Mitochondrial reactive oxygen species and Ca^{2+} signaling

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Camello-Almaraz, Cristina, Pedro J. Gomez-Pinilla, Maria J. Pozo, and Pedro J. Camello. Mitochondrial reactive oxygen species and Ca^{2+} signaling. *Am J Physiol Cell Physiol* 291: C1082–C1088, 2006; doi:10.1152/ajpcell.00217.2006.—Mitochondria are an important source of reactive oxygen species (ROS) formed as a side product of oxidative phosphorylation. The main sites of oxidant production are complex I and complex III, where electrons flowing from reduced substrates are occasionally transferred to oxygen to form superoxide anion and derived products. These highly reactive compounds have a well-known role in pathological states and in some cellular responses. However, although their link with Ca^{2+} is well studied in cell death, it has been hardly investigated in normal cytosolic calcium concentration ($[Ca^{2+}]_i$) signals. Several Ca^{2+} transport systems are modulated by oxidation. Oxidation increases the activity of inositol 1,4,5-trisphosphate and ryanodine receptors, the main channels releasing Ca^{2+} from intracellular stores in response to cellular stimulation. On the other hand, mitochondria are known to control $[Ca^{2+}]_i$ signals by Ca^{2+} uptake and release during cytosolic calcium mobilization, specially in mitochondria situated close to Ca^{2+} release channels. Mitochondrial inhibitors modify calcium signals in numerous cell types, including oscillations evoked by physiological stimulus. Although these inhibitors reduce mitochondrial Ca^{2+} uptake, they also impair ROS production in several systems. In keeping with this effect, recent reports show that antioxidants or oxidant scavengers also inhibit physiological calcium signals. Furthermore, there is evidence that mitochondria generate ROS in response to cell stimulation, an effect suppressed by mitochondrial inhibitors that simultaneously block $[Ca^{2+}]_i$ signals. Together, the data reviewed here indicate that Ca^{2+}-mobilizing stimulus generates mitochondrial ROS, which, in turn, facilitate $[Ca^{2+}]_i$ signals, a new aspect in the biology of mitochondria. Finally, the potential implications for biological modeling are discussed.

mitochondria; calcium

MITOCHONDRIA SERVE as the main source for ATP used by eukaryotic cells in the course of cellular functions. However, developments in imaging techniques have led to characterization of mitochondria involvement in other key processes, such as calcium signals or apoptosis. The number of excellent reviews and references reporting on these aspects is very high, and a fast bibliographic search will yield literally thousands of references.

A consequence of mitochondria function is the production of reactive oxygen species (ROS) during physiological and pathological states (Fig. 1). This process has also been extensively studied because ROS have a clear role in mediating functional alterations of cell physiology in inflammation, ischemia, aging, and other conditions (19, 56, 57). There is also an emerging field of study of ROS control of cellular functions (18). The present review summarizes evidence for a poorly studied aspect of mitochondria biology, the role of ROS generated during oxidative phosphorylation in Ca^{2+} signals.

Mitochondrial Production of Reactive Oxygen Species

"Reactive oxygen species" refers to a group of oxygen-containing compounds with the ability to react with reducible compounds. They comprise superoxide ($O_2^-\cdot$), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical ($\cdot OH$), although minor amounts of singlet oxygen can also be formed by cells. The initial product of the electron transport chain (ETC) is $O_2^-\cdot$, which is quickly transformed into H_2O_2 by the enzyme superoxide dismutase (SOD). H_2O_2 can be reduced to water by catalase or glutathione peroxidase or can be converted into $\cdot OH$ in presence of reduced transition metals (reduced copper or iron).

The main source of $O_2^-\cdot$ is respiratory complexes I and III located at the inner mitochondrial membrane (12, 56, 83). These complexes generate a small amount of $O_2^-\cdot$ as a side product of electron transport during oxidative phosphorylation. $O_2^-\cdot$ is released into the matrix in the case of complex I and to both the matrix and the intermembranous space by complex III. Complex III forms $O_2^-\cdot$ during cycling of the electron acceptor ubiquinone, which can donate electrons to molecular oxygen in both the internal and the external face of the inner mitochondrial membrane.

The relative importance of these sites in $O_2^-\cdot$ mitochondrial output depends on tissue and mitochondria respiratory status. For example, in fully respiring mitochondria from heart muscle (characterized by high electron flow, fast ATP synthesis, partial depolarization, and a decreased NADH-to-NAD^+ ratio, corresponding to state 3), complex III seems to be dominant, and the generation of $O_2^-\cdot$ is proportional to electron flow rate (84). In this situation oxygen, reduced substrates, and downstream electron acceptors are needed. Therefore, inhibition of electron flow into the ubiquinone cycle of complex III (by inhibition of complex I with rotenone, blockade of the cycle with myxothiazol, or inhibition of downstream electron acceptor cytochrome *c*) reduces $O_2^-\cdot$ generation, whereas antimycin enhances it by building up the partially reduced form of ubiquinone.

Complex I is the main source of $O_2^-\cdot$ in state 4, when electron transport rate and ATP synthesis are low and substrates are highly reduced (high NADH-to-NAD^+ ratio). The redox potential of complex I seems to be even higher than the NADH/NAD^+ couple, which renders it a thermodynamically unstable center prone to electron leakage to oxygen (47). In this center, $O_2^-\cdot$ production is increased in any situation leading to reduction of ETC components (for example, by application of rotenone, which blocks complex I distal to the $O_2^-\cdot$ produc-
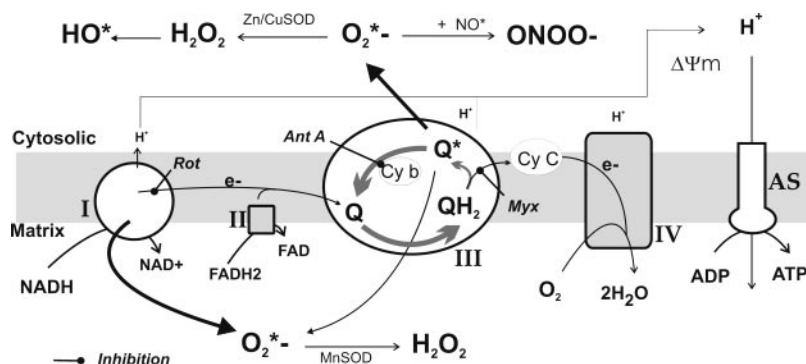

Fig. 1. Main mechanisms for mitochondrial reactive oxygen species (ROS) generation. Reduced substrates synthesized in metabolic pathways supply electrons (e^-) to complex I and II of the electron transport chain. The main centers for superoxide ($O_2^-\cdot$) formation are complex I and III, although small amounts can be formed at complex II and IV (omitted for clarity). The electron carrier of complex III ubiquinone (Q) is reduced to ubiquinol (QH_2), which transfers an electron to cytochrome c (Cy C) through a iron protein (not shown) inhibited by myxothiazol (Myx). The resulting semiubiquinone (Q^*) is oxidized back to ubiquinone by cytochrome b (Cy b), and can also transfer electrons to oxygen to form $O_2^-\cdot$. Myxothiazol reduces $O_2^-\cdot$ production because it blocks Q^* formation, whereas antimycin A (Ant A) enhances it by increasing Q^* levels. Rotenone (Rot) inhibits electron flow distal to $O_2^-\cdot$ generation, enhancing its production. The main routes for $O_2^-\cdot$ transformation are represented. SOD, superoxide dismutase; H_2O_2, hydrogen peroxide; $ONOO^-$, peroxynitrite; NO^*, nitric oxide; $\Delta\Psi_m$: mitochondrial potential; AS, ATP synthase. Only the inner mitochondrial membrane is represented.

tion point, or inhibition of electron flow at the level of cytochrome c).

Superoxide anions in the mitochondrial matrix are quickly dismutated by manganese-containing SOD, whereas those in the intermembranous space are converted by Zn- or Cu-SOD (83). If the $O_2^-\cdot$ concentration in the matrix is high enough, part of the anion escapes to the intermembranous space and cytosol via anion channels (3).

The above-described mechanisms are supported by experimental results from isolated mitochondria studies. Results from mitochondria in living, intact cells support a main role of complex III as ROS source in several systems, because rotenone or myxothiazol can impair ROS production in active cells (see, e.g., Refs. 2, 3, 10, 17). Together, these results suggest that mitochondria in these cells are in state 3. On the other hand, in other preparations administration of rotenone increases ROS production, probably through complex I, indicative of mitochondria in state 4. These opposite results can be explained by mitochondria switching between states 3 and 4 (83). In fact, it has been shown that heart mitochondria display oscillatory behavior, with cycles in mitochondrial potential and production of ROS (2, 3).

Cellular stimulation leads to fast increases in reduced substrates derived from the Krebs cycle (NADH and $FADH_2$) in the mitochondrial matrix, which has been related to cytosolic calcium concentration ($[Ca^{2+}]_i$) increases within mitochondria (28, 31, 76, 86). At the same time this situation will favor increased electron flow and rate of ATP demand (used for Ca^{2+} homeostasis, contraction, exocytosis, etc.), which would facilitate ROS production at complex III (15). Several systems have been reported to increase ROS generation in response to a variety of stimuli (10, 33, 38, 42, 43, 65, 88, 89, 91).

In any case, it has been argued that there is no convincing mechanism to explain how calcium signals can enhance $O_2^-\cdot$ generation, given that a rise in mitochondrial calcium concentration depolarizes mitochondria and should decrease $O_2^-\cdot$ formation (55, 77, 93), similar to the effect of uncouplers (24, 56, 91). Alternative proposals for Ca^{2+}-evoked increases in ROS production are direct control of ETC complexes or inhibition of complex I and IV by nitric oxide and peroxyni-

trite, which can be generated by mitochondria (6). However, the available results on this issue are conflicting, because some reports describe ROS generation in response to mitochondrial uncouplers (38) whereas others show that mitochondrial Ca^{2+} accumulation decreases it (78). Overall, the data show that calcium induces ROS production if mitochondria are treated with some inhibitor but reduces it under normal conditions (6). A possible explanation for contradictory results would be that mitochondria in vivo are under some kind of tonic inhibitory influence, allowing imported calcium to enhance $O_2^-\cdot$ formation in certain conditions (see also *Mitochondrial Production of Reactive Species Participates in Normal Ca^{2+} Signals*).

Mitochondria Participate in Ca^{2+} Homeostasis

Participation of mitochondria in calcium signal has been revealed by two main experimental approaches: *1*) mitochondria uptake and release of Ca^{2+} in the course of Ca^{2+} mobilization and *2*) inhibition of mitochondrial function modifying the shape of Ca^{2+} signals. Both fluorescent and chemiluminescent methods, including targeted optical sensors and confocal microscopy, have allowed a detailed characterization of mitochondrial calcium mobilization (71). Regarding manipulation of mitochondrial function, most studies rely on the use of specific inhibitors of the ETC or chemical uncouplers of the mitochondrial potential. A number of authoritative reviews are available (see, for example Refs. 20, 30, 55, 66, 68, 71).

Mitochondria behave as a high-capacity, low-affinity transient calcium store. Changes in $[Ca^{2+}]_i$ in response to hormones and neurotransmitters induce increases in the mitochondrial matrix calcium concentration ($[Ca^{2+}]_m$). Calcium ions enter across the inner mitochondrial membrane following its electrochemical gradient when $[Ca^{2+}]_i$ levels reach a submicromolar threshold (55, 76). The route is called the Ca^{2+} uniporter, an elusive pathway that remains poorly understood, although a recent report identifies it as a calcium-selective channel (44). In addition, it has been reported that Ca^{2+} can enter mitochondria through a "rapid mode" mechanism (75), on a millisecond timescale, as well as through a mitochondrial isoform of ryanodine receptor described in some excitable cells (4). Both sustained and transient (even oscillatory) $[Ca^{2+}]_m$

responses have been reported in multiple cell types (9, 20, 31, 70). The Ca^{2+} accumulated in mitochondria during the rising phase of the $[Ca^{2+}]_i$ signal is subsequently released during the declining phase of the signals via a Na^+/Ca^{2+} exchanger (54, 72), although both the Ca^{2+} uniporter and the permeability transition pore have also been proposed as efflux routes (29, 54, 59).

In essence, this pattern of $[Ca^{2+}]_m$ changes is considered to be due to the close apposition between mitochondria and calcium release channels of endoplasmic reticulum (68, 69) [inositol 1,4,5-trisphosphate (IP_3) and ryanodine receptors (IP_3R and RyR)], as well as proximity between mitochondria and plasma membrane (Fig. 2). This arrangement allows mitochondria to take up calcium ions entering the cytosol from internal stores or even from extracellular space. This implies that mitochondria partially buffer Ca^{2+} transients but also participate in spatiotemporal propagation of signals, because they modulate the Ca^{2+} microdomains in the immediate vicinity of calcium channels, which are known to be sensitive to calcium concentration. Thus mitochondria operate either as a barrier buffer (9, 11, 32, 60, 79, 82) or as a facilitating factor in the spreading of calcium signals (20, 73, 74), behaving as an effective shaper of calcium signals elicited by IP_3R and RyR activated during neurohormonal challenge. Although some authors report that this mechanism is also true for capacitative calcium influx in some models (32), we have shown (9) that in
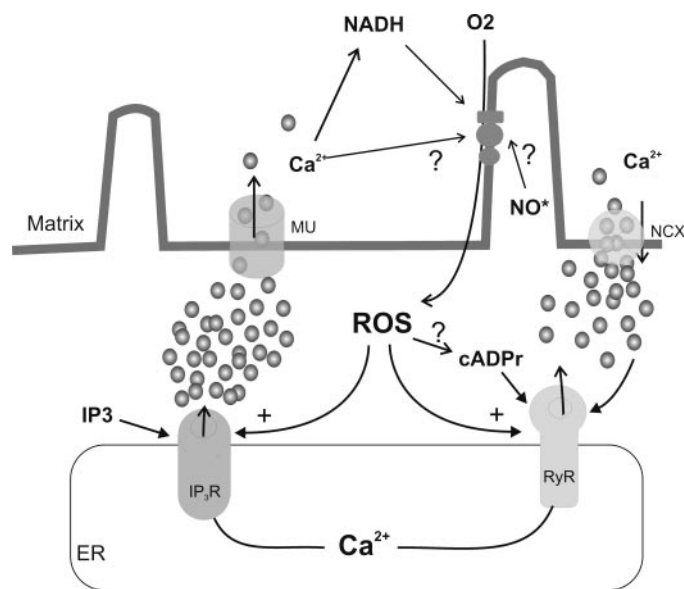
exocrine cells is a wrong conclusion, based on the misleading effect of mitochondrial buffer.

Mitochondrial calcium increases also have consequences for mitochondria, mainly acceleration of metabolic enzymes (see review in Ref. 6) and activation of the permeability transition pore, a mechanism whereby permeability of the inner membrane increases dramatically in response to factors such as oxidative stress or calcium overload. The former response increases production of reduced substrates for ETC-mediated ATP synthesis, whereas permeability transition releases not only calcium but also high-molecular-weight compounds, such as cytochrome c and other proteins, which in turn activates apoptotic signals (6). Some reports have described fast and reversible activation of permeability transition and have proposed a physiological role for this process (36, 59, 85).

Ca^{2+} Homeostasis Components Are Modified by Reactive Oxygen Species

Calcium transport systems are sensitive to redox conditions. Thus oxidants inhibit Ca^{2+} extrusion via plasma membrane Ca^{2+}-ATPase (PMCA) (48, 92) and Ca^{2+} reuptake into sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA)-operated stores (41, 49), whereas there are conflicting reports about the effects in the plasma membrane Na^+/Ca^{2+} exchange (35, 46).

Superoxide and other ROS inhibit L-type calcium channels (see, e.g., Ref. 26), acting directly on the channel, probably on the voltage sensor domain, although this conclusion is based on indirect data (46). An indirect mechanism of action of ROS on voltage-operated calcium channels through modifications of plasma membrane potential cannot be ruled out, as electrophysiological data show ROS-induced changes in potassium currents that would modify plasma membrane potential (reviewed in Ref. 46).

Regarding the main channels for calcium release from internal stores, thiol oxidants acting on sulfhydryl residues release calcium through IP_3R (5, 7, 52) and RyR (1, 23, 80), a mechanism that enhances (and even elicits) Ca^{2+} oscillations in exocrine cells (81, 90) and HeLa cells (5). This process could be due to sensitization of IP_3R-mediated calcium release (51, 52) to resting IP_3 levels, a phenomenon that has been integrated in a model to facilitate calcium oscillations (52). This effect of oxidizing reagents has been experimentally observed at the single-channel level for RyR (46). Together, the available evidence shows that the presence of sulfhydryl groups in these receptor channels enables them to respond to low levels of oxidants far below those achieved during pathological states or in extreme experimental conditions. Another example of facilitation by oxidants is the cooperative effect of H_2O_2 on the activation of TRPM2 Ca^{2+}-permeable channels by ADP ribose (45).

As a whole, the available evidence shows that ROS can induce $[Ca^{2+}]_i$ increases by release from internal stores and impairment of Ca^{2+} clearance systems. In fact, $[Ca^{2+}]_i$ increase is a constant feature of pathological states associated with oxidative stress.

Mitochondrial Production of Reactive Species Participates in Normal Ca^{2+} Signals

The relationship between oxidative stress and calcium signals has been extensively studied, given the pathological rele-

Fig. 2. Interaction between mitochondria and Ca^{2+}-release channels. After cell stimulation, inositol 1,4,5-trisphosphate (IP_3) and cADP ribose (cADPr) release calcium from endoplasmic reticulum (ER) through their respective receptors [IP_3R and ryanodine receptors (RyR)]. Local cytosolic calcium concentration ($[Ca^{2+}]_i$) increases are buffered by mitochondrial uptake through the calcium uniporter (MU) and possible alternative routes (not shown). Intramitochondrial calcium activates synthesis of reduced substrates (NADH) by metabolic pathways and accelerates the electron transport chain, increasing ROS production, which in turn facilitates Ca^{2+} release by sensitization of IP_3R and RyR. Other putative mechanisms increasing ROS generation are modification of electron transport complexes by mitochondrial calcium and inhibition by nitric oxide (NO*), which can be formed by Ca^{2+} and by mitochondria. Mitochondrial can also support Ca^{2+} release by efflux via Na^+/Ca^{2+} exchange (NCX) and by increase of cADP ribose. Outer mitochondrial membrane is not represented.

vance of the field. However, although the concept of endogenous ROS as intracellular messengers is not recent (18, 53), their involvement in physiological calcium signals has been only marginally addressed.

Physiological levels of stimulation generate in several systems transient $[Ca^{2+}]_i$ oscillations, as is the case for the pancreatic acinar cells in response to postprandial concentration of the gut hormone CCK (63). Other examples of physiological $[Ca^{2+}]_i$ signals are fast and localized, nonpropagating signals observed in excitable (Ca^{2+} sparks; Refs. 39, 64) and nonexcitable (Ca^{2+} puffs; Ref. 11) cells. These subcellular calcium signals play clear regulatory roles in several systems (e.g., control of sarcolemma potential in muscle cells or exocytosis in exocrine pancreas). Participation of mitochondria in calcium oscillations has been evidenced by the inhibitory effect of mitochondrial inhibitors and uncouplers in pancreatic acinar cells stimulated by CCK (9, 79) and gonadotropes activated by gonadotropin-releasing hormone (40). In the same line is the finding that some antihypertensive potassium channel openers activate $[Ca^{2+}]_i$ sparks in artery through mitochondria-formed ROS (91). Another recent article reports suppression of serum-induced $[Ca^{2+}]_i$ oscillations in bone marrow cells by mitochondrial depolarization (25). Following on this line of evidence, we proposed (8) a model in which mitochondrial oxidants play a cooperative role in hormonally induced $[Ca^{2+}]_i$ oscillations in pancreatic acinar cells.

To establish a role for mitochondrial ROS it is necessary, however, to demonstrate that *1*) blockade or scavenging of ROS modifies the calcium signal and *2*) ROS production takes place during physiological stimulus. Both types of results are already available.

If the model supported by these results is true, disruption of the ability of mitochondria to produce small amounts of oxidants would alter normal Ca^{2+} signals: too much ROS production would induce massive and sustained Ca^{2+} release, whereas loss of ROS production would deprive Ca^{2+} release channels of an endogenous sensitizer. In recent years several groups have reported that antioxidants or interference with mitochondrial ROS production modifies calcium signals. We have recently shown in pancreatic acinar cells that a mitochondria-targeted antioxidant (10) and mitochondrial inhibitors (9, 10) reduce CCK-evoked Ca^{2+} oscillation and ROS formation (10). A similar mechanistic evidence was provided in smooth muscle, where ROS production and Ca^{2+} sparks induced by diazoxide are blocked by rotenone and FCCP (91). Additional lines of evidence supporting this role of mitochondria are findings that pretreatment with *N*-acetylcysteine inhibits CCK-evoked oscillations (8), antioxidants reduce spark frequency in skeletal muscle (37), and $[Ca^{2+}]_i$ signals are evoked by hypoxia in pulmonary artery (88). Although not directly related to mitochondria, other reports also reinforce this concept: blockade of extramitochondrial ROS suppresses histamine-evoked $[Ca^{2+}]_i$ oscillations in endothelial cells (34), and there is a clear correlation between ROS production and spreading of calcium waves in alga cells (13). Mitochondrial blockers also prevent nongenomic effects of estrogen such as $[Ca^{2+}]_m$ increase (61) and cell proliferation (21).

The ability of cellular agonists to enhance mitochondrial ROS has been reported in several systems. We have shown (10) that postprandial levels of the gut hormone CCK increase ROS in the mitochondria of intact pancreatic acinar cells, an effect blocked by rotenone. Mitochondrial production of ROS has also been reported in cultured cells and endothelial cells for estrogen (21, 22, 61), in pulmonary arteries under hypoxia (88, 89), and in cardiac and vascular muscles and endothelial cells for angiotensin II (42, 43, 65). Similar results have been reported for hyperglycemia in rat kidney tubular cells (33) and tumor necrosis factor in carcinoma cells (38). As mentioned above, some vasodilators generate mitochondrial ROS to elicit $[Ca^{2+}]_i$ sparks in artery myocytes (91).

Although most of the evidence described above is rather correlative, some reports provide a mechanistic approach (10, 91). Together with the facilitation of Ca^{2+} release by exogenous oxidants (see *Ca^{2+} Homeostasis Components Are Modified by Reactive Oxygen Species*), the described evidence indicates that mitochondrial involvement in $[Ca^{2+}]_i$ oscillations must also be related to ROS formation by oxidative phosphorylation.

These results suggest that any Ca^{2+} mobilizing signal would become a direct stimulus for the metabolic enzymes in the mitochondria, leading to activation of metabolic pathways and respiratory chain through multiple Ca^{2+}-regulated enzymes of the mitochondrial matrix (for review see Ref. 6). Once reduced equivalents are increased the amount of $O_2^-\cdot$ increases, "pushed" by a higher number of electrons flowing from complex I and II to complex III. Indeed, there is correlation between metabolic rate and ROS generation (62). Therefore, mitochondria are suited to respond to cellular stimulation with an increase in ROS production. Agonist-induced mitochondria activation serves not only to supply the immediate energy required to extrude Ca^{2+} from cytosol (via SERCA and PMCA pumps) but also facilitates Ca^{2+} signals sensitizing the intracellular Ca^{2+} release channels.

The spatial arrangement of mitochondria with respect to Ca^{2+} release channels (IP$_3$R and RyR), which forms a privileged pathway for calcium transfer between reticulum and mitochondria (30, 67, 69), would allow fast diffusion of ROS from mitochondria to Ca^{2+} channels. In fact, the close apposition between mitochondria and the locus of endoplasmic reticulum rich in IP$_3$R and RyR is interpreted as a strategy for direct manipulation of the environment surrounding the Ca^{2+} channels' mouth. This has led to the concept of "intracellular synapse" (16). However, the mitochondrial buffer mechanism cannot explain some experimental results such as the inability of cytosolic buffers to mimic the effect of mitochondrial uptake (27).

The evidence presented above is not a negation of the "conventional" role of mitochondrial Ca^{2+} uptake effect on Ca^{2+} signals. In any case, the model presented here is compatible with the mitochondrial modulation of $[Ca^{2+}]_i$ around endoplasmic reticulum Ca^{2+} channels. Following the concept of "quasi-synaptic" transmission between mitochondria and reticulum coined by Hajnozcky's group (16), controlled amounts of mitochondrial oxidants could behave as a "neuromodulator" of the local effect of the released Ca^{2+}. In the same line, the multiple antioxidant systems present in healthy cells would terminate this "synaptic" signal, avoiding uncontrolled amplification by Ca^{2+}/ROS positive feedback. This is due not only to antioxidant barriers (SOD, catalase, etc.) but also to the high Ca^{2+} buffering capacity of the mitochondrial matrix, which limits the amplitude of mitochondrial Ca^{2+} transients.

An additional possibility is that endogenous ROS could recruit the cADP ribose pathway, because several groups have shown that cADP ribose metabolism can be modulated by redox mechanisms (58). This raises the possibility that once oscillations are started by IP$_3$ cADP ribose may cooperate to ensure their stabilization, given the sensitizing effect of this messenger on calcium signals evoked by agonists (11).

If mitochondrial ROS are confirmed as a modulatory factor in the operation of Ca^{2+} release channels under cellular stimulation, their presence must be taken into account in the development of models for Ca^{2+} signals and mitochondrial biology. Two aspects should be considered when modeling ROS and [Ca^{2+}]$_i$ signals. The most obvious is to incorporate ROS effects to [Ca^{2+}]$_i$ signaling models. Mitochondria have already been included in some calcium dynamic models (see, e.g., Ref. 50). In addition, Cortassa et al. (15) have described a detailed model of oscillations for mitochondrial O$_2^-$· production, inner membrane potential, and NADH, reproducing and predicting experimental observations performed in mitochondria from living cardiomyocytes. This model is based on a previous model from the same group describing the effect of mitochondrial calcium dynamics on energy metabolism (14). Therefore, incorporation of some aspects of this model for cytosolic [Ca^{2+}]$_i$ oscillations should give new insights into the mechanisms underlying this process and could predict the limits of the modulatory action of ROS on [Ca^{2+}]$_i$ signals.

Another issue to be considered is the effect of calcium ions on mitochondrial parameters, especially ROS production. This point has raised some uncertainty, given that mechanistic predictions indicate that [Ca^{2+}]$_m$ increases would not favor ROS production (15, 55) because they partially collapse mitochondrial potential (10, 28, 78, 87). Thus recent work has detected no effect or a slight decrease in ROS generation in response to [Ca^{2+}]$_m$ increases in isolated mitochondria (78), and Aon et al. (2, 3) report that ROS production is independent of Ca^{2+} uptake.

However, the same reports show Ca^{2+}-induced ROS formation under partial depolarization, when this process is independent from mitochondrial potential (78), and calcium can enhance ROS generation in the presence of some degree of mitochondrial inhibition (6). In fact, there is a clear mechanistic link between [Ca^{2+}]$_m$ increases and ROS generation. An increase in [Ca^{2+}] in metabolic situations where the NADH/FADH$_2$ production controls the electron transport rate enhances the respiratory flux and ROS production. The latter could be minimized in state 4 conditions by the Ca^{2+}-induced dissipation of mitochondrial potential.

Mathematical models can help us to uncover the complex relationship between mitochondrial parameters and calcium homeostasis. In addition, this approach is necessary to understand how subtle and complex changes underlie the shift from normal to pathological states, for example, when the simultaneous presence of mitochondrial Ca^{2+} increases and an otherwise nonpathological factor leads to mitochondrial dysfunction (6). This approach would support the development of new therapies or strategies. This is extremely important in cellular models under energetic stress such as cardiac and brain tissues.

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