Distinct mPTP activation mechanisms in ischaemia–reperfusion: contributions of Ca$^{2+}$, ROS, pH, and inorganic polyphosphate

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Aims

The mitochondrial permeability transition pore (mPTP) plays a central role for tissue damage and cell death during ischaemia–reperfusion (I/R). We investigated the contribution of mitochondrial inorganic polyphosphate (polyP), a potent activator of Ca$^{2+}$-induced mPTP opening, towards mPTP activation and cardiac cell death in I/R.

Methods and results

A significant increase in mitochondrial free calcium concentration ([Ca$^{2+}$]$_{m}$), reactive oxygen species (ROS) generation, mitochondrial membrane potential depolarization ($\Delta$Ψ$_{m}$), and mPTP activity, but no cell death, was observed after 20 min of ischaemia. The [Ca$^{2+}$]$_{m}$ increase during ischaemia was partially prevented by the mitochondrial Ca$^{2+}$ uniporter (MCU) inhibitor Ru360 and completely abolished by the combination of Ru360 and the ryanodine receptor type 1 blocker dantrolene, suggesting two complimentary Ca$^{2+}$ uptake mechanisms. In the absence of Ru360 and dantrolene, mPTP closing by polyP depletion or CSA decreased mitochondrial Ca$^{2+}$ uptake, suggesting that during ischaemia Ca$^{2+}$ can enter mitochondria through mPTP. During reperfusion, a burst of endogenous polyP production coincided with a decrease in [Ca$^{2+}$]$_{m}$, a decline in superoxide generation, and an acceleration of hydrogen peroxide (H$_2$O$_2$) production. An increase in H$_2$O$_2$ correlated with restoration of mitochondrial pH$_{m}$ and an increase in cell death. mPTP opening and cell death on reperfusion were prevented by antioxidants Trolox and MnTBAP [Mn (III) tetrakis (4-benzoic acid) porphyrin chloride]. Enzymatic polyP depletion did not affect mPTP opening during reperfusion, but increased ROS generation and cell death, suggesting that polyP plays a protective role in cellular stress response.

Conclusions

Transient Ca$^{2+}$/polyP-mediated mPTP opening during ischaemia may serve to protect cells against cytosolic Ca$^{2+}$ overload, whereas ROS/pH-mediated sustained mPTP opening on reperfusion induces cell death.

Keywords

Inorganic polyphosphate • Mitochondrial permeability transition pore • Ischaemia–reperfusion injury • Oxidative stress • Mitochondrial ryanodine receptor

1. Introduction

It is generally accepted that mitochondrial permeability transition (PT) is associated with the opening of the mitochondrial permeability transition pore (mPTP), a large non-specific channel in the inner mitochondrial membrane (IMM). PT represents a dramatic increase in ion conductance of the IMM. Under physiological conditions, IMM permeability is relatively low. This is required for maintenance of the tight coupling of the oxidative phosphorylation machinery. Opening of the mPTP leads to cellular and tissue damage during ischaemia–reperfusion (I/R) injury (IRI).

Ca$^{2+}$ and reactive oxygen species (ROS) are considered to be two key activators of PT during IRI. However, recent data indicate that, depending on the complex balance between cellular stress inducers and antioxidant defence systems, mPTP can undergo transient (low-conductance) or long-lasting (high-conductance) openings. Transient mPTP opening has been suggested to be involved in physiological processes such as intracellular Ca$^{2+}$ homeostasis, NAD$^+$ trafficking, and transient formation of ROS; it could also play a role in cardioprotection by ischaemic preconditioning. While a transient pore opening is typically a reversible event which is not associated with cell death, sustained mPTP opening during reperfusion may exacerbate cellular damage. 

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death, long-lasting pore opening is followed by profound alterations of cellular bioenergetics that are considered irreversible; it results in increased mitochondrial permeability to ions and solutes with mol. wt of up to 1.5 kDa, matrix swelling, loss of critical electrochemical gradients, and depolarization of mitochondrial membrane potential ($\Delta \Psi_m$).

In this condition, the $F_0F_1\text{-ATP}$ synthase actively hydrolyzes rather than synthesizes ATP, leading inevitably to cell death.11–13 Our recent studies indicate that inorganic polyphosphate (polyP),14–16 a polymer of orthophosphates linked by phosphoanhydride bonds, plays a major role in activation of $\text{Ca}^{2+}$-induced mPTP opening in cardiomyocytes. Furthermore, we found that polyP depletion prevents a transient drop in $\Delta \Psi_m$, associated with $\text{Ca}^{2+}$-induced mPTP opening.15,16 This discovery together with the fact that polyP plays multiple roles in cellular and mitochondrial function,16 including its possible participation in mitochondrial energy metabolism14–18 and in regulation of the respiratory chain activity,18 led us to hypothesize that polyP is a mediator of a transi-ent mPTP activation during reperfusion. Results are discussed in the context of the existence of two potential modes of mPTP activation during I/R: (i) a transient $\text{Ca}^{2+}$/polyP-induced mode observed during ischaemia. However, polyP depletion caused an increase in ROS production, did not inhibit mPTP opening, and potentiated ROS-mediated cell death during reperfusion, suggesting that polyP could play a protective role against oxidative stress during reperfusion. Results are discussed in the context of the existence of two potential modes of mPTP activation during I/R: (i) a transient $\text{Ca}^{2+}$/polyP-induced mode observed during ischaemia which may serve to protect cells against cytosolic $\text{Ca}^{2+}$ overload and (ii) sustained ROS/pH-induced mode observed during reperfusion associated with necrotic cell death.

2. Methods

2.1 Cell isolation and culture

Left ventricular myocytes were isolated from adult New Zealand White rabbits (3–4 month old, 2.5 kg, Myrtle’s Rabbitry, Thompsons Station, TN, USA). Rabbits were anaesthetized with sodium pentobarbital (50 mg/kg), and hearts were quickly excised, mounted on a Langendorff apparatus, and retrogradely perfused via the aorta as previously described.15 For adenoviral gene transfer, myocytes were cultured for 24–48 h on laminin-coated glass coverslips in PC-1 medium. All protocols were in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23; revised 2011) and approved by the institutional Animal Care and Use Committee.

2.2 PolyP depletion in mitochondria

To decrease mitochondrial polyP, mitochondria-targeted green fluorescent protein (GFP)-tagged exopolyphosphatase (PPX)15 that specifically hydrolyzes polyP into inorganic phosphate was adenovirally expressed in cardiomyocytes. PPX overexpression resulted in an ≏80% decrease in mitochondrial polyP levels after 24 h in culture.15 To account for the potential non-specific effects of protein overexpression in the mitochondrial matrix, control myocytes were infected with mitochondrially targeted GFP (control) adenovirus and cultured under the same conditions as PPX overexpressing cells.

2.3 Simulated I/R

Ischaemia was simulated by acidosis (pH 6.4), inhibition of glycolysis (glucose replaced with 20 mM 2-deoxyglucose), and inhibition of mitochondrial respiration (with complex IV inhibitor sodium cyanide, NaCN) by cell exposure to glucose-free modified Tyrode solution containing (in mM) 20 2-deoxyglucose, 2 NaCN, 135 NaCl, 4 KCl, 1 MgCl2, 2 CaCl2, and 10 Hesper, pH 6.4 for 20 min.19 Reperfusion was simulated by 15 min super- vention with standard Tyrode solution consisting of (in mM) 135 NaCl, 4 KCl, 10 glucose, 10 Hesper, 1 MgCl2, and 2 CaCl2; pH 7.4.

2.4 Measurement of mitochondrial function

Laser scanning confocal microscopy (A1R, Nikon) was used to follow the changes in mitochondrial free calcium concentration ($[\text{Ca}^{2+}]_m$),20–22 $\Delta \Psi_m$,20,21 ROS generation,23–25 mPTP activity,24–26 mitochondrial glutathione redox status,21,27 and mitochondrial pH (pHm),27 using specific fluorescent indicators. All fluorescence signals were recorded from individual cells and background corrected. All fluorescent indicators were obtained from Molecular Probes/Life Technologies (Grand Island, NY, USA) unless noted otherwise. Detailed experimental protocols of these experiments are described in Supplemen-tary material online.

2.5 PolyP detection in cardiomyocytes

PolyP levels were measured in control (GFP) and polyP-depleted (PPX) cells loaded with 5 μM of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) for 30 min at 37°C.15 DAPI was excited with 405 nm laser light, and emitted fluorescence was measured at 552–617 nm. Data are presented as background subtracted fluorescence collected from the whole cell and normalized to the basal level (F/F0).

2.6 Cell death

Release of lactate dehydrogenase (LDH) into the extracellular medium was measured after 20 min of simulated ischaemia and 15 min of reperfusion as an index of cell death.17 LDH release (expressed as percentage of enzyme release with respect to total cellular content and normalized to the levels of LDH release in the non-ischaemic conditions to account for cell death induced by mechanical stress due to solution replacement and suction) was measured spectrophotometrically (BioTek Synergy Mx multiplate reader) at 490 nm. The LDH assay based on the conversion of yellow tetrazo- lum salt by LDH into a red, formazan-class dye was used according to manufacturer specifications (Clontech).

2.7 Statistical analysis

Results are reported as mean ± S.E.M. for the indicated number (n) of cells or separate experiments for the LDH release assay. Individual comparisons are made using paired or unpaired t-tests; group comparisons are made using analysis of variances with post hoc comparisons using Tukey’s test. Differences are considered statistically significant at $P < 0.05$.

3. Results

3.1 ROS formation, $[\text{Ca}^{2+}]_m$, and pHm during I/R

We monitored simultaneously how I/R affects $[\text{Ca}^{2+}]_m$ and ROS generation. For $[\text{Ca}^{2+}]_m$ measurements, the mitochondrially targeted $\text{Ca}^{2+}$-sensitive protein, Mitycam,22 was adenovirally expressed in ventricular myocytes. The mitochondrial localization of Mitycam was confirmed by colocalization with mitochondria-entrapped tetramethylrhodamine methyl ester (TMRM; Figure 1A). Control experiments using permeabilized cells confirmed that this probe was sensitive to increases in mitochondrial matrix $\text{Ca}^{2+}$ and removal of the driving force for $\text{Ca}^{2+}$ uptake using the protonophore carbonyl cyanide p-(tri-fluromethoxy)phenylhydrazone (FCCP; Figure 1B). Generation of superoxide ($\text{O}_2^-$) was measured with the fluorescent probe MitoSOX Red, a cationic probe that distributes to the mitochondrial matrix, and is rapidly oxidized primarily by $\text{O}_2^-$, and to much lesser extent by $\text{H}_2\text{O}_2$ or other ROS.21,23 As shown in Figure 1C, $[\text{Ca}^{2+}]_m$
increased almost immediately with initiation of ischaemia which was followed by an increase in $O_2^{-}$ production. Upon reperfusion, $[Ca^{2+}]_{m}$ slowly declined (from $R = 0.51 \pm 0.05 - 0.34 \pm 0.06$, $n = 11$, Figure 1C and D) and no significant additional increase in $O_2^{-}$ generation was observed (the rate of $O_2^{-}$ generation actually decreased from 220 $\pm$ 21% during ischaemia to 107 $\pm$ 6% during reperfusion; $n = 11$, $P < 0.05$; Figure 1C and D). Using 2',7'-dichlorofluorescein, a fluorescent probe which preferentially detects $H_2O_2$, we found that $H_2O_2$ generation (165 $\pm$ 15% increase over the basal rate) followed the increase in $O_2^{-}$ generation already during ischaemia; however, significantly higher rates ($286 \pm 46\%$, $n = 10$, $P < 0.05$ compared with ischaemia) of $H_2O_2$ generation were observed during reperfusion (Figure 1C and D). Using 2',7'-dichlorofluorescein, a fluorescent probe which preferentially detects $H_2O_2$, we found that $H_2O_2$ generation (165 $\pm$ 15% increase over the basal rate) followed the increase in $O_2^{-}$ generation already during ischaemia; however, significantly higher rates ($286 \pm 46\%$, $n = 10$, $P < 0.05$ compared with ischaemia) of $H_2O_2$ generation were observed during reperfusion (Figure 1C and D).

We also monitored the mitochondrial glutathione redox state using the mitochondrially targeted fluorescent protein roGFP1 (mito-roGFP1). Mito-roGFP1 specifically senses changes in mitochondrial glutathione redox state. Under basal conditions, the mito-roGFP1 sensor was 51 $\pm$ 3% oxidized ($n = 10$; Figure 1E and F). During ischaemia, mito-roGFP1 oxidized further ($10 \pm 2\%$ increase from basal level, $n = 10$) reflecting the oxidative environment of the mitochondrial matrix associated with ischaemia. Additional oxidation was detected during reperfusion ($19 \pm 4\%$ increase from the ischaemia level, $n = 10$), which paralleled the enhanced $H_2O_2$ generation. Using the mitochondrially targeted pH$_{m}$ sensor mito-SypHer, we could demonstrate that the mitochondrial environment was significantly acidified (the mito-SypHer ratio signal decreased from $R = 8.90 \pm 0.64$ under basal conditions to 1.66 $\pm$ 0.09 during ischaemia; $n = 7$; Figure 1E and F). During reperfusion, pH$_{m}$ slowly recovered towards basal level and eventually became more alkaline ($R = 9.58 \pm 1.04$, $n = 7$) by the end of 15 min reperfusion. These data indicate that, similar to earlier reports, this model mimics conditions of I/R observed in vivo.

3.2 $[Ca^{2+}]_{m}$ kinetics during I/R in control and polyP-depleted cells

Mitochondrial $Ca^{2+}$ overload is an important factor contributing to mPTP activation; however, the mechanisms that lead to mitochondrial $Ca^{2+}$ increase during I/R are still controversial. Therefore, we studied $[Ca^{2+}]_{m}$ changes in control and polyP-depleted cells

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**Figure 1** (A) Mitochondrial localization of Ca$^{2+}$-sensitive protein Mitycam (left) was confirmed by colocalization with mitochondrial membrane potential-sensitive dye TMRM (middle) as reflected in the overlay image (right). (B) $[Ca^{2+}]_{m}$ changes measured with Mitycam in permeabilized ventricular myocytes in response to elevation of extramitochondrial $[Ca^{2+}]_{oj}$ from 0.1 to 0.8 and 2 $\mu$M. Addition of the mitochondrial respiratory chain uncoupler FCCP resulted in release of Ca$^{2+}$ from mitochondria. (C) Recordings of $[Ca^{2+}]_{m}$ changes monitored with Mitycam, superoxide, and $H_2O_2$ generation monitored by MitoSox Red and 2',7'-dichlorofluorescein, respectively, during I/R in intact control myocytes. (D) Summary of $[Ca^{2+}]_{m}$ ($n = 11$ cells, four animals), superoxide ($n = 11$ cells, four animals), and $H_2O_2$ ($n = 10$ cells, three animals) changes during I/R. (E) Recordings of mitochondrial pH$_{m}$ (pH$_{m}$) and redox changes during I/R monitored by genetically encoded mitochondria-targeted probes mito-SypHer and mito-roGFP1, respectively, in intact control myocytes. (F) Summary of mitochondrial pH$_{m}$ ($n = 7$ cells, three animals) and redox ($n = 10$ cells, three animals) changes during I/R.
during I/R, [Ca\textsuperscript{2+}]\textsubscript{m} was monitored using X-Rhod-1 as a Ca\textsuperscript{2+} reporter with Co\textsuperscript{2+} quenching of the cytosolic Ca\textsuperscript{2+} signal.\textsuperscript{21} We found that [Ca\textsuperscript{2+}]\textsubscript{m} increased almost immediately with the onset of ischemia, continued to increase during the entire period of ischemia, and slowly declined during reperfusion in both control and polyP-depleted cells (Figure 2A and B). The [Ca\textsuperscript{2+}]\textsubscript{m} increase during ischemia was significantly higher in control cells compared with polyP-depleted cells (Figure 2C). Blocking MCU with 1 μM Ru360 reduced the [Ca\textsuperscript{2+}]\textsubscript{m} increase during I/R by ∼50% in both control and polyP-depleted cells, but was not able to prevent the [Ca\textsuperscript{2+}]\textsubscript{m} increase completely, suggesting incomplete inhibition of the MCU or additional mechanisms for Ca\textsuperscript{2+} entry during ischemia. Cell treatment with Ru360 also changed the kinetics of the [Ca\textsuperscript{2+}]\textsubscript{m} increase during ischemia in both control and polyP-depleted cells. A sharp initial [Ca\textsuperscript{2+}]\textsubscript{m} increase was observed, followed by an additional slow increase in [Ca\textsuperscript{2+}]\textsubscript{m}. It has been reported previously that similar kinetics of [Ca\textsuperscript{2+}]\textsubscript{m} in Ru360-treated cells were mediated by additional Ca\textsuperscript{2+} entry through ryanodine receptor type 1 channels located in the IMM (mRyR1, ryanodine receptor type 1 channels located in the IMM).\textsuperscript{29,30} To test the possibility of mRyR1 contribution to the elevated [Ca\textsuperscript{2+}]\textsubscript{m} during ischemia, Ru360-treated cells were preincubated with 10 μM of the Ryr1 blocker dantrolene. Simultaneous cell treatment with Ru360 and dantrolene nearly completely eliminated the increase in [Ca\textsuperscript{2+}]\textsubscript{m} during I/R in both control and polyP-depleted cells (Figure 2C), whereas dantrolene alone did not affect [Ca\textsuperscript{2+}]\textsubscript{m} significantly during I/R (data not shown). Next, we tested for the involvement of ROS in the increase of [Ca\textsuperscript{2+}]\textsubscript{m}. Cell treatment with 50 μM MnTBAP [Mn(III) tetrakis (4-benzoic acid) porphyrin chloride], a membrane permeable superoxide dismutase mimetic, did not affect the increase of [Ca\textsuperscript{2+}]\textsubscript{m} during I/R in both control and polyP-depleted cells. A sharp initial [Ca\textsuperscript{2+}]\textsubscript{m} increase was observed, followed by an additional slow increase in [Ca\textsuperscript{2+}]\textsubscript{m}. It has been reported previously that similar kinetics of [Ca\textsuperscript{2+}]\textsubscript{m} in Ru360-treated cells were mediated by additional Ca\textsuperscript{2+} entry through ryanodine receptor type 1 channels located in the IMM (mRyR1, ryanodine receptor type 1 channels located in the IMM).\textsuperscript{29,30} To test the possibility of mRyR1 contribution to the elevated [Ca\textsuperscript{2+}]\textsubscript{m} during ischemia, Ru360-treated cells were preincubated with 10 μM of the Ryr1 blocker dantrolene. Simultaneous cell treatment with Ru360 and dantrolene nearly completely eliminated the increase in [Ca\textsuperscript{2+}]\textsubscript{m} during I/R in both control and polyP-depleted cells (Figure 2C), whereas dantrolene alone did not affect [Ca\textsuperscript{2+}]\textsubscript{m} significantly during I/R (data not shown). Next, we tested for the involvement of ROS in the increase of [Ca\textsuperscript{2+}]\textsubscript{m}. Cell treatment with 50 μM MnTBAP [Mn(III) tetrakis (4-benzoic acid) porphyrin chloride], a membrane permeable superoxide dismutase mimetic, did not affect the increase of [Ca\textsuperscript{2+}]\textsubscript{m} during I/R in both control and polyP-depleted cells (Figure 2C), suggesting that Ca\textsuperscript{2+} entry during ischemia was independent from the observed ROS formation. Furthermore, we found that cell treatment with 1 μM of the mPTP desensitizer CSA significantly attenuated the [Ca\textsuperscript{2+}]\textsubscript{m} increase in control cells, but did not affect [Ca\textsuperscript{2+}]\textsubscript{m} in polyP-depleted cells (Figure 2C). Moreover, the increase in cytosolic [Ca\textsuperscript{2+}]\textsubscript{i} that is typically observed during ischemia (see Supplementary material online, Figure S1) was further enhanced when mPTP opening was prevented either by CSA treatment or polyP depletion, suggesting that during ischemia Ca\textsuperscript{2+} can enter mitochondria through mPTP. To conclude, these data indicate that three different mechanisms contribute to the elevated [Ca\textsuperscript{2+}]\textsubscript{m} levels during ischemia: (i) Ca\textsuperscript{2+} uptake through MCU, (ii) Ca\textsuperscript{2+} entry through mRyR1 when MCU activity is inhibited, and (iii) additional Ca\textsuperscript{2+} entry through the mPTP presumably opening in a low-conductance mode.

3.3 Blocking mPTP opening during ischemia by either polyP depletion or CSA increased superoxide (O2\textsuperscript{−}) levels in mitochondria

Oxidative stress is a well-known important component of I/R injury. Therefore, we evaluated the source of O2\textsuperscript{−} generation in the mitochondrial matrix during I/R and the effect of polyP depletion on O2\textsuperscript{−} generation. Using MitosOX Red as a sensor for O2\textsuperscript{−}, we detected an increase in O2\textsuperscript{−} generation during ischemia with only a small additional increase in fluorescence observed during reperfusion (Figure 3). In both control and polyP-depleted cells, the fluorescence increase was significantly attenuated by MnTBAP (Figure 3A and B), confirming the fidelity of O2\textsuperscript{−} detection with MitosOX Red. Blocking mitochondrial Ca\textsuperscript{2+} uptake through MCU with 1 μM Ru360 completely prevented ROS generation during I/R in control (Figure 3A) and polyP-depleted (Figure 3B) cells. This indicates that Ca\textsuperscript{2+} entering via MCU stimulates mitochondrial O2\textsuperscript{−} generation during ischemia. The mitochondrial respiratory chain appeared to be the main source of O2\textsuperscript{−} generation since exposure to a ‘mock’ ischemia solution lacking sodium cyanide prevented the increase in MitoSOX Red fluorescence (Figure 3C). Moreover, we found that during ischemia either polyP depletion or CSA treatment led to an increased mitochondrial O2\textsuperscript{−} accumulation (Figure 3A and B). Taking into account that this effect was further enhanced in polyP-depleted cells in the presence of CSA (Figure 3B and C), it is likely that polyP regulation of O2\textsuperscript{−} production is not directly linked to its ability to inhibit Ca\textsuperscript{2+}-induced mPTP. Taken together, these data indicate that mitochondrial Ca\textsuperscript{2+} uptake through MCU was stimulating O2\textsuperscript{−} production in the mitochondrial matrix; however, polyP was protecting mitochondria from the excessive O2\textsuperscript{−} generation.

3.4 Role of polyP in mPTP activity and cell death under I/R conditions

While it is established that both ischemia and reperfusion can facilitate activation of mPTP, the relative contribution of these two conditions to mPTP activation and cell death is not well understood. Here, we investigated the kinetics of mPTP activity at the various stages of I/R in control cells and cells with depleted levels of the mitochondrial polyP (a known potent endogenous activator of Ca\textsuperscript{2+}-induced mPTP\textsuperscript{14,15}) in the absence and presence of the mPTP desensitizer CSA. mPTP activity was quantified as calcine red-orange release from the mitochondrial matrix compartment. As shown in Figure 4A and B, ischemia induced a profound increase in the rate of calcine release (305 ± 17%, n = 23) in control cells, indicative of enhanced mPTP opening. This calcine release was almost completely prevented by polyP depletion (103 ± 4%, P < 0.001 vs. control, n = 22); however, only partially decreased by CSA treatment (181 ± 13%, P < 0.001, n = 11). Switching from ischemia to reperfusion solution was accompanied by continued calcine release (255 ± 22%); however, in contrast to ischemic conditions, polyP depletion had no effect on mPTP activity during reperfusion (208 ± 28%), whereas CSA was very effective in blocking calcine release (123 ± 28%) at this stage. This suggests different mechanisms of action for polyP and CSA with respect to mPTP opening during I/R. Surprisingly, despite mPTP inhibition during ischemia, polyP-depleted cells did not show protection against necrotic cell death at the end of reperfusion (measured as percentage change in LDH release; Figure 4C). In fact, cell death was even more pronounced compared with control conditions. On the other hand, cell death was inhibited by 1 μM CSA in control cells; however, in polyP-depleted cells, CSA treatment brought cell death to the level observed in control untreated cells but did not decrease it further. In both control and polyP-depleted myocytes, cell death was significantly inhibited by prevention of Ca\textsuperscript{2+} entry or by scavenging of ROS (Figure 4C). No cell death was observed after 20 min of ischemia in either group (not shown), suggesting that mPTP opening during ischemia was limited, possibly occurring in a low-conductance mode or in a transient mode that was insufficient to disturb mitochondrial homeostasis to the extent to result in cell death.

3.5 PolyP depletion and CSA protect against ΔΨ\textsubscript{m} depolarization during ischemia

One of the interesting and unexpected findings described in the previous section is that mPTP inhibition did not strictly correlate with protection from cell death. Thus, we hypothesized that, in ischemia
and reperfusion, mPTP was operating in different modes where transient mPTP opening potentially in a low-conductance state during ischaemia may serve to maintain Ca\(^{2+}\) homeostasis and protect cells. Since mPTP opening in a low-conductance mode might escape the detection with the calcein assay, we further test this hypothesis by monitoring \(\Delta \Psi_\text{m}\) depolarization albeit to a lesser degree. As shown in Figure 5A, on average, \(\Delta \Psi_\text{m}\)
Figure 3  (A and B) Superoxide generation measured with MitoSox Red during I/R in control (A, n = 30 cells, 10 animals) and polyP-depleted (B, PPX, n = 27 cells, 8 animals) cells in the absence and presence of 1 μM Ru360 (n = 10 cells from four animals in control + Ru360; n = 7 cells, three animals in the PPX + Ru360 group), 1 μM CSA (n = 10 cells, three animals in control + CSA; n = 9 cells, three animals in the PPX + CSA group), and 50 μM MnTBAP (n = 7 cells, three animals in control + MnTBAP; n = 8 cells, three animals in the PPX + MnTBAP group). ‘Mock’ ischaemia solution did not contain NaCN (n = 7 cells, three animals in control; n = 8 cells, three animals in the PPX group). (C) Average amplitude of MitoSox Red fluorescence changes at the end of ischaemia and at the end of reperfusion in control and polyP-depleted cells.
decreased (depolarized) significantly to 0.79 ± 0.07 (normalized values; \( n = 23 \)) during ischaemia. mPTP closing by polyP depletion was associated with an attenuated \( \Delta \Psi_m \) depolarization to 0.95 ± 0.06 \( (P < 0.05, n = 19) \). mPTP desensitization with CSA diminished \( \Delta \Psi_m \) depolarization in control \( (0.93 ± 0.05; P < 0.05, n = 10) \) and polyP-depleted \( (0.93 ± 0.05; P < 0.05, n = 9) \) cells. During reperfusion,
an initial re-polarization was observed in all four groups (Figure 5B), with a higher degree of hyperpolarization detected in polyP-depleted cells (1.25 ± 0.08, P < 0.05, n = 19). However, at the end of reperfusion, ΔΨm in all groups was depolarized, albeit to substantially different degrees. The most pronounced depolarization of ΔΨm was observed in control (0.44 ± 0.07) and CSA-treated polyP-depleted (0.46 ± 0.07) cells. Cell treatment with CSA alone (without polyP depletion) significantly attenuated ΔΨm depolarization (0.901 ± 0.03; P < 0.05, n = 10) on reperfusion. However, polyP depletion was not as effective as CSA in preserving ΔΨm (0.67 ± 0.08; P < 0.05; n = 19) at the end of reperfusion. In summary, the ΔΨm data are consistent with the mPTP activity results obtained with the calcein red-orange assay, with the exception of the observation made in polyP-depleted cells in the presence of CSA. Under the latter condition, minimal loss of calcein red-orange was observed, while the TMRM data suggested substantial ΔΨm depolarization. This could be explained by (i) mPTP opening with a pore size of <790 Da which would not allow calcein red-orange release from mitochondria (see also Discussion on heterogeneous mPTP pore sizes) or (ii) mPTP-independent ΔΨm depolarization associated with disturbed mitochondrial metabolism in polyP-depleted cells. We found that endogenous polyP levels monitored by DAPI fluorescence increased dramatically during reperfusion in control myocytes (Figure 6A and B), demonstrating that polyP generation is associated with response to stress conditions such as I/R. Measurements in PPX-overexpressing cells did not reveal significant changes in DAPI fluorescence, confirming that these changes were indeed mediated by polyP formation. Moreover, we found that polyP generation in cardiomyocytes depended on the activity of the mitochondrial respiratory chain: (i) stimulation of the respiratory chain activity with complex I substrates 5 mM pyruvate and 2 mM glutamate led to that of polyP production, while uncoupling of the mitochondrial respiratory chain with protonophore FCCP (1 μM) decreased polyP levels (Figure 6C and E). At the same time, we found that inhibition of F1F0-ATP synthase (mitochondrial complex V) by addition of 5 μg/mL of oligomycin induced a significant increase in polyP levels (Figure 6D and E), while at the same time produced a depolarization of ΔΨm after the initial small hyperpolarization (Figure 6F and G). In cells with normal oxidative phosphorylation, ΔΨm is maintained by the proton pumping activity of the

Figure 5 (A) Changes in ΔΨm during I/R assessed by TMRM fluorescence in control and polyP-depleted (PPX) cells in the absence or presence of 1 μM CSA. (B) Average values of TMRM fluorescence at the end of ischaemia and at different reperfusion times in control (n = 23 cells, four animals), PPX (n = 19 cells, four animals), control + CSA (n = 10 cells, three animals), and PPX + CSA (n = 9 cells, three animals) groups.

L.K. Seidlmayer et al.
Mitochondrial respiratory chain. However, if respiration is impaired as it is observed during the ischaemic insult (Figure 5), the thermodynamic equilibrium favours activity of the mitochondrial complex V in reverse mode, making this enzyme acting as a proton motive $F_1F_0$-ATPase that consumes ATP and translocates protons from the mitochondrial matrix to the cytosol in order to maintain $\Delta V_m$ as long as the glycolytic supply of ATP is maintained. Since in our experimental ischaemic conditions, where glycolysis was suppressed by glucose removal and 2-deoxyglucose supplementation, the initial small increase in polyP levels during ischaemia could be explained by the decreased polyP consumption by the $F_1F_0$-ATPase. Switching from ischaemia to the reperfusion solution would restore mitochondrial respiratory chain activity and glycolysis, and therefore increase polyP generation by the respiratory chain explaining the observed ‘burst’ in polyP generation (Figure 6A).

Altogether, these data suggest that polyP production is tightly linked to the activity of the mitochondrial $F_1F_0$-ATP synthase and ATP generation/consumption. Moreover, different degrees of CSA-sensitive $\Delta V_m$ depolarization during I/R support the hypothesis of two conductance modes, which is further supported by the data shown in Supplementary material online, Figure S2. Switching pH from the acidic (pH 6.4) back to physiological levels (pH = 7.4) increased the degree of calcine red-orange release induced by oxidative stress almost three-fold, suggesting a dramatic increase in the mPTP conductivity.

4. Discussion

The main focus of the present work was to investigate the role of polyP and its interplay with Ca$^{2+}$, ROS, and pH in the induction of mPTP under stress conditions simulating I/R injury. The model which we used takes advantage of the fact that conditions of I/R can be mimicked by depriving cells of glucose and inhibiting the respiratory chain, referred to as “chemical ischaemia” conditions (for the detailed original description of the model, see Ruiz-Meana et al.19). Even though our experiments were performed at normoxic oxygen levels, we determined that conditions of “chemical ischaemia” induced a substantial increase in Ca$^{2+}$-dependent ROS generation and a depletion of the glutathione antioxidant system, which was further exacerbated during reperfusion. As a limitation of our study, we did not monitor the status of the thioredoxin system, another major antioxidant system in the mitochondrial matrix.32,33
of misfolded integral membrane proteins damaged by oxidative stress. This misfolding exposes hydrophilic residues to the bilayer phase. These hydrophilic surfaces cluster and enclose aqueous channels that conduct low mol. wt. solutes. Initially, chaperone-like proteins block conductance through these misfolded protein clusters.43 However, when protein clusters exceed the ability of chaperons to block conductance, during increased oxidative stress observed in reperfusion, unregulated pore opening in high-conductance mode occurs and Ca^{2+} is released out of mitochondria.44 Taking into account that we were able to detect a similar increase in polyP content during reperfusion as was seen in bacteria, it is tantalizing to suggest that polyP might play a universal protective role and that this mechanism was conserved from bacteria to mammalian mitochondria through evolution (see also Discussion in Gray et al.42).

Our study results are compatible with the existence of two distinct modes of mPTP activation during I/R: (i) a Ca^{2+}/polyP-induced mode observed during ischaemia and (ii) an ROS/pH-induced mode observed during reperfusion. [Ca^{2+}]_{m} levels increased almost immediately during initiation of ischaemia which was followed by a gradual increase in mitochondrial superoxide accumulation and a massive increase in H_{2}O_{2} generation during reperfusion (Figures 1 and 2). Despite the fact that mPTP opening during ischaemia is not favoured because of acidosis (Figure 1F), our study (Figures 4 and 5) and others47,48 clearly demonstrated that mPTP opening occurred already during ischaemia. Mitochondria with reduced ability to sustain a polarized ΔΨ_{m} due to reduced electron transport power and/or increased IMM leak, are more susceptible to mPTP opening because depolarization directly increases mPTP open probability.49,50 By monitoring the rate of calcein release from mitochondria and changes in ΔΨ_{m}, we determined two phases of mPTP opening during I/R which were differently affected by CSA and polyP depletion. CSA only partially affected the rate of calcein release during ischaemia, but provided an effective protection against mPTP opening during reperfusion (Figure 4A and B). Depletion of polyP, on the other hand, was very effective against mPTP opening only during ischaemia, but failed to provide protection during reperfusion. Despite the fact that both CSA treatment and polyP depletion partially decreased [Ca^{2+}]_{m} and inhibited calcein release (i.e. mPTP opening) during ischaemia (Figure 4B); however, it also led to maximal O_{2}^{-} accumulation within mitochondria (Figure 3B and C) and collapse of ΔΨ_{m} (Figure 5). The fact that mPTP inhibition during ischaemia by different interventions led to increased O_{2}^{-} accumulation and cell death on reperfusion suggests that transient mPTP opening during ischaemia may be beneficial and serves as a protective mechanism against Ca^{2+} and ROS accumulation and subsequent cell damage. Two different (low and high) conductance modes of mPTP have been shown in experiments on isolated mitochondria.51–53 Several sub-conductance levels of mPTP were detected in patch-clamp experiments,54,55 where channel flickering was detected at a half-conductance state of ~500 pS.56 This flickering 500 pS mPTP state observed in patch-clamp experiments was attributed to the low-conductance mode observed in isolated mitochondria and permeabilized cells. In our study, Ca^{2+}/polyP-triggered mPTP opening during ischaemia resulted in small CSA-sensitive membrane depolarization (Figure 5), which failed to induce necrotic cell death, thus pointing towards mPTP opening in the low-conductance mode. Moreover, according to the `misfolded proteins’ theory, the conductance by individual PT pores may vary with their varying protein
composition. This expectation is consistent with a kinetic analysis of mitochondrial swelling after the mPTP opening, which suggested that the diameters of individual pores are heterogeneous (within the 400–1450 Da range). If the polyP possesses a chaperone-like activity as it was found in bacteria (see above), it could have different effects on mPTP activity depending on the length of polyP chain.

We presented evidence that the increase in \([Ca^{2+}]_{i}\) was a trigger for superoxide generation during ischemia. We found that several mechanisms contributed to mitochondrial \(Ca^{2+}\) uptake during ischemia: \(Ca^{2+}\) uptake by MCU (blocked by Ru360), \(Ca^{2+}\) entry through mRyR1 (blocked by dantrolene), and \(Ca^{2+}\) entry through mPTP opening presumably in low-conductance mode (blocked by CSA in Figure 2). Mitochondrial \(Ca^{2+}\) uptake through mPTP under conditions of partially depolarized \(\Delta \psi_{m}\) has been reported before. No additional increase in \([Ca^{2+}]_{i}\) was observed during reperfusion. In fact, during reperfusion \([Ca^{2+}]_{i}\) slowly declined towards basal level (Figures 1B and 2A) while cytosolic \([Ca^{2+}]_{c}\) increased in a CSA-sensitive manner (see Supplementary material online, Figure S1). These data are in agreement with previous reports that cytosolic \(Ca^{2+}\) overload during reperfusion is the consequence of bioenergetic failure after mPTP opening rather than its cause.

To conclude, our data demonstrate that mPTP opened during both ischemia and reperfusion, although likely in different modes. We propose that a low-conductance or possibly transient mode of mPTP opening is mediated by a polyP–\(Ca^{2+}\) interaction during ischemia and likely plays a protective role (Figures 4 and 5). The physiological importance of transient (low-conductance) mPTP opening, which does not lead to cell death, has been suggested to mediate ischemia preconditioning-induced protection, via (i) regulation of the mitochondrial matrix \(Ca^{2+}\) concentration; (ii) induction of mild mitochondrial uncoupling; and (iii) regulation of mitochondrial ROS release.

We now suggest that inorganic polyP also contributes to the mPTP opening in a low-conductance mode. During reperfusion, however, the high-conductance mPTP mode was triggered by the combination of 
\[\text{pH}_{m}\] restoration and excessive H\(_2\)O\(_2\) production (Figure 1 and see Supplementary material online, Figure S2). It has been reported previously that pH restoration upon reperfusion presents an independent contributing factor to cell hypercontracture and death. Reperfusion performed at low pH 6.2 led to prevention of hypercontracture and necrotic death, while it did not affect ROS generation itself. Moreover, silencing mitochondrial Na\(^+\)/H\(^+\) exchanger 1 (NHE1) in rat cardiomyocytes significantly attenuated mPTP opening. Therefore, it appears that 
\[\text{pH}_{m}\] can determine the mode of mPTP opening (low vs. high conductance), while polyP determines the \(Ca^{2+}\) sensitivity of mPTP. To date, translating findings from bench to bedside has been largely disappointing, as clinical studies involving \(Ca^{2+}\) channel blockers administered on the onset of myocardial reperfusion did not show any beneficial effects. Our data demonstrate that mitochondrial \(Ca^{2+}\) actually declines during reperfusion, and only inhibition of mitochondrial \(Ca^{2+}\) uptake before the ischemic insult prevents ROS generation and subsequent cell death. Furthermore, our findings show that depletion of polyP was associated with enhanced cell death on reperfusion, indicating that stimulation of polyP production rather than inhibition of \(Ca^{2+}\) uptake on reperfusion could be beneficial for cardioprotection.

### Supplementary material

Supplementary material is available at Cardiovascular Research online.

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