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Mitochondrial ion channels in cardiac function and dysfunction

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Abstract

The study of mitochondrial physiology continues to provide new and surprising insights into how this organelle participates in the integration of cellular activities, far beyond the traditional view of the mitochondrion in energy transduction. Emerging evidence indicates that mitochondria are a centre of organization of numerous signalling pathways and are a cellular target that undergoes vast modification during both the acute and chronic phases of disease development and aging. In this context, it is also important to understand the spatial and temporal organization of mitochondrial function and how this might influence the cell's response to stress. Here, we present evidence supporting the hypothesis that 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. This intrinsic property of the mitochondria can lead to a mitochondrial 'critical' state, i.e. an emergent macroscopic response manifested as complete collapse or synchronized oscillation in the mitochondrial network under stress. The large amplitude depolarizations of $\Delta\Psi_m$ 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 an important target for potential therapeutic intervention.

Multiple non-linear control interactions govern mitochondrial oxidative phosphorylation, allowing the mitochondrial to adjust energy production to large changes in demand, while keeping the many positive and negative feedback loops in check. Thus, there must be a balance between a flexible response to the intracellular environment and a robust resistance to instability, both from the point of view of matching energy supply with demand, and to limit the toxic effects of the by-products of metabolism, including reactive oxygen species (ROS). As is often the case for highly responsive control systems, the inherent non-linear properties of a system can sometimes result in chaotic or unstable behaviour if the system is stressed beyond its normal range of behaviour. A central point of this paper is that the mitochondrial network of the heart cell has a critical threshold of oxidative stress that constitutes a breakpoint between physiological and pathophysiological domains of behaviour.

Several features of mitochondria are vital to understanding why function can fail catastrophically under stress. First, there is a large protonmotive force across the inner membrane, and the electrical potential ($\Delta\Psi_m$) makes up the bulk of this force. Second, there is a low permeability to ions across the inner membrane. Third, the mitochondrial matrix is a limited space bounded by a double membrane, therefore, there are physical constraints on ion and osmolyte movements that might influence function. Hence, there is a large electrochemical gradient for cation and anion movements across the inner membrane, but they are kept in check by relatively low permeabilities and the need for charge and volume balance to be maintained. It is easy to understand then, that if a channel opens in the inner membrane, it will dissipate energy, the magnitude of the effect being related to the conductance and specificity of the ion channel. While many mitochondrial ion channels have been proposed to be present in the inner

membrane from classical swelling experiments and electrophysiological recordings, we are still struggling to characterize their structures and physiological functions (O'Rourke 2006). This area represents an important goal for mitochondrial research in general.

From the therapeutic perspective, particularly with reference to ischaemia–reperfusion injury in the heart and brain, it is important to note that mitochondrial ion channels appear to play a role in both protection against injury and as mediators of injury. An increase in mitochondrial K^+ flux, for example, by pretreating hearts with K , ATP or K_{Ca} channel opener compounds, which are thought to activate mitochondrial K_{ATP} (mito K_{ATP}) or K_{Ca} channels (mito K_{Ca}), respectively, has been found to significantly decrease infarct size and improve recovery after ischaemia–reperfusion. On the other side of the equation, blocking mitochondrial ion channels, such as the permeability transition pore (PTP), or the inner membrane anion channel (IMAC), can prevent the loss of mitochondrial function that is a prelude to necrotic or apoptotic cell death.

Based on the novel concepts described above, in the present paper we will summarize some of the emerging ideas we have about the spatiotemporal organization of mitochondria and the role of mitochondrial ion channels in the critical transition between physiology and pathophysiology.

Mitochondrial ion channels involved in cell stress responses

While the concept of mitochondrial ion channels (or uniporters) has been around for quite some time, renewed interest has been generated as their primary roles as determinants of cell life and death have been revealed (Aon et al 2006a, O'Rourke et al 2005). Our primary focus has been on those channels involved in either protecting cardiac cells from injury, or causing ischaemia and reperfusion injury. In this context, we have employed a variety of methods to identify and characterize which ion channels may be present in isolated mitochondria and in intact cells.

K^+ channel opener compounds, including diazoxide, nicorandil and others, can protect heart cells from ischaemic or oxidative stress through a mechanism which we believe involves the opening of specific mito K_{ATP} channels on the inner membrane (O'Rourke 2004). Similarly, studies revealed a second class of K^+ channel on the mitochondrial inner membrane (mito K_{Ca}), resembling the Ca^{2+} -activated K^+ channel of the plasmalemma of certain cell types. Selective openers of the K_{Ca} channel activated this channel (e.g. NS-1619) and it was inhibited by specific toxins (Xu et al 2002). Mito K_{Ca} activation conferred protection against ischaemia–reperfusion injury, which was prevented by K_{Ca} inhibitors, providing further support for the idea that increased K^+ flux can protect the myocardium.

The role of mitochondrial Ca^{2+} uptake and ROS accumulation, and the eventual activation of the PTP, has been shown to play a prominent role in reperfusion injury (Halestrap et al 2004). While PTP is apparently triggered during reperfusion, other mitochondrial ion channels may be activated during ischaemia, to cause loss of $\Delta\Psi_m$. In particular, our recent studies have focused on the activation of IMAC, an outwardly rectifying anion channel that is modulated by mitochondrial benzodiazepine receptor (mBzR) ligands. Our current computational and experimental studies place IMAC at the centre of a mitochondrial ROS-induced ROS release mechanism that underlies the oscillatory properties of the mitochondrial network.

Mitochondria as a network of coupled oscillators

Mitochondrial criticality and large amplitude oscillation under pathological conditions

Our early studies of metabolic stress in isolated cardiac cells, in the form of substrate deprivation, revealed that energy-sensitive K^+ channels in the sarcolemmal membrane can be

activated spontaneously in an oscillatory manner (O'Rourke et al 1994). These K_{ATP} current oscillations were closely associated with whole cell metabolic oscillations in the NADH redox pool. Modulation of the cellular action potential by these metabolic oscillations led us to hypothesize that these oscillations could result in arrhythmias if present in the heart after ischaemia–reperfusion. Subsequently, we identified the mitochondria as the source of the oscillations and observed they involved the synchronized depolarization of the mitochondrial network of almost the entire heart cell (Romashko et al 1998). Based on the observation that $\Delta\Psi_m$ depolarization could either occur in individual mitochondria, clusters of mitochondria, or in the whole network, we suggested that the mitochondria may represent a network of coupled oscillators (Romashko et al 1998).

Since the mitochondrial network of the cardiac cell consists of thousands of mitochondria packed between the myofilaments in an ordered three-dimensional array (or lattice), we tested whether mitochondria behaved independently or were synchronized, and what factors might be responsible for intermitochondrial communication. High resolution, two-photon laser scanning fluorescence imaging was used to track $\Delta\Psi_m$ (with TMRE), ROS production (with matrix localized derivatives of dichlorofluorescein, a reporter of H_2O_2), and NADH (native autofluorescence) simultaneously, and a single localized high intensity laser flash was applied in a small volume of the mitochondrial network (an $8\ \mu m \times 8\ \mu m$ square, approximately $1\ \mu m$ deep). This flash rapidly depolarizes mitochondria and generates ROS in the flashed region, but no obvious effect on the remainder of the mitochondrial network is initially produced (Figs 1A and B). However, signs of spreading oxidative stress are observed: the mitochondrial matrix ROS signal increases in more and more mitochondria over the next 1–2 minutes, although $\Delta\Psi_m$ is unchanged. When a certain proportion of mitochondria (60%) in the 2D field show a ROS signal increase by 20% or more, then a global synchronized depolarization of the mitochondrial network occurs. A limit cycle oscillation in the system is then triggered, with a reproducible period of roughly 1.5 min (Aon et al 2003) (Fig. 1C). These oscillations can be prevented or even blocked acutely, by inhibiting IMAC with mBzR ligands such as 4'Cl-diazepam (Ro5 4864) or PK11195, or by DIDS, a non-specific anion transport inhibitor. Importantly, all interventions that would be expected to inhibit superoxide production by the Q cycle of complex III were also effective at stabilizing $\Delta\Psi_m$ after flash-induced oscillations were triggered (Aon et al 2003).

We referred to the state just prior to global mitochondrial depolarization as the point of 'mitochondrial criticality' (Fig. 1C). The behaviour of the mitochondrial network at criticality was consistent with the formation of a percolation or spanning cluster across the network (Aon et al 2004). This theory describes how neighbouring elements (mitochondria) in the lattice not only influence the local response, but also contribute to an emergent macroscopic response of the entire network. When a critical mass of mitochondria are at the percolation threshold, a small perturbation in any of the mitochondria of the spanning cluster can result in the whole-cell state transition. This characterization helps to account for the observation that the pattern of $\Delta\Psi_m$ depolarization is reproducible from one cycle of oscillation to the next (some mitochondria even remain polarized in the midst of widespread depolarization around them) and the lack of any specific centre of origin of each depolarization (as is often observed for spontaneous Ca^{2+} oscillations in heart cells).

Uniquely, the ability to trigger reproducible cell-wide synchronized autonomous oscillations of $\Delta\Psi_m$, NADH, ROS (and also reduced glutathione) in cardiac myocytes using a highly localized oxidative trigger enabled us to explore the mechanism and the functional implications of this spatiotemporal dynamic response in unprecedented detail. The oscillatory behaviour could also be reproduced in a mathematical model of a single mitochondrion, which incorporates ROS production by the electron transport chain, a ROS-activated mitochondrial channel (IMAC), and extramitochondrial ROS scavenging (Cortassa et al 2004) (Fig. 1D). In

this model, ROS produced by the electron transport chain accumulates to a threshold level, triggering the opening of IMAC in a positive feedback loop. IMAC activation is terminated by a reduction in ROS at the activator site of the channel as a result of membrane depolarization (decreasing ROS production and efflux from the mitochondrial matrix) and ROS scavenging by the antioxidant enzymes. Thus, the system acts as a relaxation oscillator, in which a controlling factor builds up to a critical point, and then a rapid change is triggered, with the process then repeating in a stereotypical pattern. These events, which the model suggests can occur in single mitochondria, are transmitted to the whole mitochondrial network through local ROS-dependent interactions among mitochondria.

Weakly coupled oscillations revealed by long range temporal correlations of $\Delta\Psi_m$

In an interesting example of how computational modelling can enhance interpretation of data as well as suggest new ideas to test experimentally, we observed very soon after exploring the parameter space of the mitochondrial oscillator that the frequency and amplitude of mitochondrial oscillation could span a remarkably wide range, from milliseconds to multiple hours, and μV to 100 mV (Fig. 2). We hypothesized that one of two possibilities for the transition between stable physiological behaviour and unstable pathophysiological behaviour were possible. Either the system was (i) undergoing a bifurcation in the dynamics from a stable steady state to limit cycle oscillation under stress, or (ii) the mitochondria were oscillating with high frequency and low amplitude under 'normal' conditions and there was an increase in the synchronization and coupling of the oscillators in a dominant low frequency, high amplitude mode at the critical point.

To determine if there was evidence of correlated noise due to the oscillatory dynamics of mitochondrial energetics under physiological conditions, we recorded long time series of $\Delta\Psi_m$ at a fast frame rate (~ 100 ms) and applied two methods to look for long range statistical correlations in the data (Fig. 3). The first method was to apply relative dispersion analysis (RDA), a form of detrended fluctuation analysis, to the data (Aon et al 2006b). The relative dispersion (variance standard deviation/mean) was determined using increasing window sizes for aggregating the data and the slope of the log of RD versus the log of the aggregation number was determined. In this analysis, completely random fluctuations yield a slope (or fractal dimension, D_f) of 1.5. Notably, D_f was close to 1.0 for $\Delta\Psi_m$ fluctuations under both physiological and pathophysiological conditions. This indicates that there is long term memory in the system, that is, the current state of $\Delta\Psi_m$ is correlated with $\Delta\Psi_m$ in the past, over several time scales, and this observation is consistent with the behaviour of coupled oscillators.

The second method was to use power spectral analysis (PSA) to determine if the noise was correlated. The slope (β) of the log-log plot of power vs. frequency demonstrated that $\Delta\Psi_m$ follows a power-law dependence according to $f^{-\beta}$, with β of ≈ 1.74 . Again, this slope is distinct from random noise ($\beta = 0$), and indicates that there is a broad spectrum of frequency components spanning several orders of magnitude underlying the apparently 'stable' $\Delta\Psi_m$ observed under physiological conditions.

Using the same respiratory or IMAC inhibitors employed to investigate the mechanism of the mitochondrial oscillator, we demonstrated that the same interventions that abruptly stopped the large amplitude oscillations also decreased the extent of the correlation in the PSA, particularly in the high frequency part of the spectrum. We therefore propose that the mitochondria are normally functioning as a collection of weakly coupled oscillators that, under stress, can become strongly coupled by ROS to produce a dominant slow, large amplitude oscillation in the network. The latter condition is characterized by large bursts of ROS production during the uncoupling phase of the cycle, which will eventually overwhelm the antioxidant capacity of the cell and lead to cell death.

Mitochondrial criticality as the origin of contractile and electrical dysfunction during ischaemia and reperfusion

The primary function of the cardiac cell, excitation–contraction coupling, is intimately coupled to the energetic status. In the oscillatory phenomenon described above, the repeating cycles of mitochondrial $\Delta\Psi_m$ depolarization and repolarization allow us to examine the phase relationships between $\Delta\Psi_m$, ROS bursts, and their effects on cellular action potentials and Ca^{2+} transients. The rapid phase of mitochondrial uncoupling was closely correlated with the activation of energy-sensing K_{ATP} channels of the sarcolemmal membrane (Aon et al 2003, O'Rourke et al 1994), and also with the suppression of intracellular Ca^{2+} release (O'Rourke et al 1994). This demonstrates that mitochondrial criticality scales to produce global dysfunction at the level of the whole cardiac cell. The modulation of cellular electrical excitability is particularly relevant to the generation of cardiac arrhythmias, because dispersion of repolarization of the myocardium is known to be a main factor contributing to the development of reentrant circuits.

In recent studies, we have provided evidence that failure of the mitochondrial network not only scales to the level of the whole cell, but also underlies global electrical dysfunction in the whole heart during ischaemia and reperfusion (Akar et al 2005). Isolated-perfused guinea-pig hearts were subjected to 30 minutes of global ischaemia followed by reperfusion while epicardial electrical activity was followed using a multichannel optical mapping system. In more than 90% of the control hearts, ventricular tachycardia and fibrillation was induced upon reperfusion. Exposure of the hearts to 4'Cl-diazepam, which was shown to stabilize both $\Delta\Psi_m$ and the action potential duration of cardiac cells undergoing oscillations after a laser-induced flash, completely eliminated post-ischaemic arrhythmias (Akar et al 2005). We proposed that clusters of cells that have reached mitochondrial criticality in the heart during ischaemia constitute metabolic current sinks that will impede electrical propagation due to their high K_{ATP} conductance. These regions could either be completely unexcitable, or could have marked spatiotemporal heterogeneity of the action potential duration that promotes reentry.

Studies are currently underway to determine if regional and heterogeneous conduction slowing correlates with regional heterogeneous mitochondrial depolarization. Preliminary evidence indicates that IMAC inhibition protects against the loss of $\Delta\Psi_m$ during ischaemia, prevents heterogeneity of $\Delta\Psi_m$ upon reperfusion, and preserves systolic and diastolic contractile function (data not shown).

Outstanding questions/new perspectives

Mitochondrial ROS production as a frequency and amplitude encoded signalling system

While the contribution of mitochondrial ROS to cell injury and death has been well established, the physiological relevance of ROS-dependent signalling is just beginning to be realized. A clear example is the participation of ROS in the activation of cardioprotective pathways (Oldenburg et al 2002). The presence of ROS scavengers can completely prevent ischaemic or pharmacological preconditioning. Recent evidence has also implicated mitochondrial ROS in the activation of the HIF1 α transcriptional response during hypoxia (Waypa et al 2006).

The finding that the mitochondrial network behaves as a network of coupled oscillators immediately suggests that this property might constitute a mechanism for fine modulation of ROS signals, to be decoded by redox-sensitive transcription factors, ROS-sensitive kinase/phosphatase systems, or indirectly, for example, through effects on Ca^{2+} handling. Analogies exist with other frequency-modulated systems, such as the Ca^{2+} /calmodulin dependent kinase-mediated phosphorylation pathway (Hanson et al 1994) and hormone stimulated Ca^{2+} signalling in nonexcitable cells (Hajnóczky et al 1995). In fact, the latter has recently been

shown to be dependent on mitochondrial ROS generation (Camello-Almaraz et al 2006). It has been argued that frequency-encoded systems permit the cell to more easily detect a signal above background than an amplitude modulated response.

In terms of the physiological state, it remains to be determined if and how a broad spectrum of low amplitude $\Delta\Psi_m$ oscillations, with presumably small amplitude ROS oscillations, is decoded by the rest of the cell to produce a response. We suggest that the spectral power will change as a consequence of normal cell function, for example, in response to increased workload, a change in nutrient status, or a change in second-messenger mediated signalling pathways. Much more work will be needed to establish the cause and effect relationship between such factors, but the linkage between respiration and ROS production could be a direct reporter of the global cell status, and thus uniquely positioned to bring about a response.

Of course, the positive relationship between respiration and ROS production that we have observed during mitochondrial $\Delta\Psi_m$ oscillation directly contradicts some models of ROS production by the respiratory chain, as we have previously pointed out (O'Rourke et al 2006), but is consistent with ROS production from complex III (Turrens et al 1985). So the basic question of whether mitochondrial ROS production increases or decreases with an increase in respiratory rate in intact cells needs to be resolved in future studies. It is perfectly reasonable to assume that ROS can arise from different points in the respiratory chain and may be generated on different sides of the inner membrane under different conditions, for example during hypoxia versus during reoxygenation.

How does the morphology of mitochondrial network influence cell function?

Clearly, in the cardiac cell, the mitochondrial network is highly organized, almost as a cubic array. This arrangement is probably necessary to serve the massive energy demands of muscle contraction, but it also maximizes neighbour–neighbour interaction in the network. The mitochondria are not only situated close to the myofilaments, the main sites of ATP hydrolysis, but also butt directly up to the Ca^{2+} release sites of the dyad, suggesting that local microdomain interactions may be important. Mitochondria may both shape and respond to local Ca^{2+} signals (Maack et al 2006), but may also modulate Ca^{2+} release by controlling the local redox environment or local ATP/ADP ratio, considering the fact that every one of the proteins involved in Ca^{2+} handling are sensitive to these factors.

It will therefore be interesting to investigate whether ROS-dependent mitochondrial network signalling plays a role in other cell types in which the mitochondria are distributed either as filamentous tangles, long strands, or as punctate spots. Diffusion distances should play a major role in the synchronization of these networks.

Mitochondrial ion channels: How selective are they? What are they?

A major source of controversy and frustration in the field has been the lack of molecular structure for all but a few mitochondrial ion channels (e.g. UCP, VDAC). Some of the difficulty in identifying and characterizing these channels is that they are likely to be present in low abundance, in order for the mitochondria to preserve the low permeability so essential to chemiosmotic energy transduction. Another challenge is to find a suitable method for assaying their expression. However, the ample and varied evidence that selective ion channels are present gives us hope that the hard work being carried out presently in several laboratories around the world will eventually bear fruit. Achievement of this goal will not only resolve some of the ongoing arguments, but will provide the basis for a new phase of mitochondrial research, the molecular dissection of mitochondrial ion channel function.

Conclusion

In summary, we have emphasized that the spatial and temporal organization of mitochondria is crucial to understanding the behaviour of the mitochondrial network in intact cells. In heart cells, mitochondria appear to be organized as a collection of weakly coupled oscillators that, under stress, can become strongly coupled by local ROS-induced ROS release. Synchronized oscillation of the mitochondrial network produces dramatic effects on the whole cell electrical and Ca^{2+} handling functions and these can scale to the whole organ to induce fatal arrhythmias and impaired contractile function. A particular mitochondrial ion channel, IMAC, appears to be a key player in this cascade of failures, while other mitochondrial ion channels may protect against mitochondrial dysfunction. Identification and molecular characterization of these mitochondrial ion channels will be necessary to gain a deeper understanding of the regulation of oxidative phosphorylation and intracellular signalling. It will also be important for developing novel mitochondrially targeted therapies for cardiovascular diseases, metabolic syndrome, neurodegeneration and ageing in the years to come.

DISCUSSION

Nicholls I'm missing something here. Why do the mitochondria depolarize and how does this set up an oscillation?

O'Rourke Essentially, what we have is a relaxation oscillator. There is a build up of ROS that reaches a critical threshold level and increases the opening probability of the inner membrane channel. This leads to energy dissipation and mitochondrial depolarization.

Nicholls Why would opening of an inner membrane anion channel collapse the mitochondrial membrane potential?

O'Rourke The idea would be that this channel has an equilibrium potential very far from the -150 mV mitochondrial membrane potential, which would tend to drive the membrane potential towards that equilibrium potential for anions which we are presenting as somewhere around zero. Normally the mitochondrion has controlled conductance, but not if you had a selective channel that had an equilibrium potential far from where the resting potential is.

Nicholls That happens at the plasma membrane where there is an 'infinite' pool of ions on either side. If you open up a channel, the membrane potential gets clamped. Instead you are focusing on the movement of the superoxide anion which is present in tiny amounts.

O'Rourke There are many anions present that can get through this channel. Even small metabolites such as malate have been shown to go through this inner membrane anion channel. The superoxide is going along with the flow. It is a very small concentration. This flow of anions contains a certain amount of superoxide that can be exported across the membrane. In our model we have some data to suggest that the superoxide is produced on the matrix side. First, our sensor is there, and this is getting oxidized in the matrix. We think there are oxidants produced in the matrix. Second, if we inhibit the benzodiazepine receptor we get a bigger increase in ROS in the flashed region, but we don't get any propagation outside of this. This is why we hypothesize that it is generated on the matrix face and not on the outside face of the inner membrane. The other theoretical problem with it being produced on the outside is that it is less likely to oscillate because the activator site is on the same side as the generation of the activator.

Nicholls I still have problems understanding that there is enough ion movement to significantly depolarize the mitochondria.

Bernardi This is the same point I made a couple of years ago. First of all, have you identified the charge-carrying anion, because without that you don't have a mechanism? I don't think there is more malate or glutamate inside mitochondria than there is outside.

O'Rourke There's plenty of chloride. There's 20 mM outside and other anions inside.

Bernardi As long as mitochondria are energized they will exclude chloride. If you open an ion channel that can drive chloride uptake it would hyperpolarize, not depolarize.

O'Rourke The model has an outward rectifying chloride channel based on the patch-clamp studies of Borecky and Siemen (Borecky et al 1997). You don't need many ions to move to change the membrane potential. In the sarcolemmal membrane, for example, there are very few cations moving to depolarise the membrane potential even though there is a large K^+ conductance there at all times. It depends on the impedance of the membrane.

Nicholls When I started trying to understand chemiosmosis and mitochondrial membrane potentials, I was enormously confused about what happens at the plasma membrane where the classic Hodgkin-Huxley equation holds, with infinite compartments and where the flux of a few ions can depolarise, and what happens in the mitochondrion with an 'infinitesimally' small matrix compartment.

O'Rourke It's true that there are constraints on volume and ion movement.

Brand I would be with David Nicholls and Paolo Bernardi: there is a fundamental difference between the plasma membrane and the way that it responds to an anion channel opening, and the mitochondrial membrane. You have to move large amounts of ion across the membrane if it is the mitochondrial membrane, and because of the small matrix volume, they are just not there. I don't buy into the idea that increasing the Cl^- conductance would change the potential very much because this potential is dominated by the electrogenic electron transport chain, not by secondary ion movements. It is quite different from the plasma membrane, where the potential is dominated by secondary ion movements, not by the primary electrogenic sodium pump activity.

O'Rourke Would you agree that the PTP can change the mitochondrial membrane potential? I should point out that in the swelling assay studies this was a promiscuous channel. It was not very selective even for anions and cations. It was a 4 : 1 cation : anion selectivity. I think many ions can move through this channel when it is open. I come from an ion channel background so I am glad to hear these criticisms.

Halestrap There is one way you can account for it: if phosphate goes in with a proton on the phosphate transporter with a proton and comes out on an anion channel you can effectively get a net proton movement. I don't buy this story, but I can see that it has some merit. For example, we do know that modifying single thiol groups on mitochondrial substrate transporters can turn them into channels (Dierks et al 1990). We also know that ROS can modify thiol groups. So one can conceive that a substrate transporter could become an anion channel under conditions of oxidative stress.

O'Rourke We are not making any judgement of which particular protein constitutes IMAC because we don't know the structure.

Rich What about hydroxide as a candidate anion that goes through the channel as it is there in an unlimited supply?

Lemasters Photodamage-induced injury is not the same as reperfusion injury. Photodamage is primarily mediated by singlet oxygen.

O'Rourke The only photodamage is in those 50 mitochondria in the cell.

Lemasters That is the initiator. We have looked at ischaemia–reperfusion in myocytes, and there are some interesting parallels. During ischaemia, Ca^{2+} goes up in the cytosol and the mitochondria, saturating the calcium indicators. During reperfusion, both cytosolic and mitochondrial Ca^{2+} recover quickly, and the mitochondria repolarize. The mitochondria remain relatively stable, except that they are generating free radicals at an increased and relatively steady rate. Then after 20–40 minutes, everything goes bad: the mitochondria depolarize, Ca^{2+} in the cytosol and mitochondria again increases to saturate the Ca^{2+} indicators. Some what later, the myocytes die. In these experiments, if we add permeability transition inhibitors, such as cyclosporin A or NIM811, we prevent depolarization and cell death. We also prevent the Ca^{2+} dysregulation, but we do not prevent the production of ROS. If we use antioxidants to block the ROS, we still prevent the depolarisation, Ca^{2+} dysregulation and cell death. In this model, there is a steady production of ROS after reperfusion that reaches a threshold to induce the permeability transition. Flickering of the depolarisation indicative of transient permeability transition pore opening precedes the sustained depolarization (Kim et al 2006).

O'Rourke I view it as being a hierarchy of ROS-induced targets. The IMAC is even activated during ischaemia, because with 4-chloro diazepam you can delay the depolarization of mitochondrial membrane potential in a whole heart, and get a better recovery on reperfusion, whereas cyclosporin doesn't do that. It only helps on reperfusion. In the window in the first few minutes of reperfusion we are getting a mechanism that is not really PTP dependent, and then the PTP opens after a short delay. I hope we will get to a point where not every mitochondrial depolarization is attributed to the PTP.

Jacobs Can you explain to me what is involved in propagating a signal from one mitochondria to the next, which leads to the lights going out?

O'Rourke It is ROS diffusing from one mitochondrion to its neighbours, based on this percolation theory. This is the macroscopic cue determining which mitochondria are going to depolarize.

Jacobs In this case, ROS is doing the same thing from the outside to the neighbour mitochondrion as it is doing from the inside to the one that is propagating the signal.

O'Rourke This is why we have the activator site on the channel depicted on the outside in our model. We haven't addressed the outer membrane permeability, and whether a change is needed in this. We are presuming that the superoxide can get through the outer membrane as well.

Nicholls So you are saying that mitochondria full of superoxide are waiting until they see external superoxide. They release this and a chain reaction proceeds.

O'Rourke It is a regenerative wave in the system. As long as those mitochondria are at this threshold. I wouldn't say that it is just because superoxide is sitting there: it is also a depletion of the scavenger pool. We can get to this critical state in many ways, such as substrate depletion or reduced glutathione depletion. If cells are treated with Diamide, there is a certain critical level of glutathione that is reached, and then these oscillations occur, as well as eventual depolarisation. It is the balance between the ROS production and the amount of scavenger that is present. If you load up the cytoplasm with TMPYP, or get the reduced glutathione levels high, this propagation won't occur.

Duchen For the changes in mitochondrial potential to be translated into the dysrhythmia seen at the plasma membrane, you would need very fast turnover of ATP to regulate the plasmalemmal K-ATP channel.

O'Rourke It isn't changes in ATP that matter as much as changes in the ATP : ADP ratio. We think it is mainly the increase in ADP that causes the change in sensitivity of the K-ATP channel. Mitochondrial uncoupling can rapidly activate sarcolemmal K-ATP channels, even if there is 5 mM ATP in the pipette solution. We have recently modelled this in an integrated model in which we put the mitochondria into a model of electrophysiology and Ca^{2+} handling. We can reproduce the effects on the action potential just by the burst of ADP that is produced by the mitochondria as they reverse and begin to consume ATP.

Adam-Vizi I'd like to comment on ROS generation. People usually show schemes pointing to two major sites of ROS generation: the respiratory chain and complex III. This is true if one refers to data obtained with isolated mitochondria, where complex III is blocked with antimycin and the result is huge ROS generation. But if you are discussing physiologically relevant ROS generation, this is narrowed to complex I. For complex III to produce ROS it must be blocked almost totally, which is unrealistic *in vivo*.

O'Rourke I disagree. I think the physiological cases show ROS generation from complex III but not complex I. We have never seen any state where if we reduce complex I by adding rotenone, this results in increased ROS production. We never get the production from complex I that can be obtained in an isolated mitochondrial system. This requires a very high level of reduction of complex I. Kushnareva had a paper (Kushnareva et al 2002) that suggested that it was even more reduced than NADH. This level of reduction doesn't usually occur in heart cells. For complex III, there is a paper by Turrens & Lehninger (1985) in which they took isolated mitochondria and depleted them of cytochrome c, and then added it back. They had a nice titration of VO_2 as they did this. There was a linear correlation between ROS production in complex III and VO_2 . We believe this model. It is controversial.

Adam-Vizi You can easily titrate complex III activity and measure ROS production. It can be blocked up to 70% and there is no ROS production.

O'Rourke In our experiments, blocking the entry of electrons into complex III with myxothiazol or reducing the downstream electron acceptors, e.g. with cyanide, all suppress mitochondrial ROS production and prevent mitochondrial depolarization, consistent with superoxide generation at complex III.

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FIG. 1.

Mitochondrial network depolarization after a local laser flash. (A) Upper panels: $\Delta\Psi_m$ signal before the flash, close to criticality, and after global depolarization. Lower panels: ROS signal before the flash, close to criticality, and after global depolarization. (B) The number of mitochondria with ROS above threshold increases to ~60% at criticality just prior to global depolarization and limit cycle oscillation of the network (adapted from Aon et al 2004). (C) Conceptual and computational model of the mechanism of the ROS-dependent mitochondrial oscillator (adapted from Cortassa et al 2004).

FIG. 2.

Frequency and amplitude range of the mitochondrial oscillator. Variation of a single parameter (the superoxide dismutase activity) in a computational model of the ROS-dependent mitochondrial oscillator produces a wide range of behaviours spanning from low amplitude fast oscillation (25 ms) to slow, large amplitude oscillations in $\Delta\Psi_m$ (55 min). The rapid phases of $\Delta\Psi_m$ depolarization (uncoupling) are accompanied by small pulses (fast domain) or bursts (slow domain) of superoxide release from the matrix to the intermembrane space (see Cortassa et al 2004 for details).

FIG. 3.

Relative dispersion analysis and power spectral analysis of $\Delta\Psi_m$ under physiological and pathophysiological (oscillating cell) conditions. Left panel: RDA demonstrates that under both conditions, long-range correlations in the signal are present, distinct from random noise. Similarly, the power spectrum shows a broad frequency dependence dropping off with a $1/f^{1.79}$ dependence, in this example. Both methods suggest that mitochondria are organized as a collection of weakly coupled oscillators under normal conditions (see Aon et al 2006 for details).