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Chapter 15

ROLE OF MITOCHONDRIA IN CALCIUM HOMEOSTASIS AND CONTRACTION OF SMOOTH MUSCLE CELLS

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ABSTRACT

In smooth muscle cells (SMC), Ca^{2+} regulates several key functions like cell division, growth and cell death, as well as smooth muscle contractility. Mitochondria are intracellular compartments capable of Ca^{2+} uptake and release mainly via the mitochondrial uniporter and the mitochondrial Ca^{2+}-Na^{+} exchanger, respectively. Thus mitochondria can considerably participate to Ca^{2+} homoesostasis and Ca^{2+} signalling in SMC. In addition to their rôle as Ca^{2+} store, mitochondria contribute to the control of SMC function and in particular to smooth muscle tone via reactive oxygen species (ROS) production. Mitochondrial ROS production can modulate both ion channels that influence the pattern of the calcium signal, and the sensitivity of the contractile apparatus to the calcium signal. This review gives an overview of Ca^{2+} signaling and the contrtactile apparatus of smooth muscle cells, witrh particular focus on airway and vascular smooth muscle, and summarizes (i) the rôle of mitochondria as active Ca^{2+} stores, including mitochondria-sarcoplasmic reticulum coupling and Ca^{2+} microdomains, and their

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consequence on SMC Ca^{2+} homeodynamics and signalling (ii) the rôle of mitochondria in ROS production and its subsequent modulation of smooth muscle contraction. Examples of mitochondrial involvement in smooth muscle dysfunction are also presented. Moreover, an overview is given of theoretical models that have been developed for the understanding of mitochondrial impact on the spatiotemporal pattern of Ca^{2+} signalling of the SMC.

Keywords: smooth muscle cell, mitochondria, contraction, Ca^{2+}, reactive oxygen species.

1. INTRODUCTION

Smooth muscle cells are present in a variety of contractile organs such as airways, vasculature, gastrointestinal tract, urogenital tract, and some other ones. They are under control of complex neuronal, hormonal and paracrine systems that modulate their key functions like cell division, growth and cell death, as well as smooth muscle contractility. As in other cells, Ca^{2+} is a major intracellular messenger that modulates cell activity, including contraction, and the large majority, if not all, mediators of smooth muscle contraction act via Ca^{2+} signaling. Indeed, as in striated muscle, increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) is the key event that activates the contractile apparatus. However, smooth muscle cell organization as well as the mechanism by which Ca^{2+} triggers contraction differ from striated muscle. The contractile apparatus is basically constituted of thick filaments of myosin and thin filament of actin, the thin/thick filament ratio being about 20/1 to 30/1 [1, 2]. These filaments are not organized in sarcomeres. Thick filaments are anchored on dense bodies in the cell and dense area on the plasma membrane, and actin filament are positioned between thick filaments. Dense bodies and filaments are connected by non-contractile intermediate filaments that constitute an intracellular network. Cells are connected between them by mechanical junctions, and junctions, like gap-junctions, that allows chemical communication between cells. The sarcoplasmic reticulum is often in close relation with caveolae (invaginations of the plasma membrane) and mitochondria [1, 2] $[Ca^{2+}]_i$ increase activates the formation of actin-myosin bridge by a mechanism different form that of striated muscle. Indeed, there is no troponin in smooth muscle, and cross bridge formation requires the phosphorylation of one of the myosin light chain, the regulatory 20 kD light chain (MLC_{20}). MLC_{20} phosphorylation and, as a consequence, contraction, depend on the balance of kinase and phosphatase activity, which are themselves under control of several intracellular signaling pathways that may either modify the Ca^{2+} signal or modify the decoding processes of this Ca^{2+} signal by the contractile apparatus [3]. Among them, mitochondria appear to be one of the agents that can modulate de contractile properties of smooth muscle cells, since increasing evidence show that mitochondria are not only responsible of energy production, but, as intracellular calcium stores, can participate to Ca^{2+} signaling. Mitochondria are also a source of reactive oxygen species, and these molecules, which have long been considered as toxic by-products of the oxidative phosphorylation and electron chain transfer, appear now as putative messenger that may modulate contraction.

This review will present an overview of calcium signaling and the contractile apparatus in smooth muscle cells, and summarize the role of mitochondria as active Ca^{2+} stores, and their consequence on SMC Ca^{2+} homeodynamics and signaling, and the role of mitochondria

in ROS production and its subsequent modulation of smooth muscle contraction. It will mainly focus on vascular and airway smooth muscle cells, but examples will be taken from other muscle cells.

2. CALCIUM SIGNALING AND THE CONTRACTILE APPARATUS

2.1. Ca^{2+} Signaling in Smooth Muscle Cells

As in heart and skeletal muscle, Ca^{2+} in smooth muscle cells is the primary intracellular messenger that triggers contraction. Contractile stimulation of smooth muscle results in an increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) that activates the contractile apparatus, though the mechanisms by which Ca^{2+} stimulates the formation of actin-myosin bridges critically differs in smooth muscle cells from striated muscle cells. In quiescent conditions, $[Ca^{2+}]_i$ in maintained quite low, around 100 nM, by active mechanisms of Ca^{2+} extrusion through the plasma membrane and Ca^{2+} sequestration in intracellular Ca^{2+} stores. Upon stimulation, $[Ca^{2+}]_i$ is increased via different mechanisms depending of smooth muscle cell types and/or contractile agonists [4]. Basically, increase in $[Ca^{2+}]_i$ may be due either to intracellular Ca^{2+} influx or intracellular Ca^{2+} release from internal Ca^{2+} stores, or both [3-5]. Smooth muscle cells are excitable or non-excitable cells. Excitable smooth muscle cells, e.g.., myocytes of the digestive tract, are capable of generating action potential, which may be propagated from cell to cell. This is generally the case for phasic smooth muscle, in which phases of contraction propagate along the smooth muscle. By contrast, other smooth muscle cells, e. g., airway smooth muscle cells, do not generate action potential. This is generally the case in tonic smooth muscle, in which an active tone can be maintained without phasic activity. However, these non-excitable cells are capable of membrane depolarization and voltage-dependent calcium influx. In vascular smooth muscle cells, Ca^{2+} influx through L-type voltage-operated Ca^{2+} channel (VOC) are important in cell physiology. As a consequence, membrane potential plays a key role in calcium signaling in smooth muscle cells, since they usually express VOC. Membrane potential depends on the relative conductance of the main ions present in the extracellular and intracellular medium. Normal resting membrane potential in smooth muscle cell is around -60 mV [6], and critically depends on basal potassium conductance. Indeed, due to the difference between intracellular and extracellular K^+ concentration, about 140 mM versus 5 mM, respectively, the equilibrium potential for K^+ is close to the resting membrane potential . Some K^+ channels are active at rest, and contribute to the resting K^+ conductance and hence resting membrane potential. Closure of K^+ channels tends to depolarize the plasma membrane and generate extracellular Ca^{2+} influx via VOC. By contrast, activation of K^+ channels tends to hyperpolarize the plasma membrane and inhibit VOC-dependent Ca^{2+} influx. Calcium influx and subsequent contraction primarily triggered by membrane depolarization is the so-called electromechanical coupling. Extracellular Ca^{2+} influx can be generated by voltage-independent membrane channels. Receptor-operated channels can be opened by direct binding of the agonist on the membrane receptor. For example, P2X purinergic receptors, expressed in a variety of smooth muscle cell types, are activated by binding of purinergic agonists like extracellular ATP. Since these channels are non-specific cation ones, their

opening induces extracellular calcium influx [7]. It should be noticed that voltage-dependent and voltage-independent Ca^{2+} entries are frequently cross-linked. As a matter of fact, cation influx through voltage-independent channels tends to depolarize the plasma membrane and further activate voltage-dependent Ca^{2+} entry. For example, contraction induced by activation of P2X receptors has been shown in several smooth muscle cell types to occur via VOC-dependent Ca^{2+} influx. Since P2X receptors are non-specific cation channels, opening of these channels not only allows Ca^{2+} influx but also Na^{+} entry, which depolarizes the plasma membrane and activates VOC [7]. Another source of voltage-independent Ca^{2+} entry is Ca^{2+} influx through store-operated Ca^{2+} channels (SOC). These channels, which are generally considered as members of the TRPV family, are activated by emptying of intracellular Ca^{2+} stores. Though the relative physiological importance of these channels may vary between species or smooth muscle cell types, SOC-dependent Ca^{2+} influx has been characterized in a variety of smooth muscle cells. The mechanisms by which emptying of intracellular Ca^{2+} store can activate these membrane receptors remains controversial [8].

The other source of $[Ca^{2+}]_i$ increase is Ca^{2+} release from intracellular calcium store [4]. The major Ca^{2+} store from which Ca^{2+} is released upon contraction stimulation is the sarcoplasmic reticulum, which internal Ca^{2+} concentration is in millimolar range. Calcium release from the sarcoplasmic reticulum occurs via two main types of sarcoplasmic receptors, the ryanodine receptors (RyR) and the inositol-trisphosphate receptors ($InsP_3R$). Ryanodine receptors have been so called because they are activated by ryanodine, a vegetal substance that is not a physiological agonist. Physiologically, RyR are activated by increase in Ca^{2+} concentration on the cytosolic face of the RyR receptor, or by direct mechanical coupling between L-type VOC. 3 distinct isoforms of ryanodine receptors have been identified, RyR1, 2 and 3. RyR1 can be mechanically coupled with L-type voltage channels, and is mainly expressed in skeletal muscle whereas RyR 2 is expressed is cardiac myocytes.. In smooth muscle, RyR types 1, 2 and 3 have been identified. [9-11] RyR activation by Ca^{2+} self-amplifies $[Ca^{2+}]_i$ increase. This Ca^{2+}-induced Ca^{2+} release (CICR), however, it not observed in all smooth muscle cells. In some of them, stimulation of RyR does not induce an all-or-non Ca^{2+} increase, as it would be the fact in the case of CICR, but a concentration-dependent gradual Ca^{2+} response. In human bronchial smooth muscle, for example, though present and functional, RyR do not participate the acetylcholine-induced Ca^{2+} response [12]. This can be explained by the fact that RyR type 2 is regulated by magnesium, which physiological concentration inhibits CICR. It has been shown that cyclic ADP ribose is a co-agonist of RyR can contribute to RyR-dependent Ca^{2+} release [13].

Ca^{2+} can also be released from the SR through $InsP_3R$ activated by $InsP_3$. $InsP_3$ is produced upon stimulation by contractile agonists by phospholipase C (PLC) from phosphatidylinositol phosphate [14]. As for RyR, 3 isoforms of $InsP_3R$ have been identified [15]. Activation of contraction via primary $InsP_3$ production and $InsP_3R$-operated Ca^{2+} release, a consequence of enzymatic activation of PLC, is called the pharmacomechanical coupling, in opposition with the electromechanical coupling described above. However, these 2 couplings are not independent, since initial $[Ca^{2+}]_i$ increase due intracellular Ca^{2+} release can induce membrane depolarization via Ca^{2+}-dependent membrane channels. For example, Ca^{2+}-dependent Cl^{-} channel activation tends to depolarize the plasma membrane and may induce voltage-dependent Ca^{2+} influx [16].

Basal maintenance of low $[Ca^{2+}]_i$ and Ca^{2+} removal form the cytosol upon and after stimulation is due to active mechanisms that either extrude Ca^{2+} in the extracellular medium

or uptake it in intracellular Ca^{2+} stores. Calcium extrusion is mainly due to their activity of the plasma membrane Ca^{2+} ATPase, PMCA, which uses the energy of ATP, and the Na^{+}-Ca^{2+} exchanger (NCX), which uses the energy of passive Na^{+} entry, to extrude Ca^{2+} against its electrochemical gradient [4]. Since SR is the major store from which Ca^{2+} is released upon cell stimulation, it has long been considered that the major mechanism of Ca^{2+} uptake from the cytosol was Ca^{2+} pumping back into the SR, which is due to the activity of the sarcoendoplasmic Ca^{2+} ATPase (SERCA), which pumps back Ca^{2+} into the SR using ATP. This actually occurs, and is critical for normal functioning of the cell, since, in the absence of loaded SR, Ca^{2+} release upon stimulation would nor occur. However, this does not mean that $[Ca^{2+}]_i$ decrease is mainly due to SERCA activity. Other mechanisms have been identified that tend to decrease the concentration of free cytosolic calcium. Several Ca^{2+}-binding proteins can buffer cytosolic Ca^{2+} and hence decrease $[Ca^{2+}]_i$. Though it is known that mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$) is higher than $[Ca^{2+}]_i$ and that mitochondria can capture Ca^{2+} from the cytosol, the role of mitochondria as active Ca^{2+} stores in smooth muscle has long been neglected. It was considered that mitochondria was capable of Ca^{2+} uptake only for high $[Ca^{2+}]_i$ and play a minor in Ca^{2+} homeostasis [2]. However, recent studies have reevaluated the role of mitochondria in Ca^{2+} signaling, and mitochondria should be considered as a significant Ca^{2+} store critical for Ca^{2+} homeostasis [17].

Ca^{2+} as a signal is not only the global increase in cellular Ca^{2+} pool and subsequent $[Ca^{2+}]_i$ but also, and most importantly, a dynamic change in $[Ca^{2+}]_i$. Indeed, the time-dependent variations in the shape of $[Ca^{2+}]_i$ is the actual "Ca^{2+} signal", since Ca^{2+} binding to signaling protein, e. g., calmodulin, depends on cytosolic Ca^{2+} concentration. Numerous studies have analyzed the shape of the agonist-induced Ca^{2+} responses. Overall $[Ca^{2+}]_i$ measurements generally show a transient increase in $[Ca^{2+}]_i$ (Ca^{2+} peak) followed by a progressive decay to a steady-state value (Ca^{2+} plateau) and/or by Ca^{2+} oscillations [18-21]. Changes in $[Ca^{2+}]_i$ are not uniform within the cytosol, and studies have evidenced the role of local change in Ca^{2+} signaling [22]. Ca^{2+} signaling should hence be defined as spatiotemporal changes in $[Ca^{2+}]_i$, from which depends the activity of the contractile apparatus, and other cell functions. The shape of this Ca^{2+} signal critically depends on the dynamics of Ca^{2+} fluxes between intra- and extracellular media and also between intracellular Ca^{2+} compartments. As organelles capable of Ca^{2+} uptake and release, mitochondria play a key role in Ca^{2+} signaling. Mitochondria are also source of reactive oxygen species, highly reactive molecules that have been shown to modulate several agents implicated in Ca^{2+} homeodynamics. Mitochondria are also the source of energy, critical for active movement of calcium between compartments against its electrochemical gradient.

2.2. Sensitivity of the Contractile Apparatus to Ca^{2+}

$[Ca^{2+}]_i$ increase activates the contractile apparatus via a series of enzymatic reactions, the rate of actin-myosin bridges being dependent on the level of phosphorylation of the regulatory 20 kD Myosin Light Chain (MLC_{20}). Ca^{2+} binds to the cytosolic protein calmodulin and the Ca^{2+}-calmodulin complex activates Myosin Light Chain Kinase (MLCK) that phosphorylates MLC_{20}. Phosphorylation is required for the formation of the actomyosin bridge and hence contraction. However, when formed, actomyosin bridges can be maintained

even if MLC_{20} is further dephosphorylated. In this case, myosin ATPase activity and turnover of actomyosin bridges is slowed, corresponding to the so-called “latch bridges”, and this allows the maintenance of contraction with a low level of MLC_{20} phosphorylation and low energy consumption. However, dissociation of dephosphorylated bridges is irreversible, so high level of dephosphorylation induces muscle relaxation. This dephosphorylation is achieved by Myosin Light Chain Phosphatase (MLCP). Hence, the importance of contraction depends on the balance between MLCK and MLCP activity, which are under control of several signaling pathways [23]. In parallel with the canonical MLCK-dependent MLC_{20} phosphorylation, in several smooth muscles, MLC can be directly phosphorylated by Ca^{2+}-independent kinases like ZIP kinases and Integrin-linked kinase (ILK), which can also inhibit MLCP [24-27]. Rho kinase, activated by small GTPase (Rho), which seems to be activated both by Ca^{2+}-dependent and Ca^{2+}-independent pathways, can phosphorylate directly MLC and, by inhibition of MLCP, indirectly increase MLC phosphorylation and hence regulate smooth muscle contraction [26, 28, 29]. Also, contractile agonists may, in parallel to MLCK activation, inhibit MLCP activity. Protein kinase C can inhibit MLCP, and hence modulate contraction [28]. By contrast, relaxant agonists, such as β2-agonists in airways, may act via MLCP activation and/or MLCK inhibition [30, 31]. So, in addition with the Ca^{2+}-calmoduline-MLCK pathway, other Ca^{2+}-dependent and independent enzymatic pathways regulate the contractile apparatus in smooth muscles, which status depends on the balance between MLCP phosphorylation and dephosphorylation [32]. The signaling pathways capable of modulating the contraction of a given calcium signal constitute the modulation of the sensitivity of the contractile apparatus to calcium. Since several of these pathways are sensitive to ROS, mitochondria may, in parallel with their possible modulation of the calcium signal, alter smooth muscle contraction by modulating the sensitivity of the contractile apparatus to calcium.

2.3. Energetics and Smooth Muscle Contraction

As for other muscles, smooth muscle contraction requires energy consumed by the actin-myosin cross-bridge cycle. However, energy consumption in smooth muscle is low compared with striated muscle, because ATPase activity and velocity of contraction are much lower than that observed in striated muscle. Indeed, calculation of V_{max} in smooth has shown that it is 50-100 slower than in skeletal muscle. This low energy cost and low V_{max} is greater in tonic smooth muscle, like vascular or airway smooth muscle, than in phasic smooth muscle, present for example in gastrointestinal organs [33]. Moreover, at explained above, latch bridges allow maintained contraction with low energy cost, consistent with the observation that there is a greater rate of energy turnover during force development than during maintenance of tension [33]. It should be noticed, however, than phosphorylation of MLC_{20} requires ATP, and it has been estimated that it represents about 35% of the ATP consumed during contraction. It appears then that mitochondrial energy production is not as critical in smooth muscle as it can be in striated muscle. Hence, even in chronic hypoxic conditions, it does not seem that decrease in energy production is a main way by which mitochondria modulate smooth muscle contraction. Additionally, it should be noticed that, even in normoxic conditions, glycolysis

provides a significant amount of energy production in smooth muscle cells, mainly used to support membrane ion pumping [33].

3. ROLE OF MITOCHONDRIA AS ACTIVE Ca^{2+} STORES

Mitochondria are not only important for ATP production in biological cells; they also play important role in Ca^{2+} signaling, in particular in Ca^{2+} sequestration (for review see [6, 34-36]). For a long time, however, this role of mitochondria was not recognized and in explanations of mechanisms of calcium dynamics in non-excitable cells mitochondria were not included at all. Mainly, this was a consequence of experimental evidences showing that mitochondria are able to accumulation cytosolic Ca^{2+} at concentrations of about 5 – 10 μM [37], which is much higher than the physiological Ca^{2+} concentration in the cytosol. Later the role of mitochondria was re-evaluated by experiments showing that mitochondria start to take up Ca^{2+} at average cytosolic Ca^{2+} concentrations in a range between 0.5 μM and 1 μM. [38]; This is apparently in contradiction with the previous experiments. However, since it has also been shown that mitochondria are located near the mouths of channels across the ER/SR membrane [39, 40] and in these microdomains the Ca^{2+} concentrations could be 100 – 1000 times larger than that of the average Ca^{2+} concentration in the cytosol [37, 41], mitochondria can indeed sequester the Ca^{2+} released from the ER/SR under physiological conditions [42-47]. For example, in chromaffin cells, experimental results show that around 80% of the total amount of Ca^{2+} being released from the ER is cleared first into mitochondria [48]. Mitochondria also sequester Ca^{2+} in muscle cells; for example, for vascular myocytes, several studies have shown that mitochondria play important role in Ca^{2+} sequestration, which appears as a very fast and efficient process after Ca^{2+} release from the SR, and considerably contributes to the of cytosolic Ca^{2+} regulation [43, 49-53].

Recently, the role of mitochondria in Ca^{2+} handling in airway smooth muscle cells (ASMC) has studied by a theoretical model [54] in which mitochondria were taken into account as active participants in balancing Ca^{2+} fluxes and regulating the $[Ca^{2+}]_i$ dynamics in ASMC. In this model two main mechanisms of Ca^{2+} transport across the inner mitochondrial membrane were taken into account. First, the influx mechanism represents the mitochondrial Ca^{2+} uptake by uniporters. Second, the efflux mechanism takes into account the mitochondrial Ca^{2+} release through Na^+/Ca^{2+} and H^+/Ca^{2+} exchangers. The mitochondrial Ca^{2+} uptake by uniporters is modeled by a step-like kinetics which has been proposed earlier [55-57]. For the mitochondrial Ca^{2+} release through Na^+/Ca^{2+} and H^+/Ca^{2+} exchangers, as a first approximation, a simple linear dependency on the free Ca^{2+} concentration in the mitochondria is considered, which also has been proposed in previous studies [55, 58].

In mitochondrial Ca^{2+} sequestration the role of so-called permeability transition pores (PTPs) is also well established. However, in the modeling of Ca^{2+} fluxes across the inner mitochondrial membrane [54], the opening of the mitochondrial permeability transition pores (PTPs) was not taken into account. In some previous models [59], where also PTPs were considered, their role was often correlated with the mitochondrial CICR (mCICR) as a possible candidate for the calcium spike modulation [60-62]. This indicates a rather complex interplay between the mitochondrial Ca^{2+} sequestration and an influence of Ca^{2+} signals on mitochondrial processes on one hand and the mitochondrial influence on the Ca^{2+} signals on

the other hand. Large openings of the PTPs with consequent fast electric potential depolarisations are most probably specific for individual mitochondria [63] and might play important role in extreme physiological conditions such as in case of apoptosis [46].

The important role of mitochondria in Ca^{2+} handling in ASMC was even further hypothesized. We showed that at the early stage of the cytosolic free Ca^{2+} clearance immediately after Ca^{2+} release from the SR, Ca^{2+} uptake into mitochondria may be more important than Ca^{2+} pumping back by SERCA [54]. This prediction was tested experimentally, and the experiments showed that mitochondrial inhibition indeed results in a change in the slope of the Ca^{2+} decay, indicating that mitochondrial Ca^{2+} uptake is actually involved in cytosolic Ca^{2+} clearance following Ca^{2+} release from the SR, which is in best agreement with the previous model predictions [54].

4. MITOCHONDRIAL ROS PRODUCTION AND MODULATION OF CONTRACTILE PROPERTIES

Reactive oxygen species are mostly chemicals radicals derived from oxygen, though some of them, like hydrogen peroxide, are not actual free radicals. ROS are highly reactive molecules that can oxidize proteins, DNA and membrane lipids [64]. ROS production is a natural consequence of aerobic metabolism, and has long been considered only as toxic by-products of oxidative phosphorylation and energy production. However, increasing evidence shows that ROS play also an important role in cell signaling, and they should be now considered as intracellular messengers and, in some cases, as paracrine mediators [65-67].

4.1. Mitochondrial Production of ROS

Production of energy by oxidative phosphorylation involves proton transfer across the inner mitochondrial membrane. The energy used for this transmembrane proton transfer is obtained from the electron transport chain (ETC), in which electrons and passed through a series of molecules via oxidation-reduction reactions. The last electron acceptor is oxygen, which is reduced and produces water. However, a small amount of electrons do not follow the complete ETC and contribute to the premature production of superoxide ion $O_2^{-}\cdot$ [64, 66] It is generally admitted, from studies performed on isolated mitochondria, that about 1-3% of all the oxygen consumed contributes to ROS production. However, some authors consider that this is likely an overestimate [66, 68].

The main site of $O_2^{-}\cdot$ production in mitochondria is complex III, due to the ubisemiquinone radical intermediate QH· formed during the ubiquinone-dihydroubiquinone cycle. Inhibition of complex III downstream the ubiquinone site, for example by antimycin A, increases $O_2^{-}\cdot$ production. The majority of $O_2^{-}\cdot$ is generated in the intermembrane space, from where it may pass into the cytosol [69, 70]. $O_2^{-}\cdot$ can also be produced by complex I, since the distal inhibition of this complex by rotenone can cause increase in $O_2^{-}\cdot$ generation at the matrix side of the inner mitochondrial membrane [70, 71]. The mechanism of ROS generation at the site of complex I remains unclear.

$O_2^{-}\cdot$ is a highly toxic and unstable molecule and is converted into hydrogen peroxide, either by spontaneous dismutation or via the action of superoxide dismutase (SOD) that catalyses the dismutation reaction. There are several types of SOD. Mitochondrial SOD is a tetrameric metalloprotein cofactored with manganese (Mn-SOD), whereas SOD 1 and 3, cytosolic and extracellular SOD, respectively, are metalloproteins cofactored with cupper and zinc (Cu/Zn-SOD) [66, 72]. However, Cu/Zn-SOD can be present in the intermembrane space [73]. H_2O_2 is a less toxic and more stable molecule than $O_2^{-}\cdot$. Since it is membrane-permeant, it can diffuse across the mitochondrial membrane into the cytosol. $O_2^{-}\cdot$ may also diffuse from the intermembrane space into the cytosol through the outer membrane pores, though the presence of Cu/Zn-SOD in the intermembrane space probably limits the amount of $O_2^{-}\cdot$. Cytosolic SOD are also capable of cytosolic $O_2^{-}\cdot$ dismutation into H_2O_2 [72].

4.2. Modulation of Mitochondrial ROS Production

Mitochondrial ROS generation depends on the intensity of the oxidative phosphorylation reactions and the mitochondrial membrane potential $\Delta\psi_m$. Increased respiratory turnover increases the frequency of $QH\cdot$ occurrence and hence $O_2^{-}\cdot$ generation by complex III, and it has been evidenced that ROS production correlates with metabolic rate [66]. $\Delta\psi_m$ seems also to be critical of ROS production, and mitochondrial uncouplers decrease ROS production [74, 75]. Since energy consumption is small in smooth muscle cells compared with heart and skeletal muscle cells [33], the production of ROS in smooth muscle is expected to remain low in normal conditions. However, differences in ROS production between smooth muscle have been noted. For example, ROS generation in pulmonary vasculature seems to be higher than in systemic vasculature [68].

It also seems that mitochondrial calcium concentration ($[Ca^{2+}]_m$) is capable of modulating mitochondrial ROS production, though different studies have shown opposite effect of $[Ca^{2+}]_m$ increase on mitochondrial ROS generation. It appears that Ca^{2+} may decrease ROS production in normal conditions, but this possible effect appears to be tissue-specific [66] .

The amount of $O_2^{-}\cdot$ and H_2O_2 is under control of the enzymatic activity of superoxide dismutase, which dismute $O_2^{-}\cdot$ into hydrogen peroxide, and catalase and glutathione peroxidase that reduce H_2O_2. Catalase, concentrated in peroxisomes located near the mitochondria, catalizes the formation of water and oxygen from H_2O_2. Glutathione peroxidase reduces H_2O_2 by oxidation of the small protein glutathione.

4.3. Effect of ROS on Smooth Muscle Cell Contractile Properties

Several studies have shown that ROS derived from mitochondria may alter several cell signaling pathways, in a variety of cell types, including in smooth muscle cells [66, 68, 76-81]. Mitochondria appear then as a ROS and redox signaling box. It should be noticed, however, that mitochondria is not the unique source of ROS, and that cytosolic ROS generation may also be critical for cell functioning, and it is sometimes difficult to discriminate whether the major physiologically relevant source of ROS is mitochondria or the cytosol. The cytosolic ROS production is mainly due to the activity of NAD(P)H oxidase

(nox), which catalyzes the reaction of NAD(P)H oxidation into NAD(P)+ and the generation of $O_2^{-}\cdot$, which further dismutates into H_2O, this dismutation being catalyzed by cytosolic SOD1 [67, 82, 83].

Several studies have highlighted the possible role of ROS generated by mitochondria for cell signaling, though the consequences appear to be tissue-specific. As shown in transgenic mice deficient for the adenine nucleotide translocator isoform in heart and muscle (Ant1), downstream inhibition of oxidative phosphorylation results in an increase of mitochondrial ROS production. A subsequent compensating increase in antioxidant processes, such as SOD and glutathione peroxidase, was noticed, but variable between tissues, so that the level of ROS actually differs between cell types and location [77]. As in other tissues, ROS has been shown to act as intracellular messengers, and even as paracrine mediators, in smooth muscle cells. This has been studied mainly in vascular smooth muscle, where ROS can modulate hypertrophic and proliferative pathways as well as force generation [67]. The major ROS produced are $O_2^{-}\cdot$ and H_2O_2. Though both have non specific oxidant properties, they act also via specific mechanisms. The main target of $O_2^{-}\cdot$ is nitric oxide (NO·), a relaxant messenger that plays a critical role in the modulation of the contractile state of several smooth muscle, e.g. vascular and digestive musculature. $O_2^{-}\cdot$ reacts with NO·, which is itself a highly reactive radical and produces peroxinitrite anion NO_3^{-}, itself a potent oxidant agent. Hence, increase in $O_2^{-}\cdot$ and subsequent NO· decrease tends to smooth muscle contraction with additional deleterious effects due to peroxinitrite generation. It can also interact with iron-sulfur centers of heme-containing molecules. On the other hand, H_2O_2 can oxidize cystein residues in proteins, especially in the thiolate form, in cystein sulfonic acid (Cys-SOH), and hence modulate the activity of several proteins [67]. As a consequence, ROS can affect the contractile function of smooth muscle by several mechanisms, either (i) by modifying the calcium signal, or (ii) by altering the sensitivity of the contractile apparatus to Ca^{2+}. The effects of ROS, however, on smooth muscle function seem controversial. For example, H_2O_2, according to some studies, contracts arteries, whereas other studies have shown a relaxant effect. H_2O_2 has been shown to contract several vessels such as aorta, pulmonary artery, mesenteric artery, basilar artery, cerebral artery, by mechanisms either Ca^{2+}-dependent or Ca^{2+}-independent [84-91]. On the other hand, H_2O_2 elicits relaxation in several smooth muscles such as mesenteric small artery or human coronary artery [92-96], rat bladder smooth muscle [97], and uterine smooth muscle [98]. Whether ROS production is mainly due to cytosolic NAD(P)H oxidase or mitochondrial activity is not clear, and may depend on species, tissue and location. In pulmonary arteries, however, though we cannot exclude cytosolic ROS production, several studies indicate that mitochondria are oxygen sensors capable of detecting hypoxia and generating hypoxia-induced vasoconstriction via ROS production [99-102]. It should be noticed that some authors consider that mitochondria are not the only O_2 sensors responsible for hypoxia-induced responses [103, 104]

Although there is a relative consensus that mitochondria play a key role as O_2 sensor for pulmonary vasoconstriction upon lung hypoxia, the way by which mitochondria modulates pulmonary vascular tension remains controversial. According to the so-called “redox hypothesis”, supported by Weir and Archer [68, 101], hypoxia induces a reduction in mitochondrial ROS production, by reduction of the electron transfer. According to this hypothesis, the major target of signaling ROS are voltage-dependent potassium channels such as Kv1.5. In normoxic condition, mitochondria-derived H_2O_2 activates these potassium channels, maintaining high basal potassium conductance, which contributes to basal

membrane hyperpolarization, closure of voltage-operated Ca^{2+} channels, and relaxation. In hypoxic conditions, mitochondrial ROS production is decreased, with subsequent Kv channels inhibition. This depolarizes the plasma membrane and induces extracellular Ca^{2+} entry via voltage-operated Ca^{2+} channels [68]. This hypothesis is supported by experimental measurements showing that hypoxia, even a moderate one (20 mmHg PO_2) decreases ROS production [101, 105]. However, other studies have shown opposite results, with increased ROS production under mild hypoxic conditions, supporting the alternate mitochondrial ROS hypothesis [104, 106, 107]. According to this hypothesis, ROS production, which depends on the product of the concentration of O_2 by the concentration of electron donors, may increase even if PO_2 decreases, if concentration of electron donors, such as semiubiquinone, increases more than concentration of O_2 decreases. According to the so-called "mitochondrial ROS hypothesis", mitochondria-derived ROS increase cytosolic Ca^{2+} concentration by several mechanisms acting both on extracellular Ca^{2+} entry and intracellular Ca^{2+} release [99, 100]. ROS may activate non-specific calcium channels and subsequent extracellular Ca^{2+} influx. It can also stimulate ryanodine receptor opening, possibly by enhancing the production of cyclic ADP-ribose, which is an agonist of RyR [100, 108]. Since both hypotheses are supported by contradictory experimental results, it is difficult to determine which processes actually account for the role of mitochondria in O_2 sensing and subsequent modulation of the contractile status of pulmonary artery smooth muscle cell.

As noted above, ROS can modulate the intracellular calcium concentration via extracellular calcium influx. Several putative mechanisms have been postulated or demonstrated. Several potassium channels are sensitive to $O_2^{-}\cdot$ and H_2O_2, the consequences depending on the gating properties of these channels. H_2O_2 and $O_2^{-}\cdot$ have been reported to activate large conductance potassium channels (BK_{Ca} channels) in vascular smooth muscle cells. H_2O_2 activates ATP-dependent potassium channels (K_{ATP} channels), whereas $O_2^{-}\cdot$ seems to inhibit voltage-dependent potassium channels (Kv channels) [109]. Activation of K channels tends to induce smooth muscle relaxation, whereas their inhibition tends to contraction. ROS may, as noted above, act on voltage-independent Ca^{2+} efflux via opening of voltage-independent non-selective cation channels [100]. It has been shown the ROS can also directly modulate L-type voltage-dependent Ca^{2+} channels [110]. ROS can also increase cytosolic Ca^{2+} concentration from intracellular calcium stores, in particular from the sarcoplasmic reticulum. RyR is a possible target of ROS, via increase in cyclic ADP ribose [108].

In parallel with their effect on calcium signaling, ROS may modulate the sensitivity of the contractile apparatus. In aorta, it has been shown that ROS modulate smooth muscle contraction by acting of RhoK signaling pathway. It appears that increase in ROS concentration can activate Rho and hence RhoK [111, 112]. RhoK inhibits myosin light chain phosphatase, and this increases the amount of phophorylated MLC_{20} and hence contraction. Indeed, RhoGPTase contains a redox-sensitive domain critical for guanine nucleotide dissociation [113]. This may explain the regulation of Rho activity by ROS.

It should be noticed that contraction is not the only physiological property of smooth muscle cells that are capable of hypertrophy, proliferation, or migration, all of these properties being under control of Ca^{2+} signaling and likely to be under influence of ROS, though this question is beyond the scope of this review.

Since H_2O is a relatively stable and cell-permeant molecule, it may be not only an intracellular messenger but also a paracrine mediator. This has been shown in vascular

smooth muscle cell. Non-smooth muscle cells, e.g., fibroblasts, endothelial cells, are capable of ROS production that can act as paracrine mediator on smooth muscle cells [65]. It seems that the major source of ROS under normal conditions in the vascular bed is not mitochondria but NAD(P)H oxidase. However, mitochondria activity seems to be the major source of ROS in the lung. Additionally, though their role in normal quiescent conditions is not critical, mitochondria as ROS source may be more significant in pathological conditions such as hypoxia, tissue damage, or remodeling [65]. Due to its high instability and the fact that it does not cross the lipid barrier, $O_2^{-}\cdot$ is unlikely to be a cell-to-cell messenger. However, recent studies have suggested that $O_2^{-}\cdot$ may diffuse between adjacent smooth muscle and endothelial cells through gap-junctions [114].

In summary, it appears that mitochondrial ROS should be considered as putative intracellular messengers that can modulate the contractile properties of smooth muscle cells. However, the idea that ROS may not be only by-products of the oxidative phosphorylation is relatively recent, and a lot of questions remain unclear. Independently from the mechanisms of action, investigation of the effects of ROS on smooth muscle contraction, and measurements of ROS generation, have given contradictory results. This is likely due, at least for ROS measurements, to the heterogeneity of the techniques used, but also to the fact that both ROS production by mitochondria and their effect of smooth muscle cells may depend on species, tissue and location. Additionally, the intracellular medium should not be considered as homogenous and should be considered as functionally partitioned in subcellular microdomains [115]. Existence of such microdomains may account for the fact that ROS have apparently opposite effects. Indeed, actual ROS signaling may depend on local subcellular ROS production and action, whereas experimental investigation usually analyzes the overall cellular, or even multicellular, responses.

5. MITOCHONDRIAL DYSFUNCTION AND SMOOTH MUSCLE CELL PATHOLOGIES

Mitochondrial dysfunction, Ca^{2+} homeostasis disorders, and/or altered ROS production has been observed in several pathologies in which smooth muscle functions are altered. For example, increase in $[Ca^{2+}]_i$ and change in mitochondrial ROS production is observed in chronic pulmonary hypoxia, a pathology associated with arterial wall remodeling and pulmonary hypertension [68, 99, 100, 105]. ROS generation, either by NAD(P)H oxidase activity or mitochondrial production may participate to hypertrophy and proliferation of vascular smooth muscle cells [65, 67], and contributes to hypertension [81] Excess production of superoxide, has been implicated in the altered vasomotor responsiveness observed in diabetes mellitus [116]. Both experimental and epidemiological data support the idea that mitochondrial dysfunction and increase in ROS are associated with asthma [78-80, 117]. These pathologies associate not only alteration in contractile properties but usually chronic inflammation, hypertrophy and/or proliferation, and implicate not only smooth muscle cells but other cell types: inflammatory cells, endothelial or epithelial cells, fibroblasts, etc. It is hence difficult to discriminate between these complex pathological processes what specifically depends on mitochondrial dysfunction in smooth muscle cells and the consequence of this dysfunction on smooth muscle contractility. In chronic hypoxia-induced

hypertension, however, it seems that dysfunction of mitochondria, which appear to be one of the oxygen sensors, play a key role in the cascade of events that generate chronic pulmonary hypertension. According to the "redox hypothesis", chronic hypoxia induces mitochondrial abnormalities that disrupt the O_2-sensing pathway between mitochondrial ROS production and Kv1.5 channels [68]. According to the "mitochondrial ROS hypothesis", chronic hypoxia induces chronic mitochondrial ROS production and cellular Ca^{2+} overload due to cADP ribose-induced SR emptying and subsequent SOC activation and extracellular Ca^{2+} entry. This chronic Ca^{2+} overload induces Ca^{2+} overload of the different organelles, including mitochondria, with further mitochondrial dysfunction [100].

6. CONCLUSION

In conclusion, mitochondria contribute to the modulation of the contractile properties of smooth muscle cells. Since energy cost of smooth muscle contraction, energy production by mitochondria does not appear as a limiting factor of smooth muscle contraction, even in mild hypoxic condition. However, mitochondria are involved in smooth muscle contraction as active Ca^{2+} stores and contribute to shape de Ca^{2+} response and hence contraction. Mitochondria may also modulate Ca^{2+} homeostasis via ROS production. Mitochondrial ROS production may modulate Ca^{2+} signaling by several mechanisms, e. g., alteration in potassium and calcium plasma membrane channels and extracellular Ca^{2+} influx, and Ca^{2+} release from the sarcoplasmic reticulum, possibly via cADP ribose. Mitochondrial ROS may also modulate contraction acting downstream the Ca^{2+} signal on the sensitivity of the contractile apparatus, possibly via their action of Rho/RhoK pathway. However, the precise mechanisms of action of mitochondria on smooth muscle contraction, especially via ROS production, remain unclear, sometimes with opposite experimental results and controversial hypotheses. Further studies are hence necessary to have a more clear idea of the relative importance of mitochondria in smooth muscle function, both in normal and pathological conditions.

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