Chapter 14
Sarco-Endoplasmic Reticulum Calcium Release Model Based on Changes in the Luminal Calcium Content

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Abstract The sarcoplasmic/endoplasmic reticulum (SR/ER) is the main intracellular calcium (Ca^{2+}) pool in muscle and non-muscle eukaryotic cells, respectively. The reticulum accumulates Ca^{2+} against its electrochemical gradient by the action of sarco/endoplasmic reticulum calcium ATPases (SERCA pumps), and the capacity of this Ca^{2+} store is increased by the presence of Ca^{2+} binding proteins in the lumen of the reticulum. A diversity of physical and chemical signals, activate the main Ca^{2+} release channels, i.e. ryanodine receptors (RyRs) and inositol (1, 4, 5) trisphosphate receptors (IP_3Rs), to produce transient elevations of the cytoplasmic calcium concentration ($[Ca^{2+}]_i$) while the reticulum is being depleted of Ca^{2+}. This picture is incomplete because it implies that the elements involved in the Ca^{2+} release process are acting alone and independently of each other. However, it appears that the Ca^{2+} released by RyRs and IP_3Rs is trapped in luminal Ca^{2+} binding proteins (Ca^{2+} lattice), which are associated with these release channels, and the activation of these channels appears to facilitate that the trapped Ca^{2+} ions become available for release. This situation makes the initial stage of the Ca^{2+} release process a highly efficient one; accordingly, there is a large increase in the $[Ca^{2+}]_i$ with minimal reductions in the bulk of the free luminal SR/ER $[Ca^{2+}]$ ($[Ca^{2+}]_{SR/ER}$). Additionally, it has been shown that active SERCA pumps

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are required for attaining this highly efficient Ca^{2+} release process. All these data indicate that Ca^{2+} release by the SR/ER is a highly regulated event and not just Ca^{2+} coming down its electrochemical gradient via the open release channels. One obvious advantage of this sophisticated Ca^{2+} release process is to avoid depletion of the ER Ca^{2+} store and accordingly, to prevent the activation of ER stress during each Ca^{2+} release event.

Keywords Endoplasmic reticulum (ER) · Sarcoplasmic reticulum (SR) · Ryanodine receptors (RyRs) · IP_3 receptors (IP_3Rs) · Sarco-endoplasmic reticulum calcium ATPase (SERCA pump) · Free luminal ER [Ca^{2+}] ($[Ca^{2+}]_{ER}$) · Cytoplasmic [Ca^{2+}] ($[Ca^{2+}]_i$) · Calcium buffer capacity · Kinetics on demand (KonD)

14.1 Elements of the SR/ER Involved in Ca^{2+} Release

14.1.1 Calcium Ion as Second Messenger

A transient elevation of the cytoplasmic calcium concentration ($[Ca^{2+}]_i$) leads to changes in a large array of cellular functions [1]. These are muscle contraction, gland secretion, neurotransmission, respiration, cell movement, cell proliferation, gene transcription, cell death, among others. There are two main sources of calcium ions, the external milieu and the intracellular calcium stores [2]. The latter, in turn, are formed by two main Ca^{2+} pools, the sarco-endoplasmic reticulum and the acidic Ca^{2+} stores. This review will focus on the former rather on the latter. However, before reviewing what we know on how the sarco-endoplasmic reticulum provides Ca^{2+} for different cellular events, we will discuss some principles associated with Ca^{2+} ion as one of the many different second messengers that cells use to respond to changes in the environment.

Calcium ions are toxic in principle [3], since a sustained elevation of the $[Ca^{2+}]_i$ leads to cell death. For this reason cells invest a considerable amount of energy, coming directly or indirectly from ATP hydrolysis, to keep cytoplasmic [Ca^{2+}] in the 100 nM range by actively transporting this ion to either outside the cell or inside intracellular compartments [2]. The latter are known as intracellular Ca^{2+} stores. Generally, second messengers are molecules synthesized and degraded by enzymes, which are essential in the initiation and termination of second messenger activities. However, the situation of Ca^{2+} being a second messenger is different, in this case Ca^{2+} is moved from one cell compartment to another and those concentration changes are recognized by proteins that lead to changes in cell behavior [2]. Therefore, the plasma membrane and the different intracellular membranes are endowed with a large variety of Ca^{2+} permeable ion channels that respond to different stimuli. Accordingly, the diffusion of Ca^{2+} through the open pore of these proteins occurs in response to different signals that are physical (voltage, heat, pressure) or chemical (hormones, neurotransmitters, etc.) in nature. Once the

$[Ca^{2+}]_i$ has been elevated by the increased activity of Ca^{2+} permeable ion channels, this divalent cation is bound by two different types of proteins, buffers and effectors, the former limit the increase of the $[Ca^{2+}]_i$ and this gives time to Ca^{2+} pumps to expel this ion out of the cytoplasm, while the latter are characterized by the ability of forming a Ca^{2+}-protein complex that modifies the activity of different enzymes (kinases, phosphatases, proteases, etc.) that change the cell behavior allowing cells to adapt and respond to different stimuli [2, 4].

14.1.2 Intracellular Calcium Stores

It has become evident then that intracellular Ca^{2+} pools are, at the same time, sources of Ca^{2+} in response to certain stimuli [1, 5] and also Ca^{2+} buffering compartments that help cells survive the cytotoxic effect of increased $[Ca^{2+}]_i$ [6–8]. This paradoxical situation has been the motor behind the evolution of very interesting solutions that will be reviewed below. There are basically two different types of internal Ca^{2+} stores, one represented by the sarcoplasmic/endoplasmic reticulum (SR/ER) and the other referred to as acidic Ca^{2+} store. The former is a single organelle while the latter encompasses a variety of organelles that have a luminal pH below 7.0, these are the Golgi apparatus [9, 10], lysosomes [11] and secretory granules [12], among other organelles. These two different types of stores can be observed using electron microscopy to detect intracellular sites with elevated $[Ca^{2+}]$ [13, 14]. Alternatively, these two stores can be observed by depleting the SR/ER followed by maneuvers to alkalinize and open Ca^{2+} permeable channels in the acidic organelles [10, 15].

The SR/ER is a membrane organelle that traverses all the cytoplasm. Actually, it forms the nuclear envelope and for this reason this organelle goes from the nucleus all the way to the plasma membrane at the periphery of the cell [16–18]. The reticulum is formed by tubules and saccules that are interconnected and it appears that their lumen do not have any diffusion barriers [7, 19–21]. At the same time these reticular structures are dynamic because they can move, to a certain degree, within the cytoplasm; using the microtubules as their rail-roads [17, 22, 23]. Reticular membranes also have the characteristic of being highly fusogenic, resulting in a constant reshaping of the reticulum [22–24].

The endoplasmic reticulum of muscle cells is the sarcoplasmic reticulum and, particularly in striated muscle cells, it has specialized in moving large quantities of Ca^{2+} for both muscle contraction and relaxation [25, 26]. In non-muscle cells, the endoplasmic reticulum is the main intracellular Ca^{2+} store, although this organelle also carries out many more functions such as protein and phospholipid synthesis, drug detoxification, synthesis of cholesterol, etc. [17]. The function of the sarcoplasmic/endoplasmic reticulum as Ca^{2+} store involves the following essential elements: (a) Ca^{2+} pumps to accumulate this ion in the store, against its electrochemical gradient, (b) release Ca^{2+} permeable channels and (c) luminal Ca^{2+} binding proteins that increase the capacity of the lumen to accumulate Ca^{2+} in the store. The characteristics of each one of these elements will be reviewed briefly.

14.1.3 SERCA Pumps

SERCA stands for Sarco-Endoplasmic Reticulum Calcium ATPase, is an SR/ER integral membrane protein codified by three different genes, ATP2A1, ATP2A2 and ATP2A3. All three messenger RNAs from these genes show alternative splicing in the 3′ end region, generating a large assortment of SERCA pumps. SERCA1a is the fastest pump and it is expressed in fast-twitch skeletal muscle, SERCA2a is expressed mainly in heart cells and SERCA2b is called house-keeping pump because it is present in smooth muscle cells and non-muscle cells having an ER [27].

Catalytic cycle of SERCA pumps consists of binding two Ca^{2+} ions from the cytoplasm, this in turn promotes ATP binding followed by SERCA own phosphorylation in a highly conserved aspartic residue, which results in conformational changes that close the access of cytoplasmic Ca^{2+} to the protein, and these would allow access to the lumen of the endoplasmic reticulum with a reduced affinity for Ca^{2+}. In the end, cytoplasmic Ca^{2+} is delivered into the lumen of the SR/ER in exchange for protons. This final event promotes dephosphorylation of the enzyme to reinitiate another catalytic cycle [28]. All these steps in the catalytic cycle involve large conformational changes whose structures have been determined by X-ray diffraction studies [29]. This series of conformational changes has very interesting implications for cell physiology. The slow rate of 100–1000 ions/second showed by SERCA pump [30] does not counteract the much higher Ca^{2+} rate of one million ions/sec in the RyR [31]. The leak activity of release channels then could be critical because it would impose an energy burden on the cell that could easily drain all its energy resources [32]. Accordingly, SERCA pump is one of the thermogenic sources in the body [33], to the extent that increased Ca^{2+} leakage from the SR, that in turn accelerates SERCA pump activity, can lead to malignant hyperthermia, a fatal side effect of anesthetics, like halothane, this complication can be overcome by using dantrolene, an inhibitor of Ca^{2+} release channel, by reducing the SR Ca^{2+} leak [34]. Furthermore, deletion of sarcolipin (a protein proposed to switch SERCA pump from Ca^{2+} translocation to thermogenesis) generates obese mice with the development of insulin resistance [35]. Thus, this catalytic cycle is essential for SERCA pump to produce heat and to accumulate Ca^{2+} in the lumen of the SR/ER.

It is easy to see then that any interference with the SERCA pump catalytic cycle will result in inhibition of its activity. It has been described a large variety of SERCA pump inhibitors that are either codified by the same cell or are chemicals from other sources. The former involve a series of peptides that are small, single span integral membrane proteins, that by binding to SERCA they inhibit its pump activity; these are phospholamban and sarcolipin [36], myoregulin, endorgulin, and another-regulin [37]. The second group of SERCA pump inhibitors comprises chemicals that bind to SERCA pump and inhibit the catalytic cycle, these are thapsigargin, cyclopiazonic acid and *tert*-butyl hydroquinone [38, 39]. Additionally, high levels of cholesterol and saturated fatty acids can also inhibit the activity of SERCA pump, allegedly by increasing the rigidity of the ER membrane [40]. Interestingly, it has

been found a peptide, codified by a long-noncoding RNA, named DWORF, which is able to increase the activity of SERCA pump by displacing the inhibitory peptides from the SERCA pump [41]. It is becoming clear that the activity of SERCA pump not only respond to an increase in the $[Ca^{2+}]_i$ but also to the presence of these regulatory peptides.

14.1.4 Calcium Release Channels

There are two main Ca^{2+} release channels in the SR/ER. The SR expresses the Ryanodine Receptor (RyR) while the ER is endowed with the IP_3R [42, 43]. These release channels are tetramers that form Ca^{2+} permeable, non-selective cation channels [44]. Both types of channels are activated by low cytoplasmic $[Ca^{2+}]$ and inhibited by higher concentrations of this divalent cation [45, 46]. The majority of the protein is facing in the cytoplasm and only a small fraction, in the carboxy terminal region of this protein, is inserted in the sarco-endoplasmic reticulum membrane to form the ion channel pore. Release channels are tetrameric, Ca^{2+} permeable non selective cation channels and each subunit of the RYR is more than 5000 amino acids long (the whole channel weighs around 2.2 MDa) while each subunit of the IP_3R is around 2500 amino acids long (the whole channel weight close to 1 MDa). There are three different genes for each one of these release channels and each gene produces alternative splicing isoforms. RyR1 is expressed mainly in fast-twitch skeletal muscle and is activated by what is known as mechanical coupling [47]. The membrane depolarization of the T tubules generates conformational changes in the voltage-gated calcium channels (VGCC, specifically the dihydropiridine receptor) that are directly transmitted to the RyR1 resulting in one of the fastest Ca^{2+} release events (1–2 msec time to peak). The key issue here is that RyRs in the SR require making contact with VGCC in the T tubules of the plasma membrane. Typically, each one of the subunits of RyRs connects with one VGCC, so one RyRs is connected with four VGCC. The RyRs alternate between those connected to the VGCC and those that are not connected, and the idea is that calcium-induced calcium release (CICR) would be activating those RyRs that are not connected [42]. The isoform 2 of RyR is expressed in heart cells and in this case the association with VGCCs is not that clear, RyR2s are not connected physically to VGCC, but these two proteins are located very close to each other [48]. The Ca^{2+} entering via VGCCs triggers the activation of RyR2s to produce Ca^{2+} release that results in heart cell contraction. It has been calculated that for all the Ca^{2+} involved in contraction, as much as 90% can be provided by RyR2s and only a small fraction by VGCCs, although these figures vary according to the species studied [49]. Finally, RyR3 was the last one to be cloned and is present in different type of cells, for instance in the diaphragm, brain cells and smooth muscle cells [47]. Actually, it is very interesting that smooth muscle cells express all three RyRs with different localization within the SR [50]. RyRs in smooth muscle cells appear to be involved more in relaxation than contraction [51], the idea here is that

localized Ca^{2+} release events involving the activation of RyRs, which are known as Ca^{2+} sparks, would activate Ca^{2+}-dependent, high conductance potassium channels that are heavily expressed in smooth muscle cells, this would result in membrane hyperpolarization and concomitant deactivation of VGCC, which provide most of the Ca^{2+} involved in smooth muscle contraction. This is the case because CICR is rather inefficient in smooth muscle cells [52, 53].

The IP_3R is activated by the combination of Ca^{2+} and IP_3 in a very complex manner, to the extent that IP_3Rs are activated in a very small window of IP_3 concentration [46]. This is very important because there is no correlation between the amount of IP_3 produced and the amplitude of the $[Ca^{2+}]_i$ response, actually, the main difference is time, because if an agonist produces a large amount of IP_3, this will reach the threshold for Ca^{2+} release, before the other agonist that has a much lower rate of IP_3 production [54, 55]. The IP_3 binding site is located in the amino terminus of the protein, while the ion channel is formed by the carboxy end of the IP_3R. The structure of this channel revealed by cryo-electron microscopy has shown that the very end of this protein, the carboxy terminal domain (CTD), is an alpha helix that goes all the way from the channel domain to contact the IP_3 binding region of the next subunit. This peculiar conformation of CTD might explain the allosterism displayed by IP_3 and Ca^{2+} to activate this release channel [56]. In addition to IP_3 and Ca^{2+} there are a number of proteins that can modulate the activity of IP_3Rs, examples are RACK, IRBIT, Homer, BCL2, Presenilin, Huntingtin, among others [57]. Thus, the activity of release channels is under the control of a large series of chemicals and protein interactions.

14.1.5 Luminal Calcium Binding Proteins

The $[Ca^{2+}]$ in the cytoplasm is in the submicromolar range and accordingly, the Ca^{2+} binding proteins that are present in the cytoplasm have high affinity and selectivity over Mg^{2+} to be able to bind Ca^{2+} and carry out their functions. This situation implies that the cytoplasmic Ca^{2+} binding proteins, participating in signal transduction, have evolved to display high affinity, but low capacity for Ca^{2+} ions [4].

This picture is completely different inside the lumen of the SR/ER where the free luminal $[Ca^{2+}]$ is in the submillimolar range and total $[Ca^{2+}]$ could be in the tens of millimolar range. There are two main luminal calcium binding proteins in the lumen of the reticulum, these are calsequestrin [58–60] and calreticulin [61]; the former appears to be the main Ca^{2+} binding protein in the lumen of the sarcoplasmic reticulum of striated muscles [62], while the latter is the main one in some smooth muscle cells and non-muscle cells [63]. These proteins are characterized by having low affinity but high capacity. This means that these proteins have several Ca^{2+} binding sites but their affinity for Ca^{2+} is low when compared with those proteins in the cytoplasm. The underlying idea is that these proteins are responsible for increasing the Ca^{2+} buffering power of the sarco-endoplasmic reticulum. That is, they increase the capacity of internal stores to accumulate Ca^{2+}, which allow

internal Ca^{2+} stores to be able to provide Ca^{2+} to the cytoplasm and to trigger cellular events that are driven by an increase of the $[Ca^{2+}]_i$.

Additionally, if the stores have a role in buffering the elevation of the $[Ca^{2+}]_i$, then these proteins increase the capacity of internal stores to accumulate Ca^{2+}, which in turn, avoid the cytotoxic effects of high levels of the $[Ca^{2+}]_i$. This situation has been clearly shown in neurons, where membrane depolarization activates VGCC that allow Ca^{2+} entry to the cytoplasm, and then SERCA pumps direct cytoplasmic Ca^{2+} to the lumen of the ER. Once Ca^{2+} is inside the ER, there are specific regions that accumulate this ion to concentrations as high as 40 mM [64]. These regions are adjacent to other regions of the ER that do not accumulate Ca^{2+} at all; this is not easy to explain since both regions are located within the same lumen of the ER [65]. The idea here is that the distribution of the Ca^{2+} binding proteins is not homogeneous [66], and that these proteins are trapping Ca^{2+} to reduce the activity of this ion (i.e. Ca^{2+} ions are no longer free). In the same neurons, it was observed that the inhibition of SERCA pumps with thapsigargin, leads to mitochondria Ca^{2+} accumulation due to the inability of the ER to buffer the incoming Ca^{2+} [64, 65]. Although it was not shown in this study, the increased mitochondrial matrix $[Ca^{2+}]$ would trigger mitochondria dysfunction and quite possible apoptosis [8].

Calsequestrin does not appear to be freely distributed inside the SR but it is associated with RyRs either directly [67] or indirectly via the association with triadin and junctin [47, 68]. However, it has been shown that the role of calsequestrin is more complex than the mere increase of the SR Ca^{2+} buffering capacity since it also regulates RyRs-mediated Ca^{2+} release events [58]. A very interesting example has been observed studying a single point mutation of $CASQ2^{R33Q}$, which was discovered in a patient afflicted with CPVT (catecholaminergic polymorphic ventricular tachycardia), an inherited arrhythmogenic condition that is life threatening [60]. Interestingly, the presence of CASQ2 confers RyR2s sensitivity to luminal $[Ca^{2+}]$ to the extent that there is a peak ion channel activity at 1 mM luminal $[Ca^{2+}]$ [69]. Remarkably, the mutant $CASQ2^{R33Q}$ increases the RyR2 ion channel activity by luminal Ca^{2+} at concentrations as low as 10 μM [69]. Then the presence of $CASQ2^{R33Q}$ makes RyR2 a leaky channel to the point that this results in a very low free luminal SR $[Ca^{2+}]$ in heart cells, even lower than the levels attained during normal Ca^{2+} release process [60]. Unexpectedly, this extremely low free luminal SR $[Ca^{2+}]$ with $CASQ2^{R33Q}$ results in Ca^{2+} waves and Ca^{2+} sparks of larger amplitude than those recorded in normal heart cells that have a much higher free luminal SR $[Ca^{2+}]$ [60]. Moreover, the Ca^{2+} systolic-induced contraction was only slightly smaller in myocytes expressing the mutant $CASQ^{R33Q}$ than in those expressing wild type CASQ [70]. Deletion of CASQ2 greatly decreased the amplitude of Ca^{2+} sparks despite having a normal reduction in the luminal SR $[Ca^{2+}]$ suggesting that CASQ2 is indeed the Ca^{2+} buffering protein in the SR of heart cells [60]. However, it is also clear that CASQ2 beyond being a Ca^{2+} buffering protein also has other roles in the Ca^{2+} release event since it shows a complex control of the activity of RyR2s. Assuming that the Kd for Ca^{2+} binding of $CASQ^{R33Q}$ is the same as the one for CASQ wild type, and because the free luminal SR $[Ca^{2+}]$ is

much smaller than normal, then the saturation of $CASQ^{R33Q}$ should also be much smaller, these two conditions (low free luminal SR $[Ca^{2+}]$ and smaller saturation of CASQ2) should result in a $[Ca^{2+}]_i$ response of smaller amplitude in the presence of $CASQ2^{R33Q}$. Since this is not the case [60], then it appears that $CSQ2^{R33Q}$ mutant clearly exemplifies the idea that the amount of Ca^{2+} that is released by activation of RyR2 cannot be predicted from the observed changes in the free luminal SR $[Ca^{2+}]$.

14.1.6 The Biophysical Vision of the SR/ER Ca^{2+} Stores

It is clear then that an intracellular Ca^{2+} store is a membrane compartment that forms a closed entity and involves the participation of several components, which are Ca^{2+} pumps, luminal Ca^{2+} binding proteins and Ca^{2+} release channels. The prevailing vision, particularly for mathematical models (Fig. 14.1), is that all these

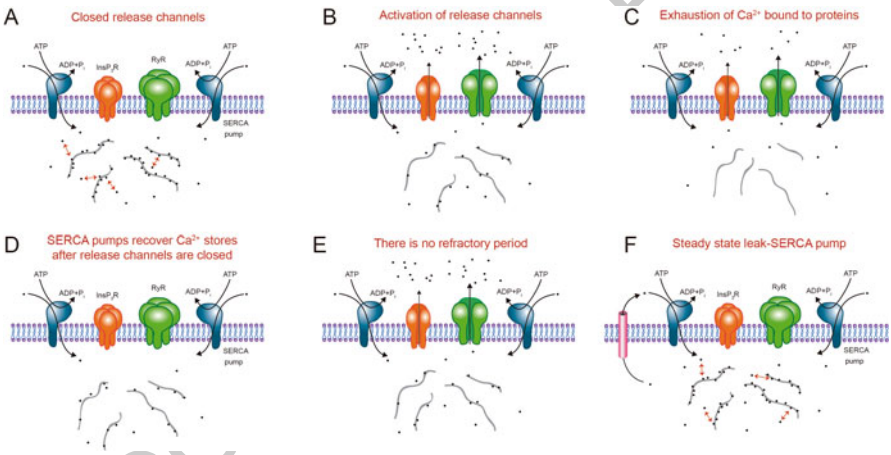

Fig. 14.1 Graphical model of Ca^{2+} release event where the free luminal SR/ER $[Ca^{2+}]$ is in equilibrium with the Ca^{2+} bound to the luminal proteins. This cartoon depicts the critical elements involved in a Ca^{2+} release event. (**a**) The Ca^{2+} bound to luminal proteins is in equilibrium with the free luminal SR/ER $[Ca^{2+}]$ (as represented by the double-headed arrows). (**b**) The activation of release channels would decrease the free luminal SR/ER $[Ca^{2+}]$ and this results in Ca^{2+} unbinding from luminal proteins, as long as the release channels stay open. This scenario cannot produce an increase of the free luminal SR/ER $[Ca^{2+}]$ in response to the activation of the release channels. (**c**) The Ca^{2+} release process terminates when all the Ca^{2+} bound to luminal proteins has been released. (**d**) SERCA pump needs that release channels to be close in order to recover the free luminal SR/ER $[Ca^{2+}]$. (**e**) This scenario cannot produce a refractory period for RyRs because the recovery of the free luminal SR/ER $[Ca^{2+}]$ implies also complete restauration of the Ca^{2+} bound to proteins. (**f**) The steady state implies that Ca^{2+} leak activity is counterbalanced by SERCA pump activity to keep constant the free luminal SR/ER $[Ca^{2+}]$ and also the total amount of Ca^{2+}. Any reduction in the free luminal SR/ER $[Ca^{2+}]$ implies a corresponding reduction in the total amount of Ca^{2+} stored in the SR/ER

three components work independently of each other and that it is only via changes in the free luminal [Ca^{2+}] that these three components communicate among each other [71, 72]. Accordingly, these models have explored the role played by free luminal [Ca^{2+}] in modulating the activity of both release channels and Ca^{2+} pumps. However, there are several experimental studies indicating that these three components are not working independently of each other. This situation was clearly unexpected particularly for SERCA pump and release channels, because they are supposed to be antagonistic elements of the Ca^{2+} pools since one increases while the other reduces the Ca^{2+} content of the intracellular store. Nevertheless, there is evidence, which will be discussed in the rest of the review, for a communication between Ca^{2+} release channels and SERCA pump to achieve an efficient Ca^{2+} release event (i.e. an increase in the $[Ca^{2+}]_i$ with minimal or no reduction in the free luminal SR/ER [Ca^{2+}]).

Mathematical models reflect our incomplete knowledge of the Ca^{2+} release event. One example can be found in a study investigating the role played by Ca^{2+} diffusion inside the SR in the termination of Ca^{2+} sparks [73, 74]. Interestingly, this model uses a previously reported Ca^{2+} diffusion coefficient in the non-junctional SR of 0.6×10^{-10} m^2/s [75], but to have an efficient Ca^{2+} release process, this coefficient has to be five-fold larger of 3.5×10^{-10} m^2/s in junctional SR [73, 74]. The problem with this difference is that the model uses a previously estimated concentration of calsequestrin of 6 mM [76], while the model needed to increase the concentration by five-fold (30 mM) in the junctional SR [73, 74]. These conditions generate a paradox because the idea is that calsequestrin is the main Ca^{2+} buffering protein in this compartment and since calsequestrin behaves as a non-mobile protein within the SR, because it is associated with the RyRs, therefore increasing the concentration of calsequestrin in the SR should lower the diffusion of Ca^{2+} ions. However, this is the opposite of what the model predicts to be happening in the junctional SR because it was increased five-fold. Thus, it appears then that our current picture of how calsequestrin is working would lead to a strong competition for the free luminal Ca^{2+} between RyRs and calsequestrin and since this does not seem to be happening in the Ca^{2+} release event, then there is still missing information on how these proteins produce an efficient Ca^{2+} release process.

14.2 Contributions of Noriaki Ikemoto to the SR Ca^{2+} Release Model

14.2.1 The Activation of RyR1 Produces an Initial Increase in the Free Luminal SR [Ca^{2+}] Before the Opening of this Release Channel

A very interesting observation made by Ikemoto's group consists in finding that stimulation of RyRs in skeletal muscle terminal cisterna SR vesicles produces an

elevation of the free luminal [Ca^{2+}] that occurs before any Ca^{2+} has come out from the store to the cytoplasm, in other words, the free luminal [Ca^{2+}] is increased in response to the activation of RyRs but before the opening of these channels [77]. The implications of this observation are so profound and paradigm changing that we are still in the process of incorporating them in our current models on how Ca^{2+} pools are working. The importance of this observation resides in the fact that current mathematical models of Ca^{2+} release cannot generate an increase in the free luminal [Ca^{2+}] in response to the activation of RyRs. The current idea on how Ca^{2+} release occurs requires the opening of RyRs to decrease the free luminal [Ca^{2+}], as an initial event, and this in turn would drive the dissociation of Ca^{2+} from calsequestrin to further amplify the amount of Ca^{2+} that is released from the SR. This model means that Ca^{2+} dissociation from calsequestrin is driven by a reduction of the free luminal [Ca^{2+}], which is the result of the opening of RyRs. This picture seems to coincide with the results obtained by Ikemoto using high concentrations of polylysine to activate RyRs [77]. However, using lower concentrations of polylysine resulted in a clear increase of the free luminal [Ca^{2+}], as measured with tetramethylmurexide, before any reduction in the total amount of Ca^{2+} (as measured with $^{45}Ca^{2+}$) stored in the SR vesicles [77]. This work suggests that activation of RyRs, before the opening of RyRs, results in the increase of the free luminal SR [Ca^{2+}] as an initial step followed then by the actual opening of these release channels.

14.2.2 Role of Calsequestrin in Producing a Transient Increase of the Luminal SR [Ca^{2+}]

Another crucial observation done by Ikemoto's laboratory concerns the role played by calsequestrin in generating an efficient Ca^{2+} release process [77]. They found that activation of RyRs with polylysine did not produce any increase in the free luminal [Ca^{2+}] when the SR vesicles were devoid of calsequestrin. This indicates that the Ca^{2+} bound to this protein is the one that is released by RyRs. However, the rather radical observation is that calsequestrin inside the SR vesicles is not enough to reconstitute the Ca^{2+} release event, but that calsequestrin needs to be associated with the RyRs of the SR vesicle to recover the increase in the free luminal [Ca^{2+}] response [77]. This signifies that the Ca^{2+} buffering power provided by calsequestrin is not enough to have a correct Ca^{2+} release event, but that RyRs and calsequestrin have to interact, either directly or indirectly, in order to reconstitute an efficient Ca^{2+} release event. Again the implications of this observation are so ample that it has been difficult to incorporate in our current thinking. The same conclusion was reached by a different group and their data was summarized in a model where there were two different RyRs, whose main difference was to have or not attached calsequestrin [78]. Those RyRs having attached calsequestrin respond very fast and produced more than 50% of the Ca^{2+} release event, while those without calsequestrin have a slower activation and provide less Ca^{2+} to the response [78]. It

is important to bear in mind that the rate of Ca^{2+} release is a critical characteristic to produce an increase in the $[Ca^{2+}]_i$ because, there are Ca^{2+} buffering proteins in the cytoplasm and Ca^{2+} pumps at the plasma membrane, that would reduce the effect of release channels on the $[Ca^{2+}]_i$. Accordingly, this means that those RyRs with calsequestrin bound would have a much higher effect on the $[Ca^{2+}]_i$ than those RyRs without calsequestrin, despite releasing similar amounts of Ca^{2+} from the SR but, the main difference is that one is faster than the other. These data are also suggesting that calsequestrin appears to be trapping Ca^{2+} next to the RyRs, meaning that this entrapped Ca^{2+} is not in equilibrium with the free luminal SR $[Ca^{2+}]$; if this is the case, then it is easy to explain why the activation of RyRs, before the opening of RyRs, produces an initial increase of the free luminal SR $[Ca^{2+}]$. Additionally, this situation also implies that the free luminal $[Ca^{2+}]$ is not a good predictor of the amount of Ca^{2+} released by activation of RyRs because the Ca^{2+} trapped by calsequestrin does not appear to be in equilibrium with the free luminal SR $[Ca^{2+}]$, which is the one reported by the fluorescent Ca^{2+} indicators. Yet another important consequence that can be derived from these works is that there will be a refractory period in the Ca^{2+} release event that would depend on the time taken by RyRs and calsequestrin to reconstitute the trapped Ca^{2+} complex. It appears that only closed conformation of RyRs allows the reconstitution of this complex, a conclusion that was derived from experiments using ryanodine in smooth muscle cells [79]. High concentrations of ryanodine lock RyRs in the open conformation but the Ca^{2+} flow is blocked because ryanodine binds to the open pore [45, 47]. In the cell, this effect of ryanodine allows SERCA pump to recover the luminal SR $[Ca^{2+}]$ because ryanodine has blocked the RyRs [79]. The most interesting observation is that in the presence of ryanodine and with normal SR luminal $[Ca^{2+}]$, the application of IP_3-producing agonists results in a much smaller increase of the $[Ca^{2+}]_i$ [79, 80]. Collectively, these data suggest that the SR Ca^{2+} store is still empty despite the presence of a high luminal SR $[Ca^{2+}]$. The interpretation for these data could be that the complex (release channel-Ca^{2+} binding protein) cannot be reassembled when the RyRs have been locked, by ryanodine, in the open conformation and this condition reduces the ability of nearby IP_3Rs to produce an efficient $[Ca^{2+}]_i$ response.

14.2.3 Evidence that There Is a Communication Between SERCA Pump and RyR1

At the end of the Ca^{2+} release event, the combined reduction in the free luminal SR/ER $[Ca^{2+}]$ and the associated increase of the $[Ca^{2+}]_i$ should lead to SERCA pump activation. At least that is the idea because high luminal $[Ca^{2+}]$ would make harder for SERCA pump the exchange of H^+ for Ca^{2+} in the lumen of the SR [81]. However, work done by Ikemoto's group has demonstrated that activation of RyR1s affects the activity of SERCA pump suggesting some kind

of communication between these two proteins [82–86]. The most interesting part is that this communication occurs right after the activation of RyRs and before any reduction in the free luminal SR [Ca^{2+}]. Even more difficult to explain is the observation that this activation of the SERCA pump occurs during the RyR-triggered increase of the free luminal [Ca^{2+}] [87]. This is quite paradoxical since it is expected that the increase in the free luminal [Ca^{2+}] should lead to further inhibition of the SERCA pump activity [81]. It is still unknown how the activation of RyRs, before their opening, results in SERCA pump activation; particularly because SERCA pump and RyRs are not necessarily localized next to each other in the SR; however, this communication could indirectly occur via luminal Ca^{2+} binding proteins [83]. Regardless how these two proteins communicate, it is easy to argue that this communication between release channels and SERCA pump is an important mechanism to avoid life-threatening situations such as ER-stress due to depletion of the ER Ca^{2+} store.

14.3 Simultaneous Recording of the Redution in the Free Luminal SR/ER [Ca^{2+}] and the Associated Changes in the [Ca^{2+}]$_i$

Since the 80's, when the organic fluorescent Ca^{2+} indicators were synthesized, it became customary to place cells in the absence of external [Ca^{2+}] to look at the [Ca^{2+}]$_i$ response induced by different stimuli, and to take amplitude of this response as an indirect measure of the amount of Ca^{2+} present in the SR/ER Ca^{2+} pools. However, this type of approach can be deceiving because there are different factors that shape the [Ca^{2+}]$_i$ response. One example of this problem was the observation in smooth muscle cells that two applications of saturating concentrations of caffeine, which are separated by only 30 s, results in a second [Ca^{2+}]$_i$ response that is 80% smaller than the first response [88]. The initial and incorrect interpretation of these data was that 30 s was not enough time to refill the SR Ca^{2+} pool, yet when using Mag-fura-2 to see the free luminal SR [Ca^{2+}], it became evident that the SR [Ca^{2+}] is fully recovered from caffeine-induced depletion after a time of only 30 s [89]. Therefore it is clear the importance of recording simultaneously the changes in the [Ca^{2+}]$_i$ and the luminal SR/ER [Ca^{2+}] with sufficiently high time resolution to be able to define an efficient Ca^{2+} release event. It is this approach what has made evident that the changes in the luminal SR/ER [Ca^{2+}] does not present a simple correlation with the corresponding changes in the [Ca^{2+}]$_i$.

Initial attempts to look at the luminal ER [Ca^{2+}] were carried out using Mag-fura-2, a low affinity Ca^{2+} indicator that does not go easily into the ER, and for this reason much of the work done with this indicator was carried out in permeabilized cells [90]. It turned out that Mag-fluo-4, a tricarboxylic low affinity Ca^{2+} indicator, goes into the ER more easily [91]. However, since Mag-fluo-4 lacks ER retention mechanism, not all dye stays in the ER, some of it goes into the Golgi

apparatus and from there goes into the vesicles of the secretory pathway [10]. The advantage of organic dyes is that they are easy to be loaded and to calibrate, their stoichiometry with Ca^{2+} is 1:1 and they are relatively insensitive to pH changes. Nevertheless, the main limitation is that they lack a targeting mechanism and this issue has been solved developing Genetically Encoded Ca Indicators (GECIs) that are targeted to different organelles, the ER/SR among others [10, 92]. These GECIs use EF-hand proteins as Ca^{2+} sensor moiety and the signal could be chemoluminescent or fluorescent in nature. The disadvantage of GECIs is that they are not homogeneously expressed in cells, their stoichiometry is not 1:1, and aequorin is irreversibly oxidized on Ca^{2+} binding, so it is a single shot Ca^{2+} indicator while GFP fluorescence is sensitive to changes in the pH [10].

Initial studies on the luminal ER $[Ca^{2+}]$ using aequorin in HeLa cells, found that the agonist-induced reduction in the free luminal ER $[Ca^{2+}]$ and the associated $[Ca^{2+}]_i$ response, were both of the same amplitude, whether the ER Ca^{2+} pool was fully loaded or practically depleted [93]. These data suggest that the Ca^{2+} released by the agonist is not in equilibrium with the free luminal ER $[Ca^{2+}]$. In pancreatic acinar cells it has been shown that a small concentration of acetylcholine (200 nM) increased the activity of Ca^{2+}-dependent Cl^- channels in the absence of any reduction in the luminal ER $[Ca^{2+}]$, despite the activation of IP_3R by acetylcholine [19]. These data indicate that activation of a small number of IP_3Rs produces no reduction in the free luminal ER $[Ca^{2+}]$ despite Ca^{2+} has been released. Again, these data suggest that the free luminal ER $[Ca^{2+}]$ is not in equilibrium with the Ca^{2+} that is released to the cytoplasm by IP_3Rs. A new cameleon targeted to the ER known as D1ER, showed that agonist-induced elevation of the $[Ca^{2+}]_i$ precedes any significant reduction in the free luminal $[Ca^{2+}]$. Actually, the majority of the reduction in the free luminal ER $[Ca^{2+}]$ occurs during the reduction of the agonist-induced $[Ca^{2+}]_i$ response [94]. These data suggest that the Ca^{2+} buffering capacity of the ER is extremely high, during the initial stages of Ca^{2+} release; later on, it switches to a form of low capacity [95–97]. This lack of correlation between the reduction in the free luminal ER $[Ca^{2+}]$ and the increase in the $[Ca^{2+}]_i$ cannot be explained by saturation of the luminal Ca^{2+} indicator. This is the case because in single uterine smooth muscle cells that were loaded with both fura-2 and Mag-fluo-4, the activation of SERCA pump increased the Mag-fluo-4 fluorescence implying that Mag-fluo-4 cannot be saturated inside the SR [91, 98]. This lack of correlation can be seen in different cell types and with activation of either RyRs or IP_3Rs. For instance, the addition of 1 mM ATP, to activate purinergic receptors in HeLa cells, results in the activation of IP_3Rs which produced a delayed reduction in the free luminal ER $[Ca^{2+}]$ with respect to the time course of the $[Ca^{2+}]_i$ response [99]. Another group studying HeLa cells that had been loaded with Indo-1 and an ER-targeted cameleon, observed that the histamine-induced increase of the $[Ca^{2+}]_i$ was associated with a rather small reduction of the free luminal ER $[Ca^{2+}]$ [100]. This was the case either in the presence or in the absence of external $[Ca^{2+}]$; indicating that the explanation for this lack of correlation is not due to the presence of Ca^{2+} entry at the plasma membrane. In heart cells, localized, fast, transient increases of the $[Ca^{2+}]_i$ known as Ca^{2+} sparks, which are due to the activation of a cluster

of RyR2s, are associated with a transient decreased of the free luminal SR [Ca^{2+}], known as blinks [101]. Remarkably, the large majority of these blinks show a slower Ca^{2+} decrease rate than the rate of Ca^{2+} increase seen with the corresponding spark [101, 102]. In skeletal muscle, the reduction in the free luminal SR [Ca^{2+}] that are associated with a Ca^{2+} spark are known as skraps [97], which also have a much slower time course when compared with their corresponding sparks [96, 97]. These data indicate that even at the subcellular level the time course of the reduction in the luminal SR [Ca^{2+}] does not coincide with the corresponding increase in the $[Ca^{2+}]_i$ [96]. To complicate matters even further, it has been shown in heart cells that activation of SERCA pump with isoproterenol produces an increase of the luminal SR [Ca^{2+}] while Ca^{2+} is simultaneously released to the cytoplasm [103, 104]. Moreover, an efficient Ca^{2+} wave propagation requires active SERCA pumps [105]. All these data indicate that there is no correlation between the changes in the free luminal SR/ER [Ca^{2+}] and the increase in the $[Ca^{2+}]_i$ despite the fact that the SR/ER is the source for the Ca^{2+} that appears in the cytoplasm.

A more detailed analysis of the Ca^{2+} release process carried out by either RyRs or IP_3Rs, in smooth muscle cells from the urinary bladder, reveals the existence of four different phases for a Ca^{2+} release event [95]. The first one involves the largest increase in the $[Ca^{2+}]_i$ with just a small reduction in the luminal SR [Ca^{2+}] (we call this an efficient Ca^{2+} release event); phase 2 is characterized by the largest reduction in the luminal SR [Ca^{2+}], without any effect on the $[Ca^{2+}]_i$; while phase three is defined by the reduction in the $[Ca^{2+}]_i$ but the luminal SR is still depleted because the release channels stay open and the fourth phase, is when release channels have been closed and the free luminal SR [Ca^{2+}] recovers to their normally high levels by the action of SERCA pumps [95]. Additionally, in the same work, it was used a low concentration of heparin, to partially inhibit IP_3Rs, and the application of carbachol, to activate IP_3Rs, results in both a transient elevation of the free luminal SR [Ca^{2+}] and a transient increase of the $[Ca^{2+}]_i$ [95]. The elevation in the $[Ca^{2+}]_{SR}$ occurred at the same time that the increase in the $[Ca^{2+}]_i$. The implication of these data is that activation of IP_3Rs unleashes Ca^{2+} trapped in luminal proteins that under normal conditions (fully activated IP_3Rs) will gain immediate access to the cytoplasm. However, with the use of heparin, to partially reduce the activity of IP_3Rs, the just liberated Ca^{2+} from luminal Ca^{2+} binding proteins, diffuses back to the bulk of the SR and it is seen as an increase of the $[Ca^{2+}]_{SR}$. This lack of correlation between the changes in the $[Ca^{2+}]_i$ and the SR/ER [Ca^{2+}] can also be detected using GECIs in cells, that are either isolated or in the tissue [92]. A newly developed GECI that uses apoaequorin to sense Ca^{2+} and GFP to have a fluorescence signal has been used together with Fura-2. Simultaneous recording of the luminal ER [Ca^{2+}] and the $[Ca^{2+}]_i$ show that the $[Ca^{2+}]_i$ response precedes the agonist-induced reduction in the luminal ER [Ca^{2+}] both in HeLa cells and neurons in hippocampal slices [92, 106]. Using a different set of GECIs, it was found that the application of both bradykinin and CPA results in a nuclear [Ca^{2+}] elevation, detected with H2B-D3cpv probe, that peaks well before the nadir of the free luminal ER [Ca^{2+}] reduction [107]. All these data undermine the idea that SR/ER is a Ca^{2+}

pool where the physical state of Ca^{2+} ions is switching from free to protein bound, but that this ion appears to be also trapped in protein complexes that release Ca^{2+} ions in response to the activation of RyRs or IP_3Rs, a picture that was portrayed by Ikemoto [77] and others [78].

The idea that Ca^{2+} does not seem to be in equilibrium inside the SR/ER has been shown using microanalysis with electron microscopes. The activation of VGCC in neurons, loads the ER with Ca^{2+} due to the activity of SERCA pump. However, the increase in Ca^{2+} was not homogenous inside the ER. The same cisterna, can have regions with high Ca^{2+} next to regions where the total amount of Ca^{2+} was not changed at all by the activity of SERCA pumps [64, 65]. Again, these data indicate that there are regions of the SR/ER where Ca^{2+} activity is reduced (meaning that Ca^{2+} has been trapped) without changing the Ca^{2+} activity of contiguous regions despite the absence of evident diffusion barriers. The easiest way to explain these observations is that Ca^{2+} is trapped by proteins and that these ions are not in equilibrium with the free luminal ER $[Ca^{2+}]$. If this is the case on how the SR/ER accumulates Ca^{2+} it might explain why there is a lack of correlation between the Ca^{2+} supplied to the cytoplasm by the SR/ER and the associated changes in the free luminal SR/ER $[Ca^{2+}]$.

A question that is still open is how luminal Ca^{2+} binding proteins are able to trap Ca^{2+} inside the SR/ER. Nevertheless, there are some hints on how this might be happening. It has been shown that calsequestrin is able to increase the number of Ca^{2+} binding sites as the Ca^{2+} concentration is increased. The underlying mechanism for the increase in Ca^{2+} binding sites depends on the ability of calsequestrin to polymerize [62]. However, since calsequestrin is not expressed by all cells and since an efficient Ca^{2+} release is a generalized cellular event, then there should be other proteins doing the same function as calsequestrin. Actually, knockout studies of both CSQ1 and CSQ2 have suggested that there must be other proteins in the lumen of the SR/ER because RyRs were still able to release Ca^{2+} [58]. Thus either calreticulin, or maybe other Ca^{2+} binding proteins in the lumen of the SR/ER, have also the property of increasing the number of Ca^{2+} binding sites in response to an increase of the luminal $[Ca^{2+}]$.

Another very interesting situation to study involves the phenomenon known as the refractory period of Ca^{2+} release that might reflect how the SR/ER is trapping releasable Ca^{2+} [88, 89, 101, 102]. If it is true that the free SR $[Ca^{2+}]$ is in equilibrium with the Ca^{2+} released by RyRs, then this time should be more than enough to fully recover the caffeine-induced $[Ca^{2+}]_i$ response. So the question remains on how to explain the presence of a refractory period for caffeine even when the free luminal SR $[Ca^{2+}]$ has reached normal levels. The same situation is observed in heart cells, where the refractory period between Ca^{2+} sparks from the same site is significantly longer than the time required for recovery of the free luminal SR $[Ca^{2+}]$ [101, 102]. Moreover, keep in mind that the inactivation of the RyRs cannot be the explanation for the presence of a refractory period because; it has been shown that the reduction in the free luminal SR $[Ca^{2+}]$ is basically the same for both Ca^{2+} release events. This observation implies that the same number

of RyRs was activated during the two Ca^{2+} release events although the second application of caffeine produced a five times smaller $[Ca^{2+}]_i$ response. Thus, all these data imply that the changes in the free luminal SR $[Ca^{2+}]$ does not reflect the total amount of Ca^{2+} that is released from the SR to the cytoplasm. If this is the case, then we think that the most likely explanation for the presence of a refractory period is that an efficient Ca^{2+} release event involves the liberation by RyRs of Ca^{2+} trapped by proteins, which are localized contiguous to the release channel [108]; however, it appears that this complex (RyRs-Ca^{2+} trapping proteins) takes a longer time to assemble than the time taken to recover the free luminal SR $[Ca^{2+}]$ (see Sect. 14.4.2).

14.4 Current View on How the SR/ER Ca^{2+} Store Is Working to Produce an Efficient Ca^{2+} Release Event

14.4.1 The Luminal Ca^{2+} Binding Proteins Compete for Ca^{2+} with the Open Release Channel

The picture depicted by Ikemoto in the '90s, on how the SR releases Ca^{2+}, appears to be a generalized situation not only for the SR but also for the ER, that is, for the IP_3Rs. However, this picture is still sketchy because we do not have enough spatial and temporal resolution to have all the elements involved in an efficient Ca^{2+} release event. For this reason, we have resorted on the use of mathematical models to find the conditions necessary to generate phase one during Ca^{2+} release from the ER/SR, as a reminder, phase one is characterized by an increase of the $[Ca^{2+}]_i$ without any reduction in the free luminal ER/SR $[Ca^{2+}]$ [95, 96]. The simplest solution we were able to figure out was reducing the number of luminal Ca^{2+} binding sites in response to a reduction of the luminal SR $[Ca^{2+}]$ [109]. We called this situation Kinetics on Demand (KonD) because the number of Ca^{2+} binding sites increases as the result of an elevation of the $[Ca^{2+}]$ while they are diminished by a reduction in the $[Ca^{2+}]$. This type of kinetics is completely different to the traditional one, where the number of Ca^{2+} binding sites is fixed and the reduction in the free $[Ca^{2+}]$ increases the number of Ca^{2+} binding sites that are unoccupied by Ca^{2+}. The mathematical model reveals that these unoccupied Ca^{2+}-binding sites, which are increasing in number during the Ca^{2+} release process, become a strong competitor for Ca^{2+} with the open release channels and naturally, this competition would slow the Ca^{2+} release process. This kind of problem would not be presented by KonD model because the number of Ca^{2+} binding sties is being reduced during the Ca^{2+} release event. We want to stress that KonD model was proposed based on the observation that the number of Ca^{2+} binding sites in calsequestrin increases as a function of its polymerization [62]. Therefore, the changes in the paradigm were based on understanding how calsequestrin is working and the main difference

consisted on assuming that the total number of Ca^{2+} binding sites are not fixed but varies depending on the presence of Ca^{2+} [62].

Phase one reflects a mechanism for an efficient Ca^{2+} release process and we think that involves Ca^{2+} trapped in luminal Ca^{2+}-binding proteins, next to release channels. This situation implies that the Ca^{2+} that is released by RyRs or IP_3Rs is not in equilibrium with the free luminal SR/ER [Ca^{2+}]. However, there could be alternative explanations for phase one, the idea that phase one is due to saturation of the luminal Ca^{2+} indicator has been discarded because there are different experimental conditions that produce an increase of the free luminal [Ca^{2+}], which argue against the idea that the Ca^{2+} indicator inside the SR/ER is saturated [91, 95, 97, 110]. In skeletal muscle it has been suggested that high cooperativity of Ca^{2+} binding to calsequestrin might explain phase one [58, 97, 111]. The main limitation with this explanation is that the high cooperativity means a smaller range of [Ca^{2+}] before reaching saturation and the ability of SR/ER to buffer Ca^{2+} is quite the opposite since it covers several orders of magnitude [64]. Mathematical models have suggested that luminal Ca^{2+} binding proteins diffuse away from the RyRs while Ca^{2+} would be diffusing towards the open release channels during the release process [73, 74]. We think this seems to be unlikely because we do not see what could be the driving force for Ca^{2+}-binding proteins to diffuse away from the RyRs. Another scenario might be a restricted diffusion of Mag-Fluo-4, which is kept in the bulk of the SR away from the RyRs, so this dye will not be in rapid equilibrium with calsequestrin, which is known to be associated with RyRs [112] and if Ca^{2+} slowly diffuses between these two compartments, then it is expected to see recovery of the free luminal SR Ca^{2+} level (provided that SERCA pumps are located where Mag-Fluo-4 is) even when the RyRs are still open. However, this scenario has not been observed. Release channels need to be in the closed conformation for SERCA pump being able to recover the free luminal SR/ER [Ca^{2+}]. This observation implies that there is no diffusion barrier for the Ca^{2+} indicator. The absence of diffusion barriers for Ca^{2+} and Ca^{2+} indicators within the lumen of the SR/ER should make it difficult to see the transition between phase one and phase two. Indeed, the fusion of these two phases in a single phase has been observed but only in particular conditions; for instance when SERCA pumps had been inhibited with thapsigargin [95] or when it has not given enough time for stores to recover from a previous release event [88, 109, 113]. These data add a new element of complexity since it argues for the importance of having an active SERCA pump to be able to see phase one separated from phase two. Additionally, our mathematical model has stressed the importance that Ca^{2+} binding proteins rapidly switch from high to low Ca^{2+} buffering capacity during the Ca^{2+} release process, a scenario that we have called Kinetics on Demand [109]. The importance of this switch is that the empty Ca^{2+}-binding sites of the luminal Ca^{2+}-binding proteins will not compete with release channels for free luminal Ca^{2+}. The absence of this competition is reflected in release channels having enough free luminal Ca^{2+} for an efficient release event even when the free luminal SR/ER [Ca^{2+}] is not decreasing. Obviously, it is important to know the molecular nature of the proteins responsible for sequestering Ca^{2+} next to

release channels and the molecular mechanism involved to switch from sequestered Ca^{2+} to free Ca^{2+} inside the SR/ER and how the activation of release channels gain access to this trapped Ca^{2+}. Based mainly, but not exclusively, on our own work and the work done by Ikemoto's group, we have developed a graphical model that might explain the differences observed for the time courses of the $[Ca^{2+}]_i$ and the $[Ca^{2+}]_{SR/ER}$ during a Ca^{2+} release event (Fig. 14.2).

14.4.2 Proposed Graphical Model on How Release Channels in the SR/ER Produce an Efficient $[Ca^{2+}]_i$ Response

The graphical model shown in Fig. 14.2 is a cartoon on how the activation of Ca^{2+} release channels leads to an efficient increase of the $[Ca^{2+}]_i$. Initially (Fig. 14.2a), release channels are closed and the Ca^{2+} lattice represents the luminal Ca^{2+} binding proteins that are present in the form of polymers attached to the RyRs [114]. These have trapped Ca^{2+} inside the lattice and accordingly, this Ca^{2+} will not be

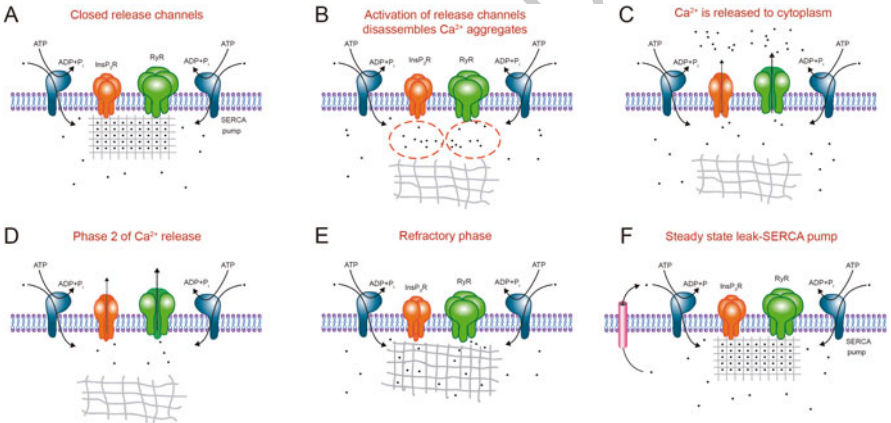

Fig. 14.2 Graphical model of an efficient Ca^{2+} release event by either RyRs or IP_3Rs in the SR/ER Ca^{2+} stores. This cartoon depicts those steps that are considered to be critical for generating an efficient Ca^{2+} release event that is reflected in a transient increase of the $[Ca^{2+}]_i$. (**a**) Ca^{2+} source is in the form of a lattice that is attached to the release channels but disconnected from the bulk of the free luminal SR/ER $[Ca^{2+}]$. (**b**) The activation of the release channels would liberate the Ca^{2+} trapped in the lattice. (**c**) The open conformation of release channels allow the movement of Ca^{2+}, down its electrochemical gradient, to the cytoplasm, without the need to reduce the bulk luminal SR/ER $[Ca^{2+}]$ (phase one). (**d**) Phase two of Ca^{2+} release, which is the inefficient part of the Ca^{2+} release event, mainly because the Ca^{2+} flux from the bulk of the SR/ER is rather slow when compared with the cytoplasmic Ca^{2+} removal mechanisms. (**e**) Once the release channels have been closed there will be a refractory period, because although the bulk of the free luminal SR/ER $[Ca^{2+}]$ has returned to normal levels, probably the Ca^{2+} lattice has not recovered just yet. (**f**) Once the Ca^{2+} lattice has reformed and reattached to the release channels, they are ready for another efficient Ca^{2+} release event

in equilibrium with the free luminal Ca^{2+} that is in the bulk of the SR/ER. This scenario might explain why there is an elevation in the free luminal SR/ER $[Ca^{2+}]$ in response to the activation of release channels; if this is the case then it is easy to see why the free luminal SR/ER $[Ca^{2+}]$ is an inadequate predictor of the amount of Ca^{2+} that the internal stores deliver to the cytoplasm. The activation of release channels (Fig. 14.2b) leads to conformational changes that disassemble the Ca^{2+} lattice producing a local elevation of the free luminal $[Ca^{2+}]$ next to the release channels. This elevation in the free luminal $[Ca^{2+}]$ might help release channels to reach the open conformation. If the release channel is not open or is blocked then this Ca^{2+} would start to diffuse to the bulk of the SR/ER, and this can be observed as a transient elevation of the free luminal SR/ER $[Ca^{2+}]$. This scenario might be the explanation behind the ability of polylysine, a substance that is both an activator and partial blocker of RyRs [115, 116], to reveal a transient elevation of the free luminal $[Ca^{2+}]$ during the activation of RyRs. Following this reasoning, we have used a low concentration of heparin to partially block IP_3Rs that had been previously activated in response to the application of carbachol in smooth muscle cells [117]. Actually, the rate of rising of the $[Ca^{2+}]_i$ when IP_3Rs had been inhibited by heparin, was strongly augmented [95]. The idea here is that Ca^{2+} would accumulate next to those partially blocked release channels and this will increase the driving force resulting in a higher Ca^{2+} exit via those few channels that were not blocked by heparin. Under normal circumstances, the release channels will be fully open (Fig. 14.2c) and this will allow free diffusion of Ca^{2+} from the SR/ER to the cytoplasm producing a significant, transient $[Ca^{2+}]_i$ response but with one very important characteristic, there will be basically no reduction of the bulk free luminal SR/ER $[Ca^{2+}]$. Since most of the Ca^{2+} indicator is in the bulk of the SR/ER, this dye will not see any reduction in the surrounding $[Ca^{2+}]$ because the Ca^{2+} next to the release channel is in a higher concentration than in the bulk of the SR/ER, at least initially in the release event. This is what we have called phase one of the Ca^{2+} release event [95], which is an efficient Ca^{2+} release process because there is an increase in the $[Ca^{2+}]_i$ with minimal or no reduction in the free luminal SR/ER $[Ca^{2+}]$. Phase one of the Ca^{2+} release process has been seen in all kind of cells, during slow or fast Ca^{2+} release events, for RyRs or IP_3Rs and using synthetic or genetically encoded Ca^{2+} indicators [96]. Phase two is observed when the Ca^{2+} supply that comes from the Ca^{2+} lattice has been exhausted and the free luminal Ca^{2+} in the bulk of the SR/ER starts to be drained via the still open release channels (Fig. 14.2d); however, in this case the Ca^{2+} movement is so slow that there is a clear reduction in the bulk of the SR/ER $[Ca^{2+}]$ but without any effect on the $[Ca^{2+}]_i$ (this is the unproductive part of the Ca^{2+} release event). Once the release channels are either closed or inactivated (Fig. 14.2e), then SERCA pump can start accumulating Ca^{2+} by hydrolyzing ATP until the high resting luminal SR/ER $[Ca^{2+}]$ has been reached, at least in the bulk of the SR/ER. However, there would be a time period larger than the time it will take to reach the normal free luminal $[Ca^{2+}]$, where it can be considered that the Ca^{2+} store has been refilled (high free luminal $[Ca^{2+}]$) but if the release channels were to be exposed to an activator in this time, the result would be an unproductive

Ca^{2+} release event [88, 95, 101, 102, 109]. We do not know the reason for this situation, but it is feasible that even when the free luminal SR/ER [Ca^{2+}] is high, close to the normal levels, the Ca^{2+} lattice appears that it has neither reassembled nor connected to the release channels yet. Thus, the characteristic of this stage is a small time window where the free luminal [Ca^{2+}] is basically normal but the activation of the release channels produces a much smaller $[Ca^{2+}]_i$ response [95, 101]. The implication of this situation is that Ca^{2+} buffering in the lumen of the SR/ER does not appear to be a reversible process. Actually, this process shows hysteresis [95, 96, 109], a situation that is not expected for proteins with a low affinity Ca^{2+} binding sites.

Activation of all RyRs, for instance with high concentrations of caffeine, displays this refractory phase in the order of tens of seconds [88, 95]. This implies that this is the time taken by RyRs to recover an efficient Ca^{2+} release event. Nevertheless, the frequency of Ca^{2+} release events in heart and skeletal muscle is much higher than the time taken by RyRs to recover an efficient Ca^{2+} release event. Then we think that muscles cope with this limitation by using only a fraction of all the RyRs present in the SR. In summary, a picture is emerging where the Ca^{2+} released by RyRs or IP_3Rs is trapped in a lattice that is not in equilibrium with the bulk of the SR/ER and it is essential for having an efficient $[Ca^{2+}]_i$ response. Additionally, we propose that the time it takes for this Ca^{2+} lattice to reconstitute itself and to attach to release channels determines the refractory period. We think that a reduction in the SERCA pump activity or the number of functional RyRs with an assembled Ca^{2+} lattice, or even worse, the presence of both conditions, lead to heart failure [118]. Under this condition, Ca^{2+} release via RyRs cannot be efficient since the Ca^{2+} flux is not fast enough to produce a large increase of the $[Ca^{2+}]_i$. One important issue here is that the formation of the Ca^{2+} lattice (Fig. 14.2f) requires both active SERCA pumps and release channels being in the close conformation. These two conditions are not met during heart failure and this might explain why the Ca^{2+} lattice would not be formed equally well and the Ca^{2+} release process is not efficient enough in this pathological condition. It is known that high concentrations of ryanodine lock RyRs in the open conformation, but at the same time, their ion pore is blocked by ryanodine [47]. This situation facilitates recovery of the high luminal $[Ca^{2+}]_{SR/ER}$ by the activity of SERCA pump after the application of caffeine, but it results in the lack of IP_3R-mediated $[Ca^{2+}]_i$ response despite the normal free luminal SR [Ca^{2+}] [79, 89, 117]. Thus, the idea is that the Ca^{2+} lattice to be reformed and attach to the release channels requires these channels to be in the closed conformation.

14.4.3 The Relevance of the Model Where Release Channels Used Ca^{2+} Trapped in the Ca^{2+} Lattice

The importance of this proposed mechanism used by the SR/ER to release Ca^{2+} is that these organelles can function as Ca^{2+} pools for cytoplasmic Ca^{2+} transients

without running the risk of triggering ER stress response due to depletion of the ER luminal [Ca^{2+}] [40, 119–121]. There would be no Ca^{2+} depletion of the SR/ER store when release channels stay open only for the period of time that encompasses phase one of the Ca^{2+} release process. The reason would be that release channels would not reach phase two of the release process and no depletion of the SR/ER would occur. The time required to reach phase two of Ca^{2+} release is much longer than the time involved in a Ca^{2+} spark or a Ca^{2+} puff. Therefore, this model of Ca^{2+} release has the compelling advantage that it can release large amounts of Ca^{2+} to the cytoplasm without eliciting depletion of the SR/ER and accordingly, without triggering the ER stress response that might limit the role of the SR/ER as Ca^{2+} pool. The price to pay for this type of Ca^{2+} release process is the presence of a refractory period because of the time needed for reassembling the Ca^{2+} lattice. However, this limitation can be overcome relatively easy by having a reserve of release channels with a Ca^{2+} lattice. The dynamic of this reserve might be compromised during the fatigue process [122].

This model of SR/ER Ca^{2+} release mechanism combines data obtained with different cell types, different Ca^{2+} indicators and with activation of either RyRs or IP_3Rs, and the observation by Ikemoto's group that activation of RyRs results in an elevation of the free luminal $[Ca^{2+}]_{SR}$ and that this effect requires calsequestrin attached to RyRs. Additionally, this model also considers that calsequestrin increases its Ca^{2+}-binding capacity as a function of its Ca^{2+}-induced polymerization [62, 109]. Despite all these data that support this model; it is still incomplete because the lack of information with sufficiently high temporal and spatial resolution to see the disassembling of the Ca^{2+} lattice induced by the activation of the release channels. To achieve these resolutions is not trivial since the distance is too short, a few nanometers, and the rate of this process is in the order of milliseconds. Additionally, it is quite feasible that calsequestrin might be one of many different proteins that can generate a Ca^{2+} lattice in association with RyRs and IP_3Rs because the double knockout of this protein was not lethal [58] and even worse, it resulted in a much smaller reduction than expected of skeletal muscle luminal Ca^{2+} buffer capacity [58]. The few attempts that have been done to see the disassembling of the Ca^{2+} lattice in real time have failed because it was tried by reducing the free luminal SR [Ca^{2+}] with ionomycin, instead of directly activating RyRs with caffeine [60]. Interestingly, the time constant for calsequestrin depolymerization was extremely slow when driven by a reduction in the luminal SR [Ca^{2+}], which made authors think that this mechanism cannot be operating in cardiac cells because systole has a much higher frequency. However, our own interpretation is that the reduction in the free luminal [Ca^{2+}] is not the signal that drives the fast disassemble of the Ca^{2+} lattice, it is the opening of the RyRs. This scenario might explain why in the absence of external Ca^{2+}, that reduces the free luminal ER [Ca^{2+}], IP_3-inducing agonists produce an $[Ca^{2+}]_i$ response that was barely decreased in amplitude by the absence of external Ca^{2+} [93]. In another study, it became clear that the spatial resolution was not good enough to see the subtle changes associated with modifications in the degree of calsequestrin polymerization during the reduction of the free luminal [Ca^{2+}] [123]. So these few attempts suggest that it will be more difficult than

previously thought to show the dynamics of the Ca^{2+} lattice associated with release channels during the Ca^{2+} release process.

14.5 Other Factors Involved in Having an Efficient Ca^{2+} Release Event

All these studies reviewed here clearly show that Ca^{2+} release from intracellular Ca^{2+} pools, particularly the SR/ER driven by release channels, is more complex than a simple analogy with a water tank (Fig. 14.1). The main difference appears to be that Ca^{2+} is trapped by luminal proteins and accordingly, it is not in equilibrium with the bulk of the SR/ER [Ca^{2+}]. It is only the activation of release channels (RyRs or IPRs) that liberates this Ca^{2+} from the lattice (Fig. 14.2). This situation implies that the change in the free luminal [Ca^{2+}] is not a good indicator of how much Ca^{2+} has been released to the cytoplasm. Additionally, the activity of release channels is under the control of both the luminal [Ca^{2+}] and the interaction with luminal proteins, as it is the case for RyRs and calsequestrin. Moreover, the distribution of release channels and Ca^{2+} binding proteins is not homogenous in the SR/ER and even when their lumen lack diffusion barriers, the tortuosity of these internal Ca^{2+} stores contribute with geometrical issues that also need to be considered. If all these factors are not adding enough complexity, there is still another layer of complexity due to the fact that all the elements involved in the Ca^{2+} release process work interconnected. In this regard, there is data showing that an efficient Ca^{2+} release process requires an active SERCA pump [89]. This was unexpected because these two activities, SERCA pump and release channels, should be antagonistic between each other and instead it turns out that an active SERCA pump is a critical element to achieve an efficient Ca^{2+} release event.

One example of this communication between SERCA pump and Ca^{2+} release channels is observed in the effect of activation of β adrenergic receptor in heart cells. In this case the phosphorylation of phospholamban by PKA dissociates this protein from SERCA pump resulting in a higher pump activity. However, this effect is reflected in a larger $[Ca^{2+}]_i$ response that paradoxically, cannot be explained by a higher free luminal SR [Ca^{2+}] [20]. Moreover, the high speed of Ca^{2+} wave propagation requires active SERCA pumps in heart cells [105], a condition that is unexpected. It has been shown that induction of Ca^{2+} release due to activation of RyR1 activates SERCA pump even during the initial phase of Ca^{2+} release when the free luminal SR [Ca^{2+}] is the highest [87], arguing against the idea that changes in the free luminal SR [Ca^{2+}] is the way that RyRs control the activity of SERCA pump [81]. Additionally, rapid inhibition of SERCA pump with thapsigargin decreases both the amplitude and the rate of rising of the cytoplasmic Ca^{2+} response to caffeine or to acetylcholine in smooth muscle cells. This is happening even when thapsigargin does not reduce immediately the free luminal [Ca^{2+}] [89]. All these data argue again for the need for active SERCA pumps to have

an efficient Ca^{2+} release event. Efficient histamine-induced Ca^{2+} release in HeLa cells also requires that SERCA pump is active [124]. Pancreatic acinar cells from RGS2 knockout mice express a compensatory twofold-increase in the expression of SERCA pump and this is associated with a larger and more sensitive $[Ca^{2+}]_i$ response to acetylcholine that cannot be explained by a larger production of IP_3 [125]. Although this type of experiment does not explain why the elimination of RGS2 has resulted in a compensatory two-fold increase in the expression of SERCA pump, these results are in line with the idea that higher activity of SERCA pump produces larger agonist-induced Ca^{2+} release events.

14.6 Ca^{2+} Signaling Between the SR/ER and Other Organelles

14.6.1 The Connection Between the ER and Mitochondria

It has been shown that close association between the ER and mitochondria plays an important role in ATP production, lipids synthesis, mitochondria dynamics and Ca^{2+} signaling [126, 127]. One of the first studies about this association showed that the majority of mitochondria, around 80%, was forming contacts sites with the ER [128]. However, using a high resolution microscope in the 90s, it was found that the mitochondrial surface in contact with the ER was estimated to be around 10% of the total mitochondrial network [129]. Subsequent works have found that the percentage of mitochondria in contact with the ER was close to 20%, and that these values can vary when cells are challenged with different experimental conditions, although this interaction has never reached 80% [130–133]. Interestingly, Scorrano's group has observed that the fraction of mitochondria interacting with the ER is near 80% [134], as it was initially described by Montisano et al. [128]. Recently, using super-resolution microscopes, it has been shown that mitochondria are the main organelle in association with the ER, although these studies did not quantify the degree of this interaction [23, 24, 135]. Collectively, these data show an uncertain scenario regarding the fraction of the mitochondrial network that is associated with the ER, since these numbers vary so widely. At the present time it is not clear the reason behind this variability among the different studies.

14.6.2 The Association Between Mitochondria and the ER Is Stabilized by a Large Assortment of Protein Tethers

At the biochemical level, it has been shown that the ER-mitochondria interaction is involved in phospholipids synthesis, this process involves the bidirectional exchange of phospholipids between the ER and mitochondria [136]. It has been shown that

this exchange occurs in specific contact sites called Mitochondria-associated ER membranes (MAMs). These structures facilitate different signaling transduction processes [137] and probably, Ca^{2+} transfer from the ER to mitochondria. Actually, it has been described several different protein tethers that modulate Ca^{2+} transfer from the ER to mitochondria [138].

It has been reported that the Voltage Dependent Anion Channel (VDAC), a resident protein in the outer mitochondrial membrane (OMM) connects with the IP_3R in the ER by interacting with the cytosolic chaperone glucose-regulated protein 75 (Grp75). This tether enhances the Ca^{2+} uptake by mitochondria [139]. Additionally, Cyclophilin D, a mitochondrial protein that modulates the opening of the permeability transition pore, interacts with the VDAC-Grp75-IP_3R complex; the loss of this protein decreases both ER-mitochondria interactions and Ca^{2+} transfer to mitochondria. This situation leads to insulin resistance in hepatic cells, suggesting that this tether might be controlling glucose homeostasis [140]. Moreover, the overexpression of α-synuclein (a central protein in Parkinson disease) in HeLa cells has promoted an increase in mitochondrial Ca^{2+} uptake by enhancing ER-mitochondria interactions. These data suggest that α-synuclein has an important role in mitochondrial Ca^{2+} homeostasis [141]. It is clear then, from these examples, that protein tethers are involved in Ca^{2+} signaling. Indeed, mitofusin 2 (Mfn-2) a GTPase that is located both in the OMM and the ER membrane, is involved in the formation of ER-mitochondria tethers. Mfn-2 ablation increases the distance between the ER and mitochondria, and this results in a reduced Ca^{2+} influx into mitochondria in response to IP_3 in both HeLa cells and mouse embryonic fibroblasts [134, 142]. Moreover, ablation of Mfn-2 leads to metabolic disorders such as glucose intolerance and impaired insulin signaling in both liver and muscle [143]. Conversely, Pozzan's group has demonstrated that silencing of Mfn-2 increases contact sites between the ER and mitochondria and augments mitochondria Ca^{2+} uptake. These data suggest that Mfn-2 appears to have a protective role because it forms a tether that prevents Ca^{2+} overload into the mitochondria [130]. Although both groups have shown that Mfn-2 is a molecular tether in the ER-mitochondria contact site, silencing this protein produces the opposite effects on Ca^{2+} signaling. Interestingly, a picture is emerging where Mfn-2 is able to increase the number of contact sites between the ER and mitochondria when they are low and it does the opposite when these sites are high. Further work is needed to understand the role played by Mfn-2 in Ca^{2+} transfer from the ER to mitochondria.

14.6.3 Mitocondrial Calcium Uniporter (MCU) Is a Finely Regulated Inner Mitochondrial Membrane Ion Channel

Ca^{2+} transfer from the ER to mitochondria is an essential event that regulates cell bioenergetics by increasing the activity of different Krebs cycle dehydrogenase; such as pyruvate dehydrogenase, isocitrate dehydrogenase and α-ketoglutarate

dehydrogenase [144]. This Ca^{2+} entry in the mitochondria matrix involves the activation of Ca^{2+} release channels from the ER, mainly the IP_3R [129]. Although the closeness between the ER and mitochondria should lead to mitochondria Ca^{2+} transient before that in the cytoplasm, it turns out that this is not the case. Indeed, GECIs targeted to mitochondria and the ER in combination with fura-2 to detect changes in cytoplasmic $[Ca^{2+}]$ have shown that despite mitochondria being close to the ER, the cytosolic Ca^{2+} increases well before it does in mitochondria [110], suggesting that closeness between these two organelles is not enough to elevate Ca^{2+} in mitochondria. Alternatively, mitochondria might have mechanisms that prevent an immediate Ca^{2+} entry in the mitochondrial matrix. The molecular identification of the Mitochondrial Ca^{2+} Uniporter or MCU [145, 146] as the mitochondria Ca^{2+} permeable channel for Ca^{2+} entry to the mitochondria matrix, has demonstrated that this channel is indeed a protein complex formed by different types of proteins, such as EMRE, MCUb and members of the MICU family [147]. MICU1, MICU2 and MICU3 are EF-hand proteins that inhibit MCU activity at low $[Ca^{2+}]_i$ (around 500 nM), thus acting as a channel gatekeeper that prevents mitochondria Ca^{2+} overload. Remarkably, MICU1 silencing leads to neurologic and muscular problems during development [148]. That is, these proteins act as natural inhibitors of MCU that block Ca^{2+} entry into the mitochondria. This might explain the mitochondria delayed Ca^{2+} increase after activation of IP_3R and increase of cytoplasm $[Ca^{2+}]$. In this regard, it has been shown that histamine, which produces a sustained Ca^{2+} release event, leads to a robust Ca^{2+} entry in mitochondria; however, glutamate, which produces a transient Ca^{2+} release event due to deactivation of IP_3Rs, produces a much smaller Ca^{2+} increase in mitochondria matrix [149]. These data suggest that Ca^{2+} entry in mitochondria requires more a sustained Ca^{2+} release from the ER than a localized elevation of cytoplasmic $[Ca^{2+}]$. Collectively, a picture is emerging showing that closeness between the ER and mitochondria is necessary but not sufficient for an elevation of the mitochondria $[Ca^{2+}]$. Moreover, an elevation of the mitochondrial $[Ca^{2+}]$ can trigger apoptosis [150]. In conclusion, the Ca^{2+} transfer from the ER to the mitochondria is important for cell respiration and for tuning ATP production while at the same time could lead to an apoptotic event. It is extremely complex how the same signal, i.e. Ca^{2+} ions, results in so divergent cell responses. Thus, essential pieces of the puzzle are still missing to fully unravel the role of Ca^{2+} in the interaction between the ER and mitochondria.

14.6.4 The Connection Between the ER and Lysosomes

The lysosome is another organelle that associates with the ER [23]. This organelle is vesicular in nature, it is filled with hydrolytic enzymes and characterized by having an extremely acidic luminal pH, around 5.0 [151]. This acidic pH is generated by the activity of a V-type proton ATPase [152, 153] and it has been considered that the activity of this pump is essential for the Ca^{2+} accumulating activity of

lysosomes [154, 155]. However, studies using Ca^{2+} indicators targeted directly to lysosomes and agonists for TRPML1 channel in lysosomes, have found that the acidic luminal pH is not essential for lysosomes to accumulate Ca^{2+} but that a still undefined mechanism allows lysosomes to accumulate Ca^{2+} that has been released by IP_3Rs from the ER [11, 156]. The dynamics of the ER and lysosomes has been recently observed with high spatial and temporal resolutions and it appears that lysosomes are able to reshape the ER [23]. However, it is still a long way to understand the regulation of Ca^{2+} transfer from the ER to lysosomes in the autophagic process [157]; although the presence of high spatial and temporal super-resolution microscopes, as GI-SIM [23], would make easier to unravel the role played by Ca^{2+} both in the lysosomes and in the ER in the activity of these acidic organelles.

14.7 Concluding Remarks

All these studies reviewed here allow us to depict a picture where an efficient Ca^{2+} release event from the SR/ER requires the activity not only of release channels, but also of SERCA pumps and the luminal Ca^{2+} binding proteins. Additionally, it is clear that the free luminal SR/ER $[Ca^{2+}]$ cannot predict the amount of Ca^{2+} that will be released to the cytoplasm, the most likely explanation is that the Ca^{2+} released during the activation of release channels involve the participation of luminal Ca^{2+} binding proteins that trap Ca^{2+} next to the release channels and that the formation of this complex requires active SERCA pumps. This scenario could explain several situations of the Ca^{2+} release event, for instance, why the amplitude of the Ca^{2+} release event in the cytoplasm does not show any correlation with the reduction in the free luminal SR/ER $[Ca^{2+}]$? Why the increase in the $[Ca^{2+}]_i$ during Ca^{2+} release is associated with a minimal reduction in the bulk free luminal SR/ER $[Ca^{2+}]$? Why is the refractory period for Ca^{2+} release longer than the recovery of the free luminal SR/ER $[Ca^{2+}]$? It is clear that we are still far from understanding how release channels produce an efficient Ca^{2+} release event but the development of the GI-SIM superresolution microscope should help [23]. This microscope has both enhanced spatial and temporal resolutions and can be used with the current Ca^{2+} indicators, so it should be easier to follow changes in the $[Ca^{2+}]$ of both the lumen of the ER and the cytoplasm to gather a better picture on how the Ca^{2+} lattice produces an efficient Ca^{2+} release event. Additionally, it is clear that the SR/ER is the main Ca^{2+} source, not only for the cytoplasm, but also for other organelles as mitochondria and lysosomes. In conclusion, it appears that an efficient Ca^{2+} release event occurs only during the initial Ca^{2+} release process and that this requires the participation of release channels, luminal Ca^{2+} binding proteins and the SERCA pump. This means that the communication among all these elements makes the Ca^{2+} release event more complex than previously envisioned but very

robust because it can successfully fulfill the role of SR/ER as Ca^{2+} source without interfering with the need of having a high luminal SR/ER [Ca^{2+}].

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