Mitochondrial calcium transporting pathways during hypoxia and reoxygenation in single rat cardiomyocytes

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Abstract

Objective: Mitochondrial [Ca$^{2+}$]$_{m}$ rises in parallel with cytosolic [Ca$^{2+}$]$_{c}$ following ATP-depletion rigor contracture induced by hypoxia in isolated cardiomyocytes. We investigated the pathways involved in the hypoxia induced changes in [Ca$^{2+}$]$_{m}$ by using known inhibitors of mitochondrial Ca$^{2+}$ transport, namely ruthenium red, an inhibitor of the Ca$^{2+}$ uniporter (the normal influx route) and clonazepam, an inhibitor of Na$^{+}$/Ca$^{2+}$ exchange, (the normal efflux route). Methods: [Ca$^{2+}$]$_{m}$ was determined from indo-1/am loaded rat myocytes where the cytosolic fluorescence signal had been quenched by superfusion with Mn$^{2+}$. [Ca$^{2+}$]$_{m}$ was measured by loading myocytes with indo-1 pentapotassium salt during the isolation procedure. Cells were placed in a specially developed chamber for induction of hypoxia and reoxygenated 40 min after rigor development. Results: 50% of control cells hypercontracted upon reoxygenation; this correlated with a [Ca$^{2+}$]$_{m}$ or [Ca$^{2+}$]$_{c}$ higher than approximately 350 nM at the end of rigor. Clonazepam completely abolished the rigor-induced rise in [Ca$^{2+}$]$_{m}$ but not [Ca$^{2+}$]$_{c}$. On reoxygenation [Ca$^{2+}$]$_{m}$ increased over the first 5 min and remained elevated whereas [Ca$^{2+}$]$_{c}$ fell. In the presence of ruthenium red a dramatic increase in [Ca$^{2+}$]$_{m}$ occurred 5–10 min after rigor development (the indo-1 fluorescence signal was saturated); [Ca$^{2+}$]$_{m}$ also increased but to a lesser extent. On reoxygenation, [Ca$^{2+}$]$_{m}$ fell rapidly even though cells hypercontracted and [Ca$^{2+}$]$_{m}$ remained elevated. Conclusions: During hypoxia following rigor development Ca$^{2+}$ uptake into mitochondria occurs largely via the Na$^{+}$/Ca$^{2+}$ exchanger rather than the Ca$^{2+}$ uniporter whereas on reoxygenation the transporters resume their normal directionality. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

There is now substantial evidence that altered Ca$^{2+}$ homeostasis plays an important role in ischaemia/reperfusion or hypoxia/reoxygenation induced myocardial cell injury [1]. An increase in intracellular [Ca$^{2+}$] precedes the irreversible cell damage occurring upon reperfusion after prolonged periods of ischaemia or hypoxia [2,3]. This rise in [Ca$^{2+}$] occurs as a result of an increase in cell [Na$^{+}$] and is probably mediated by `reverse mode' Na$^{+}$/Ca$^{2+}$ exchange [4–6]. A second key feature of irreversible cell damage is mitochondrial dysfunction [7]. Mitochondria isolated from ischemic/reperfused or hypoxic/reoxygenated tissues are functionally impaired; they have a reduced adenine nucleotide content, decreased adenine nucleotide translocase activity and reduced respiratory chain complex activity [8–11]. Intact functioning mito-
chondria are essential for mechanical recovery of the heart on reperfusion/reoxygenation; anaerobic metabolism can provide enough energy for quiescent myocardial cells but oxidative phosphorylation is required to meet the demands of the contracting heart [12,13]. However, it is as yet unclear whether Ca\textsuperscript{2+}-induced mitochondrial damage precedes cell injury or is a consequence of irreversible damage to the sarcolemma.

Ca\textsuperscript{2+} transport pathways of mitochondria have been widely studied in the isolated organelle [14,15] and revealed that normally Ca\textsuperscript{2+} influx occurs via a uniporter (sensitive to ruthenium red) and efflux via Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange (inhibited by diltiazem, clonazepam, and high external Ca\textsuperscript{2+}, [15,16]). The role of the mitochondrial Ca\textsuperscript{2+} transporters under physiological conditions appears to be to relay changes in [Ca\textsuperscript{2+}\textsubscript{m}] to the mitochondrial matrix which results in increased activity of key enzymes of the citric acid cycle, the result of which is to increase NADH and hence ATP production [17,18]. This is achieved by reaching a new steady state level of [Ca\textsuperscript{2+}\textsubscript{m}] since as extramitochondrial [Ca\textsuperscript{2+}] rises, the activity of the uniporter exceeds that of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger. ATP supply can therefore be coupled to the increased energy demand of enhanced contractile and metabolic activity. A third, non-specific, Ca\textsuperscript{2+} transporting pathway, namely the mitochondrial permeability transition pore can occur in isolated mitochondria under conditions of high [Ca\textsuperscript{2+}\textsubscript{m}], reduced ATP and ADP levels and oxidative stress [15,19]. However, the role of the pore under physiological or pathological conditions is as yet unclear.

Previously we have shown that mitochondrial [Ca\textsuperscript{2+}\textsubscript{m}] ([Ca\textsuperscript{2+}\textsubscript{m}]\textsubscript{0}) and cytosolic [Ca\textsuperscript{2+}] ([Ca\textsuperscript{2+}\textsubscript{c}]) rise following ATP-depletion rigor-contracture in hypoxic cardiomyocytes [20]. The extent of this increase determined cell recovery on reoxygenation; cells recovered (partially re-lengthened atmospheric oxygen yielding a PO\textsubscript{2} \textless ;0.02 torr. Myocytes were superfused with glucose-free Hepes-based buffer, as described previously [23]. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1985). Briefly, the heart was retrogradely perfused with low-Ca\textsuperscript{2+}, collagenase-containing, bicarbonate buffer (37°C, pH 7.2) and the perfusion terminated when the tissue became soft (approximately 30 min). The left ventricle was then surgically dissociated and the myocytes resuspended in a series of bicarbonate-based buffers with gradually increasing calcium concentrations. Cells were finally suspended in a HEPES based buffer containing, in mM, 137 NaCl, 5 KCl, 1.2 MgSO\textsubscript{4}, 1.2 NaH\textsubscript{2}PO\textsubscript{4}, 16 d-glucose, 1 CaCl\textsubscript{2}, and 20 sodium N-hydroxyethyl-piperazine-N\textsuperscript{-2}-ethansulphonic acid (Hepes; pH 7.4). Cells were maintained at 24°C and used within 8 to 10 h.

2. Methods

2.1. Myocyte isolation

Single cardiac myocytes were isolated from rat ventricles as described previously [23]. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1985). Briefly, the heart was retrogradely perfused with low-Ca\textsuperscript{2+}, collagenase-containing, bicarbonate buffer (37°C, pH 7.2) and the perfusion terminated when the tissue became soft (approximately 30 min). The left ventricle was then surgically dissociated and the myocytes resuspended in a series of bicarbonate-based buffers with gradually increasing calcium concentrations. Cells were finally suspended in a HEPES based buffer containing, in mM, 137 NaCl, 5 KCl, 1.2 MgSO\textsubscript{4}, 1.2 NaH\textsubscript{2}PO\textsubscript{4}, 16 d-glucose, 1 CaCl\textsubscript{2}, and 20 sodium N-hydroxyethyl-piperazine-N\textsuperscript{-2}-ethansulphonic acid (Hepes; pH 7.4). Cells were maintained at 24°C and used within 8 to 10 h.

2.2. Induction of hypoxia

Single myocytes were studied in a specially developed chamber which has been previously described in detail [23]. The chamber utilises a stable laminar layer of ultrahigh-purity argon to prevent the back diffusion of the anode effluent. It has been assumed previously that Ca fluxes across anode also served as the buffer outflow which remained at 24°C and used within 8 to 10 h.

2.3. Loading of mitochondria with indo-1

Myocytes were loaded with the Ca\textsuperscript{2+}-sensitive fluorescent probe indo-1 by exposing them to the membrane permeant acetoxymethyl ester of indo-1, indo-1/AM, (25...
μM) for 15 min at 30°C [5]. Following loading myocytes were washed of indo-1/AM and left for 30 min to allow complete deesterification of the probe. When myocytes are loaded with indo-1/am, about half the fluorescence is non-cytosolic and located within the mitochondria [24,25]. Perfusion of cells with buffer containing 200 μM MnCl2 allowed selective quenching of the cytosolic signal since the $K_d$ of indo-1 for Ca$^{2+}$ is about 20 times higher than that for Mn$^{2+}$. After approximately 30 min Ca$^{2+}$ transients were no longer visible and the remaining fluorescence was due to indo-1 partitioned in mitochondria. In the present study, 5 min following cessation of detectable Ca$^{2+}$ transients the Mn$^{2+}$ buffer was replaced with normal buffer and experiments started after approximately 10 min. Although Mn$^{2+}$ can affect the Ca$^{2+}$ transport pathways of isolated mitochondria [14] the concentrations needed are higher than those estimated to remain in the cytosol following the quenching procedure – 10 μM [25]. A full validation of this technique has been given by Miyata et al. [25].

2.4. Loading of cytosol with indo-1

To monitor [Ca$^{2+}$], selectively, cardiac myocytes were loaded with indo-1 pentapotassium salt [26]. Following digestion of the heart a portion of the left ventricle (approximately 5 mm$^3$) was mechanically dissociated in 1 ml Hepes buffer containing 1 mM indo-1 salt and 0.25 mM CaCl$_2$. The cell suspension was incubated at 30°C for 15 min, filtered and 2 ml Hepes buffer, containing 0.25 mM CaCl$_2$, added. The cells were allowed to sediment and gradually resuspended in Hepes buffer containing 0.5 mM CaCl$_2$ and finally 1 mM CaCl$_2$. This was found to permit selective loading of the indo-1 salt into the cytosol, presumably by diffusion through transiently permeable gap junctions. Cell loading of indo-1 was variable and cells having dye content 2–4 times background were used for experiments. These cells also fulfilled the other standard criteria for experimental selection i.e. they exhibited a rod shaped morphology with no blebbing of the cell membrane and were capable of responding to electrical stimulation with no spontaneous contractions.

2.5. Measurement of fluorescence and determination of cell morphology

A customised inverted microscope (Nikon Diaphot) was used to measure epifluorescence of cells loaded with indo-1 [27]. The myocyte to be studied was illuminated with a red light (long pass filter at 650 nm) and its image directed to a TV camera and viewed on a monitor. Indo-1 was excited by a 75 W xenon lamp at 350±5 nm. Emitted light was directed to two photomultiplier tubes selecting wavelengths of 405±17 nm and 495±10 nm, corresponding to the peak emissions of the Ca$^{2+}$ bound and Ca$^{2+}$ free forms of the indicator, respectively. The signal was passed to two amplifiers/discriminators and then to a custom-built analog circuit with anti-aliasing filters. Data was digitised at 200 Hz. Custom software was used for analysis of indo-1 data.

2.6. Calibration of the indo-1 ratio with intracellular [Ca$^{2+}$]

The fluorescence ratios obtained after subtracting cell autofluorescence (mean fluorescence at 410 and 491 nm of unloaded myocytes, [26]) were compared to ratios obtained using indo-1 pentapotassium salt in buffers of known [Ca$^{2+}$]. This calibration gives results very similar to those of an in vivo calibration [24]. The minimum ratio ($R_{\text{min}}$), obtained by measuring fluorescence from a Ca-free buffer was 0.2; the maximum ratio ($R_{\text{max}}$) obtained in micromolar Ca$^{2+}$-containing buffer was 3.5 and the apparent dissociation constant for indo-1 ($K_{d}$) was 440 nM. [Ca$^{2+}$] was obtained from the expression $K_{d}(R_{\text{min}}/R_{\text{max}}-1)$, where $R$ is the autofluorescence corrected observed indo-1 ratio.

2.7. Materials

Ruthenium red and clonazepam were obtained from Sigma Chemical Company. The ‘active ingredient’ in commercially available ruthenium red is not the red dye itself but a colourless derivative [28], the concentration of which in different batches is unknown. The ruthenium red batch used in the present study had a dye content of 36%; 20 μM ruthenium red is thus equivalent to 41 μg/ml.

Statistical analyses were performed using Student’s $t$-test (paired where appropriate) or analysis of variance (ANOVA). Results are expressed as means ± SEM of individual myocytes; control experiments were always performed on the same day as experiments on cells treated with either clonazepam or ruthenium red.

3. Results

3.1. Effects of hypoxia and reoxygenation on [Ca$^{2+}$]$_{in}$ and [Ca$^{2+}$]$_{ou}$

Approximately 30 min after the induction of hypoxia cell contraction ceased. Three to five minutes following cessation of contraction the cells rapidly shortened to roughly 2/3 of their original length as they underwent rigor contracture due to ATP depletion. This time period varied slightly for each cell and probably reflects differences in glycogen content or metabolism [23]. The mean time to rigor-contracture in control cells (cells without added pharmacological agents) was 33.5±2.7 min (n=17). No further changes in cell morphology occurred until the cells were reoxygenated when they either partially re-lengthened and recovered their ability to respond to electrical stimulation or hypercontracted into rounded
dysfunctional forms. The difference in cell morphology was very clear and therefore control cells were divided into two groups, ‘control-recovered’ or ‘control-hypercontracted’, as in previous studies [20,29]. Fig. 1 shows changes in $[\text{Ca}^{2+}]_m$ and $[\text{Ca}^{2+}]_c$ from both groups of cells. $[\text{Ca}^{2+}]_c$ is given as diastolic $[\text{Ca}^{2+}]_m$ throughout. It is apparent that cells having a $[\text{Ca}^{2+}]_m$ or $[\text{Ca}^{2+}]_c$ greater than 300–400 nM at the end of rigor hypercontracted upon reoxygenation. $[\text{Ca}^{2+}]_m$ is significantly different between the two groups of cells (using ANOVA) both for the effect of rigor ($p<0.01$) and effect of reoxygenation ($p<0.001$). At the end of rigor $[\text{Ca}^{2+}]_m$ was 280±62 nM ($n=5$) and 743±296 nM ($n=4$) for recovered and hypercontracted cells, respectively. Upon reoxygenation, $[\text{Ca}^{2+}]_m$ fell slightly in cells which recovered but not in cells which hypercontracted; both groups then showed a further rise reaching peak values of 331±110 nM ($n=5$) and 1467±522 nM ($n=4$), respectively, before falling again.

$[\text{Ca}^{2+}]_m$ changes were also significantly different between the two groups of cells; $p<0.01$ for the effect of rigor and $p<0.005$ for reoxygenation. $[\text{Ca}^{2+}]_m$ at the end of rigor was 398±120 nM ($n=3$) and 1613±465 nM ($n=5$) for recovered and hypercontracted cells, respectively. Upon reoxygenation there was an immediate decrease in $[\text{Ca}^{2+}]_m$ in both groups of cells. This was apparently due to a rapid uptake of $\text{Ca}^{2+}$ by the sarcoplasmic reticulum.

**Fig. 1.** Effects of hypoxia and reoxygenation on cytosolic and mitochondrial $[\text{Ca}^{2+}]$. Cells were loaded with indo-1 for measurement of mitochondrial or cytosolic (diastolic) $[\text{Ca}^{2+}]$ as described in Section 2. Hypoxia was induced and after a period of 30–40 min (T1, see text for values for each group of cells) cells underwent ATP-depletion rigor contracture (rigor). Cells were reoxygenated 40 min after rigor development (reoxygenation). Upon reoxygenation cells either recovered (control-recovered) or hypercontracted (control-hypercontracted) as described in the text. Mitochondrial and cytosolic $[\text{Ca}^{2+}]$ are shown in panels A and B, respectively, for cells that recovered or hypercontracted at reoxygenation. Results are expressed as means±S.E.M. Some error bars are too small to be visible.
since it was prevented by pre-treatment of cells with thapsigargin [20]. [Ca\textsuperscript{2+}]\textsubscript{i} then rose again in both groups before falling again in cells which recovered and continuing to rise in cells which hypercontracted.

There was no difference in time-to-onset of rigor between cells which recovered at reoxygenation, 34.3±2.7 min, (n=8), and those which hypercontracted, 32.8±3.4 min (n=9). Cell recovery has been shown to correlate with the time spent in rigor rather than the total period of hypoxia [23]. In the present experiments reoxygenation 40 min following rigor development resulted in approximately 50% of cells recovering. Thus experiments with added pharmacological agents were performed similarly so that any protective or deleterious effect of the added compounds could also be evaluated.

3.2. Effects of clonazepam during hypoxia and reoxygenation

Our recent studies on normoxic cells have demonstrated that clonazepam can be used in myocytes as an effective inhibitor of mitochondrial Ca\textsuperscript{2+} efflux being maximally effective at 100 μM, a concentration which had no significant effect on the contractile activity of cells or the cytosolic Ca\textsuperscript{2+} transient [30]. This agrees with previous work showing that clonazepam does not inhibit the cardiac sarcosomal Ca\textsuperscript{2+} channel, the sarcosomal Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger or sarcoplasmic reticular Ca\textsuperscript{2+} transport [31].

The compound CGP37157, although found to be a more potent inhibitor of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange in isolated mitochondria [16], gave inconsistent results when used in intact myocytes [30] and therefore was not used in the present studies.

Fig. 2A and 2B show the effect of clonazepam on [Ca\textsuperscript{2+}]\textsubscript{m} and [Ca\textsuperscript{2+}]\textsubscript{i} during hypoxia and reoxygenation. The control-recovered data from Fig. 1A are also shown in outline for comparison since none of the clonazepam treated cells hypercontracted on reoxygenation. Prior to rigor, [Ca\textsuperscript{2+}]\textsubscript{m} increased in clonazepam treated cells from 67.3±3.4 to 115±17 nM (n=6, p<0.05, paired t-test). This would be expected since, by inhibiting Ca\textsuperscript{2+} efflux from mitochondria, the presence of clonazepam would result in a new, higher steady-state level of [Ca\textsuperscript{2+}]\textsubscript{m}. However, careful analysis of Fig. 2A reveals that, paradoxically, clonazepam inhibited the hypoxia-induced rise in [Ca\textsuperscript{2+}]\textsubscript{m}. This effect is shown clearly when the changes in [Ca\textsuperscript{2+}]\textsubscript{m} are expressed as % of the pre-rigor value (taken as 100%) in Fig. 3. Thus clonazepam inhibited Ca\textsuperscript{2+} entry during rigor compared with either control-recovered cells (p=0.01, ANOVA) or control-total cells (p<0.005, ANOVA). Upon reoxygenation of cells maintained in the presence of clonazepam, [Ca\textsuperscript{2+}]\textsubscript{m} rose over the first 5 min and remained elevated after 15 min reoxygenation (Fig. 2A). The observed [Ca\textsuperscript{2+}]\textsubscript{m} values were 465±68 nM (n=6) in clonazepam-treated cells compared with control-recovered cells 92.7±19 nM (n=5), p<0.001 (unpaired t-test), and control-total cells 144±36 nM (n=9), p<0.005, (unpaired t-test).

Despite these dramatic differences in mitochondrial Ca\textsuperscript{2+} handling, clonazepam-treated cells showed changes in [Ca\textsuperscript{2+}]\textsubscript{i} during hypoxia and reoxygenation similar to control-recovered cells (Fig. 2B) with no significant differences. Comparison with control-total cells showed a significant difference for the effect of rigor, p<0.02. Clonazepam did not affect the time to rigor development, 29.5±3.2 min (n=10).

3.3. Effects of ruthenium red during hypoxia and reoxygenation

At a concentration of 20 μM, ruthenium red inhibited mitochondrial Ca\textsuperscript{2+} uptake by >95% in myocytes exposed to low [Na\textsuperscript{+}] medium (data not shown), similar to results observed by Miyata et al. [25]. We therefore used this concentration in hypoxia studies.

However, preliminary experiments showed that this concentration of ruthenium red caused all cells to hypercontract on reoxygenation and [Ca\textsuperscript{2+}]\textsubscript{m} rose dramatically following rigor saturating the indo-1 fluorescence signal (results not shown). Therefore, to eliminate the possibility that this large increase in [Ca\textsuperscript{2+}]\textsubscript{m} was due to sarcolemmal disruption, we included 20 mM butanedionemonoxime (BDM) in subsequent experiments in order to decrease the sensitivity of the myofilaments to Ca\textsuperscript{2+} [32] and inhibit the Ca\textsuperscript{2+} transient [33]. BDM did not affect significantly the changes in either [Ca\textsuperscript{2+}]\textsubscript{m} or [Ca\textsuperscript{2+}]\textsubscript{i} during hypoxia and reoxygenation, consistent with other studies showing that BDM does not delay the fall in ATP levels or alter the increases in cell [Ca\textsuperscript{2+}]\textsubscript{m} levels in hypoxic myocytes [34]. Fig. 4 shows [Ca\textsuperscript{2+}]\textsubscript{m} and [Ca\textsuperscript{2+}]\textsubscript{i} changes during and following hypoxia in cells exposed to 20 μM ruthenium red (+ BDM). [Ca\textsuperscript{2+}]\textsubscript{m} increased dramatically following rigor and the fluorescence signal reached saturating levels in all cells studied in less than 10 min. [Ca\textsuperscript{2+}]\textsubscript{i} also rose after rigor but to a lesser extent than [Ca\textsuperscript{2+}]\textsubscript{m}. On reoxygenation there was a very rapid fall in [Ca\textsuperscript{2+}]\textsubscript{m} which returned to pre-hypoxic values. [Ca\textsuperscript{2+}]\textsubscript{i} either remained elevated or fell slowly.

3.4. Combined effects of clonazepam and ruthenium red during hypoxia and reoxygenation

The above results suggest that changes in [Ca\textsuperscript{2+}]\textsubscript{m} during hypoxia following rigor and reoxygenation can be accounted for solely by the transporters affected by ruthenium red and clonazepam. As shown in Fig. 5, the simultaneous presence of ruthenium red and clonazepam resulted in almost complete inhibition of any [Ca\textsuperscript{2+}]\textsubscript{m} changes during either hypoxia or reoxygenation (p<0.01 compared with control-recovered cells for both effects of hypoxia and reoxygenation).
Fig. 2. Effect of clonazepam on [Ca\textsuperscript{2+}]\textsubscript{m} and [Ca\textsuperscript{2+}]\textsubscript{c} during hypoxia and reoxygenation. 100 µM clonazepam was present throughout hypoxia and reoxygenation. Cells were reoxygenated 40 min following rigor development (see legend to Fig. 1). Values for mitochondrial and cytosolic [Ca\textsuperscript{2+}] are shown in panels A and B respectively. The dotted lines in each graph represent ‘control-recovered’ data (actual values shown in Fig. 1) for comparison.

4. Discussion

4.1. Myocyte heterogeneity

Under the experimental conditions described here, 50% of control cells recovered (Fig. 1). Cell hypercontracture was associated with large increases in both [Ca\textsuperscript{2+}]\textsubscript{m} and [Ca\textsuperscript{2+}]\textsubscript{c} at the end of rigor. This heterogeneity in the Ca\textsuperscript{2+} handling response of individual myocytes to hypoxia or metabolic inhibition has been described previously [20,29] but the reasons for the observed difference are not yet known. It has been proposed that Ca\textsuperscript{2+} entry during hypoxia is via reversal of sarcolemmal Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange [4,5] which occurs as a result of Na\textsuperscript{+} loading, either through Na\textsuperscript{+} channels or Na\textsuperscript{+}/H\textsuperscript{+} exchange [5,35–37]. It is thus likely that differences between individual myocytes reflect alterations in one or more of these systems. This may represent the true in vivo situation since such a heterogeneous response is also observed in cultured myocytes [27], where any damage to membrane proteins during the isolation procedure would have been repaired.

4.2. Role of the mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger during hypoxia

By using clonazepam as a reasonably specific inhibitor of the mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (see [30,31]) we have dissected the role of this carrier in mediating
Fig. 3. Effect of clonazepam on \([\text{Ca}^{2+}]_m\) during rigor. Data is presented as % increase in mitochondrial \([\text{Ca}^{2+}]_m\) occurring in control and clonazepam treated cells during rigor (see legend to Fig. 1) with the pre-rigor value of \([\text{Ca}^{2+}]_m\) taken as 100% for each cell.

mitochondrial \(\text{Ca}^{2+}\) changes during hypoxia. This condition produces an increase in \([\text{Ca}^{2+}]_m\) but also causes mitochondrial de-energisation as a result of the loss of respiratory chain function [38]. In other cell types, a loss of \(\Delta \psi_m\) and \(\Delta p\text{H}\) elicited by uncouplers dramatically inhibits mitochondrial \(\text{Ca}^{2+}\) uptake via the \(\text{Ca}^{2+}\) uniport pathway [39,40]. In the heart, such a decrease in the ability of mitochondria to clear a moderate increase in cytosolic \(\text{Ca}^{2+}\) might be expected to exacerbate hypoxia-induced damage although excessive \(\text{Ca}^{2+}\) accumulation may ultimately compromise mitochondrial function. Our data using clonazepam suggest that, as an additional mechanism to allow \(\text{Ca}^{2+}\) uptake during the de-energised regime of hypoxia, \(\text{Ca}^{2+}\) uptake can occur by reversal of the \(\text{Na}^+ / \text{Ca}^{2+}\) exchanger.

Thus clonazepam treatment decreased mitochondrial \(\text{Ca}^{2+}\) uptake during hypoxia, in the face of slightly elevated \([\text{Ca}^{2+}]_m\) (Fig. 2). It should be stressed that reversal of the \(\text{Na}^+ / \text{Ca}^{2+}\) exchanger under these conditions is entirely compatible with the known properties of the channel as well as with thermodynamic considerations. A fall in \(\Delta p\text{H}\) in the de-energised cells is likely to cause a drop in the \(\text{Na}^+\) gradient across the inner mitochondrial membrane from close to 8 in energised mitochondria to 2 or less in the de-energised state [41]. The likely electroneutrality of the \(\text{Na}^+ / \text{Ca}^{2+}\) exchanger in de-energised mitochondria (2\(\text{Na}^+ : \text{Ca}^{2+}\)) means that these conditions would, in fact, be predicted to produce exchange of \(\text{Ca}^{2+}\) for \(\text{Na}^+\) in the opposite sense from that observed in normoxic cells i.e. mitochondrial \(\text{Ca}^{2+}\) uptake coupled to \(\text{Na}^+\) release.

In further support of the conclusion that the \(\text{Na}^+ / \text{Ca}^{2+}\) exchanger can operate in the reverse direction, recent work demonstrated that, in isolated mitochondria, \(\text{Ca}^{2+}\) entry via the \(\text{Na}^+ / \text{Ca}^{2+}\) exchanger could occur under conditions of membrane depolarisation [42]. In these experiments mitochondria were incubated with ruthenium red (to prevent entry via the uniporter) and 20 mM NaCl prior to addition of approximately 3 \(\mu\text{M} \text{Ca}^{2+}\). No change in \([\text{Ca}^{2+}]_m\) occurred under these conditions until the uncoupler FCCP (carbonylcyanide p-trifluoromethoxyphenylhydrazone) was added, when \([\text{Ca}^{2+}]_m\) increased from approximately 150 nM to 500 nM in less than 10 min. This entry could be inhibited by diltiazem or omission of NaCl indicating that the \(\text{Na}^+ / \text{Ca}^{2+}\) exchanger was indeed responsible.

4.3. Role of the mitochondrial \(\text{Ca}^{2+}\) uniporter during hypoxia

Our data showing that ruthenium red actually increased mitochondrial \(\text{Ca}^{2+}\) accumulation following rigor might also suggest that the \(\text{Ca}^{2+}\) uniporter, like the \(\text{Na}^+ / \text{Ca}^{2+}\) exchanger, catalysed flow of \(\text{Ca}^{2+}\) ions in the opposite direction compared with that in normoxic cells. This result does, however, need to be interpreted with caution since ruthenium red also induced an increase in \([\text{Ca}^{2+}]_m\) following rigor, although not to the same extent. Given the measured gradient of \([\text{Ca}^{2+}]_m\) during hypoxia, a reversal of the uniporter seems unlikely, requiring an inversion, i.e. positive, \(\Delta \psi_m\). Although such extreme conditions could conceivably occur during rigor, for example as a result of reversal of the normal concentration gradient of electrogenic ions, measurements of \(\Delta \psi_m\) argue against this possibility. Thus Di Lisa et al. [38] found, using almost identical conditions of hypoxia and reoxygenation, that \(\Delta \psi_m\) (measured using the fluorescent indicator JC-1) started to depolarise early in hypoxia, before the development of rigor-contracture, and reached a plateau before rising sharply again with the development of rigor to a new, higher, plateau which was maintained until reoxygenation. Under these conditions \(\text{Na}^+ / \text{Ca}^{2+}\) exchange activity, which displays a higher affinity for \(\text{Ca}^{2+}\) \((K_m=3-4\text{M}, [44])\) may well predominate over uniporter activity \((K_m=10-30\text{M}, [15])\). Furthermore, studies using
isolated mitochondria showed that, when $\Delta \psi_{m}$ is dissipated, no uniporter-mediated uptake of $\text{Ca}^{2+}$ occurred even in presence of an 8-fold gradient of $\text{Ca}^{2+}$ out:$\text{Ca}^{2+}$ in [45]. The uniporter also displays strongly sigmoidal kinetics in presence of $\text{Mg}^{2+}$ [15] and the intracellular $[\text{Mg}^{2+}]$ increases from 1 mM to 4–16 mM during hypoxia following rigor development [29] which would be expected to inhibit uniporter activity further.

4.4. Activity of the mitochondrial $\text{Ca}^{2+}$ transporters upon reoxygenation

Upon reoxygenation, the transporters appeared to resume their normal directions as evidenced by the fact that $[\text{Ca}^{2+}]_{m}$ fell rapidly in ruthenium red treated cells and rose in clonazepam treated cells. The observation that upon reoxygenation mitochondria are once again capable of taking up $\text{Ca}^{2+}$ suggests that mitochondrial membrane potential ($\Delta \psi_{m}$) is restored since $\text{Ca}^{2+}$ uptake via the uniporter is dependent on $\Delta \psi_{m}$. Again, this is in agreement with measurements of Di Lisa et al. [38] showing that upon reoxygenation there was a rapid recovery of $\Delta \psi_{m}$ even in cells which hypercontracted, indicating that at least some mitochondria had intact functioning membranes at that time. This recovery of $\Delta \psi_{m}$ on reoxygenation can account for restoration of direction of both the $\text{Ca}^{2+}$ transport pathways: the uniporter is highly dependent on $\Delta \psi_{m}$ and there is now evidence that the $\text{Na}^{+}/\text{Ca}^{2+}$

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Fig. 4. Effect of ruthenium red on $[\text{Ca}^{2+}]_{m}$ and $[\text{Ca}^{2+}]_{d}$ during hypoxia and reoxygenation. 20 $\mu$M RuR (+20 mM BDM) were present throughout hypoxia and reoxygenation. Cells were reoxygenated 40 min following rigor development (see legend to Fig. 1). Following rigor, mitochondrial $[\text{Ca}^{2+}]_{m}$ rose rapidly (panel A), the value of $>3000\text{ nM}$ at the end of rigor indicates that the indo-1 fluorescence signal was saturated (therefore no error bars are present at these points). Cytosolic (diastolic) $[\text{Ca}^{2+}]$ changes are shown in panel B. The dotted lines in each graph represent ‘control-hypercontracted’ data (actual values shown in Fig. 1) for comparison.

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4.3. Activity of the mitochondrial $\text{Ca}^{2+}$ transporters upon reoxygenation
exchanger may also be driven by ΔΨm with a probable stoichiometry of 3Na+/Ca2+ [45,46], in contrast to the electroneutral exchange in the de-energised state [42].

4.5. Protective effects of ruthenium red?

Ruthenium red has, in the past, been reported to have a protective effect against reperfusion or reoxygenation induced myocardial cell injury at concentrations of 0.1–6 μM [47–54], an effect which was attributed to its effects on [Ca2+]m. In our hands, a concentration of ruthenium red of 20 μM was required to completely inhibit the rise in [Ca2+]m seen in normoxic cells subjected to Ca2+ loading. This suggests that the observed protective effects of ruthenium red at lower concentrations may be due to properties other than direct effects on [Ca2+]m; for example Benzi and Lerch [51] found improvement of left ventricular developed pressure in rat hearts on reperfusion with ruthenium red following global ischaemia and concluded that protection was due to an increased ATP supply due to a reduced [Ca2+]c and hence [Ca2+]m. Allen et al. [53] pre-perfused isolated rat hearts with fura-2/am before induction of hypoxia and reoxygenation and found 2.5 μM ruthenium red could inhibit the reoxygenation induced rise in [Ca2+]m; however, no functional effects were reported and it is possible that this was an indirect result of a decrease in [Ca2+]c. Ruthenium red has also been claimed to have a beneficial effect during hypoxia/reoxygenation of cardiomyocytes: Stone et al. [54] found that 25 μM ruthenium red could greatly reduce the increase in total calcium seen on reoxygenation of hypoxic cell suspensions but did not report effects on cell morphology.

It is possible that the ease of permeability of ruthenium red may differ between isolated cells and whole hearts; the present study and that of Miyata et al. [25] found concentrations of at least 20 μM were required to inhibit Ca2+ entry into mitochondria in isolated myocytes. However, a study using perfused rat heart by McCormack and England [55] found that 2.5 μg/ml ruthenium red (which would correspond to about 1–2 μM) could block increases in mitochondrial pyruvate dehydrogenase activity without altering activation of phosphorylase (a cytosolic enzyme) upon stimulation of hearts with adrenergic agonists.

4.6. Role of the mitochondrial permeability transition pore?

Opening of the Ca2+ induced mitochondrial permeability transition pore has been implicated in myocardial ischemic injury, based mainly on the observations in hypoxic myocytes and ischemic hearts that cyclosporin A (a potent inhibitor of the transition in isolated mitochondria) can provide some protection against such damage [56,57].

The results of this study suggest no role for opening of the pore with regard to Ca2+ regulation in this model of hypoxia/reoxygenation damage since clonazepam and ruthenium red together prevented any changes in [Ca2+]m. The situation appears to be different in ischemia/reperfusion injury of whole hearts. Recent work shows that a non-specific pore opens upon reperfusion only and not during ischemia in isolated rat hearts [11]. It is not known whether [Ca2+]m changes during ischemia and if so, whether the same mechanisms would operate as in the present single cell model of hypoxia.
5. Conclusions

We have provided evidence that mitochondrial Na\(^+\)/Ca\(^{2+}\) exchange reverses mode during hypoxia following full ATP-depletion but appears to regain its usual directionality functioning as a Ca\(^{2+}\) efflux pathway upon reoxygenation. During hypoxia, reverse Na\(^+\)/Ca\(^{2+}\) exchange may account for the majority of Ca\(^{2+}\) uptake into mitochondria, whilst uniporter activity is dramatically reduced (but probably not reversed) under these conditions. However, during reoxygenation normal directionality of the Na\(^+\)/Ca\(^{2+}\) exchanger and uptake via the uniporter appears to be restored. Under these conditions, clonazepam appears to exert a protective effect, perhaps by allowing mitochondria to act as a Ca\(^{2+}\) ‘sink’ allowing more rapid restoration of cellular ATP levels. Although this compound may not be suitable for use in whole animals its non-mitochondrial effects on myocytes appear to be minimal [30,31] allowing its use as a tool for further studies in myocytes or possibly isolated hearts. Finally, ruthenium red at concentrations which actually inhibit changes in [Ca\(^{2+}\)]\(_{\text{mito}}\), did not show any protective effect in this model of hypoxia/reoxygenation damage.

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