Published in final edited form as:

Int J Biochem Cell Biol. 2009 October ; 41(10): 1940–1948. doi:10.1016/j.biocel.2009.02.016.

FROM MITOCHONDRIAL DYNAMICS TO ARRHYTHMIAS

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Abstract

The ROS-dependent mitochondrial oscillator described in cardiac cells exhibits at least two modes of function under physiological conditions or in response to metabolic and oxidative stress. Both modes depend upon network behavior of mitochondria. Under physiological conditions cardiac mitochondria behave as a network of coupled oscillators with a broad range of frequencies. ROS weakly couples mitochondria under normal conditions but becomes a strong coupling messenger when, under oxidative stress, the mitochondrial network attains criticality. Mitochondrial criticality is achieved when a threshold of ROS is overcome and a certain density of mitochondria forms a cluster that spans the whole cell. Under these conditions, the slightest perturbation triggers a cell wide collapse of the mitochondrial membrane potential, $\Delta\Psi_m$, visualized as a depolarization wave throughout the cell which is followed by whole cell synchronized oscillations in $\Delta\Psi_m$, NADH, ROS, and GSH. This dynamic behavior scales from the mitochondrion to the cell by driving cellular excitability and the whole heart into catastrophic arrhythmias. A network collapse of $\Delta\Psi_m$ under criticality leads to: *i*) energetic failure, *ii*) temporal and regional alterations in action potential (AP), *iii*) development of zones of impaired conduction in the myocardium, and, ultimately, *iv*) a fatal ventricular arrhythmia.

Keywords

mitochondrial oscillations; inner membrane anion channel; permeability transition; redox potential; reactive oxygen species; sarcolemmal K_{ATP} channel; action potential

Introduction

Under metabolically stressful conditions such as substrate deprivation or oxidative stress, the role of mitochondrial function becomes a key arbiter of life and death at the cellular and organ level. While under normal physiological conditions the availability of energy is fine tuned to match changes in energy demand, under stress this is not the case. Most myocardial ATP production occurs in the mitochondria through oxidative phosphorylation, and most ATP utilization occurs at the myofibrils (Cortassa et al., 2006, Saks et al., 2007, Wallimann et al.,

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2007). Direct measures of ATP synthesis through creatine kinase in the human heart demonstrated a deficit in energy supply in clinical heart failure (Weiss et al., 2005). This reduction in ATP synthesis through CK is cardiac-specific and occurs in mild-to-moderate heart failure before a significant reduction in ATP can be detected.

The remarkable non-linear properties of the mitochondrial network, and of the heart itself, make them prone to the appearance of critical phenomena and bifurcations leading to self-organized, emergent, behavior. A dramatic example of the latter is the succession of failures shown to escalate from the mitochondrial network to the whole heart resulting in reperfusion-related arrhythmias after ischemic injury, and eventually the death of the organism (Akar et al., 2005, Aon et al., 2006a, O'Rourke et al., 2005). Mitochondria from heart cells act as a network of coupled oscillators, capable of producing frequency- and/or amplitude-encoded reactive oxygen species (ROS) signals under physiological conditions (Aon et al., 2006b, Aon et al., 2007b, Aon et al., 2008). This intrinsic property of the mitochondria can lead to a mitochondrial 'critical' state, i.e. an emergent macroscopic response manifested as a generalized $\Delta\Psi_m$ collapse followed by synchronized oscillation in the mitochondrial network under stress (Aon et al., 2004). The large amplitude $\Delta\Psi_m$ depolarization and bursts of ROS have widespread effects on all subsystems of the cell including energy-sensitive ion channels in the plasma membrane, producing an effect that scales to cause organ level electrical and contractile dysfunction. Mitochondrial ion channels appear to play a key role in the mechanism of this non-linear network phenomenon and hence are a potential target for therapeutic intervention.

The loss of $\Delta\Psi_m$ is among the leading factors causing a rapid impairment of mitochondrial and cellular function that may result into necrotic or apoptotic cell death (Aon et al., 2007a, Gustafsson and Gottlieb, 2008, Slodziniski et al., 2008). Thus, maintaining $\Delta\Psi_m$ is of paramount importance. Oxidative stress is a major pathophysiological route to the collapse of $\Delta\Psi_m$ (Aon et al., 2003, Aon et al., 2004, Brady et al., 2004, Zorov et al., 2000). The toxic effects of ROS are kept in check throughout our lives by balancing the natural rates of ROS production with sophisticated antioxidant defense systems. If this balance between ROS production and ROS scavenging is disrupted, serious and often irreversible cell damage occurs (Halliwell, 1997). One such important pathological situation in the heart is reperfusion following ischemia, when ROS production accelerates and the detoxification systems are overwhelmed, resulting in the consumption of antioxidants and an increase in free radical concentrations (Aon et al., 2007a, Lucas and Szweda, 1998, Marczin et al., 2003, Slodziniski et al., 2008). This is the period during which $\Delta\Psi_m$ is most likely to become unstable, representing a major decision point between cell life or death.

Mitochondrial physiology and ion channels

The oxidation of fuels (e.g., fatty acids and glucose) leads to acetyl-CoA, the common substrate for the Krebs cycle which, in turn, drives the production of the reducing equivalents NADH and FADH₂. Electrons are passed to the electron-transport chain, where coupled redox reactions mediate proton translocation across the inner membrane to establish a proton-motive force (PMF) composed of an electrical potential and pH gradient that drives ATP synthesis by the mitochondrial ATP synthase. The PMF is the major driving force for proton influx and is used by the mitochondrial ATP synthase (F₁F₀ ATPase) to produce ATP, which is exported to the cytosol via the adenine nucleotide translocase.

Maximum coupling between proton pumping by the respiratory chain and the phosphorylation of ADP is obtained when the leak of protons across the membrane is minimized. The energy dissipated by the increased ion permeability stimulates NADH oxidation, proton pumping, and respiration. This increased permeability can be carried out by ion-selective or nonselective

mitochondrial channels that dissipate energy and alter the ionic balance and volume of the mitochondrial matrix. These ionic movements may be partly compensated by antiporters coupled to H^+ movement. Concomitant stimulation of NADH production is required to compensate for the higher rates of respiration, or else a mismatch in energy supply and demand will occur.

Several inner membrane ion channels have been described, and their prolife or –death effects highlighted (reviewed in (Aon et al., 2006a, Aon et al., 2007b, Brady et al., 2006, O'Rourke et al., 2007)). Among them are the inner membrane anion channel (IMAC), a reversible channel activated under moderate oxidative stress, and the permeability transition pore (PTP), a large, non-selective, ion channel responsible for irreversible $\Delta\Psi_m$ depolarization under high oxidative stress conditions. These ion channels have been described on the mitochondrial inner membrane, and have been shown to be responsible for fast mitochondrial depolarization. However, the reversibility of $\Delta\Psi_m$ depolarization depends on which of these two channels is activated. As a matter of fact, we have recently shown that IMAC and PTP open sequentially as a function of oxidative stress and matrix and cytoplasmic redox potentials. Under moderately low ratios of reduced glutathione (GSH) to oxidized glutathione (GSSG), a moderate increase in ROS activates IMAC and oscillations in mitochondrial inner membrane potential ($\Delta\Psi_m$) can be sustained. These can be reversed by inhibition of this channel. However, at more oxidized redox potentials, permeability transition pore (PTP) opening leads to irreversible $\Delta\Psi_m$ collapse (Aon et al., 2007a).

An IMAC was originally described in isolated mitochondria and was shown to be inhibited by cationic amphiphiles including peripheral (mitochondrial) benzodiazepine receptor (mBzR) ligands (Beavis, 1989, Beavis and Garlid, 1987). Subsequently, single channel patch-clamp studies of mitoplasts have provided evidence that anion channels are present on the inner membrane, the most common being the outwardly rectifying 108 pS (or “centum-picosiemen”) anion channel which is inhibited by mBzR antagonists. We showed that PK11195, an isoquinoline carboxamide mBzR ligand, or a structurally different mBzR ligand, 4'-chlorodiazepam (4'-Cl-DZP, or its synonym Ro5-4864) could acutely inhibit mitochondrial oscillations (Aon et al., 2003, O'Rourke, 2000). These inhibitors prevented ROS accumulation in the mitochondrial network, but actually potentiated ROS accumulation in the small laser-flashed region of the cell, leading to the proposal that IMAC might also be an efflux pathway for superoxide anion, $O_2^{\cdot-}$, from the matrix, since the latter is membrane impermeable. Moreover, induction of mitochondrial $\Delta\Psi_m$ depolarization by FGIN-1-27, an agonist that binds selectively to the mBzR, reinforced the idea that this receptor, which is thought to be present on the mitochondrial outer membrane, may be modulating IMAC (Akar et al., 2005, Aon et al., 2003). The data concerning the mechanism of the mitochondrial oscillator in heart cells, are consistent with a role for IMAC, rather than PTP, in both $\Delta\Psi_m$ depolarization and $O_2^{\cdot-}$ efflux (see (Aon et al., 2008) for a review).

Mitochondrial networks in physiology and pathophysiology

The ROS-dependent mitochondrial oscillator

When mitochondria oscillate in living cells, the asymmetry of the $\Delta\Psi_m$ depolarization-repolarization cycle is consistent with the behavior exhibited by relaxation oscillators that possess slow and fast components (Cortassa et al., 2004). The sudden, fast, depolarization phase of $\Delta\Psi_m$ during the oscillations suggested that an energy dissipating ion channel is opening, causing rapid uncoupling of oxidative phosphorylation.

An obvious candidate for rapid depolarization of $\Delta\Psi_m$ was the PTP, which can be activated by various toxic agents or metabolic changes associated with necrosis and/or apoptosis. Ca^{2+} overload is one cofactor in the opening of the PTP (Crompton et al., 1999, Di Lisa and

Bernardi, 2005, Duchen, 1999) and a model of Ca^{2+} -induced Ca^{2+} release from the mitochondrial matrix through the PTP has been proposed. After an extensive investigation of this issue we concluded that both the PTP and Ca^{2+} overload were not involved in the mechanism of whole-cell $\Delta\Psi_m$ oscillation (Aon et al., 2003, O'Rourke et al., 1994).

Besides $\Delta\Psi_m$, NADH and ROS production also oscillated in isolated cardiomyocytes (Aon et al., 2003). We hypothesized that the balance between $\text{O}_2^{\cdot-}$ efflux through inner membrane anion channels and the intracellular ROS scavenging capacity play a key role in the oscillatory mechanism. We tested the hypothesis using a computational model of mitochondrial energetics and Ca^{2+} handling including mitochondrial ROS production, cytoplasmic ROS scavenging, and ROS activation of inner membrane anion flux. The mathematical model reproduced the period and phase of the observed oscillations in $\Delta\Psi_m$, NADH, and ROS (Cortassa et al., 2004). Several predictions of the model were directly confirmed by experimental evidence. For example, short incubations with the ROS scavenger n-acetyl-L-cysteine (L-NAC), or incomplete inhibition of oxidative phosphorylation by oligomycin, prolonged the period of oscillation by approximately twofold (from 100s to 200s). Similar results were achieved by incubating myocytes with the antioxidants 2-mercaptopyrionyl glycine or reduced glutathione.

These results are consistent with a reduction in the rate of accumulation of ROS to the critical threshold level at the activation site of the channel. In the case of oligomycin, this is due to a decreased rate of $\text{O}_2^{\cdot-}$ production by the electron transport chain. In the case of ROS scavengers, the threshold is altered due to the increased oxidant buffering capacity. In the model simulations, these interventions correspond to manipulation of either the rate of ROS production or the rate of ROS scavenging (Aon et al., 2007a, Aon et al., 2003, Aon et al., 2008, Cortassa et al., 2004).

Mitochondrial network function

Mitochondria constitute an extensive subcellular network within the myocardial syncytium (Fig. 1). Pathological conditions induce synchronized, coupled oscillations across the mitochondrial network of the cardiac myocyte (Aon et al., 2003) or the whole heart (Slodzinski et al., 2008). That the cardiac mitochondrial network may be organized as a network of coupled oscillators in the physiological regime was inspired by a model prediction that anticipated the existence of the high frequency domain of the oscillator. According to the model, mitochondria are able to exhibit low amplitude (μV or a few mV) and highly frequent oscillations (in the ms range) in $\Delta\Psi_m$ and ROS (in this case, in the nM range) (Aon et al., 2008). Because of the low amplitude of the oscillations we hypothesized that such frequencies could be relevant to mitochondria operating under physiological conditions. We later confirmed the existence of the high frequency domain experimentally.

The computational studies of the mitochondrial oscillator suggested two fundamentally different views to explain this self-organization process for large amplitude oscillations in $\Delta\Psi_m$. The first possibility was that mitochondria in the "normal" state are in a dynamic steady-state and metabolic stress pushes the system towards a bifurcation point and oscillation (Aon et al., 2006a, Cortassa et al., 2004). The second possibility was suggested by the parametric analysis, which showed that low-amplitude, high-frequency oscillations were possible (Cortassa et al., 2004) (see Fig. 2A and 2C). We hypothesized that if mitochondria behaved as high-frequency oscillators under physiological conditions, small fluctuations in $\Delta\Psi_m$ may be detectable by correlation analysis of long time series recordings of $\Delta\Psi_m$.

In the absence of metabolic stress, cardiomyocytes loaded with tetramethylrhodamine methyl ester (TMRM, a fluorescent reporter of mitochondrial membrane potential) (Fig. 1), display stable $\Delta\Psi_m$ that may last for more than an hour. Using two-photon laser scanning fluorescence

microscopy, cells were imaged every ~100ms and the average fluorescence for the whole cell was calculated. By applying Relative Dispersional Analysis (RDA) and Power Spectral Analysis (PSA) to the data, we found that cardiac mitochondria behave collectively as a highly correlated network of oscillators (Aon et al., 2006b). According to RDA, the fluorescence time series exhibit long-term memory quantitatively characterized by an inverse power law with a fractal dimension, D_f , close to 1.0. This behavior, characteristic of self-similar fractal processes, is distinct from processes without memory that show completely random behavior (white or brown noise), which are characterized by an exponential (Poisson) law with a slope corresponding to $D_f = 1.5$.

Self-similar scaling was also revealed by PSA after applying Fast Fourier Transform to the TMRM fluorescence time series. The power spectrum followed a homogenous inverse power law of the form $1/f^\beta$ with $\beta \sim 1.7$. These results pointed out that mitochondrial oscillations exhibit a broad frequency distribution spanning at least three orders of magnitude (from milliseconds to a few minutes).

The results indicated that collective behavior of the mitochondrial network is a statistically fractal, self-similar, process characterized by a large number of frequencies in multiple time scales, rather than an inherent “characteristic” frequency. We proposed that these mitochondrial oscillators are weakly coupled by low levels of mitochondrial ROS leading to network behavior in the physiological state (Fig. 3). However, an increase in ROS production under metabolic stress can reach a threshold that results in strong coupling through mitochondrial ROS-induced ROS release (RIRR), and organization of the network into a synchronized cluster spanning the whole cell (Aon et al., 2004, Aon et al., 2006b). A dominant low-frequency high-amplitude oscillation ensues (Zhou et al., 2008).

When the integrated computational model of excitation-contraction coupling and mitochondrial energetics (ECME model) (Cortassa et al., 2006) was upgraded to incorporate ROS-induced ROS release and the sarcolemmal K_{ATP} channel dynamics (ECME-RIRR model), we could simulate the influence of the high frequency domain behavior of the mitochondrial network on the electrophysiology of the heart cell (Zhou et al., 2007). The effect of high frequency oscillations (ms range) in mitochondrial energetics upon the sarcolemmal action potential duration (APD) is shown in Figure 2. We can see that, within the physiological domain of the mitochondrial oscillator, increasing period (from ~130 to 200ms) and amplitude (from ~300 μ V to 20mV) in $\Delta\Psi_m$ oscillations (Fig. 2A, 2C, and 2E) decrease the APD to almost half of the control (from 170 to 80 ms) or inexcitability at full depolarization (insets, Fig. 2B, 2D, 2F). This result illustrates the link between mitochondria as a network of coupled oscillators and modulation of the electrical activity of the heart cell under physiological conditions.

The importance of preserving $\Delta\Psi_m$: The role of mitochondrial inner membrane ion channels, redox potential and ROS

Previous results showed that oxidative stress can trigger the collapse of $\Delta\Psi_m$ followed by cell-wide oscillations in isolated cardiomyocytes (Aon et al., 2003) or in whole hearts (Slodzinski et al., 2008). These oscillations in $\Delta\Psi_m$ drive changes in the action potential (AP) through activation of the sarcolemmal K_{ATP} channel in response to rapid uncoupling of oxidative phosphorylation during depolarization of $\Delta\Psi_m$. Activation of sarcolemmal K_{ATP} currents shortens the cellular AP and renders the myocyte electrically inexcitable during the nadir of $\Delta\Psi_m$ oscillations (Akar et al., 2005, Aon et al., 2003) (Fig. 2). That these effects are mediated by $\Delta\Psi_m$ depolarization was indicated by inhibition of the IMAC-mediated mitochondrial oscillations with 4'Cl-DZP, an intervention that concomitantly reestablished and stabilized the sarcolemmal AP. We proposed (O'Rourke, 2000) and provided supporting evidence (Akar et

al., 2005) that electrophysiological alterations and arrhythmias in intact hearts are in part a consequence of the failure of the cellular mitochondrial network to maintain $\Delta\Psi_m$. Inability to keep $\Delta\Psi_m$ contributes to destabilization of AP repolarization during IR in the whole heart, leading to arrhythmias.

Utilizing high-resolution optical AP mapping, we showed that 4'Cl-DZP reduces AP shortening during ischemia, prevents ventricular fibrillation (VF), and facilitates the restoration of AP duration (APD) upon reperfusion. These effects were consistent with the stabilization of $\Delta\Psi_m$ and the cellular AP when the mBzR antagonist was applied to isolated cardiac cells.

Thus, prevention of $\Delta\Psi_m$ depolarization (*a*) stabilizes the AP of metabolically-stressed cardiomyocytes, (*b*) blunts ischemia-induced AP shortening, (*c*) improves postischemic recovery of the AP, and (*d*) prevents the occurrence of spontaneous arrhythmias upon reperfusion in the whole heart. Conversely, facilitating $\Delta\Psi_m$ depolarization with a mBzR agonist accelerates ischemia-induced AP changes creating large regions of conduction block, and promotes sustained tachyarrhythmias upon reperfusion (Akar et al., 2005).

A key determinant of the approach of the mitochondrial network to criticality appears to be an imbalance between mitochondrial ROS production and ROS scavenging. The thiol oxidant diamide triggered $\Delta\Psi_m$, GSH, and NADH oscillations following depletion of cellular GSH by eliciting ROS accumulation in living cardiomyocytes (Aon et al., 2007a). Since these oscillations could be blocked by 4'Cl-DZP the results suggested the involvement of the IMAC. These results pointed out that GSH, and likely the glutathione redox potential, are main cellular variables that determine the approach of the mitochondrial network to criticality through an increase in oxidative stress by overwhelming of the antioxidant defenses. Adjustment of the glutathione redox potential in permeabilized cardiomyocytes, revealed that the critical state can be induced by partial depletion of the reduced glutathione pool and that the reversible (IMAC-mediated) and irreversible (PTP-mediated) depolarizations of $\Delta\Psi_m$ can be distinguished based on the cytoplasmic glutathione redox status; IMAC-mediated $\Delta\Psi_m$ oscillation was triggered at a GSH/GSSG ratio of 150:1–100:1, whereas PTP opening is triggered at a GSH/GSSG of 50:1. We also determined that the GSH/GSSG ratio and the total pool size of the redox couple influenced mitochondrial ion channel opening rather than the absolute glutathione redox potential and that GSH uptake and the mitochondrial matrix enzymes glutathione reductase and NADH/NADPH transhydrogenase modulate the sensitivity of mitochondrial ROS production and $\Delta\Psi_m$ depolarization to GSH/GSSG.

Extending the mechanistic findings in permeabilized cardiomyocytes (Aon et al., 2007a), the mitochondrial ROS-dependent oscillator described in living cardiac myocytes (Aon et al., 2003, Aon et al., 2004), and computational models (Cortassa et al., 2004) to the level of the myocardial syncytium, we showed that mitochondrial $\Delta\Psi_m$ oscillations could be triggered by I/R or glutathione depletion in intact perfused hearts using two photon scanning laser microscopy (Slodzinski et al., 2008). Most recently, we demonstrated that the $\Delta\Psi_m$ depolarization induced by depleting the GSH pool can induce cardiac arrhythmias even under normoxic conditions (Brown et al., 2008a). Further evidence indicating the involvement of IMAC was noted when the arrhythmias induced by GSH depletion were prevented with the IMAC blocker 4'Cl-DZP (Brown et al., 2008a).

In summary, our experimental evidence strongly supports the argument that the activation of IMAC and the PTP are distinct processes with different sensitivities to the redox state and to chemical inhibitors. PTP opening required more than 50% depletion of the GSH pool and was correlated with very high rates of ROS production. In permeabilized cardiomyocytes, although 4'Cl-DZP prevented $\Delta\Psi_m$ loss, the rate of oxidation of the ROS probe CM-DCF was enhanced in the mitochondrial matrix. Unlike 4'Cl-DZP, CsA did not affect ROS production or the GSH/

GSSG ratio at which PTP opening was triggered, although the latter was delayed (Aon et al., 2007a). In this sense, IMAC can be thought of as an upstream “instigator” of the PTP, because it promotes depletion of the mitochondrial GSH pool and increases ROS loads. The finding that matrix oxidation is increased even though $\Delta\Psi_m$ is more polarized in the presence of 4'Cl-DZP, can be explained by inhibition of the $O_2^{\cdot-}$ efflux rate when IMAC is blocked (Aon et al., 2007a, Cortassa et al., 2004), leading to enhanced oxidation of the matrix-localized CM-DCF.

Coupling of mitochondrial oscillations to cellular electrical excitability

Synchronized mitochondrial oscillations drive the sarcolemmal action potential

Early studies showed that cardiomyocytes subjected to energetic stress by substrate deprivation display spontaneous oscillations (period ~1–3 min) in sarcolemmal currents that were attributed to the cyclical activation and deactivation of ATP-sensitive potassium current ($I_{K,ATP}$) (O'Rourke et al., 1994). Since the period and amplitude of the $I_{K,ATP}$ oscillations were the same in electrically-stimulated or quiescent cells, a Ca^{2+} - or plasma membrane potential-dependent source of oscillation was ruled out.

The activation of $I_{K,ATP}$ was correlated with, and slightly preceded by, oxidation of the intracellular NADH/NAD⁺ redox couple, indicating that large changes in energy metabolism were occurring. In addition to $I_{K,ATP}$ activation, the amplitude of the Ca^{2+} transient was suppressed, highlighting the global effects of metabolic oscillation on the integrated function of the cell. This was also illustrated by the shortening and suppression of the AP of myocytes undergoing oscillations in $I_{K,ATP}$, leading us to propose that this mechanism may be of pathophysiological relevance as a trigger of arrhythmias related to ischemia-reperfusion (I/R) injury (O'Rourke, 2000, O'Rourke et al., 1994).

The abovementioned early studies also showed that metabolic oscillations have a profound effect on the excitability and Ca^{2+} handling properties of the cardiac cell. The oscillatory uncoupling of mitochondria depletes cellular ATP levels and drives the activation of ATP-sensitive K^+ (K_{ATP}) channels in the sarcolemma. This will, in turn, produce cyclical changes in the action potential of the cardiomyocyte, and this heterogeneity was proposed to be a possible source of ischemia-related arrhythmias.

Using the laser flash-induced oscillation, we demonstrated that the APD was tightly coupled to the mitochondrial energetic state. At the onset of $\Delta\Psi_m$ depolarization, the AP rapidly shortens and the cell quickly becomes inexcitable; this is followed by the parallel recovery of both the metabolic and electrical signals (Akar et al., 2005, Aon et al., 2003) (see Fig. 2E and 2F). Stabilizing $\Delta\Psi_m$ with 4'Cl-DZP, an antagonist of the mBzR, not only inhibited the oscillations in mitochondrial energetics, but also stabilized the AP (Akar et al., 2005, Aon et al., 2008). Action potentials were strongly affected only during the synchronized whole-cell mitochondrial oscillations and not when single, or small clusters of, mitochondria were depolarized.

This crucial evidence gave us a direct insight into the mechanism of mitochondrial-driven sarcolemmal AP oscillations. This led us naturally to the prediction that blockage of mitochondrial oscillations should stabilize the AP and suppress arrhythmias in whole hearts in a reperfusion scenario after ischemic injury. Indeed, arrhythmias can be blocked by 4'Cl-DZP whereas the use of an IMAC opener could induce arrhythmias led by a more extensive period of electrical silence than in the control. The PTP blocker cyclosporin A (CsA) also recovers the AP but with more prolonged periods of electrical silence on reperfusion, and to a lesser extent as compared with the IMAC blocker.

The mitochondrial origin of post-ischemic arrhythmias

After ischemic injury, the early reperfusion phase would be expected to favor mitochondrial criticality since a burst of ROS production and antioxidant depletion are known to occur (Bolli and Marban, 1999, Slodzinski et al., 2008). Optical mapping studies of isolated perfused guinea-pig hearts subjected to 30 minutes of ischemia demonstrated that persistent ventricular tachycardia and/or fibrillation occurs within minutes of reperfusion (Akar et al., 2005). In this experimental system we determined that the mBzR antagonist 4'Cl-DZP could prevent post-ischemic arrhythmias. Moreover, using two-photon microscopy in perfused hearts subjected to I/R, we could verify that 4'Cl-DZP treatment could completely prevent $\Delta\Psi_m$ depolarization occurring during 30 min I/R (Slodzinski et al., 2008). These studies bridged the existing gap between cellular studies and whole-heart mapping and arrhythmias that we have reported earlier (Akar et al., 2005).

4'Cl-DZP-mediated protection against arrhythmia correlated with protection against mechanical dysfunction (Brown et al., 2008b). Administration of 4'Cl-DZP delayed the onset of ischemic contracture and also led to significantly improved recovery of left ventricular developed pressure (LVDP) at the onset of reperfusion (Fig. 4). 4'Cl-DZP also had significant effects on the duration of the cellular AP, the peak calcium current, and the intracellular calcium transient (Brown et al., 2008b). We hypothesized that the stabilizing effect of 4'Cl-DZP on mitochondrial function helps to maintain the energy supplies required for Ca^{2+} homeostasis both during and after ischemia.

Administration of 4'Cl-DZP provided protection against arrhythmias and mechanical dysfunction also when administered only at the onset of reperfusion (Fig. 4C), an observation of significant clinical relevance (Brown et al., 2008b). While a wide number of experimental compounds are cardioprotective when administered before an index ischemia, very few can protect the heart when administered before reperfusion alone. This observation provides further evidence that targeting the mBzR complex is a uniquely relevant strategy to mitigate reperfusion arrhythmias.

By comparison, CsA, the PTP inhibitor, had no effect on ischemic electrical parameters, and did not provide substantial protection against reperfusion arrhythmias (Akar et al., 2005, Brown et al., 2008b). Arrhythmia scores for hearts treated with CsA did not differ from hearts that received no treatment (Brown et al., 2008b) (see Fig. 4D). The mBzR ligand FGIN-1-27, which enhanced mitochondrial depolarization in single cell studies, shortened the time to inexcitability and conduction block in ischemic hearts, but had no effect in normoxic hearts (Akar et al., 2005).

These findings supported the idea that loss of $\Delta\Psi_m$ during ischemia and early upon reperfusion, is due to the opening of IMAC, and can be inhibited by mBzR antagonists.

Metabolic sink/block as a mechanism of conduction failure and arrhythmias

The results described in the previous section support the idea that “metabolic sinks” develop during I/R, and that this pattern is related to individual cells experiencing high levels of oxidative stress.

The $\Delta\Psi_m$ loss occurring on reperfusion when coupled with activation of sarcolemmal K_{ATP} channels may create spatial and temporal AP heterogeneity that can be a substrate of ventricular reentry (Akar et al., 2005). Within this rationale we postulated that the failure of mitochondrial energetics can create “metabolic sinks” in the reperfused myocardium that may constitute sites of functional conduction block. The formation of areas of the myocardium undergoing regional or temporal changes in $\Delta\Psi_m$ constitutes a metabolic current sink (Fig. 5). In this case, the

propagating wave of depolarization encounters clusters of cells in which the mitochondrial network is depolarized and the sarcolemmal K_{ATP} channels are open. These cells are rendered inexcitable because of the large background K^+ conductance locking the sarcolemmal membrane potential close to the diffusion potential for K^+ rather than by their inability to conduct current; i.e., they are powerful current sinks.

This metabolic sink/block mechanism is distinct from (but could be occurring in parallel with) blocks caused according to existing paradigms of electrical dysfunction in the heart. Block of electrical propagation in the myocardium can be attributed to 3 main cellular mechanisms (Kleber and Rudy, 2004): *i*) loss of cell-cell coupling by closure of gap junctions, *ii*) regional uncoupling by anatomical barriers to conduction (e.g. scar tissue), *iii*) dynamic functional block due to heterogeneity of intrinsic electrophysiological restitution properties. For the sake of comparison between mechanisms; whereas with gap junctional block, an increase in voltage at the wave front could result in propagation via a bypass path (i.e., propagation has a high safety factor (Kleber and Rudy, 2004) the opposite could occur when a current sink is present. The “safety factor” (SF) is a way to measure successful conduction in the myocardium. Equation 1 shows how SF is calculated:

$$SF = \frac{\int_A I_c dt + \int_A I_{out} dt}{\int_A I_{in} dt} \quad A|Q_m > 0$$

I_c is the capacitive current of the cell, and I_{in} and I_{out} are the axial currents *in* and *out* of the cell, respectively. The charge Q associated with each current is computed by the time integral of that current over an interval A during which the net membrane charge Q_m is positive. Initially, $V_m = V_{rest}$ and $Q_m = 0$.

In the equation, the numerator is the sum of the charges that the cell generates for its own depolarization (Q_c) and for the depolarization of downstream cells (Q_{out}). The denominator (Q_{in}) is the charge that the cell receives from the upstream tissue. $SF > 1$ indicates that more charge is produced during cellular excitation than the charge required to cause the excitation.

Metabolic sink/block will dramatically decrease the SF for conduction since the cell will be unable to generate charge due to depolarized $\Delta\Psi_m$ (Fig. 5) and inexcitability due to opening of the sarcolemmal K_{ATP} channels (Aon et al., 2003) (Fig. 2). In contrast, gap-junctional block will increase the SF because the charge of the depolarized cell will build up when an electrically uncoupled cell is encountered due to the reduced electrotonic interaction. Consequently, the higher voltage increases the likelihood that the wave of depolarization will bypass the region of block via an alternative conduction path.

Final remarks: Escalation of failures from the mitochondrial network to the whole heart

A central insight derived from the Complex Systems Approach (Aon and Cortassa, 2009) has been to show that complex systems, including physical, social, economic, or biological networks, can collapse, crash, or rupture when stressed (Sornette, 2000). The mitochondrial network of cardiac cells is no exception. Figure 6 shows the sequence of events, from the mitochondrion to the whole organ, leading to arrhythmias. Under oxidative stress mitochondria accumulate high levels of ROS that - when the network attains criticality - produces the transition to pathophysiological behavior. This transition drives the mitochondrial network, first, into a $\Delta\Psi_m$ collapse, and, secondly, to high amplitude, low frequency whole cell

oscillations. During the transition from physiology to pathophysiology the mitochondrial network exhibits a drastic reduction in the richness of the frequency spectrum. The self-organized oscillation of mitochondrial energy state induces alternation between energetic blackout and recovery - this effect scales to the whole cell to dramatically alter the AP morphology or to render the cell completely inexcitable. When this behavior is extended to cell neighbors, regions of depolarization (“islands of inexcitability”) composed of whole group of cells with depolarized $\Delta\Psi_m$, develop in the myocardium potentially leading to conduction block and fatal arrhythmias, such as demonstrated in reperfused hearts after ischemia.

Acknowledgments

This work was supported by NIH grants R37-HL54598, R33-HL87345 and P01-HL081427(BO'R)

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Figure 1.

Two-photon laser scanning fluorescence microscopy was used to image the epicardium of isolated perfused hearts loaded with 100nM TMRM. The (sub)cellular resolution of the two photon image of the $\Delta\Psi_m$ signal (TMRM fluorescence, yellow pseudocolor) shows the myocytes still connected through gap junctions (arrows) in the myocardial syncytium and the extended mitochondrial network. Bar, 20 μm .

Figure 2.

Simulation of mitochondrial oscillations effect on sarcolemmal action potential (AP) under physiological and pathophysiological conditions performed with the ECME-RIRR model. Panels A, C, E, show $\Delta\Psi_m$ oscillatory behavior in the physiological (A: 138ms period; C: 203ms period) and pathophysiological (E: 770ms period) domains. Panels B, D, F, show the sarcolemmal AP, V_m . The control without oscillations had an APD of 170ms. The simulation parameters were: the fraction of electron transport diverted to ROS production, shunt=0.1 (i.e. 10%), and the amount of superoxide dismutase, EtSOD=0.8, 0.9 and 1.0 μM , respectively. All other parameters were as described in previous publications (Aon et al., 2006b, Cortassa et al., 2006, Cortassa et al., 2004).

Figure 3.

Simulation of mitochondrial network oscillatory behavior in the physiological domain. Panel A, oscillations of ~5mV amplitude and ~130 ms period were obtained with shunt = 0.1 and EtSOD = 0.8 mM conditions. Panel B shows a zoom within two oscillatory cycles to highlight the sequential $\Delta\Psi_m$ depolarization as well as repolarization. Shown are the results obtained with a linear network of eleven mitochondria. All other parameters were as described in previous publications (Aon et al., 2006b, Cortassa et al., 2006, Cortassa et al., 2004).

Figure 4.

Representative rabbit heart left ventricular pressure tracings and ECG waveforms for the last 5s of reperfusion in: (A) control hearts, (B) hearts receiving 24 μM 4'Cl-DZP beginning 10 min prior to ischemia thru the duration of reperfusion, (C) hearts receiving a bolus of 4'Cl-DZP at the onset of reperfusion, and (D) hearts receiving 0.2 μM cyclosporin-A 10 min prior to ischemia through the duration of reperfusion. Reprinted from Brown et al. *Cardiovascular Research* (2008) **79**, 141–149 (with permission from Oxford University Press).

Figure 5. Two-photon laser scanning fluorescence microscopy of the epicardium of a ~20min reperfused guinea pig heart after 30min global ischemia

The heart was loaded with 100nM TMRM and 4 μ M CM-DCF before being subjected to the I/R protocol (see (Akar et al., 2005, Slodzinski et al., 2008)). The first two rows correspond to the $\Delta\Psi_m$ (TMRM fluorescence, yellow fluorescence) and ROS (CM-DCF fluorescence, green fluorescence) signals whereas the autofluorescence (NADH, blue fluorescence) belongs to the row at the bottom. The sequence captures an extended region (from left to right) of the myocardial syncytium (~400 μ m diameter: third frame from the left) with cells exhibiting completely depolarized mitochondria (first row) and a relatively oxidized NADH pool. We consider this region to be an “island of inexcitability” or “metabolic sink”. The cells surrounding the “metabolic sink” contain polarized mitochondria, high levels of ROS, and a reduced redox pool. The levels of ROS within the sink appear to be low probably because of release of the ROS probe after permeability transition pore opening (see (Aon et al., 2007a)). Bar, 40 μ m.

Figure 6. From mitochondrial dynamics to arrhythmias

When subjected to oxidative stress mitochondria accumulate high levels of ROS until a threshold is reached, and the network attains *criticality*. Under these conditions, the slightest perturbation will drive the cell-wide spanning cluster containing high levels of ROS into an energetic blackout ($\Delta\Psi_m$ depolarization) followed by high amplitude self-organized oscillations. This behavior escalates to the whole cell which is rendered inexcitable at the nadir of $\Delta\Psi_m$ depolarization that, when extended to regions of depolarization in the myocardium like in I/R injury (see Fig. 5), leads to reperfusion-related arrhythmias.

(The mitochondrion schematic on the far left was taken from www.cartage.org.lb/.../Mitochondria.htm whereas the panel on the far right was reprinted slightly modified from: Akar, F.G., Roth, B.J., and Rosenbaum, D.S. (2001) Am J Physiol Heart Circ Physiol 281:533-542, 2001)