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Characteristics and Possible Functions of Mitochondrial Ca^{2+} Transport Mechanisms

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Abstract

Mitochondria produce around 92% of the ATP used in the typical animal cell by oxidative phosphorylation using energy from their electrochemical proton gradient. Intramitochondrial free Ca^{2+} concentration ($[Ca^{2+}]_m$) has been found to be an important component of control of the rate of this ATP production. In addition, $[Ca^{2+}]_m$ also controls the opening of a large pore in the inner mitochondrial membrane, the permeability transition pore (PTP), which plays a role in mitochondrial control of programmed cell death or apoptosis. Therefore, $[Ca^{2+}]_m$ can control whether the cell has sufficient ATP to fulfill its functions and survive or is condemned to death. Ca^{2+} is also one of the most important second messengers within the cytosol, signaling changes in cellular response through Ca^{2+} pulses or transients. Mitochondria can also sequester Ca^{2+} from these transients so as to modify the shape of Ca^{2+} signaling transients or control their location within the cell. All of this is controlled by the action of four or five mitochondrial Ca^{2+} transport mechanisms and the PTP. The characteristics of these mechanisms of Ca^{2+} transport and a discussion of how they might function are described in this paper.

Keywords

mitochondria; calcium uptake; calcium efflux; calcium signaling; permeability transition; reactive oxygen species

Introduction

Since the discovery of transport of Ca^{2+} by mitochondria from mammals and other higher vertebrates in the early 1960's [1,2], we have learned a considerable amount about how Ca^{2+} is transported into and out of mitochondria, and sometimes have been able to infer why it is done as it is. Mitochondria in higher eukaryotes today carry out many functions (cytosolic $[Ca^{2+}]$ buffering, partial control of apoptosis [3,4], β oxidation of fatty acids [5], role in the urea cycle [5], role in synthesis and metabolism of iron-containing proteins [6,7], etc.) in

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addition to oxidative phosphorylation. However, production of ATP by oxidative phosphorylation was surely the initial and is still the primary function of mitochondria. Oxidative phosphorylation produces around 92% of the ATP used in the typical mammalian cell. In times past, production of sufficient ATP in times of stress must have exerted considerable evolutionary pressure on the rate limiting steps of oxidative phosphorylation so that these steps have been accelerated by evolutionary change to a point where there is no single rate limiting step. Today, rate limitation is shared by a number of steps [8–10], which has led to a situation in which activation of a single partially rate limiting step doesn't do much to increase the rate of the overall process. Yet it is vital to all life to produce and use ATP slowly when only a little energy is needed but to be able to produce and use it very quickly to escape danger. Therefore, it is crucial to be able to control the rate of oxidative phosphorylation. What is needed to do this is a single signal which can accelerate all of the partially rate limiting steps simultaneously and a transient increase in intramitochondrial free Ca^{2+} concentration ($[Ca^{2+}]_m$) is a very good choice. Ca^{2+} is one of the most common second messengers, modulating many processes which increase the use of energy including muscle contraction. By using the same type of signal, a transient increase in intracellular and intramitochondrial $[Ca^{2+}]$, to increase both the use of energy and energy production, evolution produced what may be the world's first on time delivery system. For more complete descriptions of the arguments which underlie these concepts see earlier reviews [11–13].

We have known for some time of the elaborate set of mechanisms and processes controlling Ca^{2+} transport both inward and outward across the mitochondrial inner membrane – three mechanisms or modes of influx and two of efflux. There is also the mitochondrial permeability transition (MPT) mediated by the permeability transition pore (PTP), which is Ca^{2+}-induced and makes the membrane leaky to all small, freely-diffusible ions and molecules [14–17]. The most studied of the transport mechanisms is the mitochondrial Ca^{2+} uniporter, the mechanism mediating the Ca^{2+} influx which led to the initial discovery of Ca^{2+} uptake by mitochondria [1,2]. Another mechanism or mode of uptake is called the rapid mode or RaM, which was discovered much more recently and has received the least attention of all of these transport mechanisms [18]. The third Ca^{2+} uptake mechanism is the mitochondrial ryanodine receptor (mRyR) that was identified recently in excitable cells [19]. The two mechanisms of Ca^{2+} efflux are the Na^+-dependent and the Na^+-independent mechanisms, which were discovered in the 1970's[20,21]. During the years when these transport mechanisms were discovered and initially studied, it was felt that the outer mitochondrial membrane was freely permeable to small ions through the voltage dependent anion channel (VDAC); however, more recent data has suggested that VDAC can provide a barrier to free diffusion under some conditions [22]. Curiously, VDAC provides a higher Ca^{2+} permeability in the closed states [23] Finally, the MPT, also discovered in the 1970's [14–16,24–26], was initially associated with pathological consequences, but is today viewed as possibly also having physiological roles.

Isolated Mitochondria vs Mitochondria in Cells

We would like to understand how Ca^{2+} affects mitochondria in the living animal, but even studies in tissue are very difficult. Partly because there were no convenient probes or techniques available prior to the mid 1980's that allowed studies of these mechanisms at the cell or tissue levels, the earlier work in identifying mitochondrial Ca^{2+} transport mechanisms was carried out using isolated mitochondria, usually from liver, heart, or brain. Today, with the development of many fluorescent probes, which can be selectively directed to specific parts of the cell, including the mitochondria, endoplasmic reticulum, etc., most of the work is being carried out at the cell level [27]. However, there are advantages and disadvantages to working at the isolated mitochondrial and at the cell levels that should be considered logically. An obvious advantage of working at the cell level over the isolated mitochondrial level is that mitochondrial structures and the concentrations of components of medium around the

mitochondria are more like those in vivo than they are with an isolated mitochondrial suspension. However, they are not necessarily the same as those in vivo. The metabolism of isolated cells in culture, particularly of immortalized cell lines, is often very different from that of similar types of cells in vivo [28–31]. It is common for transformed cell lines to produce more ATP through glycolysis than similar types of cells would in tissue. There are other organelles and structure at the cell level that could show important interactions with mitochondria; however, even this cell structure may be modified from that found in vivo. Concentrations of trace constituents of the intracellular medium are undoubtedly more similar to those found in vivo than to the artificial media used with isolated mitochondria. As an example of a component that can significantly modify the behavior of both the uniporter and the RaM, physiological levels of spermine or other polyamines [32–34] can cause large changes in the transport characteristics of these mechanisms. These polyamines are always present in experiments carried out in cells, but with a few exceptions, they haven't been in experiments with isolated mitochondria. A major negative factor that affects all work with isolated mitochondria is that the process of isolating mitochondria from tissue not only selects for healthy mitochondria, but also breaks up filaments of mitochondria which then reseal in a non physiological way. While this resealing is rapid, there may be some deenergization and exchange of internal for external medium involved in this process. We know that mitochondrial fission and fusion are normal physiological processes that occur continually with mitochondrial populations inside of cells [35,36]; however, the fragmentation which takes place during mitochondrial isolation is different from that which takes place physiologically.

Not all of the advantages lie with work at the cell level, however. Even where fluorescent probes can be successfully targeted to specific organelles such as mitochondria, only free ion concentration can be estimated through the fluorescence techniques. We refer to the results of measuring [Ca^{2+}] in mitochondria inside cells as estimates because it is very difficult to accurately calibrate the K_d for binding of Ca^{2+} to the fluorescent probe inside of mitochondria inside cells, particularly for nonratiometric probes [37]. It is sufficiently difficult that very few investigators in the field even attempt to do this. Absolute errors in concentration can be over a factor of two [37]. By using radioactive isotopes as well as fluorescent probes, both free and total ion concentrations can be measured. This provides easily measured, direct transport data with isolated mitochondria, while this type of experiment is much more difficult and contains more caveats with intact cells. This can be an important advantage for working with isolated mitochondria. Other types of measurements such as membrane potential, oxidation rate, intramitochondrial pH, etc. are also valuable for an understanding of mitochondrial Ca^{2+} influx or efflux and these measurements can often be made simultaneously and much more accurately with a larger variety of techniques with isolated mitochondria than with mitochondria within cells. As a result, a considerable amount of quantitative data is available from the literature from measurements on isolated mitochondria while measurements on mitochondria within cells are generally more qualitative. This quantitative data is very useful for calculating estimates of necessary energies or effects of processes that are difficult to measure directly in intact cells. While more recent data at the intact cell level has provided very important insights and modifications to the earlier data obtained from experiments on isolated mitochondria, particularly with respect to the interactions of mitochondria with endoplasmic reticulum and other cell structures [27,38], the concepts of mitochondrial Ca^{2+} transport today are still similar to the concepts drawn from the isolated mitochondrial data. At the very least, measurements on isolated mitochondria tell us what type of behavior can be mediated by the mitochondria themselves, independently of the presence of the rest of the cell, e.g. very rapid uptake through the RaM mechanism. It is useful to have both types of information and to evaluate results with the limitations of the techniques used in mind.

Characteristics of the Influx Mechanisms: *The Uniporter*

The mitochondrial Ca^{2+} uptake mechanisms appear reversible; however, it is still proper to refer to them as "influx mechanisms" unless the mitochondria are deenergized because of the effects of the strong, internally negative mitochondrial membrane potential on the direction of transport.

Mitochondria from all vertebrate organs tested show Ca^{2+} uptake via a mechanism which has similar characteristics in all of these types of mitochondria, those of a Ca^{2+} uniporter [17]. A uniporter is a mechanism which facilitates passive transport of Ca^{2+} down its electrochemical gradient without coupling Ca^{2+} transport to the transport of another ion. This mode of transport begins during embryonic development at a time that is species- and possibly organ-dependent [39]. Mitochondria in yeast, plants and lower animal species are also often able to sequester Ca^{2+}; however, the characteristics of transport in these species can differ significantly from those of the mitochondrial Ca^{2+} uniporter of higher animals [40]. Recognition that this mechanism of Ca^{2+} uptake was a uniporter came in stages. The first was Mitchell's recognition that the mitochondrial inner membrane pumped protons outward to produce an electrochemical proton gradient composed of a large internally negative membrane potential and an internally alkaline pH gradient [41,42]. An internally negative membrane potential could also be produced by ATP hydrolysis on the F_1 ATPase or by efflux of intramitochondrial K^+ using the ionophore valinomycin. Both of these means of producing an internally negative potential also induced Ca^{2+} uptake by the mitochondria [43,44]. In an elegant series of swelling experiments, that identified the combinations of ions which, with Ca^{2+}, would permit swelling under passive conditions, Selwyn et al. showed that the influx of Ca^{2+} was not directly coupled to the transport of another ion [45]. Finally, it was shown that the membrane potential dependence of Ca^{2+} uptake via this influx mechanism closely fit the predictions of the equations for electrochemical diffusion [17,46]. Taken together, these observations identified the transporter as a passive uniporter whose driving force is the electrochemical Ca^{2+} gradient

$$\left(\Delta\mu_{Ca}=RT\ln\left(\frac{[Ca^{2+}]_{out}}{[Ca^{2+}]_{in}}\right)+2F(\psi_{out}-\psi_{in})\right)\ [17]. \tag{1}$$

The initial measurements of the Hill coefficient (i.e. the number that gives the order of transport) of the uniporter gave sigmoidal plots of transport velocity vs $[Ca^{2+}]$ and showed that the mechanism was second order in $[Ca^{2+}]$ [47,48]. While there was some controversy over the value of 2 for the Hill coefficient, that value is generally accepted today partly because it is also strongly supported by the results of Vinogradov and Scarpa showing that the uniporter has both a transport site and a separate activation site [49]. These sites have different binding affinities and the activation site can be activated by other divalent ions and also by trivalent lanthanides, such as Pr^{3+}, or by Ca^{2+}. The sigmoidicity of the plot of transport velocity of Mn^{2+} vs $[Mn^{2+}]$ disappears, for example, in the presence of small amounts of Ca^{2+}, which bind more tightly to the activation site, activate transport of Mn^{2+}, and convert the plot to an apparent first order plot [49]. These results suggest that the $[Ca^{2+}]$ dependence of the mitochondrial Ca^{2+} uniporter is given by a second order Hill Eq. of the form

$$v=V'_{max}\frac{[Ca^{2+}]^2}{K^2_{0.5}+[Ca^{2+}]^2}, \tag{2}$$

where V'_{max} is a function of membrane potential. It is generally agreed that measured values of V'_{max} are lower than the actual value due to rate limitation by electron transport under conditions of very fast Ca^{2+} uptake [17]. The fastest reported V'_{max} (1750 nmol/mg/min) was measured in dog heart mitochondria prepared using the nagarse method [50]. There is also no real agreement in the published values of $K_{0.5}$ with values in the literature ranging from 1 to 189 μM [49,51–57]. However, since low values of $K_{0.5}$ are generally reported where the reported value of V'_{max} was also low, it is likely that the values of $K_{0.5}$ are distorted by the same problem affecting the reported value of V'_{max}. Therefore, it is likely that the true value of $K_{0.5}$ lies nearer to the upper end of the range of reported values.

The membrane potential dependence of Ca^{2+} uptake is also an important characteristic of uniporter behavior. Since a uniporter facilitates the transport of an ion down its electrochemical gradient without coupling it to the transport of any other ion, the membrane potential dependence should be consistent with the equations for electrochemical diffusion. Where $\Delta\varphi \equiv 2bF(\Delta\psi - \Delta\psi_0)/RT$, this membrane potential dependence has been shown in liver mitochondria to fit the form $[e^{\Delta\varphi/2}\Delta\varphi/2]\,[\sinh(\Delta\varphi/2)]$ (shown in Fig. 1), which is derived from the equations of electrochemical diffusion through the Goldman flux equation [17,46]. In the above, $\Delta\psi$ is the mitochondrial membrane potential in millivolts, F, R, and T are the Faraday constant, the gas constant and the Kelvin temperature, respectively, and b and $\Delta\varphi_0$ are fitting parameters, which for the set of data shown in Fig. 3 of Gunter and Pfeiffer [17], rapidly converge to values of 1 and 91 mV, respectively. While taking data for a recently published paper on mitochondrial proton leak in ischaemia/reperfusion injury [58], Paul Brookes' laboratory also measured the membrane potential dependence of the uniporter in heart mitochondria and found results very similar to those described above for liver mitochondria (P. S. Brookes, personal communication). The combined concentration and membrane potential dependence is then given by

$$v = V_{max}\left(\frac{[Ca^{2+}]^2}{K_{0.5}^2 + [Ca^{2+}]^2}\right)\left[e^{\Delta\varphi/2}\left(\frac{\Delta\varphi}{2}\right)\right]\left[\sinh\left(\frac{\Delta\varphi}{2}\right)\right]. \quad (3)$$

The uniporter is Ca^{2+} selective but also transports other cations with a selectivity series $Ca^{2+} > Sr^{2+} > Mn^{2+} > Ba^{2+} > La^{3+}$ [59–63]. Lanthanides such as La^{3+}, Gd^{3+} and Pr^{3+} are only transported very slowly and act as effective competitive inhibitors although they may activate the uniporter's activation site at low concentrations and speed up transport of other ions [49, 63–65]. The most commonly used inhibitor is the hexavalent polysaccharide stain, ruthenium red [66]. Ruthenium red, which is often used as a stain for glycoproteins and binds to many Ca^{2+} binding sites, is actually a set of related ruthenium compounds [67]. Some of the components of this set are more effective in inhibiting mitochondrial Ca^{2+} uptake than others. One of them, Ru360, is available in purified form and is over an order of magnitude more effective than ruthenium red in inhibiting mitochondrial Ca^{2+} transport [68]. A number of drugs, such as the cardioactive drugs quinidine, alprenolol, propranolol, oxyfedrine, and tetracaine [69], the diuretic, ethacrynic acid, amiloride analogs and derivatives [70], and the antibiotic gentamicin [71] have also been shown to inhibit Ca^{2+} uptake via the mitochondrial Ca^{2+} uniporter. The uniporter has recently been reported to be regulated by cytosolic Ca^{2+} [72,73], and inactivated by a sustained $[Ca^{2+}]_c$ increase. This inactivation is increased by matrix acidification by proton influx through the F_0 component of the F_1F_0 ATP synthase and reduced by matrix alkalinazation [74].

Inorganic phosphate (Pi) can speed the rate of uptake of Ca^{2+} via the Ca^{2+} uniporter. It does this partly because it precipitates with Ca^{2+} in the mitochondrial matrix, lowering the free

Ca^{2+} concentration there, and partly because the loss of H^+ from the Pi in the mitochondrial matrix makes proton pumping by the electron transport chain easier and rebuilds the membrane potential more rapidly; however, Pi has also been found to increase the rate of Ca^{2+} uptake even at constant membrane potential [75]. At lower Ca^{2+} concentrations, polyamines such as spermine and spermadine significantly increase the rate of Ca^{2+} influx through the uniporter [32]. The amount of these polyamines varies somewhat between cell types, being approximately 1 mM in the cytoplasm of hepatocytes and about one third of this level in the cytoplasm of cardiac myocytes [76].

Isolation and purification of the mitochondrial Ca^{2+} uniporter would open many new and important approaches to mitochondrial research such as to interesting molecular biological experiments. Therefore, there have been numerous efforts to isolate and purify the mitochondrial Ca^{2+} uniporter but unfortunately without success. The primary difficulty is that the uniporter represents a very small fraction of the protein in the mitochondrial inner membrane. While the components of the electron transport chain may be present in amounts of nanomoles of each component per mg of mitochondrial protein, estimates of the amount of the uniporter from titration studies with lanthanum and ruthenium red are around 0.001 to 0.01 nmole/mg protein [77,78]. Probably the most important conclusion reached in 35 to 40 papers published on attempts to isolate and purify the uniporter is that the uniporter or at least a major component is probably a glycoprotein in the molecular weight range between 33 and 42 kD [79,80]. For the purposes of this review, it is best to provide references in which this literature is reviewed more extensively earlier [17,81] and discuss only the most recent work in this area.

Wolfgang Graier's laboratory has recently published very interesting results in which they report that overexpression of the uncoupler proteins UCP2 or UCP3 in short term cultured human umbilical vein endothelial cells and other cell types results in an increase in mitochondrial Ca^{2+} uptake. Specifically, exciting these endothelial cells in which either UCP2 or UCP3 were overexpressed with histamine produced Ca^{2+} transients. Following this, the ratiometric mitochondrial Ca^{2+} indicator, pericam, reported 33% increased mitochondrial Ca^{2+} over that in control cells for UCP2 overexpression and 76% increased mitochondrial Ca^{2+} over control for UCP3 overexpression [82]. The strong implication of these data and further controls is that increasing the amount of UCP2 or UCP3 in the mitochondria significantly increases the activity of the Ca^{2+} uniporter [82]. Furthermore, this uptake also appeared to be sensitive to inhibition by ruthenium red. However, expression of UCP2 or UCP3 in cells of the yeast, *saccharomyces cerevisiae*, did not cause mitochondria isolated from these cells to show ruthenium red sensitive Ca^{2+} uptake, showing that more than UCP2 or UCP3 alone were necessary to induce the ruthenium red sensitive Ca^{2+} uniporter in mitochondria [82]. The results of Trenker et al. have been challenged by Brookes et al. [83] on a number of grounds, including new data, and have been defended by the original authors in a new set of papers. Several of the points made by Brookes et al. were 1) that if the conclusions of Trenker et al. were correct, the primary effect would be on the rate of Ca^{2+} uptake, while the data of Trenker et al. primarily showed effects on increased $[Ca^{2+}]_m$, but minimal effects on uptake rate, 2) that in other systems, overexpression of UCP2 and UCP3 had resulted in a nonfunctional uncoupled phenotype thought to be due to misfolded proteins, 3) that the results of Trenker et al. suggested that only part of the activity of the Ca^{2+} uniporter was ruthenium red inhibitable while both Brookes' data and earlier data showed that all of the uniporter's activity was ruthenium red inhibitable, and 4) in new data submitted by Brookes et al. there was no effect of UCP2 or UCP3 knockout on either Ca^{2+} uptake or membrane potential depolarization in mitochondria from liver, skeletal muscle, heart, or kidney. Furthermore, the wide variation in UCP expression between these tissues would be expected to result in a matching variation in Ca^{2+} uniporter activity between tissues, but this was not the case.

Patch clamping of mitoplasts from Cos-7 cells has revealed an inwardly-rectifying ion channel with most of the properties of the Ca^{2+} uniporter [84]. For example, this channel is inhibited by ruthenium red and ruthenium 360 and shows the selectivity series of the uniporter [84]. This type of measurement permits higher precision and accuracy than the older measurements using intact mitochondria and provides the additional findings that this channel has multiple subconductance states between 2.6 and 5.2 pS, a $K_{0.5}$ of 19 mM, and a maximum ion flux of 5×10^6 Ca^{2+}/sec, which is considerably higher than that estimated earlier for the uniporter [17,84]. These results will be discussed further after a discussion of the properties of the rapid mode of uptake or RaM.

Satrustegui's laboratory and others have investigated Ca^{2+} binding motifs of mitochondrial proteins using molecular biological techniques in an effort to identify important Ca^{2+} binding sites and transporters [85]. A recent, excellent review of this work is now available [85].

The mitochondrial Ca^{2+} uniporter is a relatively fast mechanism. Because of the low number of Ca^{2+} uniporters found per mg protein (around 0.001 nmoles/mg protein reported [77]) and the large value of the estimated V_{max} [approximately 1200 nmol Ca^{2+}/(mg·min)] the turnover of Ca^{2+} per site can be calculated to be about 2×10^4 Ca^{2+}/(site·sec) [17]. This is a little smaller than that estimated by Brahm [86] for the anion exchanger of the erythrocyte membrane of around $4–5\times10^4$ ions/(site·sec) for a mechanism which is believed to be a gated pore. However, the turnover per site of the mitochondrial Ca^{2+} uniporter is orders of magnitude slower than the turnover per site estimated by Brahm [86] for K^+ transport through the gramicidin channel [2×10^7 ions/(site·sec)] or through the urea transporter of the red blood cell (1×10^7 molecules/(site·sec), which are thought to be simple channels. In terms of turnover per site, the Ca^{2+} uniporter behaves more like a gated pore.

The RaM

By the late 1980's, it was clear from experiments using fluorescent Ca^{2+} indicators such as fura-2, that Ca^{2+} signaling in the cell cytosol was not via shifts in the steady state level of intracellular [Ca^{2+}] ($[Ca^{2+}]_c$) but via transient changes in $[Ca^{2+}]_c$. After exposure of many types of cells to hormones or other factors, the cells would respond by passage of a change in $[Ca^{2+}]_c$ from a low level of 85 to 100 nM to a much higher level (up to levels above 1 μM) which could vary over the volume of the cell. The characteristics of these Ca^{2+} transients, or pulses, varied somewhat from cell type to cell type and also varied with the type and concentration of the activating agent. Nevertheless, over many types of cells and activating agents, the average $[Ca^{2+}]_c$ only varied from around 500 nM to about 1000 nM. In special locations, however, such as near the release sites from intracellular stores (microdomains), the concentrations could be much higher [87–89]. The RaM was discovered through experiments designed to determine how mitochondria responded to transients of Ca^{2+} like those seen in the cytosols of many types of cell [18,33].

In order to study mitochondrial uptake of Ca^{2+} from Ca^{2+} transients, a computer-controlled automatic pipettor was set up to alternately deliver a pulse of buffered Ca^{2+} and then a strong Ca^{2+} chelator, producing a pulse of free Ca^{2+} concentration in a cylindrical cuvette in the sample chamber of a fluorescence spectrometer. Using rapid mixing with this apparatus, one could generate pulses or transients of free [Ca^{2+}] in the cuvette like those seen in the cytosols of cells [18]. By generating "square wave" pulses and using a small amount of $^{45}Ca^{2+}$ in the Ca^{2+} injected into the cuvette, one could measure the [Ca^{2+}] to which the suspension of mitochondria were exposed through the fluorescent Ca^{2+} indicator, fura-2, and after ending the pulse with the Ca^{2+} chelator, could separate the mitochondria in the cuvette from the supernatant and measure Ca^{2+} uptake into the mitochondria by measuring the $^{45}Ca^{2+}$ in the mitochondria. By varying the length of the square wave pulses at each [Ca^{2+}], a plot like that shown in Fig. 2 could be obtained. By putting a line of best fit through data for each [Ca^{2+}],

several interesting observations could be seen. First, the slopes of the lines of best fit gave an accurate representation of the velocity of the Ca^{2+} uniporter under each set of conditions that was consistent with older results. Second, the uniporter didn't transport Ca^{2+} when the [Ca^{2+}] was below about 200 nM. Look at the slope of the line of fit to the data taken at [Ca^{2+}] = 165 nM; it has a slope of zero. To within the accuracy of the data this zero slope was observed for data where the [Ca^{2+}] was up to between 200 and 250 nM. This is an important result that is often overlooked. Third, something different from uniporter kinetics must have occurred prior to the first time point at each [Ca^{2+}], because instead of the lines of fit going through zero Ca^{2+} uptake at zero time, they intersect the ordinate at a positive value indicating uptake at zero time. Since, clearly no uptake can occur with zero time of exposure to Ca^{2+}, we have to interpret this as saying that some rapid process has occurred before the first time point. From the nature of the experiment there can be only three possible explanations. First, rapid binding of labeled Ca^{2+} to the mitochondrial exterior could cause this type of effect; however, that should have been eliminated by the Ca^{2+} chelator added to end the square wave pulse. Second, since exchange can be faster than net transport on some transporters, this effect could be caused by rapid exchange of external labeled Ca^{2+} for internal unlabeled Ca^{2+} by the Ca^{2+} uniporter. Third, rapid net transport could also be the explanation. These possibilities were tested in a series of experiments described by Sparagna et al. which showed that the results were caused by rapid net uptake [18].

This rapid net uptake was referred to as the rapid mode of uptake or RaM. Experiments have established many of the characteristics of the RaM in liver and heart mitochondria [18,33,34] and some of its characteristics in brain mitochondria [12]. Uptake of Ca^{2+} via the RaM showed strong similarities in liver and heart mitochondria, but also showed significant differences. In both liver and heart mitochondria it was found that binding of Ca^{2+} to an external binding site at a [Ca^{2+}] above about 140 to 160 nM inhibited further uptake via the RaM mechanism after a brief period of rapid uptake [33,34]. The presence of physiological levels of spermine greatly increased Ca^{2+} uptake via the RaM with liver mitochondria [33] and also increased Ca^{2+} uptake via the RaM with heart mitochondria [34]. Ruthenium red inhibited Ca^{2+} uptake via the RaM in both liver and heart mitochondria, but its effects with heart mitochondria were more complex [33,34]. After Ca^{2+} uptake via the RaM was inhibited by Ca^{2+} binding to the external inhibition site, the Ca^{2+} could be removed from the inhibition site in liver mitochondria in less than a second permitting further Ca^{2+} uptake via the RaM mechanism whereas it took more than a minute to completely remove the Ca^{2+} bound to the inhibition site in heart mitochondria [33, 34]. Keep in mind, however, that there is still a significant amount of Ca^{2+} uptake via the RaM in heart mitochondria for another pulse a second or less after an earlier pulse. The Ca^{2+} uptake via RaM per pulse (as much as 8 nmoles/mg per 600 nM pulse) was much greater for liver mitochondria than that for heart mitochondria; however, because there are many more Ca^{2+} pulses per unit time in heart than in liver, the uptake via RaM per unit time is not so different between these two types of mitochondria [33,34]. This amount of Ca^{2+} uptake per pulse via the RaM of liver mitochondria is significantly greater than the amount necessary to activate the intramitochondrial Ca^{2+}-dependent metabolic reactions which activate ATP production, estimated by McCormack to be around 4 nmoles/mg protein [90]. By using "caged Ca^{2+}" to produce shorter pulses of free Ca^{2+} concentration and a more sophisticated system for fluorescence detection and data storage and analysis than was used in the experiments described above, the period of Ca^{2+} uptake via the RaM was shown to be completed in 25 to 30 msec, making the velocity of Ca^{2+} uptake via the RaM mechanism approximately 1000 times faster than that via the uniporter (Fig. 3) [81]. If the number of RaM transporters is similar to the number of uniporters, this suggests a turnover per site similar to those of the simple pores described by Brahm [86].

Additional studies were carried out by Paul Territo in Robert Balaban's laboratory, using a different system for generating pulses of free Ca^{2+} concentration with isolated pig heart

mitochondria. In this study, Ca^{2+} uptake was measured using an intramitochondrial fluorescence indicator, Rhod-2, NADH production using NADH fluorescence, and fast oxygen use by calibrating hemoglobin as an O_2 indicator. The time resolution of the pulse generating system was around 100 msec [91]. These results showed 1) rapid Ca^{2+} uptake within the 100 msec time resolution of the system, activation of NADH production within 200 msec and rapid use of O_2 for oxidative phosphorylation within 270 msec [91]. In addition, these results showed that these mitochondria responded by rapid oxidative phosphorylation more quickly to Ca^{2+} addition than to any other stimulus (such as ADP addition) and responded more strongly to addition of 535 nM pulses of Ca^{2+} than to either higher or lower concentrations of Ca^{2+} [91]. These results clearly demonstrate a rapid response like that induced by Ca^{2+} uptake via the RaM, and show that the heart mitochondrial system responds very strongly to pulses of average size within the cytosol. With RaM uptake, it is not necessary for the $[Ca^{2+}]_c$ to reach high levels (50 μM) like those within microdomains [87–89] to observe the strongest response. These results show that in heart as well as liver, rapid Ca^{2+} uptake from average sized Ca^{2+} pulses can activate oxidative phosphorylation very rapidly. By this means, all of the mitochondria within a cell can be activated to produce ATP at a faster rate not just those in microdomains near the Ca^{2+} release sites of endoplasmic (ER) or sarcoplasmic reticulum (SR).

The observations described above [33,34,91] are not the only ones which have reported rapid Ca^{2+} uptake by mitochondria. For example, Gallitelli et al. using flash-freezing techniques with electron probe microanalysis observed rapid mitochondrial Ca^{2+} uptake in myocytes [92]. Their data suggest that Ca^{2+} uptake maximizes around 20 ms after the peak of the cytosolic pulse consistent with the short burst of Ca^{2+} uptake seen via the RaM mechanism [81,92]. Other observers using a variety of cell types with fluorescent probes located in the mitochondria have also provided evidence for rapid Ca^{2+} uptake [93–95].

Another characteristic of the RaM which has been observed in data from mitochondrial Ca^{2+} uptake in cells is mitochondrial uptake when cytosolic $[Ca^{2+}]$ is low. As discussed above and as shown in Fig. 2, the uniporter doesn't transport Ca^{2+} at concentrations below a threshold in the 200 to 250 nM range, while the RaM can transport Ca^{2+} in this concentration range. Several laboratories have published evidence for mitochondrial Ca^{2+} uptake where the extramitochondrial concentration of Ca^{2+} was around 200 nM [96–101]. Furthermore, in some cases activation of NADH production has been shown to follow this uptake from low cytosolic Ca^{2+} concentrations [98,99,101]. Clearly, it is not necessary that mitochondria be in microdomains in order to observe mitochondrial Ca^{2+} uptake and activation of the steps of ADP phosphorylation [102].

The results on Ca^{2+} uptake via the RaM and uniporter mechanisms and also the existence of the mitochondrial ryanodine receptor, discussed below, beg the question of why two or more mechanisms of uptake are necessary and what the role of each may be. This will be discussed after describing the efflux mechanisms, the MPT, and production of reactive oxygen species (ROS).

The Mitochondrial Ryanodine Receptor

The identification of a ryanodine receptor (RyR) in the inner membrane of mitochondria (IMM) of cardiac muscle cells represents a potential third mechanism of mitochondrial Ca^{2+} import [19,103,104]. The simple explanation for finding RyR in these mitochondria, that mRyR is a contaminant from sarcoplasmic reticulum (SR), was investigated very carefully. The approach was to use multiple techniques whose strengths complemented each other to rule out significant SR contamination. These techniques included 1) immunogold particle labeling of RyR in isolated mitochondria with confirmation of its existence by electron microscopy, 2) Western blot analysis to substantiate the specific detection of RyR-like protein in IMM preparations, 3) a binding assay with [^{3}H]ryanodine to isolated heart mitochondria, 4) detection of ryanodine

or dantrolene sensitive mitochondrial functions such as Ca^{2+} uptake, Ca^{2+}-dependent O_2 consumption, swelling, and cytochrome c release, 5) patch clamping of mitoblasts and recording a ryanodine-sensitive single channel conductance, 6) RT-PCR verification for the mRNA of all three RyR subtypes in cardiac muscle cells, 7) subtype-specific antibody detection of RyR1 in mitochondria from rat and mouse hearts but not in mitochondria isolated from RyR1 knock out mice, and 8) reconstitution of sucrose-purified mitochondrial fractions into lipid bilayers yielding large conductance (500–800 pS) channels with signature properties of RyRs. This mechanism was not found in liver mitochondria. Recent reports from other laboratories have also suggested the existence of RyR in mitochondria [105,106]. One report used type-specific antibodies, immunogold particles, and electron micrographs and demonstrated RyR3 present on the cristae of mitochondria in sinus endothelial cells [105]. Another used immunogold particles with monoclonal antibodies that recognized RyRs. Electron micrography indicated the existence of RyRs in the zone of perinuclear mitochondria [106].

[^{3}H]ryanodine binding to isolated heart mitochondria showed high affinity (K_d = 9.8 nM). This binding was modulated by Ca^{2+} and inhibited by Mg^{2+}(IC_{50} = 0.33 mM) and ruthenium red (IC_{50} = 105 nM). Interestingly, it also showed the bell-shaped Ca^{2+} dependency of [^{3}H] ryanodine binding with a half activation concentration of approximately 2μM, peaked at 40 μM and inactivated at higher Ca^{2+} concentrations [19,103,104]. The bell shaped Ca^{2+} dependency of [^{3}H]ryanodine binding suggests that at sub to low μM concentrations, mRyR becomes activated. However, at higher concentrations (> 50 μM), the ranges of [Ca^{2+}] that favor activation of the Ca^{2+} uniporter, mRyR becomes inactivated. As discussed below, Ca^{2+} concentrations in the microdomains between ER/SR and mitochondria could fluctuate between less than 1 μM and up to 100 μM during cytosolic Ca^{2+} transients. In the lower half of this range of [Ca^{2+}] (<50 μM), mRyR would be activated, while in the higher half of this range ([Ca^{2+}] > 50 μM), mRyR would be inactivated. Therefore, the mRyR are poised to take up Ca^{2+} from transients in the microdomains more effectively than the mitochondrial Ca^{2+} uniporter.

Work on the characterization of mRyR is still in its infancy in comparison to what has already been done on SR-RyRs. Two fundamental questions that are still to be addressed are 1) is the mRyR modulated by mitochondrial proteins such as cyclophilin D (cyp D) and VDAC?, and 2) how is the mRyR targeted to the mitochondrial inner membrane? In the future, work using RyR knock out or knock in mice to study the molecular properties and functional significance of mRyR will help to move this field forward.

Mechanisms of Efflux: *Energy Considerations*

Both a Na^+-dependent and a Na^+-independent mechanism of efflux are found in mitochondria. While the Na^+-independent efflux mechanism is dominant in liver and kidney mitochondria [20,107], these mitochondria also contain some Na^+-dependent efflux [17]. Similarly, mitochondrial Ca^{2+} efflux in heart and brain mitochondria is dominated by the Na^+-dependent mechanism [108–110]; however, these mitochondria also show some Na^+-independent Ca^{2+} efflux [17]. In the earliest work on mitochondrial Ca^{2+} transport, influx was thought to be an active process and efflux passive [111]. However with the acceptance of the chemiosmotic hypothesis of oxidative phosphorylation and the realization that mitochondria had an internally negative membrane potential and an internally alkaline pH gradient, it became clear that Ca^{2+} influx was energetically downhill and efflux of Ca^{2+} required a source of energy. The minimum energy required to transport a mole of Ca^{2+} ions from the mitochondrial matrix space to the intermembrane space is given by

$$\Delta\text{Energy} = RT\ln\left(\frac{[Ca^{2+}]_{out}}{[Ca^{2+}]_{in}}\right) + 2F\Delta\psi. \quad (4)$$

Where $[Ca^{2+}]_{out}$ is 100 nM, $[Ca^{2+}]_{in}$ is 200 nM, $\Delta\psi$ is 180 mV and T = 21 C or 294 K, this gives ΔEnergy = −1.69427 + 34.73280 = 33.04 kjoules/mole. This energy can come either from some active source, such as ATP hydrolysis or oxidation of substrates using the electron transport chain, or from coupling efflux of Ca^{2+} to the transport of some other ion that can move down its electrochemical gradient or from some combination of these sources of energy. Outward pumping of protons sets up an inwardly directed proton electrochemical gradient, which can be calculated through an expression similar to that above for Ca^{2+} as

$$\Delta\text{Energy} = 2.303RT\log\left(\frac{[H^+]_{out}}{[H^+]_{in}}\right) + F\Delta\psi = 2.303RT\Delta\text{pH} + F\Delta\psi \quad (5)$$

= 23.0 kjoules required for each mole of H^+ ions pumped outward. In this calculation, everything is as it was for the Ca^{2+} calculation above, and ΔpH is 1/2 of a pH unit. Because of tight coupling of the H^+ and Na^+ gradients through a Na^+/H^+ exchanger, inward transport of Na^+ would yield about the same amount of energy as that for protons [112,113]. Mechanisms of coupling to the transport of other ions are also theoretically possible; however, the $[K^+]$ of the mitochondrial matrix is almost as high (around 140 mM) as that of the cytosol in most cells [114] and so coupling of Ca^{2+} efflux to K^+ uptake would require a larger number of K^+ ions being transported, making this type of mechanism more complex. Perhaps this is why the known efflux mechanisms utilize Na^+ and H^+ instead of K^+.

Na^+-Dependent Efflux

A Ca^{2+}/nNa^+ exchanger is the primary mechanism of Ca^{2+} efflux in brain, heart, skeletal muscle and many other types of mitochondria [21,108–110]. On this exchanger, Li^+ may substitute for Na^+ and Sr^{2+} may substitute for Ca^{2+} [21]; however, Mn^{2+} is not transported via this mechanism [115]. The kinetics of this mechanism in both heart and liver mitochondria have been determined to be first order in Ca^{2+} and second order in Na^+ [21,108,116]. These kinetics can be described mathematically as

$$v = V_{max}\left(\frac{[Na^+]^2}{K_{Na}^2 + [Na^+]^2}\right)\left(\frac{[Ca^{2+}]}{K_{Ca} + [Ca^{2+}]}\right) [116]. \quad (6)$$

For liver mitochondria, V_{max} has been found to be 2.6 ± 0.5 nmol/(mg·min), K_{Ca} to be 8.1 ± 1.4 nmol/mg protein, and K_{Na} to be 9.4 ± 0.6 mM [116]. For heart mitochondria, the corresponding parameters have been found to be 18 nmol/(mg·min), around 10 nmol/mg protein, and 7–12 mM Na^+, respectively [21,109], whereas for brain mitochondria, V_{max} has been found to be around 30 nmol/(mg·min) [109]. It is likely that these differences in V_{max} simply represent different amounts of the protein complex responsible for this transport in each type of tissue.

The amount of energy available to transporters like either of the mitochondrial Ca^{2+} efflux mechanisms can be obtained by what might be called an “efflux null point” experiment [117]. In this type of experiment, the rate of net efflux is measured as the external $[Ca^{2+}]$ is

raised until the point is reached at which the efflux mechanism no longer has sufficient energy to transport the Ca^{2+} outward against the Ca^{2+} gradient. This is the efflux null point. Under the conditions of the null point, the amount of energy available from various ion gradients such as the H^+ gradient and the Na^+ gradient are determined. The hypothetical exchangers that might account for the Ca^{2+} efflux mechanism are considered and equations for the null point for each of these hypothetical exchangers derived. Then the theoretical null points are compared with the experimental null point to see which types of mechanism are consistent with the data. This type of experiment was carried out by Baysal et al. [118] for efflux via the Na^+-dependent mechanism of heart mitochondria. For a passive $Ca^{2+}/2Na^+$ exchanger, the null point would be reached when

$$\left(\frac{\{Ca^{2+}\}_{out}}{\{Ca^{2+}\}_{in}}\right)=\left(\frac{\{Na^{+}\}_{out}}{\{Na^{+}\}_{in}}\right)^{2}, \tag{7}$$

where the curly brackets indicate activity, while for a passive $Ca^{2+}/3Na^+$ exchanger the null point would be reached when

$$\ln\left(\frac{\{Ca^{2+}\}_{out}}{\{Ca^{2+}\}_{in}}\right)=3\ln\left(\frac{\{Na^{+}\}_{out}}{\{Na^{+}\}_{in}}\right)+\left(\frac{F\Delta\psi}{RT}\right). \tag{8}$$

Intramitochondrial free Ca^{2+} concentration ($[Ca^{2+}]_m$) and pH were determined using fura-2 and cSnarf-1 measurements, respectively. External pH was measured with a glass electrode, and external $[Ca^{2+}]$ ($[Ca^{2+}]_{out}$) was varied to determine the null point. Membrane potential was measured using safranine as a potential sensitive probe [118]. The Na^+ gradient was assumed to be very close to the H^+ gradient because of the very active Na^+/H^+ exchanger present in these mitochondria [112,113]. The results of 20 separate experiments showed that the experimental null points exceeded the value predicted by the equation for a $Ca^{2+}/2Na^+$ exchanger by 15 to 100 fold, depending on conditions. However, these null points were consistent with the null points predicted by the equations for a $Ca^{2+}/3Na^+$ exchanger. While the Na^+-dependent Ca^{2+} efflux mechanism had been reported to be electroneutral [119], its electroneutrality was reexamined by the Brierley laboratory and it was concluded that the exchanger was electrogenic with a stoichiometry of $Ca^{2+}/3Na^+$, consistent with the null point data [120].

Many substances inhibit the Na^+-dependent efflux mechanism. One of the most commonly used inhibitors of this mechanism is CGP37157[121]; however, tetraphenyl phosphonium (TPP^+) [116], diltiazem [122], trifluoperazine [123], verapamil [124], clonazepam [122], amiloride [125], bepridil [122] and many other inhibitors of the Na^+-dependent mechanism have been identified.

Na^+-Independent Efflux

The Na^+-Independent efflux mechanism transports Ca^{2+} [20,126], Sr^{2+} [126], Ba^{2+} [127] or Mn^{2+} [20,115,126] outward across the mitochondrial inner membrane against the Ca^{2+} electrochemical gradient. This mechanism appears to be nonelectrogenic [128–130]; however, no cation flux has been directly shown to be coupled to Ca^{2+} efflux via this mechanism. Since $[H^+]$ would be dominated by the $H^+ + OH^- = H_2O$ equilibrium and pH buffers, and the H^+ flux via the transport mechanism could not be easily measured, this mechanism has been suggested by many workers to be a simple passive $Ca^{2+}/2H^+$ exchanger [107,130–133].

Nevertheless, some of the data obtained in studies of this mechanism do not fit this passive exchanger model [117,129,134–136]. A passive mechanism is defined here to mean that the energy available to the mechanism at the site of transport is restricted to that from the electrochemical gradients of the ions which are transported. The sodium-independent Ca^{2+} efflux mechanism was the first system studied using the efflux null point method described above [117]. In these measurements, $[Ca^{2+}]_m$ was measured using fura-2 fluorescence [137], and ΔpH was measured using a radio label of the weak acid, acetate, along with simultaneous mitochondrial water volume measurements. Net Ca^{2+} efflux was determined using antipyrylazo III, and medium $[Ca^{2+}]$ was increased until a null point, i.e., the $[Ca^{2+}]$ at which this mechanism could no longer mediate net Ca^{2+} efflux, was reached [117]. The null point for a passive $Ca^{2+}/2H^+$ exchanger should be reached when

$$\left(\frac{\{Ca^{2+}\}_{out}}{\{Ca^{2+}\}_{in}}\right)=\left(\frac{\{H^+\}_{out}}{\{H^+\}_{in}}\right)^2 \text{ or where } [Ca^{2+}]_{out}=[Ca^{2+}]_{in}\left(\frac{\gamma_{in}}{\gamma_{out}}\right)10^{2\Delta pH}. \quad (9)$$

The ratio of calcium activity coefficients was estimated using the extended Debye-Huckel and Davis approximations. The results showed that for different conditions, the experimentally measured null point was between 3.5 and 47 times larger than that obtained by applying the theoretical equation for a passive $Ca^{2+}/2H^+$ exchanger [117]. This suggested that at least 9.5 kjoules per mole was available to this Na^+-independent mechanism over and above that which would be available to a passive $Ca^{2+}/2H^+$ exchanger. Since the mechanism appears to be nonelectrogenic [128–130], and there is evidence that inhibitors of electron transport also inhibit this mechanism independently of effects on ΔpH, the simplest explanation is that this mechanism is an active $Ca^{2+}/2H^+$ exchanger, which obtains some energy from electron transport [17,138]. This is consistent with earlier data obtained by Rosier et al., who carried out a partial reconstitution of this mechanism with results which suggested an active mechanism [139]. These points are discussed at more length in an earlier review [140].

The calcium kinetics for this mechanism in liver mitochondria were worked out by Wingrove [138] who showed that the mechanism was second order in Ca^{2+} fitting an Adair-Pauling or nonessential activation model. This can be expressed mathematically as

$$v=V_{max}\left(\frac{[Ca^{2+}]^2+a[Ca^{2+}]}{K_m^2+[Ca^{2+}]^2+2a[Ca^{2+}]}\right), \quad (10)$$

where V_{max} = 1.2 ± 0.1 nmol/(mg·min), K_m = 8.4 ± 0.6 nmol/mg, and a = 0.9± 0.2 nmol/mg [138].

The Na^+-independent efflux mechanism is inhibited by Sr^{2+} [126,141], Mn^{2+} [115,142], low levels of uncouplers such as DNP, CCCP. and FCCP [17,139,143–145], by very high levels of tetraphenyl phosphonium (TPP^+) and triphenyl methyl phosphonium (TPMP) [116,138], by CN^-[17], and by very high levels of ruthenium red [116,138].

The Mitochondrial Permeability Transition

The mitochondrial permeability transition, or MPT, is a very important mechanism which has been closely associated with cell death through both apoptosis and necrosis [3,4,146,147]. It has been thoroughly reviewed in the earlier literature and will not be treated in as much detail here [17,148–150]. The MPT is not, strictly speaking, a Ca^{2+} transport mechanism; however,

it can be induced by high $[Ca^{2+}]_m$, and it can transport Ca^{2+} along with many other ions and molecules. Intramitochondrial $[Ca^{2+}]_m$ can activate ATP production by increasing the rate of NADH production [151] and the turnover of the F_1F_0 ATP synthase [91,152]; however, it can also induce the MPT [17]. Intramitochondrial Ca^{2+} then can signal an increase in the rate of ATP production, often necessary for the function and survival of the cell, and can also signal the MPT, which can lead to the death of the cell. Clearly, control of the exposure of the mitochondrial matrix to Ca^{2+}, controlled by the mechanisms of mitochondrial Ca^{2+} transport and the MPT, is crucial to cell function [11,17,81,153]. For these reasons, we believe that consideration of the properties of the MPT is necessary for an understanding of why the mitochondrial Ca^{2+} transport system has evolved with the properties that are observed.

Recognition that the damaging effects of Ca^{2+} on mitochondria were caused by a mechanism that had the characteristics of opening a large pore in the mitochondrial inner membrane actually took place in stages. By the middle 1970's, it was recognized that a Ca^{2+}-induced transformation occurred in adrenal cortex mitochondria which allowed NADPH and NADH to directly enter the mitochondrial matrix and that this transformation was inhibited by Mg^{2+} [14,15,26]. This transformation caused swelling and ultrastructural changes in the mitochondria [14,15,26]. It was recognized in work on heart mitochondria that energy coupling was lost in a related configurational transformation and that Ca^{2+} release was involved with this transformation [16,154].

These observations and others were described as a set of related mitochondrial behaviors which began to be called the Ca^{2+}-induced mitochondrial permeability transition (MPT) [24,25, 155,156]. Hunter and Haworth noted that what was known about this MPT could be caused by opening of a proteinaceous pore (the permeability transition pore or PTP) in the mitochondrial inner membrane. This turned out to be a very important insight [24,25,155, 156]. After opening of the PTP, mitochondria can not maintain an electrochemical proton gradient and can not phosphorylate ADP; however, pore opening is reversible by Ca^{2+} removal [17,157]. Removing Ca^{2+} with chelators, such as EGTA or BAPTA, has been found to be an effective way of reversing PTP opening if the PTP has not been open for too long a time [17]. From the perspective of an individual mitochondrion, this PTP opening is a rapid event, with loss of matrix components and initiation of swelling in a fraction of a second; however, from the perspective of an ensemble of mitochondria, the transition can take place over a period of minutes, since PTP opening in each mitochondrion occurs separately [17,157]. The probability of opening the PTP is increased by the presence of a number of compounds called inducing agents, which can be either endogenous or from external sources. These inducing agents lower the amount of Ca^{2+} necessary for PTP opening [17]. Inorganic phosphate (Pi), oxaloacetate, and acetoacetate are common endogenous inducing agents, while Mg^{2+}, ADP, and ATP act as endogenous inhibitors of PTP opening [17,157]. Decreasing the amount of Pi increases the lag time between addition of the inducing agent and PTP opening [158]. PTP opening is aided by oxidized thiol groups and increased production of reactive oxygen species [17,157]. An acid pH has been found to be a potent inhibitor of the MPT and a high mitochondrial membrane potential also inhibits PTP opening [159,160]. Inducing agents which are often used experimentally include tert butyl hydroperoxide, phenylarsine oxide, atractyloside, carboxyatractyloside, and N-ethyl maleimide [17,157]. The most commonly used MPT inhibitors are bongkrekate, adenine nucleotides, chelation of Ca^{2+}, and cyclosporin A (cys A), discussed in more detail below [17,157].

Bongkrekate, an inhibitor of the adenine nucleotide translocase (ANT), which holds the ANT in the "m" conformation facing the matrix, is a potent inhibitor of PTP opening. Atractyloside and carboxyatractyloside, inhibitors of the ANT, which hold this exchanger in the "c" conformation, facing the cytosol, activates PTP opening. This was first noticed by Hunter and Haworth [24] who suggested a role for the ANT in the MPT. Further work confirmed that

agents which held the ANT in the “m” conformation inhibited PTP opening while those which held the ANT in the “c” conformation activated PTP opening [161–165]. This led to a model of PTP structure as involving a complex of the ANT with VDAC, the voltage-dependent anion channel, which is the largest pore in the mitochondrial outer membrane at contact sites between the inner and outer mitochondrial membranes [165]. Some held that the ANT was the pore protein while others held that the data only showed that the ANT was located topographically near the pore protein [148,149,166]. Early work by Hunter and Haworth using various sized polyethylene glycols, showed that molecules with a molecular weight smaller than 1500 Daltons can pass freely through the PTP in heart mitochondria [155], equivalent to a hydrodynamic radius between 1.2 and 1.5 nm [167]. However, there was strong evidence for a variation of sizes for PTP formed under different conditions [168,169]. There was even evidence for loss of matrix proteins after passage of time [170]. Finally, Wallace’s laboratory was able to obtain livers from newborn mice whose mitochondria did not contain the ANT and found that these mitochondria showed the usual characteristics of the MPT, showing that the ANT was not essential for the MPT [171]. Today most believe that while the ANT is involved with most PTP’s other membrane protein complexes in addition to the ANT can also be involved in PTP opening.

A discovery, key to elucidating the molecular nature of the MPT, was the finding that cyclosporin A (cys A), an immunosuppressant used in organ transplants, was a potent inhibitor of the MPT which blocked pore opening by a different mechanism from that which made it an immunosuppressant [172,173]. Cys A inhibition of PTP opening is caused by binding of cys A to the matrix protein cyclophilin D (cyp D), a peptidyl-prolyl cis-trans isomerase that acts as a chaperone in controlling the folding of specific matrix proteins [166]. Ca^{2+} activates cyp D binding to the inner mitochondrial membrane and to the ANT inhibiting the binding of ADP and ATP [163,174,175] and this is believed to be a crucial step in PTP opening. Cys A is believed to bind to cyp D and prevent cyp D from binding to the mitochondrial membrane and to the ANT thereby inhibiting PTP opening.

Patch clamping and other electrophysiological studies have been carried out on mitochondrial membranes and on preparations reconstituted from mitochondrial membrane proteins [84, 176,177]. Results of these studies have identified a large channel called the mitochondrial megachannel which many believe to be the PTP. This channel has been shown to “flicker” or to open and close transiently [176–179]. Indications of this flickering behavior has also been seen at the isolated mitochondrial and cell levels [180,181]. This flickering may correspond to the reported characteristics of a lower conductance state of the PTP, which is induced by free radicals and is insensitive to the usual inhibitors of PTP opening, during which the membrane becomes leaky to H^+, K^+, and Ca^{2+} but not to larger molecules [182–184]. This PTP flickering may play a physiological role, as will be discussed later.

Recent work on the MPT has involved the use of transfected mice, which do not produce cyp D [185–187]. Mitochondria from the livers of these mice are much more resistant to the MPT than are mitochondria from normal mice. It has also been shown that the much lower amount of cyp D present in brain mitochondria is responsible for the higher resistance of brain mitochondria to induction of the MPT than other types of mitochondria [188]. Also, while the MPT can be induced in the presence of other counter ions such as arsenate, vanadate, or bicarbonate, Pi is necessary for the inhibitory effects of cys A or of cyp D ablation on the MPT to be seen [189]. These observations suggest that only Pi and not other anions such as arsenate or vanadate can bind to an MPT regulatory site, which is not accessible when cyp D is bound to the pore protein [189].

Opening of the PTP causes dissipation of the mitochondrial electrochemical proton gradient and obviates ADP phosphorylation by oxidative phosphorylation, which could lead to a loss

of about 92 % of the ATP production in a cell. Nevertheless, the MPT is conserved and observed in every type of vertebrate mitochondrion in which it has been studied. What can be so important to the cell that it is tolerated in the face of loss of production of 92% of the cell's ATP? Long term opening of the PTP leads to rapid mitochondrial swelling which has been shown to lead, in turn, to loss of cytochrome c, Smac/Diablo, AIF, and other components of the mitochondrial intermembrane space into the cytosol, and to represent one of the pathways to apoptosis [4]. However, there are other pathways to apoptosis in addition to MPT induction [190,191]. In some cases, the death and replacement of a cell is the appropriate pathway to survival of the organism. This may represent one possible physiological role of the MPT.

A question very important to inferring how the complex mitochondrial Ca^{2+} transport system and the MPT functions is - how does the amount of Ca^{2+} necessary to activate phosphorylation of ADP compare with the amount necessary to induce the MPT? Clearly, only a small amount of Ca^{2+} uptake is sufficient to activate ATP production. It can be done by Ram uptake of a single pulse of around 500 to 600 nM peak height by either liver [33] or heart mitochondria [91], consistent with McCormack's earlier estimate that 4 nmoles/mg is probably sufficient [90]. On the other hand, at least 20 nmoles/mg protein is probably required to activate the MPT, even when the Ca^{2+} is aided by the presence of an inducing agent [13]. This has been discussed more completely earlier [13]. Thus, small amounts of Ca^{2+} uptake could activate ADP phosphorylation, while significantly larger amounts and longer times would be necessary to induce PTP opening.

Recent Work at the Cell Level That has Broadened Our Understanding of Mitochondrial Ca^{2+} Transport and the Effects of This Transport

There has been considerable progress in understanding the Ca^{2+} environment of mitochondria inside the cell and mitochondrial interactions with other organelles such as ER or SR and endosomes. This work has utilized carefully-crafted fluorescent probes and molecular biological techniques, and has yielded detailed information on the interactions of mitochondria with other intracellular organelles, particularly with ER and SR in specific cell types. While an in-depth discussion of this area is beyond the scope of this review, excellent recent reviews are available (see Pozzan (this issue), Rizzuto (this issue) or [102]). Many cell types have been observed to have a preponderance of mitochondria around the nucleus and in close proximity to the ER or SR [192]. This has been shown to lead to the formation of "microdomains" or regions of higher $[Ca^{2+}]_c$ near the release sites from the Ca^{2+} stores in the ER or SR in which the $[Ca^{2+}]_c$ can exceed 50 μM during pulses [27,38,87,193,194]. The high concentrations of Ca^{2+} within these microdomains would be expected to lead to uptake via the mRyR and the uniporter in preference to the RaM. While there might be a small amount of RaM uptake at the beginning of the pulse, this mechanism would soon be inhibited by the high $[Ca^{2+}]$ in the microdomain. There is probably sufficient uptake via the mRyR and the uniporter in these regions of the cell for Ca^{2+} activation of ADP phosphorylation to occur via activation of αKGDH, PDH, and the F_1F_0ATP synthase [87,193,194]. There are cellular mechanisms that control the distribution of mitochondria within the cell [195], and the larger concentration of mitochondria in these regions is probably because of the high ATP consumption of both the nucleus and of the ER or SR. This mitochondrial distribution, which is modulated by Ca^{2+}, helps insure an adequate supply of ATP [195,196]. There could also be deleterious effects of a large Ca^{2+} uptake, which will be discussed below.

Important recent work using these sophisticated fluorescent probes has shown three types of interaction between mitochondria and ER which could be an indication of a special relationship between Ca^{2+} release by the ER and uptake by the mitochondria [102,197,198]. These are: 1) attachment of both mitochondria and ER to microtubules and microfilaments, 2) tethers linking the outer mitochondrial membrane to ER, and 3) complexes involving the IP3 receptors in the

ER and the VDAC pore on the outer mitochondrial membrane [102]. Recent work has suggested that ryanodine receptors in the ER may also be involved in targeted transfer of Ca^{2+} from ER to mitochondria [199]. Another set of observations, also using fluorescent probes has suggested a special relationship between mitochondria and endosomes important in delivering iron to the mitochondria that is used in the formation of functional heme proteins and other iron-containing proteins [6,7]. These are examples of important transport relationships between mitochondria and other organelles which could only be made at the cell or tissue levels, since any such “tethering” or association would be disrupted by isolation of mitochondria.

There is clear evidence, from fluorescence probe data at the cell level, for mitochondrial modification of the shape, magnitude, and distribution of Ca^{2+} transients [200–202]. Some of these effects are caused by local modulation of the processes that cause Ca^{2+} transients. Examples are the suppression by mitochondrial Ca^{2+} uptake of Ca^{2+} feedback activation of 1,4,5 inositol trisphosphate receptor-induced Ca^{2+} release and maintenance of store-operated Ca^{2+} influx by mitochondria through limitation of Ca^{2+}-induced desensitization [202,203]. The localization of mitochondria very close to these channels modifies the Ca^{2+} feedback to the channels and adjusts the activity of the channels [204]. An extreme example of spatial modification of Ca^{2+} transients occurs in polarized pancreatic acinar cells where a layer of very high mitochondrial density confines Ca^{2+} transients to the apical third of the cell where they are needed for secretion of secretory granules [205,206]. Ca^{2+} uptake via the mitochondrial Ca^{2+} uniporter is likely involved in causing many of these effects on Ca^{2+} transients [204].

The Relationship Between Mitochondrial Production of Reactive Oxygen Species (ROS), Ca^{2+} Transport, and the MPT

In the sections above, we have discussed the strong relationship between mitochondrial Ca^{2+} transport and induction of the MPT. Mitochondrial production of ROS are also intimately involved with both Ca^{2+} transport and with the MPT and these relationships should be discussed.

Mitochondrial ROS generation

Cellular homeostasis of ROS is determined by the balance between ROS generation and ROS annihilation. Physiological levels of ROS are critical in controlling redox signaling of cells [207]. However, unregulated ROS levels can lead to oxidative stress, which is a major cause for cellular damage, and eventually cell death [208,209]. ROS generated in cells include superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hydroxyl radical ($OH^{\bullet}$), and singlet oxygen ($^1O_2$). These ROS are formed as a consequence of endogenous enzymatic and nonenzymatic reactions within the cell and within mitochondria. Although non-mitochondrial ROS generation pathways such as NADPH oxidase can be important in certain cell types [210], we will focus on mitochondrial sources of ROS and the defense mechanisms that maintain physiological oxidative homeostasis.

The primary ROS made by mitochondria is $O_2^{\bullet -}$, mostly due to the slippage of electrons to oxygen. Complex I and complex III are the two dominant sites for $O_2^{\bullet -}$ generation. Reduced sites within complex I, including flavin mononucleotide (FMN), iron sulfur clusters, and semiquinones have been suggested to be potential sites responsible for $O_2^{\bullet -}$ production. The generation of $O_2^{\bullet -}$ by complex I faces the matrix side of the inner membrane [211–213], where Mn-SOD converts it to H_2O_2. Electrons entering at complex II (succinate dehydrogenase) can flow backward through complex I to make ROS [211]. This pathway is usually prevented by forward electron flow through complex I from NADH, except under pathological conditions

in which NADH is depleted. The key step in ROS generation at complex III is the ubisemiquinone radical intermediate ($QH^{\bullet}$) formed during the Q cycle at the Q_0 site [212]. The rate of ROS production is accelerated by inhibition of complex III at sites distal to Q_0 (e.g. antimycin A) and further down the respiratory chain at the level of complex IV (e.g. cyanide). The majority of $O_2^{\bullet -}$ from Q_0 is made facing the intermembrane space [212], leading to suggestions that $O_2^{\bullet -}$ may be released through voltage-dependent anion channels (VDAC) into the cytosol [214].

$O_2^{\bullet -}$ production is stimulated by high mitochondrial $\Delta\psi_m$ generated by respiratory chain electron transport [215]. On the other hand, mild uncoupling of $\Delta\psi_m$ such as the proton leak mediated by uncoupling proteins (UCPs) decreases ROS production [216,217]. This decrease in ROS production may be one of the reasons why the proton leak is cytoprotective in several models of ischemic injury [218]. Interestingly, recent results from several laboratories show that a transient depolarization of $\Delta\psi_m$ due to the brief opening of mitochondrial permeability transition pores (PTP) causes a transient burst of mitochondrial ROS production [209,219, 220]. The molecular mechanisms for describing this PTP-mediated ROS generation are still not clear. Among the proposed mechanisms are: changes in electron transport chain (ETC) activity, dislocation of cytochrome c, release of mitochondrial glutathione (GSH), and changes in mitochondrial volume [209,220]. It has been hypothesized that brief PTP opening results in a massive electron leakage from the ETC due to altered membrane rigidity [221] and conformational changes of ETC proteins [222] giving rise to brief bursts of superoxide production. Another intriguing hypothesis is the possible interaction between the PTP and mitochondrial fission/fusion. Recent evidence shows that the PTP interacts with mitochondrial fission/fusion proteins in regulating cristae remodeling [223]. This interaction is manifested by protein-protein interaction among proteins involved in fission (DLP1 and hFis1), proteins involved in apoptosis (Bcl2/BclxL and Bax/Bad), and proteins involved with the PTP (VDAC and the adenine nucleotide translocator, ANT). Recent work has shown that cells transfected with the dominant-negative mutant DLP1-K38A maintain tubular mitochondria without mitochondrial fission. These cells are able to survive a hyperglycemic insult by sustaining normal cellular ROS levels without a significant opening of the PTP and subsequent induction of apoptosis [224]. These results demonstrate that mitochondrial fragmentation is an upstream factor stimulating ROS overproduction and cell death under prolonged high glucose conditions. Finally, although still controversial, the PTP is thought to consist of VDAC, ANT, and cyp D [225,226]. The recent generation of cyp D knock out mice by several groups provides a useful model for studying the functional role of the PTP in ROS generation [185–187,227]. Another interesting possibility is that the accumulation of ROS in the mitochondrial matrix may reach a critical level in a certain fraction of the mitochondrial network that causes the activation of inner-membrane anion channels (IMACs) [228]. This opening of IMACs leads to ROS release from mitochondria via IMACs that affects neighboring mitochondria. At the stage of "mitochondrial criticality", $\Delta\psi_m$ becomes unstable, undergoing a rapid depolarization or a self-sustained oscillation [228]. These mitochondrial depolarizations cause bursts of ROS production that propagate through the population of mitochondria in the network. In addition, the oscillatory uncoupling of mitochondria depletes cellular ATP levels that cause activation of ATP-sensitive K^+ channels in the sarcolemmal membrane. As a result, the duration of cardiac action potential becomes oscillatory and increases the susceptibility for cardiac arrhythmias including post-ischemic arrhythmias [229]. Taken together, it appears that two different profiles of ROS generation exist; one is a continuous or constitutive process during mitochondrial respiration, and the other is a transient stochastic process triggered either by PTP or IMAC mediated $\Delta\psi_m$ depolarization. The crosstalk between these two modes of ROS generation could be critical in determining physiological ROS signaling in localized cellular microdomains and pathological oxidative stress in global cellular areas.

ROS annihilation

Superoxide is quickly converted to hydrogen peroxide (H_2O_2) either by spontaneous dismutation or by the enzyme superoxide dismutase (SOD). H_2O_2 can then either be converted to H_2O and O_2 by glutathione peroxidase or catalase or be further transformed to $OH^{\bullet}$ in the presence of metal ions by a Fenton reaction [211,230]. The glutathione redox couple (GSH/GSSG) is the primary cellular redox buffer and is maintained at a very high GSH:GSSG ratio (greater than 30:1) [231]. GSH is the most abundant cellular antioxidant (~ mM range). It is a cysteine containing tripeptide that can directly scavenge ROS or act as a cofactor for glutathione peroxidase, which oxidizes glutathione to reduce H_2O_2. GSSG is subsequently reduced by glutathione reductase, which uses NADPH as a substrate. In mitochondria, NADPH is generated at the expense of NADH by NADH transhydrogenase. The NADH/NAD^+ redox couple also plays a role in redox buffering [232]. Thioredoxin is a disulfide-containing protein that can directly scavenge H_2O_2 as part of the thioredoxin reductase and thioredoxin peroxidase system [233].

Physiological and pathological roles of ROS

Our understanding of the importance of mitochondrially-derived ROS has been transformed in recent years. Previously ROS were thought to be only a detrimental by-product of respiration, responsible for oxidative damage [234]. However, recent evidence has shown that mitochondrially-derived ROS are important for multiple cell signaling processes [209]. Indeed, recent evidence indicates that an array of cellular functions (e.g., channel/transporter activity, kinase/phosphatase activity, gene expression), are highly redox sensitive [235]. Therefore, the spatial and temporal distributions of cellular ROS concentrations are very dynamic. It is conceivable that the physiological and pathological roles of ROS are dictated by the subtle balance of their dynamics as a function of time and space. Hence, a basal or tonal concentration of ROS, especially at the level of the mitochondrion, is essential for basic cell signaling processes. In other words, all ROS are not created equal, and compartmentalization and concentration gradients are highly important. For example, the observation that cytosolic Cu/Zn-SOD−/− mice are viable, whereas mitochondrial Mn-SOD−/− mice are not suggests a much more localized and subtle role of mitochondrial ROS in redox signaling [236,237]. It is important to recognize that the abolishment of all cellular ROS by vigorous use of antioxidants may not be beneficial and, indeed, may prove harmful. The requirement for a basal ROS tone for cell signaling may explain why many antioxidant-based therapies have failed [238].

Regulation of mitochondrial ROS generation by Ca^{2+}

The fact that mitochondria use intramitochondrial Ca^{2+} to control the rate of ATP production implies that Ca^{2+} may also serve as a key regulator for mitochondrial ROS generation because ROS are by-products of energy-producing processes. The underlying mechanisms for Ca^{2+}-mediated mitochondrial ROS generation can be summarized based upon the present literature [209]: 1) High mitochondrial Ca^{2+} induces the MPT leading to cytochrome c and NADH release and an inhibition of complexes III and IV, resulting in enhanced ROS generation at the Q cycle [239]; 2) High mitochondrial Ca^{2+} induces the MPT leading to release of mitochondrial GSH [240]; 3) High cytosolic Ca^{2+} competes for binding to cardiolipin on the inner mitochondrial membrane, displacing cytochrome c and enhancing ROS generation in a manner similar to (1) above [241]; 4) High mitochondrial Ca^{2+}, together with $NO^{\bullet}$, inhibits complexes I and IV, causing them to generate ROS [242,243]; 5) Ca^{2+} activates the TCA cycle causing an increase in NADH concentration and subsequent electron flow, promoting electron slippage to O_2 and thus ROS generation [244]; 6) Mitochondrial Ca^{2+} increase shifts the mitochondrial morphology dynamics toward the direction of fission, which favors more ROS generation [224,245]. It is difficult to select the operant mechanisms from the list above because 1) the variation in the mitochondrial Ca^{2+} transport mechanisms and ROS generation/eradication

mechanisms in different cell types is difficult to assess, 2) the distinction between physiological and pathological Ca^{2+}-dependent ROS generation is scarcely delineated, 3) the availability of probes for spatial and temporal profiles of ROS in intact cells is limited, and 4) the conceptual and experimental framework for ROS microdomains is undeveloped.

The isolated mitochondrial preparation provides a simple system for investigating Ca^{2+}-mediated mitochondrial ROS generation. A very consistent observation is that treating isolated mitochondria with high concentrations of Ca^{2+} (e.g. 50 μM) leads to a CsA-sensitive swelling of mitochondria with a concomitant ROS increase in brain [246], liver [247], and heart [248]. The mechanisms responsible for this increase in ROS levels are the release of cytochrome c and/or mitochondrial GSH due to the opening of the PTP. Indeed, the release of cytochrome c and GSH from mitochondria has been widely accepted as key steps in mitochondrially-mediated apoptotic and necrotic cell death, which may also contribute to cancer, neurodegenerative diseases, stroke, muscular dystrophy, and cardiac reperfusion injury [249]. Interestingly, as described above, there are a number of reports suggesting that the MPT could also open under physiological conditions and serve as a physiological Ca^{2+} efflux and ROS generation mechanism. In adult rat ventricular myocytes, it has been shown that periodic transient opening of the PTP during heartbeats provide a pathway for mitochondrial Ca^{2+} extrusion under relatively normal conditions [250]. This PTP-mediated Ca^{2+} efflux has been shown to involve a transient increase in cytosolic Ca^{2+} in the microdomains which can regulate localized ion channel activities and Ca^{2+} homeostasis [249,251], as discussed above. In several cell types, local Ca^{2+} release from endo/sarcoplasmic reticulum (ER/SR) leads to a transient elevation in intramitochondrial Ca^{2+} concentration termed a 'Ca^{2+} mark' [252] and a transient mitochondrial depolarization termed a 'flicker' [253]. There is evidence suggesting that these flickers of $\Delta\psi_m$ are the results of transient PTP opening [180,254]. These results raise three interesting questions: 1) How can mitochondria sequester Ca^{2+} so rapidly? 2) What mechanism (s) is (are) then responsible for bringing out the sequestered Ca^{2+} rapidly? 3) If the PTP is involved in the rapid efflux of Ca^{2+}, is it also involved in physiological ROS signaling? As described earlier, RAM and mRyR have the ability to transport Ca^{2+} on a millisecond time scale. Another factor that may favor mitochondria having the capability to sequester Ca^{2+} rapidly during physiological Ca^{2+} pulses is the existence of a micro-domain of high Ca^{2+} near the mitochondrial Ca^{2+} influx transporters. Physiological Ca^{2+} pulses originate from release and reuptake of Ca^{2+} by ER/SR in most cell types. Measurements of Ca^{2+} concentrations formed by localized ER/SR Ca^{2+} release (Ca^{2+} sparks) have been estimated to be between 20 and 100 μM [255,256]. For mitochondria to respond to these localized high Ca^{2+} concentrations, they must be close to ER/SR. This has been shown to be true in numerous cell types including adult cardiac ventricular myocytes [27]. Our published data show that the shortest distance between cardiac SR and mitochondria is approximately 30 nm [257]. In fact, peptide linkages have been observed that may help to position mitochondria near Ca^{2+} release channels, leading to a distance between ER and mitochondria of between 5–30 nm at the junctions [258]. The proximity of these two organelles creates a privileged Ca^{2+} exchange between the ER/SR and mitochondria, as discussed above. This transport of Ca^{2+} from ER/SR to mitochondria cannot be effectively decreased by exogenous Ca^{2+} chelators such as EGTA or BAPTA [257,259]. The next question is how can mitochondria release the sequestered Ca^{2+} rapidly so that they won't reach to the injurious stage of Ca^{2+} overload? One can envision that rapid influx of Ca^{2+}, either through RAM or mRyR, would transiently create a region of high Ca^{2+} in the mitochondrial matrix near these two Ca^{2+} transport proteins. This is conceptually similar to the theoretical analysis showing that Ca^{2+} concentrations can reach 100 μM within 20 nm of open plasma membrane Ca^{2+} channels [259]. Hypothetically, if the PTP locates in proximity to RAM or mRyR, the high Ca^{2+} in this microdomain could induce the opening of PTP and lead to "Ca^{2+}-induced Ca^{2+} release" via the PTP. While speculative, this may be the mechanism behind the occurrence of PTP flickers. Interestingly, it has been shown that the PTP has a low- and high-conductance state [254]. A fast rate of mitochondrial Ca^{2+}

uptake promotes the PTP to operate in its low-conductance conformation leading to rapid Ca^{2+} release, thus ensuring physiological Ca^{2+} homeostasis. However, a tonic slow mitochondrial Ca^{2+} uptake results in significant Ca^{2+} overloading, which promotes the PTP to operate in its high-conductance conformation leading to apoptosis/necrosis of cells. As described above, opening of the PTP could lead to ROS generation. Taken together, these results suggest that the PTP may play a dual role in physiological Ca^{2+} as well as ROS signals. Finally, in addition to Ca^{2+}-triggered transient opening of the PTP, several other studies have shown that constitutive electron leakage from the ETC sets the physiological level of ROS that triggers brief, infrequent openings of the PTP which can feedback to promote transient bursts of ROS production, a phenomenon termed “ROS-induced ROS release” (RIRR) [221,260].

Regulation of Ca^{2+} transport mechanisms by redox environments

It is now recognized that several ion channels, including RyR, IP3 receptors, and L-type Ca^{2+} channels are regulated by the redox environment [261,262]. Physiologically, this redox regulation may serve as a mechanism to provide optimal channel function. For instance, it was suggested that redox regulation of SR-RyR1 provides a positive feedback mechanism to enhance skeletal muscle contraction during exercise [263,264]. Pathologically, the redox regulation of ion channels may also contribute to disease processes. For example, excessive ROS may enhance L-type Ca^{2+} channel activity and promote arrhythmogenic afterdepolarizations [265]. Interestingly, redox regulation of Ca^{2+} signaling may also be localized in discrete cytosolic microdomains (e.g. the narrow spaces between mitochondria and SR). For example, ROS specifically derived from mitochondria dilate cerebral arteries by activating Ca^{2+} sparks from the SR [266]. Similarly, in adult ventricular myocytes, inhibition of basal mitochondrial ROS production decreases spontaneous Ca^{2+} spark production by as much as 60%, suggesting that baseline mitochondrial ROS production plays an important role in maintaining normal Ca^{2+} spark activity [267]. Some indirect evidence that ROS can modulate mitochondrial Ca^{2+} transport is the observation that mitochondria become overloaded with Ca^{2+} under oxidative stress conditions. However, presently very few studies report the redox regulation of mitochondrial Ca^{2+} transport even though mitochondria are the primary producers for cellular ROS. One of the key reasons for this much slower progress is the lack of molecular information about mitochondrial Ca^{2+} transport proteins. Identification of the molecular and structural nature of mitochondrial Ca^{2+} transporters and their regulation by nearby signaling molecules such as protein kinases will help to move this field forward.

What is the Relationship Between the RaM, the Uniporter, and the mRyR and of the Two Efflux Mechanisms and What Does Each of These Mechanisms Do?

Rapid Ca^{2+} uptake (RaM behavior) has been observed in both mammalian and avian mitochondria and in mitochondria from liver, heart, and brain [12,18,33,34,91]. Therefore, the RaM like the other mechanisms of mitochondrial Ca^{2+} transport seems to be generally present in vertebrate mitochondria. Why do mitochondria need multiple mechanisms of Ca^{2+} influx? Let us consider the physiological roles with which mitochondrial Ca^{2+} influx is thought to be involved [12,81]. First, and perhaps most important, is the activation of the rate of ATP production by oxidative phosphorylation. Second, is transport of sufficient Ca^{2+} to induce the MPT and apoptosis when that is called for. Third, is sequestering or buffering cytosolic [Ca^{2+}] and modifying the shape of cytosolic Ca^{2+} transients when that is not adequately controlled by cytosolic mechanisms [12,81,200,203,205,206,268]. In carrying out these physiological roles, it should be noted that mitochondria support the life and function of the cell through their ATP production, they may control the death of the cell through apoptosis, and they modify cell signaling through shaping of cytosolic transients [81,147,269]. The RaM

mechanism limits the Ca^{2+} that it can transport severely by binding of Ca^{2+} to an external inhibition site so that it can't transport the amounts of Ca^{2+} needed to induce PTP opening, or to have much effect on buffering cytosolic $[Ca^{2+}]_c$, or modulating the shape of cytosolic transients. What it does do is to sequester a small amount of Ca^{2+} very rapidly. Nevertheless, that small amount of Ca^{2+} does seem to be enough to activate ADP phosphorylation [33,90, 91]. Indeed the RaM seems to have evolved to avoid sequestering enough Ca^{2+} to induce the MPT.

In contemplating how the RaM might function to induce a maximum effect on the rate of ATP production with a minimum amount of Ca^{2+}, it was suggested that because RaM uptake is so fast, it might allow Ca^{2+} to bind to the metabolic sites involved with this activation prior to equilibration [81]. Because of the many low affinity binding sites for Ca^{2+} inside mitochondria, only about 1% of intramitochondrial Ca^{2+} is free at equilibrium [20,137,270,271]. Therefore, if transport and binding of Ca^{2+} to the metabolic sites is very rapid, much less Ca^{2+} would be needed to activate ATP production than if transport is slower. Two advantages of this would be a much smaller likelihood of inducing the MPT and less energy cost for Ca^{2+} transport, making the process more efficient. The rapid binding of Ca^{2+} to each of the activation sites (PDH, αKGDH, ICDH, and the F_1F_0 ATP synthase) would of course be due to the characteristics of each of these complexes, and it along with the extremely fast transport via the RaM would permit activation of these sites very rapidly. The results of Territo et al. [91] strongly suggest that enough Ca^{2+} is sequestered rapidly via the RaM mechanism to carry out this activation. The increase in efficiency and protection against induction of the MPT comes as a result of the unusual RaM characteristic of Ca^{2+} inhibition of further Ca^{2+} transport by Ca^{2+} binding to the inhibition site. In this case, it inhibits Ca^{2+} transport that is unnecessary for activation of ATP production, and could induce the MPT.

Because of the high $[Ca^{2+}]$ around mitochondria in or near microdomains, it is inevitable that uptake via the uniporter and/or the mRyR would also be involved with mitochondria in these locations and so this rapid uptake hypothesis might be called the RaM plus uniporter hypothesis to differentiate it from the hypothesis that holds that all of the Ca^{2+} uptake involved in activating ATP production is via the uniporter. According to this RaM plus uniporter hypothesis, mitochondria throughout the cell could be activated to produce ATP at a faster rate through Ca^{2+} uptake via the RaM where $[Ca^{2+}]_c$ is lower and via the uniporter or mRyR inside the microdomains. There is a clear need for activation of ATP production by an uptake mechanism with kinetics different from those of the uniporter because in many types of cells a large fraction of the mitochondria are outside of the microdomains. In Hela cells, for example, only 5 to 21% of the mitochondrial membranes are close to release sites in the ER [193]. Also in nerve axons and dendrites where a large fraction of the energy used for ion transport is expended, the mitochondria are very distant from the microdomains near the ER. In a recent review, Szanda et al. have outlined the case for Ca^{2+} uptake which can lead to activation of ATP production outside of microdomains [272].

Another point which should be made about sequestration of Ca^{2+} in order to activate ATP production is that it is common to observe a series of Ca^{2+} transients in cells and not just a single transient [11]. For situations in which there are a large number of Ca^{2+} transients or pulses in a sequence, particularly in heart where there can be millions of transients in the sequence, it is important that the Ca^{2+} in the cell maintain a kind of steady state with the same amount of Ca^{2+} entering the cell through the influx mechanisms that leaves the cell during the period between pulses [81,273]. Otherwise there would be a continuous increase or decrease in the amount of Ca^{2+} in the mitochondria. An increase of this type could eventually lead to induction of the MPT. Notice in the data given above on the mitochondrial Ca^{2+} influx and efflux mechanisms that the V_{max} for even the uniporter, which is much slower than the RaM, is much larger than that for the efflux mechanisms. Whether one favors the RaM plus uniporter

hypothesis or the uniporter hypothesis of activation of ATP production, this strongly suggests that within the microdomains it is likely that a $[Ca^{2+}]_m$ will be reached in which the MPT can be induced. It seems natural to assume that at least one reason for evolution of and conservation of the MPT is that it is necessary to function, perhaps in its low conductance form and perhaps through flickering, as an additional efflux mechanism to preserve the steady state of $[Ca^{2+}]_m$ within these microdomains. No other mechanism currently known would avoid this dilemma. Furthermore, in cases where there is too much damage caused by Ca^{2+} overload and by the accompanying ROS production, the further evolutionary development of this process to irreversible MPT induction and to apoptosis could be beneficial to the organism.

Calculations suggest that Ca^{2+} efflux through transient PTP opening might be energetically favored within the microdomains. First, consider how much energy it would take to transport 20 nmoles Ca^{2+}/mg protein (i.e. the minimum amount of Ca^{2+} estimated to possibly induce the MPT [81]) from the matrix of a mitochondrion to the external space via an efflux mechanism. If the conditions were similar to those used in the calculation in "Mechanisms of Efflux: Energy Considerations" above, i.e.100 nM external $[Ca^{2+}]$, 200 nM internal $[Ca^{2+}]$, 21 C, and $\Delta\psi$= 180 mV, the minimum energy would be about 33 kjoules/mole, as calculated above. For a mitochondrion like an isolated mitochondrion described by Lehninger [274], that has a matrix water volume of about 1 μl/mg protein, an inner membrane surface area of about 16 μm^2, a membrane thickness of about 40 Angstroms, and about 3×10^9 mitochondria per mg protein, 20 nmoles/mg would represent $(20\times10^{-9})/(3\times10^9) = 6.667\times10^{-18}$ moles of Ca^{2+} per mitochondrion. Therefore, a minimum of $(6.667\times10^{-18})(33.\times10^3)$ or **2.20×10^{-13} joules/mitochondrion** would be required to transport this Ca^{2+} out of the matrix. If the MPT were transiently induced, opening of the pore and deenergization would allow rapid equilibration of internal Ca^{2+}, K^+, and H^+ with external Ca^{2+}, K^+, and H^+. This is a passive process that would require no energy; however, it would leave the mitochondrion deenergized. We should consider the minimum amount of energy necessary to reenergize the mitochondrion. Since the membrane potential depends only on the net charge difference across the membrane, we should start by calculating the number of protons which must be pumped to regenerate a membrane potential of 180 mV. Using capacitance, $C = A\varepsilon/d$ to estimate the capacitance of a mitochondrion, along with the inner membrane area, A = 16 μm^2, the membrane permittivity, $\varepsilon = 1.77\times10^{-11}$ farad/meter, and the membrane thickness, $d = 40\times10^{-10}$ meters yields $C = 0.708\times10^{-13}$ farads. Then, using Q = CV, where Q is the charge necessary to charge the capacitor to voltage, V = 0.180 volts, yields 0.127×10^{-13} coulombs, or $(0.127\times10^{-13})/(1.6\times10^{-19}) = 7.97\times10^4$ charges or H^+ ions pumped, or 1.323×10^{-19} moles of protons, where 1.6×10^{-19} coulombs is the charge on an electron or proton. The minimum energy necessary to pump the first proton would be very small, but the minimum energy necessary to pump the last proton would be the same as that calculated in the section on Mechanisms of Efflux, above or about 23 kjoules/mole, based on ΔpH = 1/2. Since it is unlikely that the biological system would be able to take advantage of the low energy necessary to pump protons out of deenergized mitochondria, we should simply use the energy needed to pump the last proton (23 kjoules/mole) for the calculation. So the energy required to reenergize the mitochondrion would be $(23\times10^3)(1.323\times10^{-19})$ = **3.04×10^{-15} joules/mitochondrion**, almost two orders of magnitude less than the minimum necessary to transport the Ca^{2+} out via an efflux mechanism. Several things should be noted with respect to these rough calculations: First, outward pumping of 1.323×10^{-19} moles of protons would drive the intramitochondrial pH up to around 10.6 if it were not for intramitochondrial buffering and reequilibrations of ions which would go on as the protons were being pumped. A ΔpH of 1/2 pH unit was used because empirically that is a typical mitochondrial pH gradient for energized mitochondria [117] and the reason that it is so low is due to intramitochondrial buffering and equilibration of ions, particularly K^+ through the K^+/H^+ exchanger. Second, these processes of equilibration that would follow reenergization of the mitochondrion are passive processes that would require no further expenditure of energy. Finally, no energy would be saved by opening the pore for very small

loads of Ca^{2+}. The only reason that the calculation showed that opening of the pore would save energy was that a large enough amount of Ca^{2+} to induce the MPT was used.

Mitochondria control their volume through the K^+ uniporter and K^+/H^+ exchanger mechanisms. Under normal conditions, because of the higher osmotic pressure caused by the very high concentrations of proteins, nucleic acids, etc within the mitochondrial matrix, the $[K^+]$ in the mitochondrial matrix is kept slightly lower than that of the cytosol to help balance the osmotic strength contribution of the macromolecules. This sets up a situation in which opening of the pores in the inner membrane, which allows rapid equilibration of K^+ and other small ions and molecules, leads to rapid swelling because the macromolecules can't equilibrate across the inner membrane and osmotic swelling ensues upon equilibration of the small ions and molecules. If the pore remains open for very long, this would be expected to lead to rupture of the outer membrane and dumping of inner membrane space proteins into the cytosol leading to apoptosis. For those situations in which irreparable damage had been done to the mitochondrion's ability to produce sufficient ATP, apoptosis was the answer to the evolutionary pressure to get rid of the damaged cells and replace them with newly divided cells.

It is also valuable to discuss how the mRyR may fit into this scheme of Ca^{2+} dynamics discussed above. The published data show that the mRyR is most abundant in excitable cells especially in heart [19]. The majority of mitochondria in adult cardiac myocytes are adjacent to SR [257], thus they are constantly exposed to high Ca^{2+} microdomains during excitation-contraction coupling processes. Intriguingly, the cardiac myocytes cultured from RyR1 knock out mice no longer respond to Ca^{2+} stimulated oxygen consumption [103]. The bell shape Ca^{2+}-dependent mRyR activation curve (see Figure 5 in Beutner et al., 2005) and the well-known extremely-rapid kinetics of RyR provide the scientific basis that mRyR may contribute to the excitation-contraction coupling in cardiac muscle cells.

Both the RaM and the mitochondrial Ca^{2+} uniporter appear to be uniporter mechanisms, that is, they facilitate the transport of Ca^{2+} down its electrochemical gradient without coupling that transport to the transport of any other ion. It is interesting that the RaM and uniporter are both inhibited by ruthenium red and are both activated by spermine. It is also interesting that the RaM is inhibited by Ca^{2+} binding to the external inhibitor site and following this no longer functions as a Ca^{2+} transporter in a Ca^{2+} concentration range not far below that at which the uniporter begins to transport Ca^{2+}. Furthermore, if the number of RaM transporters is similar to that of the uniporter, the turnover per site of the uniporter is in the range for gated pores while that for the RaM is in the range of simple pores or channels. It is tempting to speculate that binding of Ca^{2+} to the RaM's inhibitor site causes a conformational change in the transport complex, rotating a gate into the channel, converting it into the uniporter, a gated pore, and that the RaM and the uniporter are the same protein complex in two distinct conformational and conductance forms. This would be consistent with the results of the patch clamp data of Kirichok et al., which showed multiple conductance states and a maximum ion flux much greater than that of the "classical" uniporter, if these were characteristics of the RaM conductance state as opposed to the uniporter conductance state [84]. We must await molecular biological data to determine whether or not this speculation is true. Regardless of whether or not this is true, it is important to distinguish between these two modes of transport because the transport kinetics of these forms of transport are vastly different. The names, RaM and uniporter, should be associated with the transport kinetics and characteristics of these modes of transport not simply with the protein complex or complexes mediating the transport.

The situation with the mitochondrial Ca^{2+} efflux mechanisms is very different. The Na^+-dependent and Na^+-independent efflux mechanisms are distinctly different transporters with different types of kinetics, different selectivity series, many different inhibitors, and primarily

important in different types of tissue. One seems to be electroneutral and the other electrogenic. Why do mitochondria need these two types of Ca^{2+} efflux mechanisms? Consider that the Na^{+}-independent mechanism is dominant in liver and kidney, tissue involved with the elimination of many ions and toxins from the body. Interestingly, in the selectivity series for this mechanism, the transport velocities of Ca^{2+}, Sr^{2+}, and Mn^{2+} are not very different. Perhaps the Na^{+}-independent mechanism should be looked upon not as a simple Ca^{2+} efflux mechanism, but as a more general mechanism for ridding the mitochondrion of divalent ions so that they can be eliminated from the cell [12].

In summary, we know of four or five transporters of Ca^{2+} across the mitochondrial inner membrane. The PTP can also transport Ca^{2+}. We know quite a bit about the transport characteristics of the uniporter, the RaM, and the two efflux mechanisms in isolated mitochondria, but not very much about their molecular identity. We know quite a bit about the molecular identity of the mRyR, but not as much about its transport characteristics in situ in the mitochondrial membrane and draw on its similarity to the RyR's of SR and ER. The function of the RaM must be to transport Ca^{2+} for activation of ADP phosphorylation. We conclude this because: 1) It has been shown to activate ADP phosphorylation in isolated mitochondria [91], and it doesn't transport enough Ca^{2+} to do anything else [33,34]; 2) It seems to limit the amount of transported Ca^{2+} in order to avoid inducing the MPT [33,34]; and 3) Because the amount of transported Ca^{2+} is limited, it is more economical energetically [81]. The uniporter and mRyR can clearly transport the Ca^{2+} necessary to activate ADP phosphorylation in the microdomains, as well as to affect the shape of cytosolic Ca^{2+} transients, and to shield cytosolic regions from the Ca^{2+} transients in some types of cells [87,102,193,194]. However, because of the slow rate of the efflux mechanisms compared with influx, the problem caused by uniporter or mRyR uptake is that in the microdomains that probably allows Ca^{2+} to accumulate to levels which could induce the MPT [81]. This may be why flickering of the MPT developed, i.e. to function as an additional mitochondrial Ca^{2+} efflux mechanism under conditions of Ca^{2+} overload [176–181]. The further evolution of the full MPT then allowed mitochondria to play an additional role in induction of apoptosis under the appropriate conditions. The mRyR may be an effective Ca^{2+} influx mechanism within microdomains in excitable tissue like myocytes. It is likely that while the Na^{+}-dependent efflux mechanism is a Ca^{2+} transport mechanism, the Na^{+}-independent mechanism is a general efflux mechanism for divalent cations in tissue where clearance of ions and toxins from the body is a priority [12].

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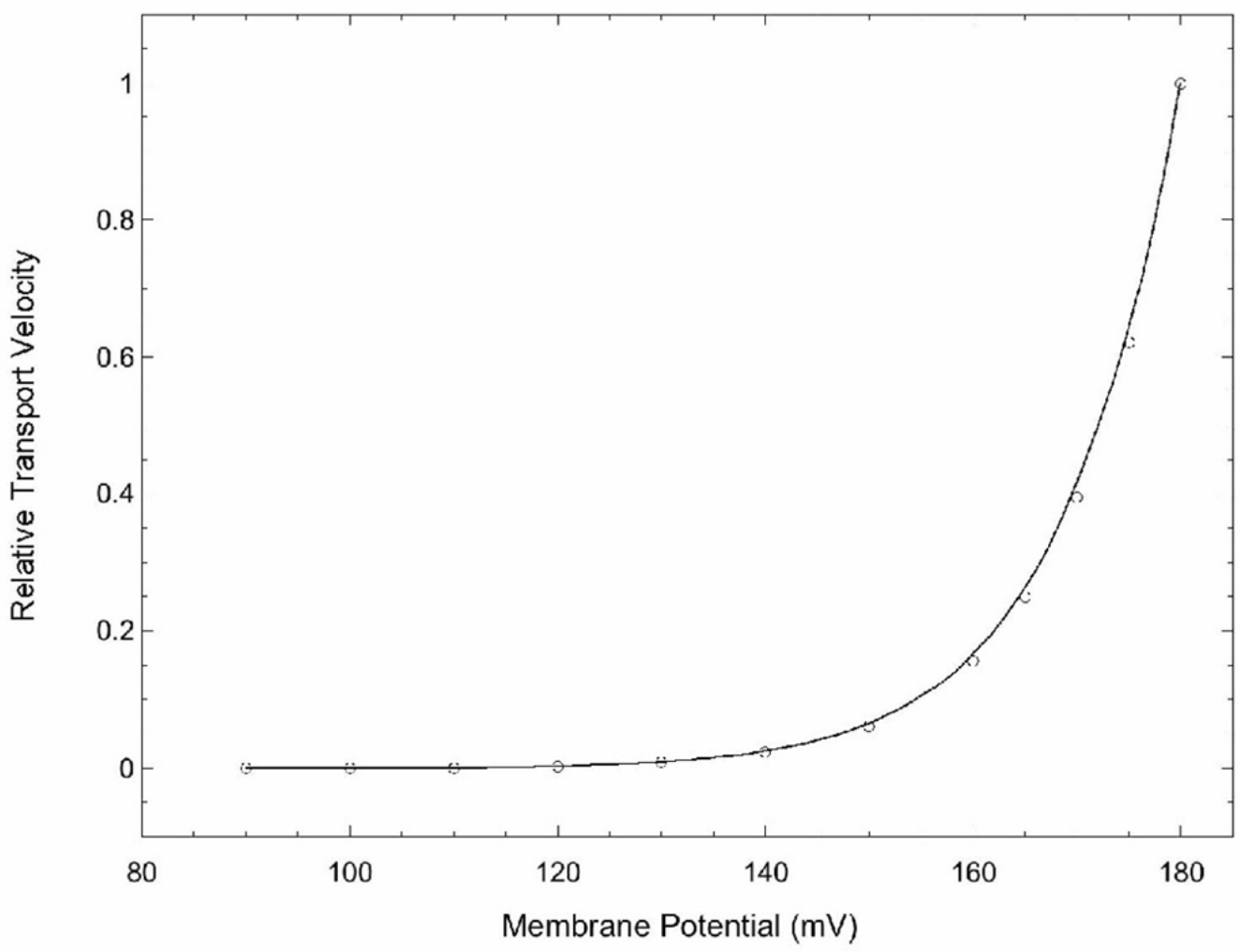

Figure 1.

A plot of the membrane potential dependence of the mitochondrial Ca2+ uniporter which is the function $[e^{\Delta\varphi/2}\Delta\varphi/2]\{\sinh(\Delta\varphi/2)\}$, where $\Delta\varphi = 2bF(\Delta\psi - \Delta\psi_0)/RT$, and where $\Delta\psi$ is the membrane potential in mV. F, R, and T are the Faraday constant, the gas constant, and the Kelvin temperature, respectively. b is a fitting parameter = 1.0, and $\Delta\psi_0$ is a fitting parameter = 91 mV. This plot has been normalized to give a value of 1.0 at 180 mV.

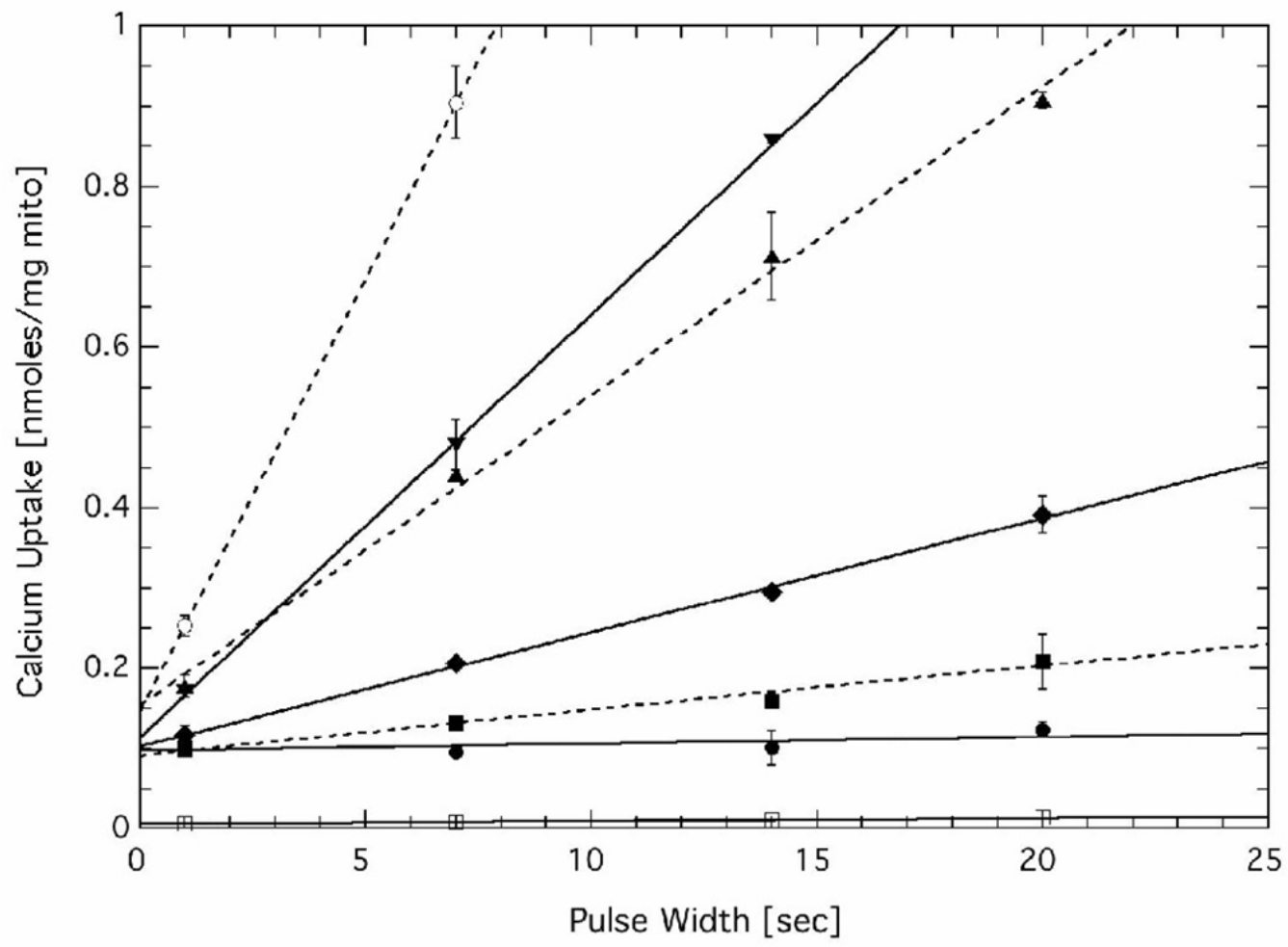

Figure 2.

Ca uptake vs pulse width as a function of pulse height for rat liver mitochondria. Pulses were made using 2.4 mM EGTA and 2.7 mM $^{45}CaCl_2$-buffered with HEDTA to a free calcium in the pulse medium of 1μM. The experiment was run in HEPES-buffered 150 mM KCl (pH 7.2) with 5 mM Ksuccinate present with mitochondria at a concentration of 0.5 mg/ml. the pulse heights are as follows: □, 474 +/− 14 nM with ruthenium red; ●, 165 +/− 12 nM; ■, 307+/−7nM; ◆, 408 +/− 8 nM; ▲, 567 +/− 8 nM; ▼;, 719 +/− 2 nM; ○, 877 +/− 9nM. Error bars are 1 standard deviation. (Reprinted from Gunter, T. E., Buntinas, L., Sparagna, G. C., and Gunter, K. K. (1998) The Ca^{2+} Transport Mechanisms of Mitochondria and Ca^{2+} uptake from Physiological Type Ca^{2+} Transients. Biochim. Biophys. Acta 1366, 5–15 with permission from Elsevier Science.)

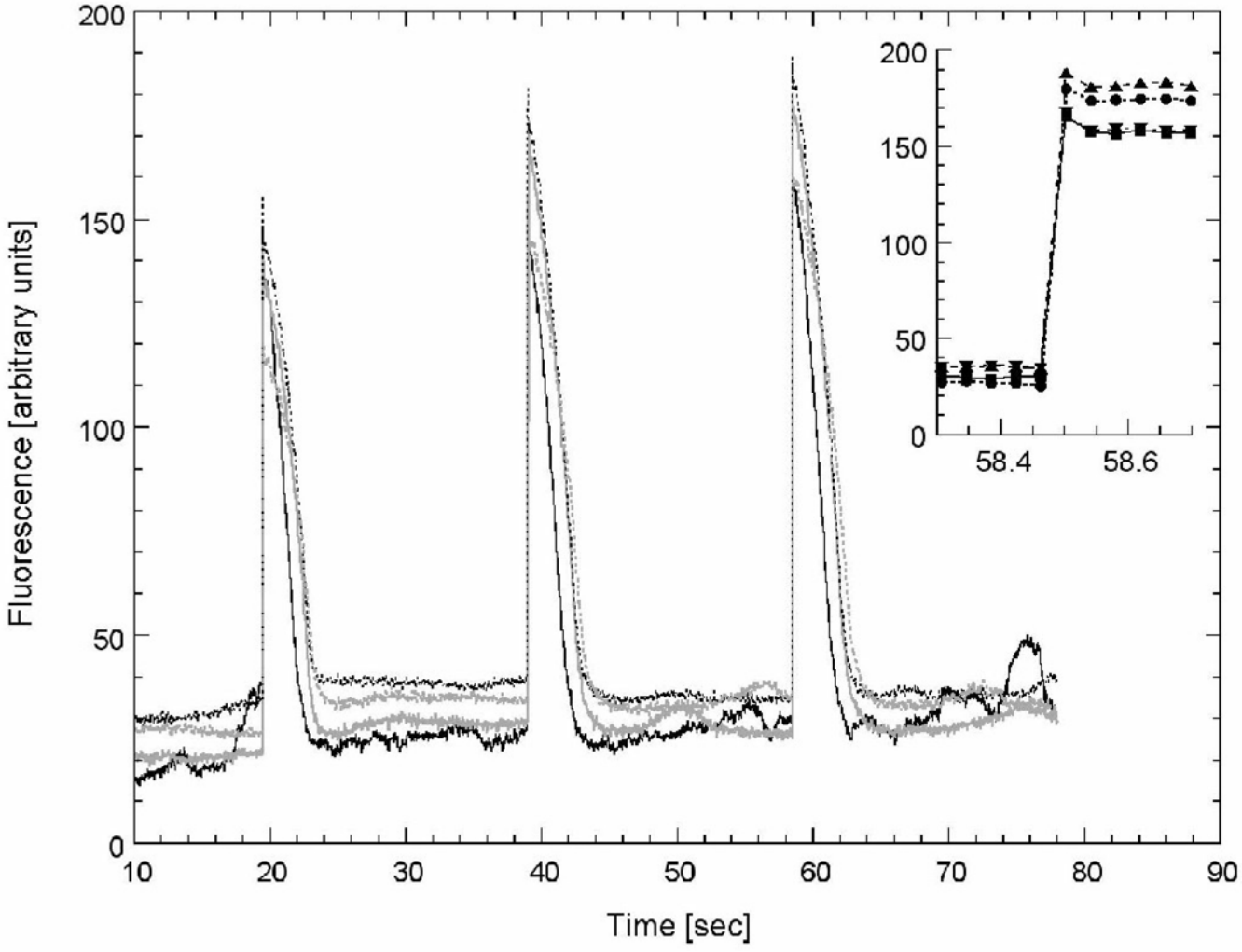

Figure 3.

Fluorescence response (Fluo 4) indicating intramitochondrial free Ca^{2+} concentration in four individual mitochondria during 3 UV pulses of 0.5 ms duration, which release Ca^{2+} from "caged Ca^{2+}". Ram uptake is complete in less than 40 ms; however, $[Ca^{2+}]_m$ remains high in the mitochondrial matrix for seconds. The inset shows a time expanded view of uptake during the third pulse. ((Reprinted from Gunter, T. E., Yule, D. I., Gunter, K. K., Eliseev, R. A., and Salter, J. D. (2004) Calcium and Mitochndria. FEBS Lett. 567, 96–102 with permission from Elsevier Science.)