force in isolated single skeletal muscle fibres following an extended period of severe hypoxia and subsequent reoxygenation suggest that an irreversible state of \(\text{Ca}^{2+}\) release impairment from the SR was associated with a permanent loss of contractility. Exposure to 100 \(\mu\)M TMZ prevented increases in relative basal \([\text{Ca}^{2+}]_c\) during 15 min of hypoxia, suggesting preservation of SR \(\text{Ca}^{2+}\)-ATPase re-uptake activity. However, exposure to TMZ failed to prevent decreases in force or relative peak \([\text{Ca}^{2+}]_c\), following hypoxia, and also failed to increase force or peak \([\text{Ca}^{2+}]_c\) recovery above control levels following 3 min of reoxygenation. These results suggest that: (1) a 15 min period of hypoxia induces severe contractile dysfunction and surpasses the threshold of these muscle fibres to withstand severe changes in intracellular homeostasis, resulting in an inability to completely restore functionality following reoxygenation; and (2) the activity of TMZ during acute H/R in skeletal muscle includes modulation of intracellular \(\text{Ca}^{2+}\) kinetics as a mechanism of significance, but that simply attenuating the increase in resting \([\text{Ca}^{2+}]_c\) does not protect skeletal muscle from the deleterious effects of H/R.


