Use of ruthenium red as an inhibitor of mitochondrial Ca^{2+} uptake in single rat cardiomyocytes

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Abstract With the current resurgence of interest in the role of mitochondrial $[Ca^{2+}]$ in energy production and cellular Ca^{2+} signalling, ruthenium red (RR) is being increasingly used as an inhibitor of mitochondrial Ca^{2+} uptake. In the present study, the effects of RR on cell and mitochondrial $[Ca^{2+}]$, and on cell contractility were determined in isolated rat ventricular myocytes subjected to adrenergic and electrical stimulation. At low concentrations, 0–1 μM, RR inhibited mitochondrial Ca^{2+} uptake but this was a secondary effect due to a reduced total intracellular $[Ca^{2+}]$, a conclusion supported by the ability of RR to inhibit cell shortening. 5 μM RR completely inhibited cell contraction, whereas higher concentrations, 10–25 μM, induced spontaneous Ca^{2+} oscillations and contractile waves. These results indicate that great care must be taken when using RR in intact cells, and in interpreting any effects as resulting from a primary inhibition of mitochondrial Ca^{2+} uptake.

Key words: Indo-1; Mitochondrion; Calcium; Ruthenium red

1. Introduction

There is currently a resurgence of interest in the role of mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_m$) in both cell energy production and intracellular Ca^{2+} signalling (reviewed in [1–3]). In non-cardiac cell types, subpopulations of mitochondria situated near sites of Ca^{2+} release, such as those of the endoplasmic reticulum, are capable of rapidly accumulating large amounts of Ca^{2+} [4–6]. In heart cells, however, the question of how rapidly mitochondria take up Ca^{2+} is controversial, especially whether $[Ca^{2+}]_m$ changes during a single contraction [7–9]. Accumulation of Ca^{2+} by mitochondria can rapidly activate dehydrogenases of the citric acid cycle to increase NADH, and hence ATP, production [1,4,5]. In heart cells, mitochondrial Ca^{2+} transport is especially significant since the heart is absolutely dependent on ATP from oxidative phosphorylation for contraction. Furthermore, the large proportion of mitochondria within heart muscle gives them the potential to modulate cytosolic Ca^{2+} levels, as found in other cell types [5,10]. However, this has yet to be demonstrated during the normal excitation–contraction coupling cycle of the heart. In disease states such as ischaemia/reperfusion injury, mitochondrial Ca^{2+} uptake may act as a double-edged sword; it can buffer cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$), and thus protect the cell from Ca^{2+} overload, but may also induce the mitochondrial permeability transition pore, which would then prevent ATP resynthesis upon reperfusion (reviewed in [11–13]).

Studies attempting to dissect the role of mitochondrial Ca^{2+} transport in both physiological and pathological functioning of the heart have relied largely on the use of inhibitors of the mitochondrial Ca^{2+} transporters: ruthenium red (RR), which inhibits Ca^{2+} entry via the Ca^{2+} uniporter, and drugs like diltiazem, clonazepam and CGP 37157, which inhibit Ca^{2+} efflux by the mitochondrial Na^+/Ca^{2+} exchanger [14–16]. All these inhibitors are effective in isolated mitochondria, but the situation is more complicated in intact cells or organs. Diltiazem cannot be used since it is an inhibitor of sarcolemmal Ca^{2+} channels, CGP 37157 has been used in some non-cardiac cell types, but appears to be unreliable for use in isolated myocytes, and although clonazepam has been used successfully in isolated rat myocytes, it has yet to be tested in cells from other species, or in whole hearts [16,17].

RR has, however, been widely used in both isolated hearts and myocytes. Although known to affect sarcoplasmic reticular (SR) Ca^{2+} transport, its action in protecting against ischaemia/reperfusion injury has been attributed to its ability to inhibit mitochondrial Ca^{2+} uptake [18–23]. The aim of the present study was to determine whether low concentrations of RR (0–5 μM) could inhibit mitochondrial Ca^{2+} uptake in isolated myocytes independently of effects on $[Ca^{2+}]_c$ and changes in cell contraction.

2. Materials and methods

2.1. Myocyte isolation

Single cardiac myocytes were isolated from rat ventricles by collagenase digestion [8]. 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

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Abbreviations: RR, ruthenium red; $[Ca^{2+}]_i$, intracellular $[Ca^{2+}]$; $[Ca^{2+}]_m$, mitochondrial $[Ca^{2+}]$; $[Ca^{2+}]_c$, cytosolic $[Ca^{2+}]$

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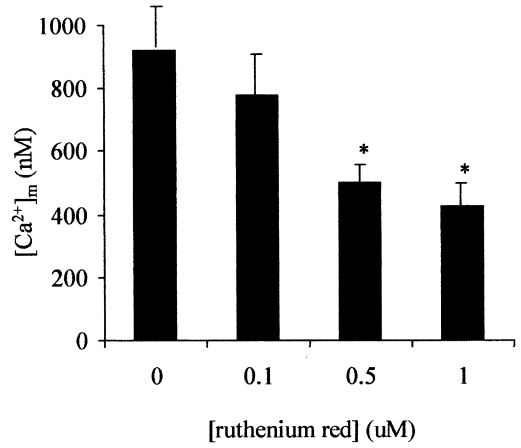

Fig. 1. The effect of RR on mitochondrial Ca^{2+} uptake. Single myocytes were electrically stimulated to contract at a rate of 6 Hz in presence of 0.25 μM noradrenaline in order to induce mitochondrial Ca^{2+} uptake. $[Ca^{2+}]_m$ was then measured using indo-1 fluorescence (see Section 2), after reaching a peak (in about 1 min). When present, RR was added 10 min before the start of the stimulation protocol. $n = 4$–11 cells from 3–6 hearts in each group. $^*P < 0.05$ versus 0 [RR].

150 μM $CaCl_2$. After filtration the cells were allowed to sediment in this buffer for 7 min. 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 isolation buffer plus 2 mM $CaCl_2$.

2.2. Measurement of [Ca^{2+}]

$[Ca^{2+}]_m$ and total intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) were determined using indo-1/AM. Cells were incubated with 10 μM indo-1/AM for 15 min at 30°C, then centrifuged (1 min at $500 \times g$) and resuspended in isolation buffer (containing 2 mM $CaCl_2$); under these conditions the dye partitions approximately equally between cytosolic and mitochondrial compartments [7,8].

For determination of total $[Ca^{2+}]_i$, 1 mM probenecid was then added to prevent leakage of indo-1 from the cytosol during cell storage.

For determination of $[Ca^{2+}]_m$, cells were incubated (without probenecid) at room temperature for 2 h, followed by 1.5 h at 37°C. This process, termed 'heat-treatment', promotes loss of cytosolic, but not mitochondrial, indo-1 through sarcolemmal anion channels. Full details of the method and experiments to confirm the mitochondrial origin of the remaining fluorescence are given in Griffiths et al. [8].

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 contained (in mM): 137 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 16 D-glucose, 1 $CaCl_2$, 20 HEPES pH 7.4 (using NaOH), temperature 37°C. The 'low-Na' solution used to induce cytosolic Ca^{2+} loading (see Section 3) contained 5 mM NaCl, 132 mM choline chloride (pH 7.4 using KOH). The myocyte to be studied was illuminated with a red light and its image visualised with a TV camera and monitor. Indo-1 was excited at 340–390 nm and emission detected at 410 ± 5 and 490 ± 5 nm, corresponding to the peak emissions of the Ca^{2+}-bound and Ca^{2+}-free forms of the indicator, respectively. 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. Calculation of [Ca^{2+}]

To obtain values of $[Ca^{2+}]_i$, the fluorescence ratios obtained after subtracting background fluorescence were compared to ratios obtained using a solution of indo-1 salt in saline and containing buffers of known $[Ca^{2+}]$; this method gives results very similar to an in vivo calibration [24]. The minimum ratio (R_{min}), obtained by measuring fluorescence from a Ca-free buffer was 0.2; the maximum ratio (R_{max}) obtained in micromolar Ca^{2+}-containing buffer was 3.5. Cellular $[Ca^{2+}]$ was then calculated from the equation using $440(R-R_{min})/(R_{max}-R)$, where 440 is the dissociation constant in nM.

2.5. Materials

Fluorescent dyes were obtained from Molecular Probes Limited. Collagenase was Worthington type I (supplied by CamBio, UK) and protease Sigma type XIV. Other reagents were obtained from Sigma, BDH or Boehringer Mannheim. RR was obtained from Sigma as the crude preparation; no attempt was made to purify this to allow comparison with previous studies where the crude preparation was also used.

2.6. Statistical analyses

Statistical analyses were performed using Student's *t*-test. Data are presented as means ± S.E.M.

3. Results

In isolated rat myocytes, $[Ca^{2+}]_m$ is normally maintained at a slightly lower concentration than that in the cytosol [7,8]. This did not change significantly even when cells were electrically stimulated to contract at high (although physiological) rates of 6 Hz (results not shown). However, superfusion with 0.25 μM noradrenaline, together with a stimulation rate of 6 Hz, did induce a significant increase in $[Ca^{2+}]_m$ (Fig. 1). Under these conditions, $[Ca^{2+}]_m$ does not change during a

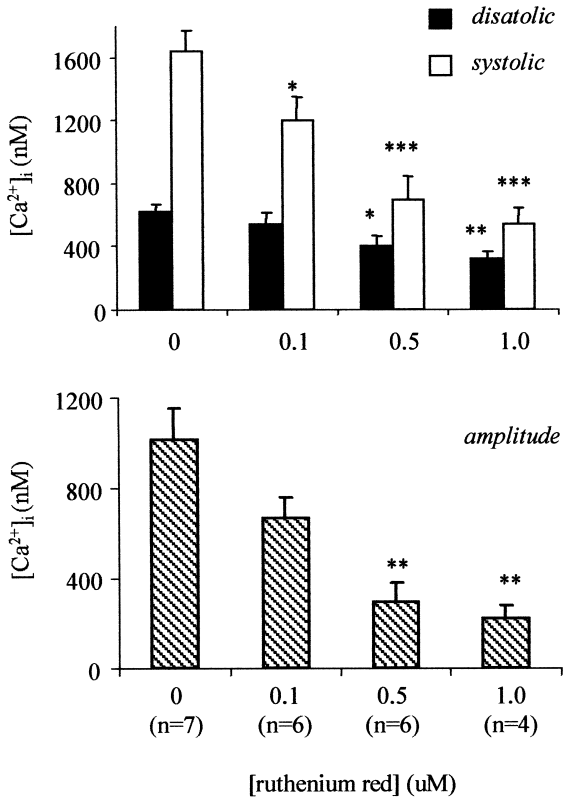

Fig. 2. Effect of RR on $[Ca^{2+}]_i$. Myocytes were subjected to the same stimulation protocol as described for Fig. 1, except that total $[Ca^{2+}]_i$ was determined using indo-1 fluorescence (see Section 2), for diastolic and systolic $[Ca^{2+}]$ (top panel), and the amplitude of the Ca^{2+} transient (systolic−diastolic, lower panel). $n = 4$–7 cells from 3–6 hearts in each group. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus corresponding value at 0 [RR].

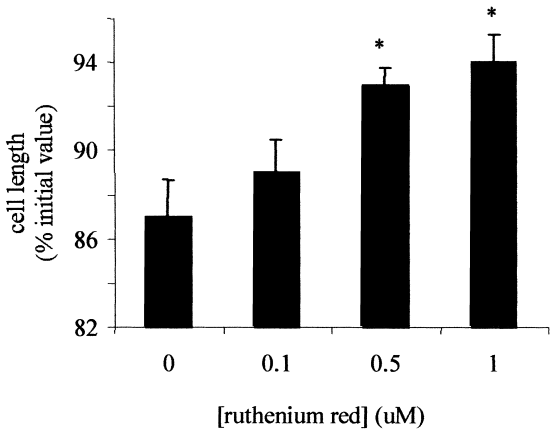

Fig. 3. Effect of RR on maximal cell shortening. Cells were subjected to the stimulation protocol described for Fig. 1. Cell length was measured using an edge tracking device, and is expressed as %(maximal shortening at peak stimulation)/(initial cell length). $n = 6$–18 cells from 3–11 hearts in each group. $^*P < 0.05$ versus 0 [RR].

single contraction, but does increase more slowly, reaching a maximum in approximately 1 min (see [8]). The effect of various concentrations of RR on $[Ca^{2+}]_m$ was then tested using this protocol. Fig. 1 shows that whereas 0.1 μM RR had no significant effect on the increase in $[Ca^{2+}]_m$, both 0.5 and 1 μM RR significantly reduced $[Ca^{2+}]_m$.

In order to determine whether this was independent, or a secondary consequence, of a decrease in $[Ca^{2+}]_c$, the protocol was repeated in cells where total $[Ca^{2+}]_i$ was measured. Fig. 2 (top panel) shows that the peak systolic $[Ca^{2+}]$ was reduced by all concentrations of RR, and 0.5 and 1 μM RR also reduced diastolic $[Ca^{2+}]$. Since this method measures total $[Ca^{2+}]_i$, it could be argued that the decreases in diastolic and systolic $[Ca^{2+}]$ were simply a reflection of the decreased $[Ca^{2+}]_m$. However, the observation that the amplitude of the Ca^{2+} transient was also reduced by RR (Fig. 2, lower panel), indicates that this is not the case. Furthermore, the maximum cell shortening induced in response to high stimulation plus noradrenaline was also reduced by RR at 0.5 and 1 μM, indicating that RR was indeed affecting $[Ca^{2+}]_c$ (Fig. 3).

In attempting to use higher concentrations of RR, it was found that 5 μM completely inhibited cell contraction, whereas at 10 μM and above, the cells underwent spontaneous contractions and $[Ca^{2+}]_i$ oscillations, suggesting that RR has a dual effect on $[Ca^{2+}]_i$.

In order to determine whether RR can in fact inhibit mitochondrial Ca^{2+} uptake, cells were exposed to a solution containing low $[Na^+]$, which induces cellular Ca^{2+} overload by reversal of the sarcolemmal Na^+/Ca^{2+} exchanger, and contributions of SR Ca^{2+} transport were abolished using thapsigargin. Fig. 4 shows that, using this procedure, a concentration of 25 μM RR was required to completely prevent the increase in $[Ca^{2+}]_m$; this was not a secondary result of a reduction in $[Ca^{2+}]_c$ since all cells underwent spontaneous contractile oscillations, indicative of cytosolic Ca^{2+} overload.

4. Discussion

The results of this paper indicate that, at low concentrations (0.1–1 μM), the inhibition of mitochondrial Ca^{2+} uptake by RR is secondary to a reduction in cytosolic, notably systolic, $[Ca^{2+}]$, suggesting an inhibition of Ca^{2+} release from the SR. This conclusion was supported by the ability of RR to also inhibit cell shortening; indeed at 5 μM RR, cell contraction was completely abolished. Inhibition of mitochondrial Ca^{2+} uptake was observed at a [RR] of 25 μM in response to a Ca^{2+} loading protocol which was independent of SR Ca^{2+} release (Fig. 4). However, at 10 μM and above, RR induced spontaneous Ca^{2+} and contractile oscillations, which are indicative of cytosolic Ca^{2+} overload. Thus, RR appears to have dual effects on $[Ca^{2+}]_i$; at low concentrations RR inhibits the release of Ca^{2+} from the SR, and thus also inhibits cell contraction, whereas at higher concentrations, RR induces cytosolic Ca^{2+} overload. In either case, normal excitation–contraction coupling is disrupted.

In previous studies, the concentrations of RR used have varied greatly, from 0.025 to 50 μM [7,18–23,25–28]. Very few of these studies have actually measured $[Ca^{2+}]_m$, and of those that have, concentrations in excess of 20 μM have generally been used to inhibit mitochondrial Ca^{2+} uptake [7,25]. The effects of lower concentrations were not reported in these studies. In the other studies, where 0.025 to 5 μM RR were used, effects were attributed to its action as an inhibitor of mitochondrial Ca^{2+} uptake. However, the results of the present study indicate that this is likely to be due to a secondary effect of a reduced $[Ca^{2+}]_c$, and not to an effect on mitochondrial Ca^{2+} uptake per se.

The mechanism of the Ca^{2+} overload induced by RR at higher concentrations is not known. RR has also been found to increase $[Ca^{2+}]_i$ in neuronal cells in a dose- and time-dependent manner [29], and mitochondrial oxidative function was also depressed by RR in this study. This latter observation agrees with previous work using isolated rat liver mitochondria which showed that high concentrations of RR inhibited respiration [30]. One explanation is that RR may act as an electron carrier itself and transfer electrons from NADH to cytochrome *c*, thus bypassing the normal pathways of respiration and oxidative phosphorylation [31]. Any such inhibition of ATP synthesis in the heart would be expected to result in disruption of Ca^{2+} homeostasis, and lead to eventual cyto-

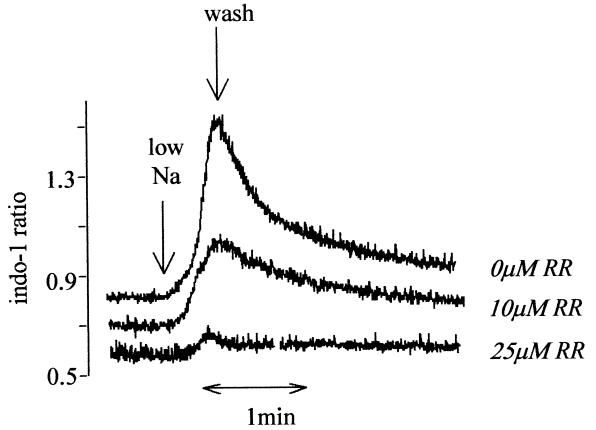

Fig. 4. Effect of RR on $[Ca^{2+}]_m$ during a cell Ca^{2+} overloading protocol. Cells were not stimulated and were pre-treated with 1 μM thapsigargin prior to the experiment. Cells were then briefly exposed to 'low Na' solution (see Section 2) before returning to normal buffer. $[Ca^{2+}]_m$ was measured as described in Section 2, and results are presented as raw data tracings from single cells (typical of three such experiments).

solic Ca^{2+} overload. In addition, RR is a polycationic molecule, and could conceivably displace bound intracellular Ca^{2+} by a non-specific action.

With the current interest in the role of $[Ca^{2+}]_m$ in many aspects of physiological and pathological cell functioning, RR is increasingly used with any effects on cell function or intracellular Ca^{2+} signalling attributed to inhibition of mitochondrial Ca^{2+} uptake. The results of the present study caution strongly against the indiscriminate use of this agent in such studies, and that measurements of both cytosolic and $[Ca^{2+}]_m$ should ideally be made before any conclusions can be drawn.

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