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Reversal of mitochondrial Na/Ca exchange during metabolic inhibition in rat cardiomyocytes

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Abstract During hypoxia of isolated cardiomyocytes, Ca^{2+} entry into mitochondria may occur via the Na/Ca exchanger, the normal efflux pathway, and not the Ca-uniporter, the normal influx route. If this is the case, then depletion of myocyte Na^+ should inhibit Ca^{2+} uptake, and collapse of the mitochondrial membrane potential ($\Delta\psi_m$) would inhibit the uniporter. To test these hypotheses, isolated rat myocytes were exposed to metabolic inhibition, to mimic hypoxia, and $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ determined by selective loading of indo-1 into these compartments. $\Delta\psi_m$ was determined using rhodamine 123. Following metabolic inhibition, $[Ca^{2+}]_m$ was significantly lower in Na-depleted cells than controls ($P < 0.001$), $[Ca^{2+}]_c$ was approximately the same in both groups, and mitochondria depolarised completely. Thus Na-depletion inhibited mitochondrial Ca^{2+} uptake, suggesting that Ca^{2+} entry occurred via Na/Ca exchange, and the collapse of $\Delta\psi_m$ during metabolic inhibition is consistent with inactivity of the Ca-uniporter.

Key words: Mitochondrial calcium transport; Ca-uniporter; Mitochondrial membrane potential; Indo-1; Rhodamine; Hypoxia

1. Introduction

Under normal conditions in the heart, mitochondria have specific transport pathways for Ca^{2+} uptake and release. Studies on isolated mitochondria revealed that Ca^{2+} uptake occurred via a uniporter, inhibited by ruthenium red and driven by the highly negative mitochondrial membrane potential [1,2], whereas Ca^{2+} efflux occurred via an Na/Ca exchanger, inhibited by diltiazem, clonazepam and CGP 37157 [3]. However, only clonazepam is effective when used in intact myocytes due to non-mitochondrial effects of the other two inhibitors [4]. One role of these transporters under physiological conditions is to co-ordinate ATP supply and demand: increases in cytosolic Ca^{2+} ($[Ca^{2+}]_c$), which occur upon increased cardiac workloads or adrenergic stimulation of the heart, are relayed to the mitochondrial matrix via the transporters and activate enzymes of the citric acid cycle, resulting in increased rates of oxidative phosphorylation and hence ATP supply [5].

Mitochondria have the capacity to accumulate large amounts of calcium with very little deleterious effect under normal conditions. However, in pathological states such as hypoxia or ischaemia, accumulation of Ca^{2+} by mitochondria has been associated with the transition from reversible to irreversible cell injury [6–9]: when the heart is reoxygenated or reperfused, the effects of Ca^{2+} on mitochondria are exacerbated due to free radical generation, for example, this can lower the threshold of $[Ca^{2+}]$ needed for inducing the permeability transition pore [10]. If the mitochondria are damaged, ATP synthesis on reperfusion will be impaired and the heart will be unable to recover mechanical function since ATP from aerobic metabolism is necessary for normal contractile activity [11,12]. Therefore, preventing or delaying mitochondrial Ca^{2+} uptake during ischaemia/hypoxia may be protective.

In attempting to design strategies to inhibit mitochondrial Ca^{2+} uptake during hypoxia, myself and colleagues found very recently that significant alterations in the transport pathways may occur since the increase in $[Ca^{2+}]_m$ during hypoxia could be prevented by clonazepam but not by ruthenium red [13]. This suggested that Ca^{2+} entry during hypoxia was occurring via reversal of the Na/Ca exchanger whereas the uniporter was largely inactive.

The present study investigates the mechanism of the changes in mitochondrial Ca^{2+} transport (using a model of metabolic inhibition to mimic hypoxia) without use of inhibitors. This strategy avoids any possible interference from non-mitochondrial systems by non-specific effects of the inhibitors. Isolated myocytes were exposed to metabolic inhibition following depletion of intracellular $[Na^+]$ ($[Na^+]_i$); this should remove the driving force for mitochondrial Ca^{2+} uptake if it is indeed occurring via reversal of the Na/Ca exchanger. Mitochondrial membrane potential ($\Delta\psi_m$) was also measured to determine whether any changes could account for the proposed inactivity of the Ca-uniporter.

2. Materials and methods

2.1. Myocyte isolation and measurement of indo-1 fluorescence

Single cardiac myocytes were isolated from rat ventricles by collagenase digestion [10,13]. Male Wistar rats (200–250 g) were killed by cervical dislocation and the heart removed and placed in ice-cold 'isolation buffer' plus 0.75 mM $CaCl_2$. Isolation buffer contained, in mM: 20 sodium *N*-hydroxyethylpiperazine-*N*′-2-ethansulphonic acid (HEPES), 130 NaCl, 4.5 KCl, 5 $MgCl_2$, 1 NaH_2PO_4, 21 glucose, 5 Na-pyruvate, pH 7.25 with NaOH. The heart was perfused with isolation buffer plus 0.75 mM $CaCl_2$ at 37°C for 4 min before switching to Ca^{2+}-free buffer (isolation buffer plus 90 μM EGTA) for 4 min. The perfusate was then switched to 'enzyme solution' consisting of 50 ml isolation buffer plus 50 mg collagenase (Worthington, type I), 5 mg protease (Sigma, type XIV) and 15 μM $CaCl_2$. The enzyme solution was continued until the tissue felt soft; approximately 15 min. The heart was then washed with isolation buffer plus 150 μM $CaCl_2$, ventricles removed, sliced approximately 10 times and shaken for 5 min at 37°C in 20–25 ml isolation buffer plus 150 μM $CaCl_2$. After filtration the cells were allowed to sediment in this buffer for 7 min.

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The supernatant was removed and cells resuspended in 0.5 mM $CaCl_2$, the process repeated and cells finally resuspended in approximately 15 ml of 2 mM $CaCl_2$.

2.2. Measurement of [Ca^{2+}] and mitochondrial membrane potential ($\Delta\psi_m$)

Mitochondrial and cytosolic [Ca^{2+}] were determined by selective loading of indo-1, and $\Delta\psi_m$ using rhodamine 123 (indicators from Molecular Probes Inc.). Loading conditions for the indicators was as follows:

2.2.1. Mitochondrial loading of indo-1. Three ml suspension was incubated with 10 μM indo-1/am for 15 min at 30°C when the dye partitions approximately equally between cytosolic and mitochondrial compartments [14]. The cells were centrifuged for 1 min at 500 rpm and resuspended in 5 ml isolation buffer (containing 2 mM $CaCl_2$). The cells were then incubated at room temperature for 2 h, shaken gently at 37°C for 1.5 h, sedimented by centrifugation, resuspended and stored at room temperature. This process, termed 'heat-treatment', promotes loss of cytosolic, but not mitochondrial, indo-1 through sarcolemmal anion channels, and can be inhibited by probenecid. Full details of the method and experiments to confirm the mitochondrial origin of the remaining fluorescence are given in Griffiths et al. [15].

2.2.2. Cytosolic loading of indo-1. Three ml cell suspension was incubated with 10 μM indo-1/am for 5 min at 37°C. These conditions have been reported by other workers to result in approximately 90% cytosolic loading of the indicator fluo-3 [16]. The cells were then centrifuged for 1 min at 500 rpm and resuspended in 5 ml isolation buffer (containing 2 mM $CaCl_2$) plus 1 mM probenecid to prevent dye leakage from the cytosol. In order to confirm the distribution of indo-1 in the present experiments, cells were subjected to cytosolic loading conditions, except that no probenecid was present in the final resuspension solution. Fluorescence of a sample of these cells was measured, and the remainder subjected to the heat-treatment procedure. For each preparation, the mean fluorescence of the heat-treated cells was expressed as a percentage of the initial fluorescence (10–14 cells measured before and following heat-treatment in each case). This gave a very consistent loading with 85 ± 0.7% ($n = 3$ hearts) of the dye being located in the cytosol.

2.2.3. Loading of rhodamine 123. Rhodamine 123 is a fluorescent indicator which partitions into the mitochondrial membrane due to the highly negative membrane potential [17]. Cells were incubated with 10 μg/ml rhodamine 123 for 30 min at 37°C. The cells were then centrifuged for 1 min at 500 rpm and resuspended in 5 ml isolation buffer (containing 2 mM $CaCl_2$). Cells were maintained at room temperature for at least 1 h prior to use to allow for accumulation of rhodamine by mitochondria. The mitochondrial location of rhodamine was confirmed by addition of FCCP, a mitochondrial uncoupler, which dissipated $\Delta\psi_m$. See Fig. 4 for full details.

2.3. Measurement of fluorescence and cell length

A small portion of the loaded cells was placed in an experimental chamber which was mounted on the stage of an inverted microscope (Nikon Diaphot 300). The normal superfusate ('normoxia') contained, in mM: 137 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 16 D-glucose, 2 $CaCl_2$, 20 HEPES pH 7.4 (using NaOH), temperature 37°C. The myocyte to be studied was illuminated with a red light and its image visualized with a TV camera and monitor. Indo-1 was excited at 340–390 nm and emission detected at 410 ± 5 nm and 490 ± 5 nm, corresponding to the peak emissions of the Ca^{2+} bound and Ca^{2+} free forms of the indicator, respectively. Rhodamine 123 was excited at 450–490 nm and emission detected at 535 ± 10 nm. Fluorescence of the whole cell was collected on-line by a Newcastle Photometric Systems Photon Counting System (Newcastle, UK). Light was collected at a rate of up to one data point/10 ms from a single myocyte following subtraction of background fluorescence. Cell length changes were monitored using a Crescent Electronics Video Edge Motion Detector.

2.4. Experimental protocol

Where indicated in Section 3, cells were depleted of Na by superfusion with '0 Na' buffer where the 137 mM NaCl in the normal superfusate was replaced with 137 mM choline chloride (pH 7.4 using KOH). The following superfusion protocol, based on that described by Siegmund et al. [18] was used to prevent cell hypercontracture during Na-depletion (1 μM thapsigargin was present throughout to eliminate interference from the sarcoplasmic reticulum): normoxia, 5 min; 0 Ca (+90 μM EGTA), 5 min; 0 Ca, 0 Na, 60 min. Na-depletion was confirmed by returning to normoxia (in presence of 2 mM $CaCl_2$) when no Ca^{2+} entry occurred. Metabolic inhibition was induced by superfusion with normoxic solution containing 2.5 mM KCN and no glucose.

2.5. Expression of results and statistical analyses

[Ca^{2+}] is expressed as indo-1 ratio, presented as means ± S.E. unless raw data tracings are shown. Calibration of the indo-1 signal in terms of absolute values of calcium was not attempted since this would provide no advantage over ratio values in the present experiments. Rhodamine fluorescence is expressed in arbitrary units, this in a non-ratiometric indicator and the signal cannot be calibrated in terms of absolute values of electrical potential. In Fig. 4B the results are expressed as % increase in rhodamine fluorescence over initial values, no bleaching of the dye occurred under the experimental conditions used. Statistical analyses were performed using Student's *t*-test, paired where appropriate.

3. Results

3.1. Mitochondrial and cytosolic [Ca^{2+}] changes during metabolic inhibition in presence and absence of sodium

Upon exposure of isolated myocytes to either metabolic inhibition or anoxia, cells undergo ATP-depletion rigor-contracture, where the cell contracts to approximately 60% of its original length [12,19,20]. Only after this point do both $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ begin to increase. Cell recovery upon subsequent reoxygenation (or washout of inhibitors) occurs only in cells where $[Ca^{2+}]_m$ remains below 300–400 nM during hypoxia [7,13].

In the present study, the effect of metabolic inhibition on $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ was determined firstly in control cells, i.e. experiments performed in presence of Na^+. Control cells had initial levels of $[Ca^{2+}]_m$ that were slightly lower than $[Ca^{2+}]_c$, Fig. 1, agreeing with previous observations on rat myocytes [14,15]. Following exposure of control cells to metabolic inhibition, no change in either $[Ca^{2+}]_m$ or $[Ca^{2+}]_c$ occurred prior to rigor-contracture. However, following rigor both $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ increased significantly over their respective initial values and there was no significant difference between cytosolic and mitochondrial levels (Fig. 1).

In order to determine whether the Na/Ca exchanger was indeed responsible for the mitochondrial Ca^{2+} uptake observed following rigor-contracture, cells were depleted of Na^+ as described in Section 2 prior to induction of metabolic

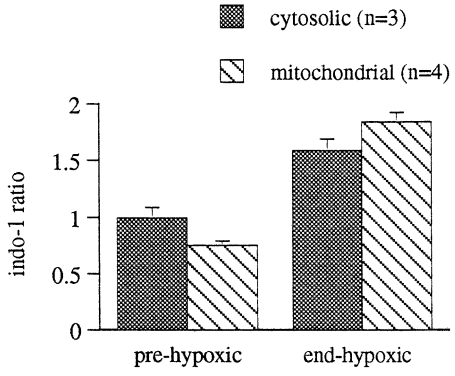

Fig. 1. Cytosolic and mitochondrial [Ca^{2+}] changes during exposure of myocytes to metabolic inhibition in presence of sodium. Data show mean values for indo-1 ratios prior to metabolic inhibition and following rigor development. There are no significant differences between mitochondrial and cytosolic values.

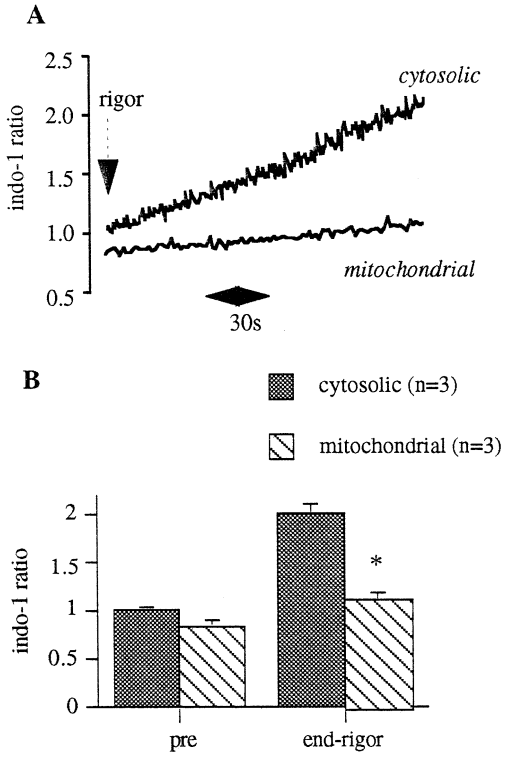

Fig. 2. Cytosolic and mitochondrial [Ca^{2+}] changes during exposure of myocytes to metabolic inhibition in absence of sodium. A: Raw data from single cells showing changes in indo-1 ratio following rigor-contracture (no changes occurred prior to rigor). B: Mean values for indo-1 ratios prior to metabolic inhibition and following rigor development. *$P < 0.05$ vs. cytosolic value.

inhibition (which was also performed in absence of Na^+). There was no increase in either $[Ca^{2+}]_m$ or $[Ca^{2+}]_c$ before rigor development, as found in control cells. However, following rigor $[Ca^{2+}]_c$ increased rapidly and significantly as shown in Fig. 2. In contrast, $[Ca^{2+}]_m$ increased only very slowly and was significantly lower than $[Ca^{2+}]_c$ at the end of rigor (Fig. 2). Thus Na^+ appeared to be necessary for entry of Ca^{2+} into

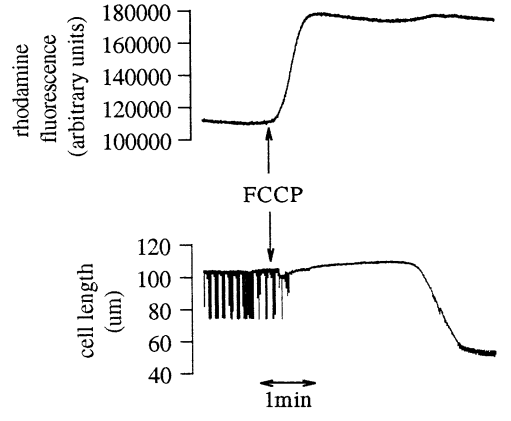

Fig. 3. Changes in rhodamine 123 fluorescence and cell length upon addition of FCCP. Simultaneous measurement of cell length and rhodamine fluorescence in a single myocyte stimulated to contract at 1 Hz.

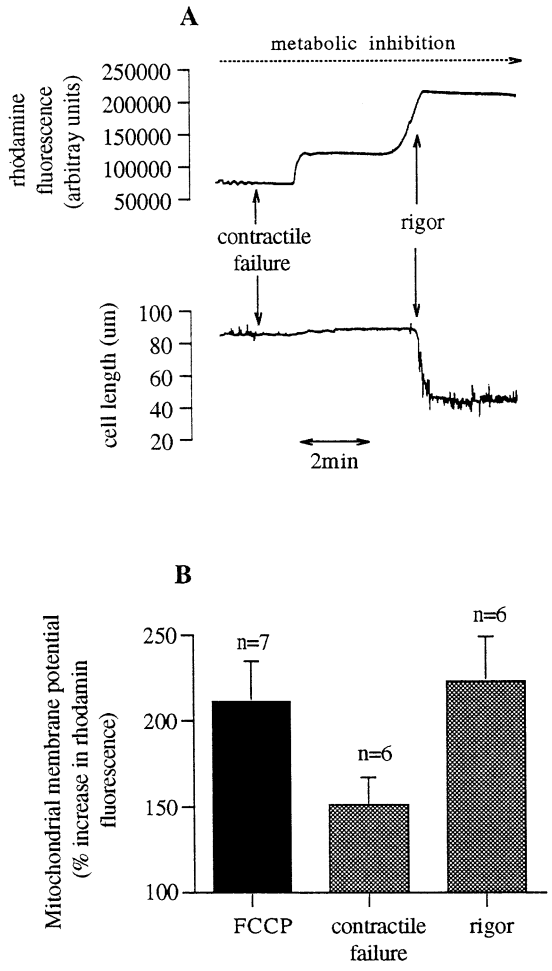

Fig. 4. Changes in mitochondrial membrane potential during metabolic inhibition. A: Simultaneous measurements of rhodamine fluorescence and cell length during exposure of a single myocyte to metabolic inhibition. B: Mean values of experiments shown in Fig. 3 and A. Data are presented as the % increase in rhodamine fluorescence over initial values. The change in $\Delta\psi_m$ achieved during rigor was identical to that observed using FCCP.

mitochondria, but not into the cytosol, during metabolic inhibition following rigor-contracture.

3.2. Changes in mitochondrial membrane potential ($\Delta\psi_m$) during metabolic inhibition

Cells were loaded with rhodamine for measurement of $\Delta\psi_m$ as described in Section 2. In order to confirm the mitochondrial localisation of the indicator, cells were exposed to FCCP, an uncoupler of oxidative phosphorylation, which completely depolarises the mitochondrial inner membrane. Fig. 3 shows the changes in rhodamine fluorescence and cell length following exposure of a single cell to FCCP. Following addition of FCCP, $\Delta\psi_m$ began to increase just prior to contractile failure and reached a peak before rigor development (indicated by cell shortening). There appeared to be very little effect of rigor development on rhodamine fluorescence. The large increase in rhodamine fluorescence following addition of FCCP confirms the mitochondrial localisation of the indicator. Since the rhodamine signal cannot be calibrated, results are subsequently expressed in either arbitrary units or as % of initial fluorescence.

The changes in $\Delta\psi_m$ during exposure to metabolic inhibition for a single cell are shown in Fig. 4A together with simultaneous measurement of cell length. $\Delta\psi_m$ increased slightly following contractile failure and began to increase further just prior to rigor development. Following rigor, $\Delta\psi_m$ reached a plateau and remained at this level throughout. The peak value of $\Delta\psi_m$ following rigor development was identical to that observed using FCCP, as shown in Fig. 4B which compares the % changes in $\Delta\psi_m$ observed during metabolic inhibition to those seen using FCCP. Thus mitochondria were completely depolarised following rigor development.

4. Discussion

The results presented here indicate that Na^+ is necessary for entry of Ca^{2+} into mitochondria, but not cytosol, following rigor development in cells exposed to metabolic inhibition. Under control conditions (Na^+ present), $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ increased to approximately the same extent following rigor development (Fig. 1). However, when cells were depleted of sodium prior to exposure to metabolic inhibition, entry of Ca^{2+} into mitochondria was greatly inhibited (Fig. 2), indicating that the pathway of Ca^{2+} entry into mitochondria during metabolic inhibition is Na-dependent. This, together with previous work which showed that Ca^{2+} entry into mitochondria of hypoxic myocytes could be inhibited by clonazepam [13], provides compelling evidence that the route of Ca^{2+} entry is indeed the Na/Ca exchanger, the normal efflux pathway.

The mechanism of the observed increase in $[Ca^{2+}]_c$ in absence of Na^+ is unknown. Ca^{2+} entry into cells during hypoxia has been proposed to be largely due to the sarcolemmal Na/Ca exchanger following an increase in $[Na^+]_i$, via either Na/H exchange or Na-channels [21]. This was based on experiments showing that hypoxic Ca-loading can be reduced by nickel, an inhibitor of Na/Ca exchange [22] and by studies showing that inhibitors of Na/H exchange reduced Na-loading with a corresponding decrease in Ca-loading [23]. The results of the present study indicate that Ca^{2+} can enter the cytosol of Na-depleted myocytes during metabolic inhibition. A previous study [18], found a similar result in hypoxic myocytes. These workers suggested that their observation did not disprove the previous conclusions that the major route of Ca^{2+} entry was by Na/Ca exchange, but rather that under conditions of Na-depletion another, as yet unidentified, pathway became active. The present results support this conclusion but also indicate that no similar pathway exists for Ca^{2+} entry into mitochondria during Na-depletion.

Upon exposure of myocytes to metabolic inhibition, $\Delta\psi_m$ partially depolarised prior to rigor and reached a peak following rigor. The $\Delta\psi_m$ achieved during rigor was identical to the $\Delta\psi_m$ produced by FCCP allowing the conclusion that mitochondria were completely depolarised at this time. The changes in $\Delta\psi_m$ occurring during metabolic inhibition were very similar to those reported to occur in hypoxic myocytes [24].

$\Delta\psi_m$ is normally maintained at highly negative values, approximately -100 mV, with respect to the cytosolic potential [1] and isolated mitochondria, uptake of Ca^{2+} via the Ca-uniporter is driven by $\Delta\psi_m$. In absence of $\Delta\psi_m$ generation by respiration, no Ca^{2+} uptake into isolated mitochondria could be observed even in the presence of an 8-fold gradient of $[Ca^{2+}]$ [25] (Na^+ was not present in these experiments). These workers concluded that Ca^{2+} uptake through the uniporter would not occur in absence of $\Delta\psi_m$ even though the process would not be thermodynamically restricted. In experiments where Ca^{2+} uptake into isolated mitochondria was studied in presence of Na^+, and when $\Delta\psi_m$ was dissipated with FCCP, Jung et al. [26] found that rapid Ca^{2+} uptake into mitochondria did indeed occur. The Ca^{2+} uptake was inhibited by diltiazem, suggesting that the Na/Ca exchanger was the route of Ca^{2+} entry under these conditions. The exchange was electroneutral in contrast to the situation during normal, energised conditions where the Na/Ca exchanger is now thought to be electrogenic with a stoichiometry of $3Na^+:Ca^{2+}$ [26,27].

The present results underline the importance of studying basic mechanisms of Ca^{2+} transport in designing protective strategies against hypoxic/ischaemic injury. In attempting to limit Ca^{2+} uptake by mitochondria during hypoxia, previous studies have assumed that the route would be via the uniporter [28–31]. We have now shown that conditions during hypoxia are such that the uniporter is largely inactive, and that Ca^{2+} entry occurs, instead, by the Na/Ca exchanger.

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