

## Mitochondrial Ion Channels: Gatekeepers of Life and Death

Brian O'Rourke, Sonia Cortassa,  
and Miguel A. Aon

Institute of Molecular Cardiobiology, Division of Cardiology,  
The Johns Hopkins University, Baltimore, Maryland  
bor@jhmi.edu

Continuous generation of ATP by mitochondrial oxidative phosphorylation is essential to maintain function in mechanically active cells such as cardiomyocytes. Emerging evidence indicates that mitochondrial ion channels activated by reactive oxygen species can induce a mitochondrial "critical" state, which can scale to cause electrical and contractile dysfunction of the cardiac cell and, ultimately, the whole heart. Here we focus on how mitochondrial ion channels participate in life-and-death decisions of the cell and discuss the challenges ahead for translating recent findings into novel therapeutic applications.

We live in a dangerous environment, continuously immersed in a medium containing 21% oxygen, a highly reactive molecule. Fortunately for us, this impediment to life has been turned back on itself and has actually been harnessed by the cell to generate the stored chemical energy necessary to meet the major demands of cell physiology. Mitochondria are the organelles providing this protection, compartmentalizing and controlling the oxidation of metabolic fuels to produce ATP, the high-energy phosphate used to maintain ion gradients, synthesize new molecules, actively dispose of old molecules, and perform mechanical work. In the heart, the energetic demands are extreme: the heart is required to pump roughly 75 gallons of blood per hour, unceasingly, for up to 100 years. To meet this workload, the heart accounts for nearly 10% of the total oxygen consumption ( $V_{O_2}$ ) of the body at rest, and this demand increases four- to fivefold during exertion. Thus it is vital for the cardiac cell to continuously adjust the fine balance between energy production and energy consumption and to limit the ill effects of the byproducts of metabolism, which include reactive oxygen species (ROS). Over the physiological range of activity, the multiple nonlinear control interactions governing mitochondrial oxidative phosphorylation elegantly and robustly adapt to large changes in demand while keeping the many positive and negative feedback loops in check to prevent instability in the metabolic network.

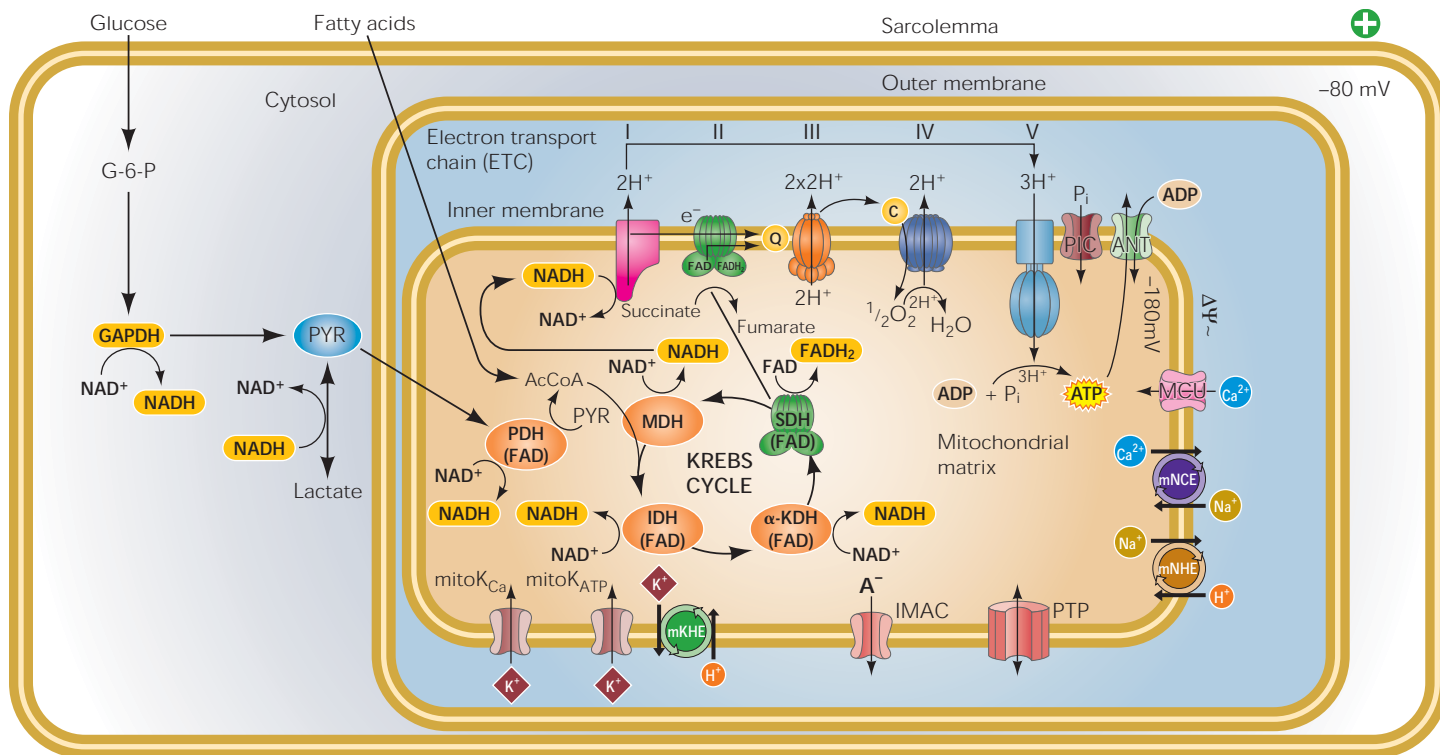
Unfortunately, age and/or disease can introduce a variety of stressors that can, under certain circumstances, result in the collapse of mitochondrial function, causing necrotic or apoptotic cell death. Failure at the level of the organelle can scale to the mitochondrial network and have a major impact on the basic excitation-contraction coupling processes of the cardiomyocyte. As a consequence of the syncytial nature of the myocardium, this cas-

cade of failures can scale to level of the organ and, ultimately, determine the life or death of the organism. Intervention into this process requires a detailed understanding of the mechanisms underlying metabolic control, the factors responsible for loss of control, and the built-in mechanisms that might protect the cell from injury. Emerging evidence suggests that ion channels on the mitochondrial inner and outer membranes are key participants in the decision between cell life and death. Despite the central role of mitochondrial ion channels in cell injury, remarkably little effort has been directed toward developing specifically targeted therapeutic agents to modulate their activity. Furthermore, the molecular structures of key mitochondrial ion channels and/or transporters have not been determined, hampering progress toward a complete understanding of their roles. Hopefully, recent discoveries and new techniques for identifying mitochondrial proteins and assaying mitochondrial ion channel activity will spur new initiatives to resolve long-standing questions in the field of bioenergetics and will inspire the development of tools for manipulating mitochondrial function in the future.

### Mitochondrial Bioenergetics and ROS Production

#### *Overview of cardiac energy metabolism*

Investigating the effects of mitochondrial ion channels and transporters on bioenergetics requires a basic understanding of energy metabolism in the heart, as depicted schematically in **FIGURE 1**, which emphasizes the mitochondrial processes involved in oxidative phosphorylation. Glucose and fatty acids, the primary metabolic substrates of the heart, are sequentially oxidized to produce acetyl-CoA, the common intermediate driving the production of the reducing equivalents NADH and



**FIGURE 1. Overview of oxidative phosphorylation in the cardiac cell**

The sequential oxidation of fuels (e.g., fatty acids and glucose) leads to the common substrate for the Krebs cycle, acetyl-CoA, which drives the production of the reducing equivalents NADH and FADH<sub>2</sub>. Electrons are passed to the electron-transport chain, where coupled redox reactions mediate proton translocation across the inner membrane to establish an electrical potential and pH gradient (proton-motive force) that drives ATP synthesis by the mitochondrial ATP synthase. Ion-selective or nonselective mitochondrial ion channels dissipate energy and alter the ionic balance and volume of the mitochondrial matrix, which is partly compensated by antiporters coupled to H<sup>+</sup> movement. See text for further details. ANT, adenine nucleotide translocase; G-6-P, glucose-6-phosphate; IMAC, inner-membrane anion channel; MCU, mitochondrial Ca<sup>2+</sup> uniporter; mitoK<sub>Ca</sub>, mitochondrial Ca<sup>2+</sup>-activated K<sup>+</sup> channel; mitoK<sub>ATP</sub>, mitochondrial ATP-sensitive K<sup>+</sup> channel; PIC, phosphate carrier; PTP, permeability transition pore; PYR, pyruvate; KHE, K<sup>+</sup>/H<sup>+</sup> exchanger; NHE, Na<sup>+</sup>/H<sup>+</sup> exchanger; NCE, Na<sup>+</sup>/Ca<sup>2+</sup> exchanger; IDH, isocitrate dehydrogenase; KDH, α-ketoglutarate dehydrogenase; MDH, malate dehydrogenase; PDH, pyruvate dehydrogenase; SDH, succinate dehydrogenase.

FADH<sub>2</sub> by the Krebs cycle (tricarboxylic acid cycle). NADH feeds electrons to the electron-transport chain (respiratory chain) through the NADH:ubiquinone oxidoreductase (complex I), which reduces ubiquinone (coenzyme Q). The reduced flavin moiety of succinate dehydrogenase (complex II), which is also a Krebs-cycle enzyme, passes electrons to ubiquinone directly. Ubiquinone is a lipid-soluble mobile carrier that transfers electrons to the cytochrome bc1 complex (complex III), which, in turn, reduces the peripherally associated carrier cytochrome c. In the terminal redox reaction, electrons are passed to cytochrome c oxidase (complex IV) and ultimately to molecular oxygen. The redox reactions occurring at complexes I, III, and IV are coupled to proton (H<sup>+</sup>) translocation across the mitochondrial inner membrane, establishing a proton-motive force (Δp), composed of the pH gradient (ΔpH) and the mitochondrial membrane potential (ΔΨ<sub>m</sub>). This large driving force for proton influx is used by the mitochondrial ATP synthase (F<sub>1</sub>F<sub>0</sub> ATPase) to produce ATP, which is exported to the cytosol via the adenine nucleotide translocase (ANT).

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. An increase in the ion permeability of the mitochondrial inner membrane can occur in the presence of protonophoric chemical uncouplers, in response to an increase in matrix ADP (through stimulation of proton flux through the ATP synthase), or as a consequence of opening mitochondrial ion channels. The energy dissipated by the increased ion permeability stimulates NADH oxidation, proton pumping, and respiration. 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.

### Sites of mitochondrial ROS production

Although the respiratory chain quite efficiently uses O<sub>2</sub> in the controlled oxidation of the redox carriers, the superoxide anion (O<sub>2</sub><sup>-</sup>) is an unavoidable byproduct of oxidative phosphorylation, making mitochondria a major site of ROS production. It has been estimated that ~1–5% of the electrons flowing through the electron-transport chain leak into the production of ROS (83). The dual effects of



mitochondria.

Since these models of mitochondrial ROS production are almost exclusively based on data from isolated mitochondria, the question remains as to which site is important in intact myocytes or muscles. We have provided evidence that in intact myocytes, the majority of mitochondrial  $O_2^-$  production in cells subjected to local oxidative stress is from complex III, most likely from the matrix-oriented site (see Ref. 3 and below). A similar transition from low electron flow to high electron flow would be expected to occur early in reperfusion after ischemia and could contribute to the well-known burst of ROS produced during this period. It remains to be determined whether other sites of ROS production, both intrinsic and extrinsic to the mitochondria, are activated under specific conditions, such as during hypoxia.

### Loss of Mitochondrial Function: Sequelae of Ischemia

There is widespread agreement that a reproducible series of events occurs in the myocardium during ischemia (FIGURE 3), although the cause-and-effect relationship of each with respect to ischemic injury is often difficult to ascertain. At the onset of ischemia, anaerobic metabolism is activated within 8 s of flow cessation (65), and contraction declines within 20 s. The myocardial electrical properties also change rapidly; the cellular action potential shortens, and the ST segment of the EKG is elevated within the first minute of ischemia. Intracellular pH declines and anaerobic glycolysis then becomes inhibited. Over the course of many minutes of ischemia, intracellular  $Na^+$  increases (2), in part through  $Na^+/H^+$  and  $Na^+/Ca^{2+}$  exchanger action, and the cytoplasmic and mitochondrial compartments load with  $Ca^{2+}$  (82). The  $\Delta\Psi_m$  declines progressively during the ischemic period (30). Interestingly, when hearts are preconditioned (that is, subjected to one

or more short periods of ischemia with reperfusion before a long ischemia),  $\Delta\Psi_m$  may drop even more than with ischemia alone, yet the functional recovery is improved upon reperfusion (93).

Upon reperfusion, the  $Na^+$ -loaded heart fosters additional cellular  $Ca^{2+}$  overload via reverse-mode  $Na^+/Ca^{2+}$  exchange, and a major burst of ROS is evoked. During this important period, the course of cardiomyocyte survival or death is set, determined in large part by the ability of mitochondria to recover. The activation of destructive mitochondrial ion channels plays a role in this decision. Chemical or ischemic preconditioning of the heart appears to blunt most of the detrimental changes in cellular parameters, and mitochondrial function is better preserved. A number of intracellular signaling pathways and effects on oxidative phosphorylation, ROS production, and  $Ca^{2+}$  handling have been implicated in this native protective mechanism (84). A central role for the mitochondrial ATP-sensitive  $K^+$  (mito $K_{ATP}$ ) channel is widely supported (63), but the precise mechanism of protection is still being investigated.

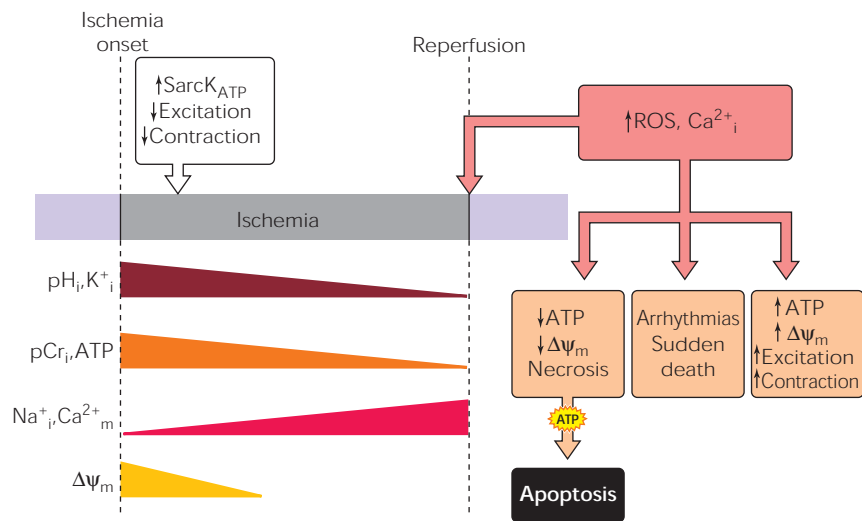
### Mitochondrial Ion Channels

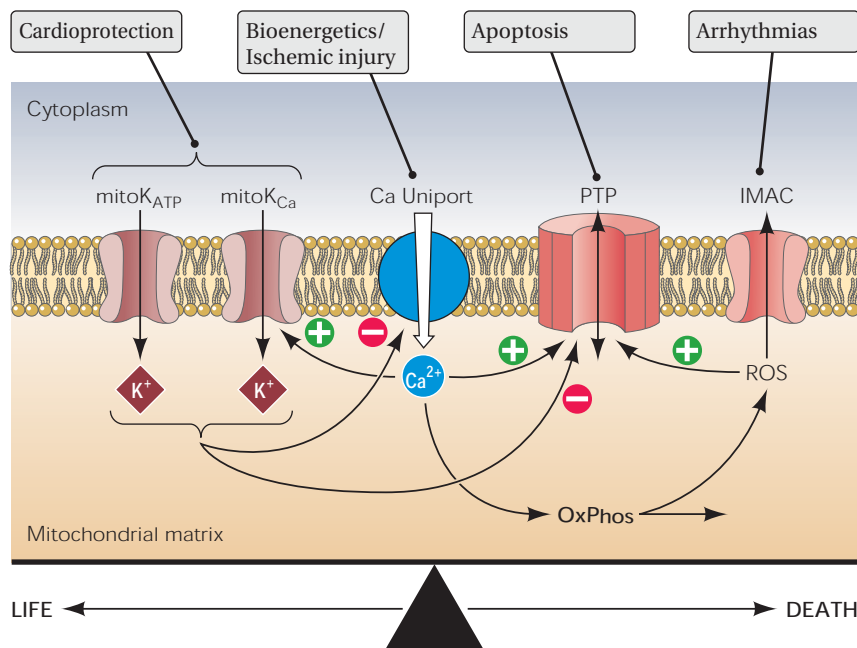
#### Function of mitochondrial ion channels

The physiological roles of many inner-membrane ion channels are largely unknown. For example,  $K^+$ -selective and/or anion-selective pores are believed to be important for regulating mitochondrial volume (31), but in vivo confirmation of this function has been difficult to obtain (63). Mitochondrial swelling and contraction has been proposed to modulate the rate of substrate oxidation (36) under normoxic conditions and could be an important factor in determining the extent of ischemia-reperfusion injury (44, 49, 50). At a minimum, energy dissipation due to channel opening will increase the flux through the electron-transport chain, and if this effect is uncompensated by increased sub-

**FIGURE 3. Sequelae of ischemia and reperfusion**

Loss of electrical and contractile function of the heart occurs rapidly after the onset of ischemia. Changes in intracellular ions and high-energy phosphates occur throughout the ischemic period, contributing to injury upon reperfusion. The time course of mitochondrial membrane potential ( $\Delta\Psi_m$ ) depolarization is not well defined, but failure to recover  $\Delta\Psi_m$  upon reperfusion is likely to be the main determinant of cellular life or death. A burst of reactive oxygen species (ROS) and cellular and mitochondrial  $Ca^{2+}$  overload is known to occur within the first 5 min of reperfusion. Sarc $K_{ATP}$ , sarcolemmal  $K_{ATP}$  channel; pCr, phosphocreatine.





**FIGURE 4. Protective and destructive mitochondrial ion channels participating in the life-and-death decisions of the cell**

Activation of either of two protective mitochondrial  $\text{K}^+$  channels ( $\text{mitoK}_{\text{ATP}}$  and  $\text{mitoK}_{\text{Ca}}$ ) has been shown to protect the heart against ischemia and reperfusion injury, whereas the opening of the PTP or the IMAC can result in the collapse (or oscillation in the case of the latter) of  $\Delta\Psi_{\text{m}}$ . The protective channels are known to inhibit PTP opening. This could occur either as a result of inhibition of the factors that cause PTP opening (e.g., ROS,  $\text{Ca}^{2+}$ ) or perhaps by direct physical interaction with the PTP protein complex.

strate production, then net oxidation of the matrix, as well as a change in ROS production, will occur. Thus one potential physiological role of mitochondrial channels could be redox regulation, which is known to be an important intracellular signaling mechanism influencing transcription, translation, phosphorylation/dephosphorylation cascades, and programmed cell death. Paradoxically, net reduction of the cellular redox state can also activate transcription factors; for example, oxygen deprivation activates the transcription factor hypoxia inducible factor-1 (HIF-1), leading to an increased expression of adaptive enzymes (72). Recent evidence suggests that hypoxia might also increase ROS production (86), but the precise site of ROS production and the downstream effects on cell function are still under investigation.

Mitochondrial swelling assays and later direct patch-clamp studies of mitoplasts (which are mitochondria stripped of their outer membranes) have identified a number of ion-conductive pores on the inner membrane (94). In general, these mitochondrial ion channels have been associated with either protective or destructive effects on cells during metabolic stress, as illustrated schematically in **FIGURE 4**.

### ***Ca<sup>2+</sup> uniport***

The  $\text{Ca}^{2+}$  uniporter of the mitochondrial inner membrane has perhaps the most well-understood role in physiology. This ruthenium red-sensitive  $\text{Ca}^{2+}$  uptake pathway is the primary route for  $\text{Ca}^{2+}$  entry into the mitochondrial matrix, and there is strong evidence that an increase in matrix  $\text{Ca}^{2+}$  is important for stimulating oxidative phosphorylation at several sites, including the  $\text{Ca}^{2+}$ -sensitive

dehydrogenases of the Krebs cycle (26) and one or more sites in the electron-transport chain (7, 21). Although recent evidence supports the notion that the  $\text{Ca}^{2+}$  uniporter is a highly  $\text{Ca}^{2+}$ -selective, low-conductance ion channel (46), the molecular structure of this channel has not yet been determined. Intriguing recent studies have proposed that type I (skeletal type) ryanodine receptors (RyR1) are present on the cardiac mitochondrial inner membrane and may participate in either  $\text{Ca}^{2+}$  influx or efflux (12). Several major discrepancies between the behavior of the  $\text{Ca}^{2+}$  uniporter and RyR1 will need to be resolved before the ryanodine receptor can be considered as a viable candidate for the protein responsible for  $\text{Ca}^{2+}$  uniport activity in heart cells.

The  $\text{Ca}^{2+}$  uniporter has also been implicated in the pathophysiological consequences of ischemia and reperfusion. Mitochondrial  $\text{Ca}^{2+}$  loading has been associated with cell injury (58), although the relative contributions of cytoplasmic vs. mitochondrial  $\text{Ca}^{2+}$  overload are often difficult to assess individually due to their interdependence, and there is not always a clear correlation between mitochondrial  $\text{Ca}^{2+}$  and survival (34). Although mitochondria are normally capable of tolerating large amounts of  $\text{Ca}^{2+}$  uptake with minimal effects on oxidative phosphorylation, there is ample evidence demonstrating that mitochondrial  $\text{Ca}^{2+}$  loading under simulated or true ischemia-reperfusion conditions can trigger a mitochondrial permeability transition (37). The latter may depend on the simultaneous contribution of multiple factors such as  $\text{Ca}^{2+}$ , ROS, ADP,  $\text{NAD}^+$ , etc. (67).

### ***Cytoprotective potassium channels***

There are at least two types of potassium channels

in the mitochondrial inner membrane that have been shown to be protective against ischemia-reperfusion injury. An ATP-sensitive channel (mitoK<sub>ATP</sub>) with properties somewhat resembling those described for surface membrane K<sub>ATP</sub> channels has been found to be present on intact mitochondria (42) and in bilayers reconstituted with purified mitochondrial proteins (66). The mitoK<sub>ATP</sub> observed in these electrophysiological studies is thought to be the ion channel corresponding to ATP-sensitive and K<sup>+</sup> channel opener/blocker-sensitive K<sup>+</sup> fluxes studied in isolated mitochondria, proteoliposomes (10, 43), and mitochondrial swelling studies (24). MitoK<sub>ATP</sub> activation has been implicated in the mechanism of protection against ischemic injury induced by ischemic or chemical preconditioning and in protecting cells against apoptosis and PTP activation (1, 38, 44). Several questions and controversies related to the specificity of certain K<sup>+</sup> channel openers and the mech-

*“PTP activation likely represents an irreversible terminal event for the mitochondria.”*

anism of protection by mitoK<sub>ATP</sub> activation are the subject of ongoing investigation, as discussed in recent reviews of the topic (35, 60, 63).

A second mitochondrial inner membrane K<sup>+</sup> channel (mitoK<sub>Ca</sub>), having properties resembling the Ca<sup>2+</sup>-activated K<sup>+</sup> channel (K<sub>Ca</sub>) of the surface membrane, has also been identified in cardiac (92) and glioma cell (73) mitochondria. This channel is blocked by the scorpion-venom toxins charybdotoxin and iberiotoxin and by paxilline, known blockers of K<sub>Ca</sub> channels in other cell types. MitoK<sub>Ca</sub> channel openers have been shown to confer protection against ischemia-reperfusion injury in a manner analogous to that of mitoK<sub>ATP</sub> (56), but through distinct pathways (71). A role for the mitoK<sub>Ca</sub> channel in delayed preconditioning, which is manifested 24–72 h after a preconditioning treatment, has also been suggested by a recent study (89). The molecular structure of this mitochondrial channel is currently under investigation, but a 55- to 80-kDa protein cross-reacting with anti-BKCa channel antibodies was identified in purified mitochondrial membranes using 2D-PAGE analysis (92). Future studies will be required to establish the role of mitoK<sub>Ca</sub> in the normal physiology of the cell, but we propose that this channel might be activated at high mitochondrial Ca<sup>2+</sup> loads, acting as a relief valve to prevent further Ca<sup>2+</sup> accumulation or to fine-tune mitochondrial volume to optimize oxidative phosphorylation.

In addition to the two K<sup>+</sup> channels mentioned above, a voltage-activated K<sup>+</sup> channel of the Kv1.3 type has recently been identified in lymphocyte mitochondria (80). This channel, however, has been suggested to participate in apoptotic cell death, since knockout of the channel prevented cytochrome c release and  $\Delta\Psi_m$  depolarization induced by actinomycin D (15).

**Channels contributing to dysfunction under stress**

PERMEABILITY TRANSITION PORE. The inner-membrane ion channel receiving the most attention is the permeability transition pore (PTP), a large-conductance (~1 nS) pore that permits the passage of solutes up to 1,500 Da nonselectively (67). It is thought to be activated by oxidative stress, Ca<sup>2+</sup>, and depolarization and is strongly inhibited by cyclosporin A (CsA). The PTP described in mitochondrial suspension studies is believed to be identical to the mitochondrial mega- (or multiconductance) channel (MMC) observed in electrophysiological studies (81). From a structural point of view, it has been argued that the PTP is a multi-protein complex comprised of (at least) the mitochondrial ANT, the voltage-dependent anion channel of the outer membrane (VDAC), the F<sub>1</sub>F<sub>0</sub> ATPase, and cyclophilin D, the receptor for CsA. The pore structure has not been conclusively identified, but it has been observed that, in high Ca<sup>2+</sup>, the ANT can be converted into a large channel (~600 pS) that is nonselective with respect to cations and partially selective for cations over anions (P<sub>K</sub>/P<sub>Cl</sub> ~4:1) (17). This channel is inhibited by bongkrekic acid and ADP, but not carboxyatractyloside, resembling the profile of the PTP. Furthermore, a recombinant bacterial ANT type protein can form similar channels when expressed in *Escherichia coli*, excluding the argument that contaminating proteins in the mitochondrial preparation could have been responsible for the channel activity (18). On the other hand, knockout of ANTI in mice does not eliminate the mitochondrial permeability transition in response to metabolic stress but alters its sensitivity to Ca<sup>2+</sup> and ANT modulators (48). In addition, the MMC can still be observed in mammalian cells lacking mitochondrial DNA ( $\rho^0$ ) and in yeast in which the VDAC or ANT have been knocked out (53, 54).

There is considerable evidence that the PTP opens during ischemia-reperfusion, and it has been linked to cytochrome c release and the triggering of apoptosis (23). Griffiths et al. (33) reported that the PTP does not open during ischemia but is activated upon reperfusion. Since the PTP opens when there is significant cellular Ca<sup>2+</sup> overload and severe energy depletion, and it mediates loss of matrix constituents, PTP activation likely repre-

sents an irreversible terminal event for the mitochondria. There are several reports that PTP opening can be visualized in intact cells (62, 67, 68) or in isolated mitochondria (40) by monitoring the fluorescence of voltage-sensitive dyes (indicating membrane polarization) or calcein (indicating permeability transition) loaded into the mitochondrial matrix compartments. PTP opening can also mediate fast  $\text{Ca}^{2+}$  release from  $\text{Ca}^{2+}$ -loaded mitochondria, perhaps through a  $\text{Ca}^{2+}$ -induced release mechanism; this has led to the concept of mitochondria as excitable organelles with  $\text{Ca}^{2+}$  representing the signaling molecule (41). A model of ROS-induced ROS release involving the PTP has also been described by Zorov et al. (95). We have also recently described mitochondrial ROS-induced ROS release (discussed below) in cardiac cells subjected to local oxidative stress, but the mechanistic details appear to be quite different from the previously described studies implicating the PTP.

**ANION CHANNELS.** The mitochondrial inner membrane is also home to several anion-selective ion channels with undetermined physiological roles. Anionic conductances of 15 pS, 26 pS (SMAC), 57 pS (INMAC), and 107 pS (centum pS channel) have been reported. The 107-pS channel was the first channel identified in patch-clamp studies (76). This channel, also referred to as the centum picosiemens channel or mCS (19, 61), is only slightly anion selective ( $P_{\text{Cl}}/P_{\text{K}} = 4.5$ ) and is strongly voltage sensitive, being driven to the closed state by hyperpolarization. Thus it could be a prime candidate for positive-feedback activation of ion flux under conditions of  $\Delta\Psi_{\text{m}}$  depolarization. Although the 108-pS channel has been tentatively associated with the IMAC (16) characterized in swelling assays of isolated mitochondria (9), this match is not definitive, and other candidates (e.g., the 15-pS channel) have been proposed. IMAC is permeable to a variety of anions and small anionic metabolites (e.g.,  $\text{Cl}^-$ , malate, oxaloacetate, malonate, citrate, etc.), including ATP. It is blocked by a wide variety of amphipathic compounds that can accumulate in the mitochondria, including many clinically effective classes of drugs: antiarrhythmics (amiodarone), anesthetics (dibucaine),  $\beta$ -adrenergic antagonists (propranolol), antidepressants (amitryptiline), and benzodiazepines (clonazepam) (9). Reactive thiols are also present on IMAC, and it is regulated by pH, Mg, and  $\text{P}_i$ . Importantly, it is not blocked by CsA, which permits one to discriminate between IMAC vs. PTP opening (3). Moreover, IMAC does not allow passage of fluorescent markers with sizes of  $\sim 500$  mol wt (e.g., calcein), and its opening is independent of  $\text{Ca}^{2+}$  (for other evidence that distinguishes IMAC from the PTP, see Ref. 3).

**OUTER-MEMBRANE CHANNELS.** Patch-clamp and bilayer-reconstitution studies have also detected ion channels in the mitochondrial outer membrane, which was originally shown to be highly permeable to large solutes and protein. In recent years, the outer membrane has been the target of intense interest, with the discovery that cytochrome c (as well as other factors) can be released from the intermembrane space to induce apoptosis (88). Although there is a paucity of molecular information on mitochondrial inner-membrane ion channels, the sequence and isoform distribution of the major outer membrane protein, VDAC (or porin), is known (55). Changes in VDAC permeability have been implicated in the cell death process and activation of the PTP, and they can modulate the rate of metabolite transport in and out of the mitochondria (70, 87). Current investigations have focused on determining if the apoptogenic proteins Bad, Bax, or tBid independently form channels that allow passage of cytochrome c to the cytoplasm, or if they modulate VDAC activity. Recent patch-clamp studies suggest that either Bax or Bad can directly form cytochrome c-permeable channels, referred to as mitochondrial apoptosis-inducing channels, in the outer membrane (25). Several studies have also recently reported that an increase in outer membrane permeability does not always require a concomitant permeability transition on the inner membrane (88).

Mitochondrial ion channels can also be formed by the proteins involved in the translocation of mitochondrial protein precursors. The translocase of the outer membrane and its partner on the inner membrane can form ion-conductive pores that are blocked by mitochondrial polypeptide-targeting fragments (59). It remains to be determined if these pores are permeable to ions in the intact cell and when they may be active. A variety of other ion-channel conductances have also been observed in the outer membrane (57), but they have not been well characterized or attributed to a specific function.

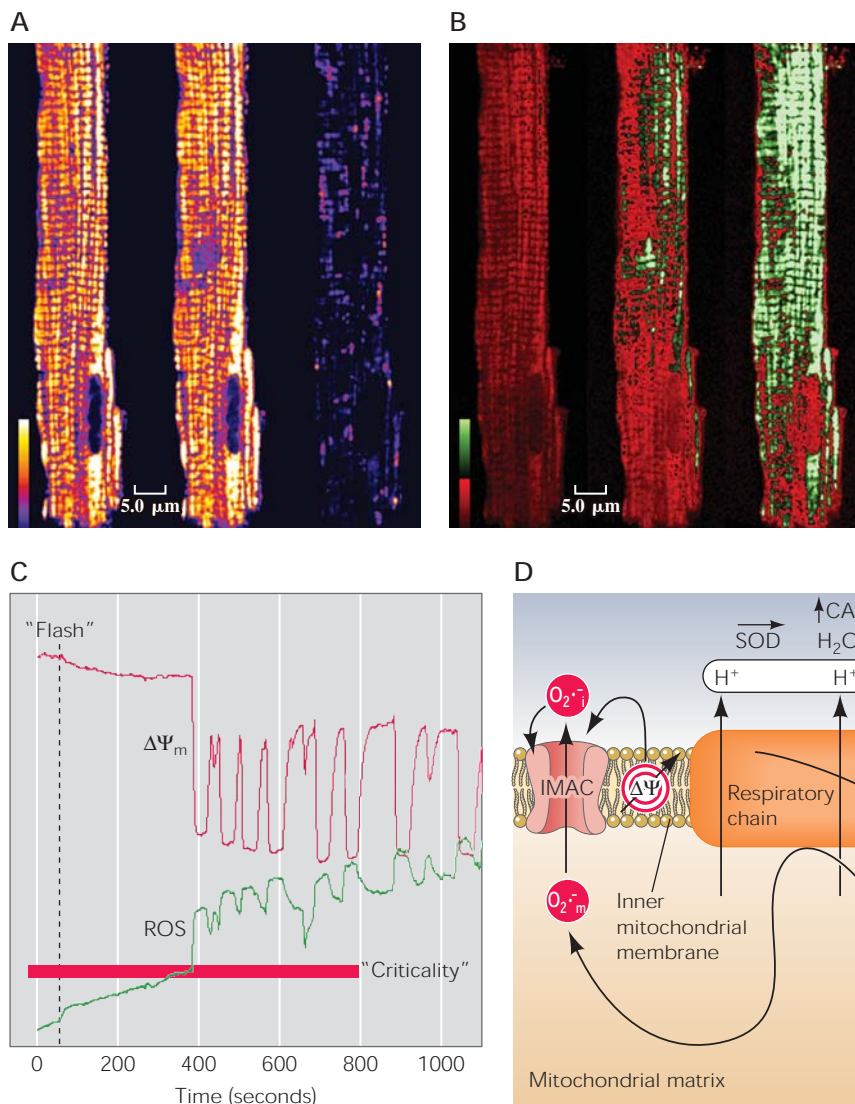
### Mitochondrial Criticality and Emergent Network Behavior

The mitochondrial network of cardiac cells consists of thousands of mitochondria packed between the myofilaments in an ordered, three-dimensional array (FIGURE 5A, LEFT). This arrangement is required to place the sites of ATP production close to the sites of ATP consumption (e.g., the myosin ATPase of the thick filament and the ion pumps required for  $\text{Ca}^{2+}$  sequestration and ion homeostasis). This spatial organization begs the question of whether mitochondria behave independently or are synchronized and what factors might be

responsible for intermitochondrial communication. These questions apply to both the normal range of function, when the bioenergetic status must rapidly adapt to changes in workload to maintain a constant supply of ATP (that is, mitochondria must be both flexible and robust), and to pathological situations, when metabolism becomes severely compromised by ischemia, reperfusion, or other forms of metabolic stress (e.g., oxidative stress, substrate deprivation). In these situations, the inherent vulnerability of the mitochondrial network is revealed, as previously reported (39, 41, 74) (for review see Refs. 23, 28).

The idea that biological systems can operate at the edge of dynamic instability is a new way to view physiological control (6, 45) (for review see Ref. 77). It is thought that this adaptation may have evolved to favor a quick and flexible response to environmental change. However, the downside of this type of nonlinear response can be the appearance of critical behavior when cells are exposed to pathological conditions, leading to deadly outcomes

such as apoptosis or necrosis (51, 69). It is under these circumstances that the mitochondrial network can display a remarkably rich set of dynamic spatial and temporal patterns that are, in many ways, reminiscent of other physical or manmade connected systems. For example, networks of nonlinearly coupled elements that are subjected to excessive loads or to failure of parts of the system can approach a critical state, which is characterized by an extremely large susceptibility to external factors and strong correlation between individual components. Consequently, new emergent macroscopic behavior can appear, including spatiotemporal patterns of self-organization, visualized as oscillations and/or waves in activity or the level of an intermediate. Our recent experimental and theoretical work shows that such conditions are met when mitochondrial ROS accumulates to a critical threshold in a certain fraction of the mitochondrial network (FIGURE 5, B AND C). At this point of mitochondrial criticality (4), oxidative metabolism becomes unstable, undergoing a rapid transition



**FIGURE 5. Critical behavior of the mitochondrial network induced by oxidative stress**

**A:** images of  $\Delta\Psi_m$  in an isolated guinea pig cardiomyocyte before local mitochondrial oxidative stress (left), after local  $\Delta\Psi_m$  depolarization, but before whole-cell depolarization of the mitochondrial network (middle; note square region of low  $\Delta\Psi_m$ ), and during global depolarization (right). **B:** approach to mitochondrial criticality depends on the history of oxidative stress in the mitochondrial matrix, reported by the oxidation of the mitochondrially located fluorophore, chloro-methyl dichlorofluorescein (CM-DCF). When a significant fraction (~60%) of the network shows evidence of elevated ROS production, a small further perturbation can lead to the catastrophic cell-wide depolarization of  $\Delta\Psi_m$  (image sequence corresponds to the same time points for the images shown in A). **C:** time course of whole-cell  $\Delta\Psi_m$  oscillation triggered by the local oxidative stress (red trace) and ROS production (indicated by the rate of change of the CM-DCF signal shown in green).

Experiment was carried out as described in Aon et al. (3) using two-photon laser scanning fluorescence imaging. **D:** the mitochondrial oscillator, as previously described in the computational modeling study of Cortassa et al. (22). SOD, superoxide dismutase; GPX, glutathione peroxidase; CAT, catalase.

involving  $\Delta\Psi_m$  depolarization or self-sustained oscillation (FIGURE 5, A–C) reflected by a coordinated response of almost the entire population of mitochondria in the network.

Uniquely, the ability to trigger reproducible cell-wide synchronized autonomous oscillations of  $\Delta\Psi_m$ , NADH, ROS, and glutathione in cardiac myocytes using a highly localized oxidative trigger has enabled us to explore the mechanism and the functional implications of this spatiotemporal dynamic response in unprecedented detail (3, 22). The oscillatory behavior 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 (FIGURE 5D). 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 (akin to the buildup of force in the earth's crust prior to an earthquake), 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 neighbor-neighbor interactions among mitochondria, synchronized and spatially organized by a process that can be described as a percolation lattice (4). The emergent macroscopic patterns therefore depend on the spatial arrangement and the proximity of the mitochondria to each other; hence, the tight and ordered packing of mitochondria in the cardiac cell provides a unique three-dimensional lattice to explore this type of interaction. Similar investigations in other cell types, with various mitochondrial distributions, should in the future yield more information on the importance of organization in the propagation of mitochondrial signals.

### ***Linkage to integrated cell function***

The biological complexity of the control mechanisms described above does not end at the level of the mitochondria, for the integrated functions of the cardiac cell are intimately coupled to the energetic status. Both the electrical and  $\text{Ca}^{2+}$ -handling subsystems of the myocyte are strongly influenced by the energy state of the mitochondria. In the oscillatory phenomenon described above, the repeating cycles of mitochondrial  $\Delta\Psi_m$  depolariza-

tion and repolarization allow us to examine the phase relationships between  $\Delta\Psi_m$ , ROS bursts, and their effects on cellular action potentials and  $\text{Ca}^{2+}$  transients. The rapid phase of mitochondrial uncoupling was closely correlated with the activation of energy-sensing  $\text{K}_{\text{ATP}}$  channels of the sarcolemmal membrane (3, 64), and also with the suppression of intracellular  $\text{Ca}^{2+}$  release (64), demonstrating that mitochondrial criticality scales to produce critical behavior in the primary function of the cardiomyocyte, excitation-contraction coupling. These effects are presumed to be the result of the mitochondria switching from energy-producing to energy-consuming organelles. The drop in the ATP/ADP ratio will affect multiple intracellular targets, including the  $\text{K}_{\text{ATP}}$  channel, the sarcoplasmic reticulum  $\text{Ca}^{2+}$  pump, the ryanodine receptor, the L-type  $\text{Ca}^{2+}$  channel, and energy-dependent ion pumps. The repetitive switching of the cell between electrically excitable and inexcitable states would be expected to raise havoc with whole-heart electrical propagation. Thus we had previously proposed that if these transitions in the energy state are present in hearts subjected to metabolic stress, they could set the stage for global arrhythmias (64). Recent experiments from our laboratory have provided support for this hypothesis and have suggested a potentially important new therapeutic target in the mitochondrial membrane.

### ***Scaling to the whole heart: ischemia-related arrhythmias***

**MECHANISM OF ISCHEMIA-RELATED ARRHYTHMIAS.** Many investigators have explored the mechanisms underlying ischemia- or reperfusion-induced arrhythmias, and a number of hypotheses have been promulgated (reviewed in Refs. 47 and 90). Both reentrant and nonreentrant mechanisms have been implicated, and the mechanism may differ for arrhythmias occurring during ischemia vs. after reperfusion. Triggered automaticity is thought to be related to cellular electrical disturbances, including early or delayed depolarization, whereas reentry has been attributed to heterogeneous conduction block in the myocardial syncytium (47). The latter can occur as a result of an anatomic obstruction (47) or dynamic instabilities related to the restitution properties of the cellular action potential (91). It has been well documented that an increase in regional action potential (AP) dispersion predisposes the heart to ventricular tachycardias and fibrillation. Although ample descriptive evidence is available about the initiation and propagation of reentrant arrhythmias, there is much less consensus about the cellular mechanisms that make the electrical substrate more vulnerable to abnormal conduction. Certainly, altered ion homeostasis is important, including extracellular  $\text{K}^+$

accumulation, acidification of the cytosol, and Na<sup>+</sup> and Ca<sup>2+</sup> loading during ischemia. Especially for reperfusion-induced arrhythmias, sarcolemmal K<sub>ATP</sub> channels have been implicated, and K<sup>+</sup> channel blockers have been reported to decrease the incidence of postischemic arrhythmias (13, 14). Mitochondrial  $\Delta\Psi_m$  depolarization could be involved in the activation of K<sub>ATP</sub> channels during ischemia and reperfusion, but few studies have investigated how or when this occurs. In a recent study, whole-heart  $\Delta\Psi_m$  measurements suggested that mitochondrial depolarization and a lack of  $\Delta\Psi_m$  recovery upon reperfusion may contribute to fibrillation through a mechanism that is largely insensitive to CsA (11). Thus it is possible that the mechanism of mitochondrial criticality that we have described above might contribute to these postischemic arrhythmias.

EFFECTS OF THE MITOCHONDRIAL BENZODIAZEPINE RECEPTOR INHIBITORS ON POSTISCHEMIC ARRHYTHMIAS. Ongoing studies in our laboratory are now testing the hypothesis that failure of the mitochondrial network could be the basis of postischemic arrhythmias. With respect to the ROS-induced ROS-release mechanism, the early reperfusion phase would be particularly conducive to the mitochondrial critical state, since a burst of ROS production and antioxidant depletion is known to occur. Preliminary data from our lab employing two-photon fluorescence imaging in intact perfused hearts suggests that spatial and temporal heterogeneities of  $\Delta\Psi_m$  are, in fact, present in the postischemic heart (75). The complete collapse or oscillation of  $\Delta\Psi_m$  leading to modification of the cardiac action potential could potentially introduce enormous gradients of electrical excitability and repolarization across individual cells or groups of cells that have reached mitochondrial criticality. These “islands” of inexcitability could constitute

regions of metabolic block that could result in reentry and ventricular fibrillation. Recently, we tested whether inhibitors of the mitochondrial benzodiazepine receptor (mBzR), which are known to inhibit IMAC (8) and can block  $\Delta\Psi_m$  oscillations by stabilizing  $\Delta\Psi_m$  (3), could alter the electrophysiology of isolated perfused hearts exposed to 30 min of ischemia followed by reperfusion. Epicardial action potentials were mapped using the fluorescent voltage-sensitive indicator di-4-ANEPPS and a high-resolution photodiode array system. Although almost all (~86%) of the control hearts displayed ventricular tachycardia and/or fibrillation upon reperfusion, stable postischemic action potentials were observed in hearts treated with 4-Cl-diazepam throughout the ischemia-reperfusion protocol or given as a bolus just prior to reperfusion. Moreover, arrhythmias were observed in <10% of the treated hearts (1a).

### Mitochondria as Therapeutic Targets

These data support the central hypothesis that mitochondrial ion channels play a central role in the decision between life and death. The activation of energy-dissipating mitochondrial ion channels can result in the uncoupling of oxidative phosphorylation at the level of the individual mitochondrion, which can scale to cause the failure of the mitochondrial network, loss of cardiomyocyte function, and whole-heart dysfunction (FIGURE 6). In contrast, the opening of K<sup>+</sup>-selective mitochondrial ion channels could protect against cell injury by activating intracellular pathways that resist the approach to the critical state. One must always keep in mind that the mitochondrial channels are probably there for physiological reasons we do not yet understand. For example, IMAC may modulate mitochondrial volume to counteract excessive

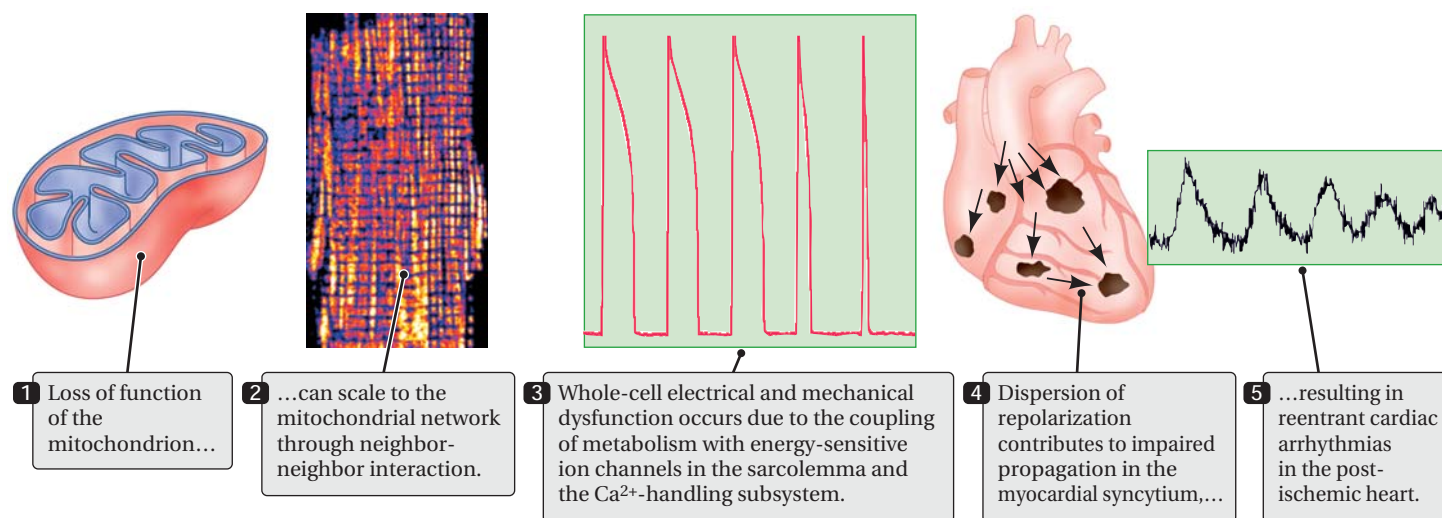


FIGURE 6. Scaling from the level of the organelle to the organ

swelling (9), or it may play a role in ROS-mediated intracellular signaling, so one must proceed with caution before recommending chronic inhibition to protect against ischemia-reperfusion injury.

Continued investigative effort will be required to devise ways of exploiting this newfound knowledge to develop novel therapeutic strategies. There are several challenges ahead that stand as impediments to progress in this area. First and foremost is the lack of identification of the molecular structures responsible for protection (e.g., the mitochondrial  $K^+$  channels), mitochondrial ion homeostasis (e.g., the  $Ca^{2+}$  uniporter and  $Na^+/Ca^{2+}$  exchanger), or injury (e.g., the PTP and IMAC). With regard to the widely reproduced model of the PTP as a multiprotein complex of the ATP synthase, ANT, the inorganic phosphate carrier, VDAC, mBzR, and cyclophilin, contradictory results from knockout mice (48) and gene deletions in yeast (53, 54) need to be resolved. New methods for genomic and proteomic analysis of mitochondrial targets, as well as gene-deletion techniques, will facilitate progress in this area.

A second consideration is that our recent work indicates that the collapse of  $\Delta\Psi_m$  can occur through the opening of mitochondrial ion channels distinct from the classical PTP, highlighting the importance of finding out which specific conditions or factors are responsible for the opening of reversible and irreversible pores in the inner membrane. More potent and specific tools will be required to characterize existing and novel ion-conductive pores in mitochondrial membranes. A difficult, but not impossible, job lies ahead in designing compounds that can cross the surface membrane to target the mitochondria without affecting other cellular sites.

From the point of view of both basic science and therapeutic development, it will also be imperative to identify the important control sites of metabolism and ROS production and how they are remodeled by cardiovascular disease. Thus a parallel course of discovery lies ahead to answer long-standing questions in bioenergetics while focusing on the essential questions of life. ■

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