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ARTICLE *in* TRENDS IN CARDIOVASCULAR MEDICINE · NOVEMBER 2001

Impact Factor: 2.91 · DOI: 10.1016/S1050-1738(01)00123-2 · Source: PubMed

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Calcium Signal Transmission between Ryanodine Receptors and Mitochondria in Cardiac Muscle

György Csordás, Andrew P. Thomas, and György Hajnóczky*

Ryanodine receptor (RyR) mediated Ca^{2+} signals play a central role in excitation-contraction coupling in cardiac muscle. To support the rhythmic contractile activity there is a need for continuous tuning of cellular oxidative energy generation in the mitochondria to the actual work-load. Evidence has emerged that RyR-mediated cytosolic Ca^{2+} signals are efficiently transmitted to the mitochondria, providing a means for coupling cardiac muscle excitation to oxidative energy production, through activation of Ca^{2+} sensitive mitochondrial dehydrogenases. Recent data suggest that the Ca^{2+} signal transmission between RyR and mitochondria is dependent on local Ca^{2+} interactions between subdomains of sarcoplasmic reticulum (SR) and mitochondria. Here we give a short overview of the determinants and spatio-temporal organization of Ca^{2+} signal transmission between SR and mitochondria. (Trends Cardiovasc Med 2001;11:269–275).

In the heart, excitation-contraction coupling involves Ca^{2+} influx through voltage-sensitive (L-type) Ca^{2+} channels of the plasma membrane and, in turn, Ca^{2+} release from the SR Ca^{2+} store via Ca^{2+}-induced Ca^{2+} release channels (ryanodine receptors: RyR). The increase in cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) triggers the contractile apparatus through activation of the troponin-tropomyosin regulatory complex. The demand for contractile activity implicates a demand to meet the associated energy expenses in parallel. Muscle cells have a high capacity and rapidly mobilizable store of energy in the form of the high-energy phosphate groups of phosphocreatine. In addition, a reserve of glycogen exists for anaerobic ATP production under conditions of relative hypoxia or limitation in aerobic substrate supply. However, in continuously working cardiac muscle, mitochondrial oxidative energy production must keep pace with changing rates of ATP utilization imposed by a dynamic workload. To synchronize contractile function and mitochondrial metabolism, the following mechanisms are available:

1. Feedback regulation of ATP phosphorylation potential: Increased $[ADP]+[P_i]$ and [creatine] (depletion of energy reserves) exert positive feedback on mitochondrial ATP production and O_2 consumption (see references in Territo et al. 2001).
2. Feed-forward control by excitation-oxidative metabolism coupling: The $[Ca]_c$ spikes that elicit contraction, derived from SR RyRs, are also transmitted to the mitochondria, giving rise to mitochondrial matrix $[Ca^{2+}]$ ($[Ca^{2+}]_m$) signals, which evoke activation of Ca^{2+}-sensitive steps of oxidative metabolism.

Excitation-metabolism coupling can also occur at the level of glycogenolysis and glycolysis in the cytosol, since these pathways are also regulated by $[Ca^{2+}]_c$.

The significance of Ca^{2+}-mediated excitation-oxidative metabolism coupling has been a subject of skepticism until the last decade. This is because the global $[Ca^{2+}]_c$ increases usually elicited by activation of the RyRs are at or below the minimum level required for mitochondrial Ca^{2+} uptake. Recently, $[Ca^{2+}]_m$ spikes coupled to agonist-induced Ca^{2+} release from the intracellular reticular stores (SR/ER) have been demonstrated in several muscle and non-muscle cell types. Also, evidence has emerged that subdomains of the reticular Ca^{2+} stores are in close apposition with mitochondria, thus allowing a local control of $[Ca^{2+}]$ between reticular Ca^{2+} release sites (IP_3 receptors or RyRs) and mitochondrial Ca^{2+} uptake sites concentrated in these regions (reviewed in Hajnóczky et al. 2000a, Rizzuto et al. 2000). When the release sites are activated, the $[Ca^{2+}]$ in their immediate vicinity rises much higher than the global $[Ca^{2+}]_c$, and the resulting high $[Ca^{2+}]$ microdomain may produce a much larger activation of the interfacing mitochondrial Ca^{2+} uptake sites than could be predicted from the global $[Ca^{2+}]_c$ increase. In hepatocytes, the local $[Ca^{2+}]$ control allows mitochondria to respond to prolonged stimuli delivered to mitochondria in the form Ca^{2+} oscillations and to tune out sustained $[Ca^{2+}]_c$ signals (Hajnóczky et al. 1995). In the heart, rhythmic contractions are always established by RyR-mediated $[Ca^{2+}]_c$ oscillations. However, as the heart rate increases the diastolic $[Ca^{2+}]_c$ may also increase. Inefficient transmission of small amplitude, sustained global $[Ca^{2+}]_c$ elevations into the mitochondria may be important to prevent continuous mitochondrial Ca^{2+} accumulation, since mitochondrial Ca^{2+} overload can ignite processes that lead to cell death. In order to adjust ATP production quickly to the demand imposed by the changing cardiac contraction, it may also be a benefit if mito-

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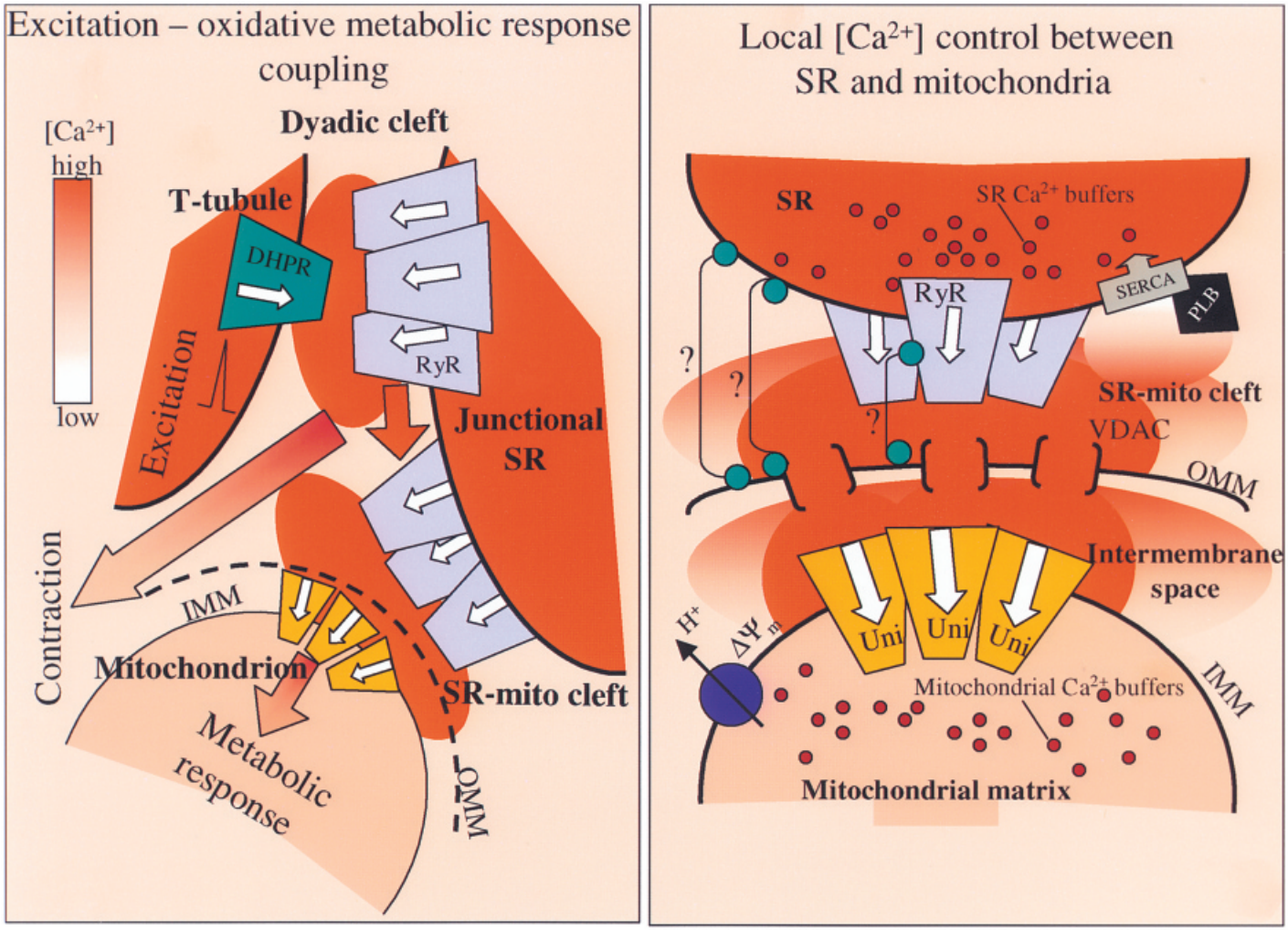

Figure 1. Excitation-oxidative metabolism coupling and the underlying local [Ca^{2+}] control between the SR and mitochondria. The relative [Ca^{2+}] in the different compartments are coded as shades of red. The abbreviations represent: DHPR, dihydropyridine receptor (L-type Ca^{2+} channel); RyR, ryanodine receptor (Ca^{2+}-induced Ca^{2+} release channel); SR, sarcoplasmic reticulum; OMM/IMM, outer/inner mitochondrial membrane; SERCA, sarco-endoplasmic reticulum Ca^{2+} ATP-ase; PLB, phospholamban; VDAC, voltage-dependent anion channel (porin); Uni, the mitochondrial Ca^{2+} uniporter; $\Delta\Psi_m$, mitochondrial (inner) membrane potential (maintained by the H^+ pump activity of the electron transport chain). The interconnected green structures represent putative mechanical coupling elements between the two organelles.

chondria rapidly sense Ca^{2+} release in the vicinity of RyR.

- **Spatial Determinants of the $[Ca^{2+}]_m$ Signal Coupled to RyR Activation**

The schematic in Figure 1 shows the local control model of the excitation-oxidative metabolism coupling (left) and Ca^{2+} signal transmission between RyRs and mitochondria (right). The key elements of the postulated arrangement of the Ca^{2+} channels are:

1. Both the activation of RyRs by Ca^{2+} entry through the L-type Ca^{2+} channels and the activation of mitochondrial Ca^{2+} uptake sites (Ca^{2+} uniporters) by Ca^{2+} release through the RyRs are facilitated by a local [Ca^{2+}] control in the intermembrane clefts. The former is established predominantly between the plasma membrane T-tubules and junctional SR (dyads, triads, or couplons, Franzini-Armstrong et al. 1999), the latter is between the SR and mitochondria.
2. Distribution of RyRs is mostly restricted to the junctional SR (Franzini-Armstrong et al. 1999).
3. Lateral diffusion of Ca^{2+} is highly restricted in the cleft between sarcolemma and junctional SR because of the strong local Ca^{2+} buffering and the mechanical effect of the protruding membrane protein molecules (see references in Peskoff and Langer 1998, Soeller and Cannell 1997). However, local Ca^{2+} movements may be facilitated by the local surface charges (Soeller and Cannell 1997).
4. Because of the size of mitochondria, discrete groups of RyRs are likely to participate in the local [Ca^{2+}] control at the dyadic cleft and at the SR-mitochondrial cleft. Most likely the Ca^{2+} released by the RyRs activated in the dyadic cleft activates neighboring RyRs that are in close apposition to the mitochondrial Ca^{2+} uptake sites.

• SR Ca^{2+} Store

[Ca^{2+}] in the SR ([Ca^{2+}]$_{SR}$)

The $[Ca^{2+}]_{SR}$ is determined by the balance between the Ca^{2+} uptake by the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA), Ca^{2+} binding by the luminal buffers and Ca^{2+} leak out of the SR caused by RyR activity and possibly by reverse mode of the SERCA pump (Shannon et al. 2000). The resting $[Ca^{2+}]_{SR}$ is in the range of 500–1000 μM. With use of a low-affinity fluorescent Ca^{2+} probe $[Ca^{2+}]_{SR}$ was estimated to be about 7000 times higher than $[Ca^{2+}]_c$ in rat cardiomyocytes (700 μM vs. 100 nM, Shannon and Bers 1997).

SR Ca^{2+} Uptake

The large $[Ca^{2+}]$ gradient between the SR lumen and cytosol is maintained by a high-affinity and high-capacity Ca^{2+} uptake mechanism, the SERCA pump. For the cardiac subtype, SERCA 2a, the K_d for Ca^{2+} is ~400 nM and the V_{max} is ~200 nmol Ca^{2+} (mg protein)$^{-1}$ min^{-1} in the absence of phospholamban (PLB; Misquitta et al. 1999, Toyofuku et al. 1994). The SERCA pumps are under negative regulation by the accessory protein, PLB in cardiac and skeletal muscle. PLB can be phosphorylated (e.g., by β adrenergic agonists acting through cAMP), and this reverses its inhibitory effect on the SERCA pump, resulting in a positive inotropic effect. The physiological significance of this pathway has also been underscored by studies with PLB-deficient mice, and PLB gene knock-out has been proposed as a potential therapeutic tool to increase heart muscle contractility in heart disease (see references in Frank and Kranias 2000).

Ca^{2+} Buffering

The Ca^{2+} buffering system in the SR lumen is of low-affinity, high-capacity (the concentration of Ca^{2+} binding sites is ~14 mM and the K_d for Ca^{2+} is ~0.638 mM $[Ca^{2+}]_{SR}$ in isolated rat cardiac microsomes; Shannon and Bers 1997). The Ca^{2+} buffering is predominantly due to proteins with specialized calcium binding sites [calsequestrin (CSQ), calreticulin]. CSQ can bind up to ~40 mol/mol Ca^{2+} with an apparent K_d of ~0.4–1 mM. It is concentrated at the terminal cisternae, and it may be bound to the RyRs directly or through its accessory proteins (triadin, junctin) creating a "ready-to-go" Ca^{2+} reserve linked to the Ca^{2+} release sites (reviewed in Bers and Perez-Reyes 1999, MacKrill 1999). Overexpression studies showed that increased CSQ leads to severe cardiac hypertrophy in mouse and revealed interaction between the control of CSQ expression and protein components of the SR Ca^{2+} release channel complex (decreased expression of RyRs, triadin and junctin, Jones et al. 1998).

Ca^{2+} Release

The distribution and regulation of the RyR Ca^{2+} release channels have been reviewed recently (Bers and Perez-Reyes 1999, Franzini-Armstrong et al. 1999, Marks 2000). Here we highlight the properties of the RyR that appear to be important in delivery of Ca^{2+} to the mitochondrial Ca^{2+} uptake sites. The RyR is a large homo-tetrameric protein that is visible in electron micrographs as the "feet" of the terminal cisternae of the SR. In cardiac muscle, its main physiological activator is the increase in $[Ca^{2+}]_c$ resulting from L-type Ca^{2+} channel opening. However, its activation can be modified by a number of factors, directly or through accessory proteins, and in Ca^{2+}-dependent and independent ways. The gating of the RyR exhibits a bell-shaped dependency on $[Ca^{2+}]_c$, with activation in the nanomolar to micromolar range and inhibition by millimolar $[Ca^{2+}]_c$. A recently recognized feature of the RyRs is the so-called "coupled gating," which refers to the capability for coordinated openings of channels organized in clusters. An extraluminal accessory protein that also binds the immunosuppressant FK506 (FKBP12 and FKBP12.6) and is necessary for the synchronous subunit-openings in the channel, has been proposed to be important for coupled gating (Marx et al. 1998). Synchronization of the openings of individual channels is a particularly effective way to maximize the net Ca^{2+} flux through the channel cluster giving rise to a local high $[Ca^{2+}]$ microdomain, which may support activation of strategically positioned low-affinity Ca^{2+} sensors (Bers and Fill 1998). In addition to the gating properties and synchronization of the RyRs, the net flux through the channels is also controlled by the driving force of Ca^{2+} release, namely the concentration gradient between $[Ca^{2+}]_c$ and $[Ca^{2+}]_{SR}$. An increase in the Ca^{2+} loading status of the SR leads to larger Ca^{2+} efflux by increasing the driving force and providing a more extensive reservoir of Ca^{2+} for release. In addition, increased $[Ca^{2+}]_{SR}$ has been reported to exert a direct positive effect on RyR open probability (Györke and Györke 1998). Interestingly, increasing the Ca^{2+} load was reported to result in a supralinear increase of the caffeine-releasable fraction of Ca^{2+} in voltage-clamped rabbit cardiomyocytes (Shannon et al. 2000). Thus, factors influencing the Ca^{2+} loading state of the SR may have a profound effect on the locally high $[Ca^{2+}]$ microdomain that develops in the cytoplasmic domain in proximity to the activated RyR channel. Notably, impaired Ca^{2+} accumulation into the SR has been proposed to play a role in the etiology of certain forms of dilatative cardiomyopathy and heart failure (Marks 2000, Meyer et al. 1995).

• Mitochondrial Ca^{2+} Store

Here we focus specifically on the mechanisms contributing to the Ca^{2+} signal transmission from RyR to mitochondria in the heart. For comprehensive reviews on mitochondrial cation transport pathways, see Bernardi (1999), Duchen (1999), Gunter and Pfeiffer (1990).

Quantification of $[Ca^{2+}]_m$ in intact cells is difficult owing to uncertainties about the intramitochondrial environment and the properties of Ca^{2+} indicators in this milieu. During RyR activation evoked by depolarization in cardiomyocytes $[Ca^{2+}]_m$ was estimated to increase from a basal level of 100–200 nM to a maximum of ~600 nM with the use of a variety of fluorophores and correctional calculations (Miyata et al. 1991, Ohata et al. 1998, Zhou et al. 1998). However, when a fraction of extracellular Na^+ was replaced with K^+, this evoked an increase in the amplitude of $[Ca^{2+}]_c$ spikes and inhibited the Na^+-dependent mitochondrial Ca^{2+} efflux pathway, causing $[Ca^{2+}]_m$ increases as high as 2 μM upon stimulation. Nevertheless, these values are far below those obtained in some non-muscle cell lines with the use of low-affinity Ca^{2+} probes (10–500 μM, reviewed in Rizzuto et al. 2000).

Mitochondrial Ca^{2+} Uptake

Ca^{2+} entering the mitochondria from the cytosol must cross two membranes. The outer mitochondrial membrane (OMM) is generally considered freely permeable to inorganic ions and small molecules owing to the presence of transmembrane pore proteins. By contrast, the inner mitochondrial membrane (IMM) has a very limited permeability to ions, and a great mechanical expandability owing to its extensive invagination to form the cristae.

In the outer mitochondrial membrane, the pore proteins of the OMM are predominantly members of the VDAC (voltage dependent anion channel or porin) family. Although VDAC proteins are envisioned to be abundant in the OMM, it is not clear yet if the OMM represents any limitations for localized high $[Ca^{2+}]_c$ increases to pass through. One may speculate that a decrease in the density or Ca^{2+} permeability of VDAC could attenuate activation of Ca^{2+} uptake sites in the IMM. On the other hand, an increase in the VDAC channel density or possible insertion of additional pore-forming proteins (e.g., certain members of the bcl-2 family proteins) may facilitate the transfer of high $[Ca^{2+}]$ microdomains into the mitochondrial intermembrane space. Interestingly, under certain predisposing conditions, the $[Ca^{2+}]_m$ signal triggers apoptosis in addition to the metabolic response (reviewed in Hajnóczky et al. 2000b, Pacher et al. 2001), and in this case insertion of pore-forming pro-apoptotic proteins into the OMM may facilitate $[Ca^{2+}]_m$-dependent activation of the apoptotic machinery.

In the inner mitochondrial membrane, Ca^{2+} enters the mitochondrial matrix through the mitochondrial Ca^{2+} uniporter. The uniporter exhibits half-maximal saturation at $\sim$10 μM and the uniporter-mediated respiration-dependent uptake rate is in the range of 200–600 nmol Ca^{2+}/mg protein/min (reviewed in Gunter and Pfeiffer 1990). Although the uniporter's transport activity is well characterized functionally in cell-free systems, its molecular identity remains elusive. The uniporter displays cooperativity (Hill-coefficient $\sim$2) between two putative Ca^{2+} binding sites: a low-affinity "transport site" in the channel pore and a higher affinity allosteric "activation site" located on the extraluminal segment (reviewed in Gunter and Pfeiffer 1990). Ca^{2+} binding to the activation site potentiates Ca^{2+} transport (increases the affinity of the transport site) and removal of Ca^{2+} from this site blocks Ca^{2+} transport through the uniporter (Litsky and Pfeiffer 1997). In addition to Ca^{2+}, the uniporter is also regulated negatively by adenine nucleotides and Mg^{2+} and positively by spermine (Litsky and Pfeiffer 1997). The dominant driving force for the mitochondrial Ca^{2+} uptake is the highly negative membrane potential ($\Delta\Psi_m \sim -200$ mV) and, to a smaller extent, the $[Ca^{2+}]$ gradient (reviewed in detail by Bernardi 1999, Gunter and Pfeiffer 1990). Exposure of the mitochondrial Ca^{2+} uptake sites to high local $[Ca^{2+}]$ during Ca^{2+} release via neighboring RyRs is expected to contribute to activation of the uniporter and to the driving force for Ca^{2+} uptake. However, the short lifetime of the Ca^{2+} microdomains generated by RyRs may be a limiting factor in the activation of mitochondrial Ca^{2+} uptake. In cell-free systems, the uniporter has been reported to display relatively slow deactivation (Gunter and Pfeiffer 1990). Such a mechanism could conserve the activation state of the uniporter shortly after exposure to high $[Ca^{2+}]$ and keep it in a "sensitized state" to $[Ca^{2+}]_c$ increases. In support of this, we found that synchronized activation of the reticular Ca^{2+} release sites resulted in a robust and slowly decaying increase in uniporter permeability, as estimated by the rate of Mn^{2+}-quench of compartmentalized fura2FF in the mitochondria of permeabilized mast cells (G. Csordás and G. Hajnóczky, unpublished observation). In the case of the beating heart, this type of plasticity of uniporter activation could ensure a frequency-dependent modulation of the Ca^{2+} permeability of the IMM.

A recently described Ca^{2+} uptake mechanism in isolated liver and cardiac mitochondria, termed the rapid uptake mode (RaM), has been reported to mediate fast uptake of short Ca^{2+} pulses in the range of 400 nM (reviewed in Gunter et al. 2000). This is believed to be mediated by the uniporter or a pharmacologically related entity. Although the existence of RaM in intact cells is not yet established, it could enhance the efficacy of Ca^{2+} signal transmisson from RyRs to the mitochondria during the short $[Ca^{2+}]_c$ spikes underlying each heartbeat. The minimum $[Ca^{2+}]$ requirement for activation of the mitochondrial Ca^{2+} uptake (uniporter) is a subject of controversy. In patch-clamped myocytes, Zhou et al. (1998) could not detect mitochondrial Ca^{2+} uptake below $[Ca^{2+}]_c$ of 300–500 nM. By contrast, in isolated rat heart mitochondria Kapus et al. (1991) reported a $[Ca^{2+}]$ threshold for uniporter-mediated uptake as low as 15 nM. Recently, significant mitochondrial Ca^{2+} uptake activity and compensatory release fluxes were reported at 100–300 nM $[Ca^{2+}]_c$ in voltage-clamped frog sympathetic neurons (Colegrove et al. 2000). Thus, the lack of detectable changes in $[Ca^{2+}]_m$ during small increases of $[Ca^{2+}]_c$ may be due to a balance between uptake by the activated uniporter and consequent stimulation of Ca^{2+} efflux pathways.

Ca^{2+} Buffering

The molecular composition and possible regulation of the mitochondrial matrix Ca^{2+} buffering system is not well characterized. The mitochondrial phospholipid, cardiolipin, binds Ca^{2+} with high affinity and other anionic phospholipids can also bind Ca^{2+}. Much of the dynamic Ca^{2+} buffering is probably contributed by carboxy-anion-containing metabolites of the Krebs cycle (citrate, oxalo-acetate) and inorganic phosphate (P_i), which can also form relatively poorly soluble salts with Ca^{2+}. The properties of these Ca^{2+} buffers are linked to mitochondrial bioenergetics through the pH gradient across the IMM, which forms part of the chemiosmotic proton motive force. The Ca^{2+} binding affinity of anions is sensitive to matrix pH, and the IMM pH gradient also sets the distribution of the carboxylated anions and phosphate between the mitochondria and cytosol. The availability of these anions is also affected by the metabolic state of the cell. Thus mitochondrial matrix Ca^{2+} buffering is subject to dynamic regulation. In contrast to the SR, mitochondrial proteins specialized for Ca^{2+} buffering and storage have not been identified in the heart.

Mitochondrial Ca^{2+} Efflux

The egress of Ca^{2+} from the mitochondrial matrix is mediated by ion-exchangers or by opening of the permeability transi-

tion pore (PTP). The two major cation-exchangers in the IMM are the $3Na^+/Ca^{2+}$ and $2\text{-}3H^+/Ca^{2+}$ exchanger. The Na^+/Ca^{2+} exchanger pathway predominates in heart muscle mitochondria (reviewed in Bernardi 1999, Gunter et al. 2000). The V_{max} is ~18 nmol Ca^{2+} (mg protein)$^{-1}$ min^{-1} for Na^+-dependent efflux in the heart. This rate is smaller by an order of magnitude than that of the uniporter-mediated Ca^{2+} uptake (see above), so that during $[Ca^{2+}]_c$ spikes Ca^{2+} uptake mediated by the uniporter may override the physiological efflux pathways by several-fold. Nevertheless, once the active release of Ca^{2+} into the cytosol ceases, the exchanger-mediated efflux pathways may dominate during the decay phase of $[Ca^{2+}]_{c/m}$. Although an inhibitor of the PTP has been reported to attenuate Ca^{2+} release in the heart (Altschuld et al. 1992), the role of the PTP in mitochondrial Ca^{2+} extrusion under physiological conditions remains elusive.

• SR-Mitochondrial Cleft

[Ca²⁺]

Given that the local microdomain of high $[Ca^{2+}]_c$ in the putative SR-mitochondrial cleft is continuous with the cytosol, it would be expected to equilibrate rapidly with $[Ca^{2+}]_c$. Notably, recent data suggest that Ca^{2+} uptake by SERCA pumps concentrated at the cleft may insulate mitochondria from modest $[Ca^{2+}]_c$ elevations originating outside the SR/ER-mitochondrial junctions (Csordás and Hajnóczky 2001). Activation of RyRs facing the mitochondria is estimated to result in a short-lasting $[Ca^{2+}]$ increase of up to >100 μM at <10–20 nm distance from the release sites, and about 10 μM elevation of $[Ca^{2+}]_c$ at 100 nm distance (Csordás et al. 1999). However, direct measurement of $[Ca^{2+}]$ in the microdomain ($[Ca^{2+}]\mu$) has proven difficult. After closure of RyRs, the $[Ca^{2+}]\mu$ appears to decay to the level of global $[Ca^{2+}]_c$ perhaps in a few tens of milliseconds. When an aequorin Ca^{2+} tracer targeted to the mitochondrial intermembrane space was used, histamine-induced $[Ca^{2+}]$ spikes were reported to be 1 μM larger in the intermembrane space than the simultaneously measured $[Ca^{2+}]_c$ spike (3.5 vs. 2.5 μM) in HeLa cells (Rizzuto et al. 1998). As an alternative approach to determining the magnitude of the local $[Ca^{2+}]\mu$, we calibrated the caffeine-induced mitochondrial Ca^{2+} uptake rate with Ca^{2+} additions in permeabilized H9c2 cardiac myotubes and estimated that $[Ca^{2+}]\mu$ peaks at ~30 μM during caffeine-induced release (Szalai et al. 2000). The measurements of intermembrane $[Ca^{2+}]$ could underestimate $[Ca^{2+}]\mu$ since $[Ca^{2+}]$ was averaged for the total intermembrane volume and the microdomain may be restricted to the vicinity of the SR-mitochondrial junctions, or only occur at contact sites between the inner and outer mitochondrial membranes. By contrast, calibration with Ca^{2+} addition could result in overestimation of $[Ca^{2+}]\mu$, if the added Ca^{2+} enters the clefts slowly.

Ca^{2+} buffering in the SR-mitochondrial cleft has yet to be characterized. By analogy to the dyadic cleft, the junctional space may exhibit stronger buffering than the global cytosolic buffer owing to the local effect of membrane-bound Ca^{2+}-binding proteins. Furthermore, diffusible buffering components (especially P_i) may have different concentrations in the SR-mitochondrial cleft than in the cytosol (e.g., possible close apposition of ATP-hydrolyzing SERCA pumps and mitochondrial P_i carriers).

Dimensions

An important determinant of the local coupling is the distance that Ca^{2+} travels from RyRs to mitochondrial uptake sites. Electron microscopic studies estimated the distance between RyRs and mitochondria at 40–180 nm in rat myocardium (Ramesh et al. 1998), and between the SR and mitochondria at the "contact sites" in the range of 25–100 nm in H9c2 cardiac myotubes (Pacher et al. 2000). Close proximity is also suggested by the observation that the caffeine-induced $[Ca^{2+}]_m$ signal was only slightly attenuated in the presence of the fast Ca^{2+} chelator, BAPTA (1 mM) in skinned rat cardiomyocytes (Sharma et al. 2000). These data raise the issue of whether the close appositions between the SR and mitochondria result from positioning of the corresponding organelles by cytoskeletal elements, or whether they are established by more specific coupling elements holding subdomains of the organelles together. Recently, Wang et al. (2000) identified autocrine motility factor (AMF) as a Ca^{2+}-dependent connecting element between subdomains of ER and mitochondria in a kidney cell line. However, thus far there is no evidence for a role of AMF in $[Ca^{2+}]_m$ signals, and no other molecular bridges have been identified.

There are no data available on the density of SR Ca^{2+} release sites in the SR-mitochondrial cleft. Assuming a similar spatial distribution to that inside the dyadic/triadic clefts, the center-to-center distance between RyRs would be in the range ~29 nm (Franzini-Armstrong et al. 1999). Although specific labeling has not been established to trace the position of the uniporters in the IMM, functional data demonstrate that RyR-mediated Ca^{2+} release can result in near maximal activation of mitochondrial Ca^{2+} uptake in H9c2 myotubes (Szalai et al. 2000). Based on this observation and considering that only a part of the total mitochondrial surface is in close contact with the SR, it is likely that the mitochondrial Ca^{2+} uptake sites are concentrated to the contact areas where mitochondria and SR are in close apposition.

• Control of Oxidative Metabolism by $[Ca^{2+}]_m$ Signals

The best recognized physiological targets of $[Ca^{2+}]_m$ signals are the Ca^{2+} sensitive mitochondrial dehydrogenases (CSMDH) that transfer reducing equivalents to NAD. The NADH formed is a primary electron donor feeding the electron transport chain (reviewed in Denton and McCormack 1980, Hansford 1994). This generates the chemiosmotic H^+ gradient that gives rise to the proton motive force responsible for driving mitochondrial ATP synthesis. In hepatocytes, the discrete $[Ca^{2+}]_c$ spikes that comprise agonist-induced $[Ca^{2+}]_c$ oscillations are delivered efficiently into the mitochondria as $[Ca^{2+}]_m$ spikes (Hajnóczky et al. 1995, Robb-Gaspers et al. 1998). In these cells, the activity of CSMDH can be regulated over a broad range by the frequency of the oscillating $[Ca^{2+}]_m$ signal (Hajnóczky et al. 1995). One of the most controversial aspects of mitochondrial Ca^{2+} signaling in heart is whether $[Ca^{2+}]_m$ and the activity of CSMDH are under a beat-to-beat control, effectively paralleling $[Ca^{2+}]_c$ transients (reviewed in Hüser et

al. 2000). The alternative is that $[Ca^{2+}]_m$ is a time-average of cardiac $[Ca^{2+}]_c$ that is modulated over longer periods by the frequency and amplitude of the RyR-mediated Ca^{2+} release pulses. Restricted frequency-modulation of $[Ca^{2+}]_m$ was demonstrated in rat and ferret myocytes, but beat-to-beat transients of $[Ca^{2+}]_m$ were not (Miyata et al. 1991, Zhou et al. 1998). By contrast, $[Ca^{2+}]_m$ oscillations coupled to $[Ca^{2+}]_c$ spikes were reported in paced rabbit and guinea-pig heart muscle cells (Griffiths 1999, Ohata et al. 1998), indicating possible species-dependent differences. Importantly, the frequency range used in these studies included the physiological and pathological range of the human heart (0.25–4 Hz), but in the small mammals studied the heart rate is 5 Hz or more. Thus even the high end of the tested frequency range modeled a bradycardia. The CSMDH (NAD(P)H fluorescence) response evoked by increases of the pacing frequency from 0–0.5 Hz to 3–5 Hz was examined in paced (rat and ferret) cardiac muscle cells. The NAD(P)H fluorescence displayed an initial rapid decay followed by a relatively slower (temperature- and Ca^{2+}-dependent) recovery (Brandes and Bers 1999, White and Wittenberg 1995). The speed of this recovery appeared to be faster at higher frequencies (Brandes and Bers 1999). These changes in mitochondrial metabolism can be explained by a metabolic feedback without significant contribution of $[Ca^{2+}]_m$ changes. However, other studies (Denton and McCormack 1980 and 1990, DiLisa et al. 1993, Hansford 1994, Hansford and Zorov 1998) have provided evidence for Ca^{2+}- and frequency-dependent activation of the CSMDH in heart. Although a single $[Ca^{2+}]_{c/\mu}$ spike may not evoke sufficient net mitochondrial Ca^{2+} uptake, multiple spikes with high frequency could sensitize the uniporter (through the mechanism discussed earlier), resulting in a slower increase in $[Ca^{2+}]_m$. The latter model would predict that the $[Ca^{2+}]_m$ response follows an increase in the pacing frequency with a delay of several contractile cycles.

• Future Directions in the Studies of Ca^{2+} Signal Transmission between the SR and Mitochondria

Although the relationship between the Ca^{2+} loading status of the SR and RyR-mediated Ca^{2+} release has been characterized, it is not known how $[Ca^{2+}]_m$ signals depend on SR Ca^{2+} loading. Moreover, impaired SR Ca^{2+} accumulation in the failing heart may affect the control of oxidative energy metabolism by $[Ca^{2+}]_m$ signals. To understand the dynamic regulation of mitochondrial Ca^{2+} targets, it is also important to determine whether changes of $[Ca^{2+}]_m$ buffering occur during the contractile cycle in myocytes. In order to evaluate the relative contribution of local SR-mitochondrial Ca^{2+} signaling and global $[Ca^{2+}]_c$ to the control of mitochondrial Ca^{2+} uptake in cardiomyocytes, the spatial relation between SR and mitochondrial surfaces and the distribution of mitochondrial Ca^{2+} uptake sites needs to be determined. This will also raise the questions of whether the close associations between the organelles are controlled dynamically, and whether anchoring elements stabilize the interactions between SR and mitochondria. Based on indirect evidence, mitochondrial Ca^{2+} uptake has also been proposed to be activated by Ca^{2+} release events restricted to a few RyRs (Duchen et al. 1998). Thus, further studies to resolve perimitochondrial and intramitochondrial $[Ca^{2+}]$ signals at the single mitochondrial level will be important. Overall, such studies will help to dissect the mechanisms underlying local Ca^{2+} signal transmission between the SR and mitochondria.

In a recent publication, Roberts et al. (2001; EMBO J 20:4998–5007) used a green fluorescent protein-based fluorescent Ca^{2+} indicator targeted to the mitochondria and reported beat-to-beat $[Ca^{2+}]_m$ oscillations in single, spontaneously beating ventricular myocytes, further supporting the beat-to-beat model of $[Ca^{2+}]_m$ regulation.

• Acknowledgments

This work was supported by grants from American Heart Association and NIH (to G.H.). G.H. is a recipient of a Burroughs Wellcome Fund Career Award.

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PII S1050-1738(01)00123-2 **TCM**