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Role of Mitochondrial Ca^{2+} in the Regulation of Cellular Energetics

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Abstract

Calcium is an important signaling molecule involved in the regulation of many cellular functions. The large free energy in the Ca^{2+} ion membrane gradients make Ca^{2+} signaling inherently sensitive to the available cellular free energy, primarily in the form of ATP. In addition, Ca^{2+} regulates many cellular ATP consuming reactions such as muscle contraction, exocytosis, biosynthesis and neuronal signaling. Thus, Ca^{2+} becomes a logical candidate as a signaling molecule to modulate ATP hydrolysis and synthesis during changes in numerous forms of cellular work. Mitochondria are the primary source of aerobic energy production in mammalian cells and also maintain a large Ca^{2+} gradient across their inner membrane providing a signaling potential for this molecule. The demonstrated link between cytosolic and mitochondrial [Ca^{2+}], identification of transport mechanisms as well as proximity of mitochondria to Ca^{2+} release sites further supports the notion that Ca^{2+} can be an important signaling molecule in the energy metabolism interplay of the cytosol with the mitochondria. Here we review sites within the mitochondria where Ca^{2+} plays a role in the regulation of ATP generation and potentially contributes to the orchestration of the cellular metabolic homeostasis. Early work on isolated enzymes pointed to several matrix dehydrogenases that are stimulated by Ca^{2+}, which were confirmed in the intact mitochondrion as well as cellular and *in vivo* systems. However, studies in these intact systems suggested a more expansive influence of Ca^{2+} on mitochondrial energy conversion. Numerous non-invasive approaches monitoring NADH, mitochondrial membrane potential, oxygen consumption and workloads suggest significant Ca^{2+} effects on other elements of NADH generation as well as downstream elements of oxidative phosphorylation including the F_1F_O-ATPase and the cytochrome chain. These other potential elements of Ca^{2+} modification of mitochondrial energy conversion will be the focus of this review. Though most of specific molecular mechanisms have yet to be elucidated, it is clear that Ca^{2+} provides a balanced activation of mitochondrial energy metabolism which exceeds the alteration of dehydrogenases alone.

Introduction

Calcium plays a central role in cell signaling at numerous levels. The remarkably high potential energy in the gradient of Ca^{2+} between the cytosol, extracellular space and specialized cellular compartments (ΔG_{Ca}) approaches the ATP free energy (ΔG_{ATP}) available for cellular work(1-3) with ΔG_{Ca} and ΔG_{ATP} linked together through the Ca^{2+}ATPase and ion transport processes. The large ΔG_{Ca} can quickly change the local [Ca^{2+}] several fold in milliseconds with changes in membrane resistance. The cell uses this rapid and very high gain system as an important trigger in regulating many differentiated cell processes that consume ATP, such as muscle contraction, exocytosis, neuronal transmission and cellular motility. As such, linking Ca^{2+} concentration to the production of ATP would make a logical feed-forward or parallel control network to maintain ΔG_{ATP} during the activation of these processes, but also to support ΔG_{Ca} required for the Ca^{2+} signaling used to trigger these processes.

Supporting this notion, it has been appreciated for many years that that the cell has a remarkable ability to match the rate of ATP production and utilization with little or no change in metabolic intermediates including ADP and Pi (4;5). This phenomenon of constant [ATP] and ΔG_{ATP} during work transitions, especially in heart and muscle, has been termed a metabolic homeostasis (6-8) as it is implied that the metabolic regulation of the cell strives to maintain ΔG_{ATP} constant at the time it needs it most, during increases in workload. Ca^{2+} has been proposed as a key element in the feed-forward or parallel system for the production of mitochondrial ATP and maintenance of metabolic homeostasis in tissues due to its dual role in activating ATPase activity as well as metabolism (6;9-14). In this review, we will focus on the role of Ca^{2+} in modulating the mitochondrial energy conversion processes as it relates to the maintenance of cellular metabolic homeostasis.

The mitochondrion plays a critical role in cellular energy conversion of the differentiated cell by generating ATP from reduced carbon substrates. The mitochondrial membrane maintains a very large ΔG_{Ca}, however, unlike the endoplasmic reticulum or plasma membrane, this potential energy is largely supplied by the -180 to -200 mV membrane potential ($\Delta\Psi$) across the inner membrane in addition to a Ca^{2+} concentration gradient. The large ΔG_{Ca} across the mitochondrial inner membrane is consistent with a role for Ca^{2+} in signaling across this membrane. In addition, the volume of the mitochondrial matrix is very small, on the order of 1 to 15% of the cell cytosol, effectively amplifying the movement of few Ca^{2+} molecules into large concentration changes in the matrix space. Thus, the combination of a large ΔG_{Ca} across the inner membrane and a small volume results in a very high gain signaling potential for Ca^{2+} in the mitochondrial matrix.

While very controversial for many years, numerous genetically generated optical probes, as well as exogenous probes, have demonstrated that the mitochondrial Ca^{2+} concentration ($Ca^{2+}{}_m$) responds to rapid changes in gross or regional changes in cytosolic Ca^{2+} ($Ca^{2+}{}_c$), implying a dynamic import and export system for Ca^{2+}(15-20). The contributions by Pozzan and Rizzuto using genetically coded probes for different cellular domains (18;21-23) cannot be minimized in demonstrating the role of micro-domains of Ca^{2+} in overcoming some of the kinetic limitations of Ca^{2+} signaling in mitochondria. These local regions of high Ca^{2+} release near mitochondria add a cellular geometry aspect to mitochondrial metabolic regulation. Finally, the transporters for Ca^{2+} across the mitochondrial membrane have just recently been identified (24;25) almost 50 years after the description of Ca^{2+} uptake by mitochondria(26). The Ca^{2+} uniporter system is apparently a protein complex (27) that will likely have a wide variety of regulatory mechanisms for changing its conductance. The export system in heart and brain is believed to be primarily an exchange mechanism with Na^+, while in liver and kidney, Na^+-independent efflux is dominant(28). However, the large discrepancy between reported influx and efflux V_{max} values suggests other mechanisms may also be at play (28). For the purposes of this review, we will assume that $Ca^{2+}{}_c$ influences $Ca^{2+}{}_m$, though the temporal fidelity of this relationship may vary (see (29)).

Given that Ca^{2+} signaling occurs across the mitochondrial membrane, we will concentrate on the identified and potential targets for Ca^{2+} signaling within the mitochondrion that influence the energy conversion processes.

Isolated Enzyme Studies

One of the classic systems of metabolic regulation is phosphorylation of mitochondrial pyruvate dehydrogenase (PDH) originally described by Linn et al. who, in a series of papers in 1969 using ^{32}P labeling and activity measurements (30;31), identified a protein kinase (PDHK) and phosphatase activity (PDHP) within the PDH complex. Soon thereafter, Randle's lab demonstrated that insulin can modulate the phosphorylation of PDH in fat cells (32;33) providing the first evidence that this was a regulated process. In very short order,

Denton et al. (34) demonstrated that PDHP was Ca^{2+} sensitive and that physiological levels of Ca^{2+} can increase PDH activity by enhancing de-phosphorylation. Similar results were also found by Siess and Wieland (35). These demonstrations were the first direct linkage of Ca^{2+} to the activity of a mitochondrial energy conversion enzyme.

The PDH complex is a huge ~10 megadalton molecular machine that has three catalytic components, i.e. pyruvate dehydrogenase (E1), dihydrolipoyl transacetylase (E2), and dihydrolipoamide dehydrogenase (E3), a core protein E3 binding protein (E3BP), and two regulatory components, pyruvate dehydrogenase kinase (PDHK) and pyruvate dehydrogenase phosphatase (PDHP). The regulatory elements are minor constituents of the complex (~10%). There are 4 isoforms of PDHK that are tightly bound and two weakly bound isoforms of PDHP(36). The regulatory phosphorylation sites on the E1 α chain are Ser-292, Ser-299 and Ser-231(all amino acid positions are referenced to the porcine; each mammalian species has slight shifts in position) (37;38). Though there is some controversy on the relative effectiveness of these sites, the Ser-292 is apparently the most potent inhibitory site followed by Ser-299 and Ser-231. These phosphorylation sites have recently been confirmed by mass spectroscopy (39;40) while Gnad et al. (40) also identified Ser-294 and Thr-230 as sites. Though the activity of the PDHK is under numerous different controls including transcriptional control of isoforms in the tissue, attenuation by ADP, NAD^+, and pyruvate, and activation by ATP, NADH and acetyl-CoA via the reduction and acetylation of the lipoyl groups of E2 (for review see(41)), there is no direct link of the PDHK isoforms activity with Ca^{2+}. The activation of PDH via Ca^{2+} is apparently solely dependent on dephosphorylation of the E1 subunit by the activation of PDHP isoform 1 (PDP-1). The PDP-1 Ca^{2+} -binding domain enhances its association with the E2 subunit increasing phosphatase activity (42-44). PDHP-2 is the primary isoform in liver and adipose tissue (36) and is activated by polyamines but not Ca^{2+} {Damuni, 1984 DAMUNI1984 /id;Pezzato, 2009 PEZZATO2009 /id;Huang, 1998 HUANG1998 /id}.

McCormack and Denton screened most of the citric acid cycle enzymes of the porcine heart in 1979 (47) searching for other interactions sites of Ca^{2+}. They found no effect of Ca^{2+} on aconitase, glutamate dehydrogenase, malate dehydrogenase, NADP-isocitrate dehydrogenase, succinate dehydrogenase and the non-phosphorylated form of PDH. Note that PDH was already in its active form in this screen. A modest increase in the citrate synthase affinity for oxaloacetate was detected but likely not significant in the presence of physiological Mg^{2+}. However, a very large increase in the affinity of the α-ketoglutarate dehydrogenase complex (αKDH, also known as oxoglutarate dehydrogenase) for α–ketoglutarate with no change in V_{max} was observed and confirmed by later studies in bovine kidney (48). Conversely, inhibition of αKDH by high concentrations of Ca^{2+} has been reported in the brain (49). The interaction of Ca^{2+} with ADP, ATP, NADH, and Pi levels can alter the rate of αKDH by over 100–fold, *in vitro* (48;50). However, while αKDH is controlled and regulated by substrates and cofactors (see review (51)), the precise mechanisms of Ca^{2+} modulation of αKDH activity are not known.

Calcium and ADP increase the substrate affinity for NAD^+ linked isocitrate dehydrogenase (ICDH) with no change in V_{max}(52). The mechanism for this process is also unknown. A sophisticated ICDH serine phosphatase/kinase system is present in *Escherichia coli* (see review (53)), however, no homologue in eukaryotic systems has been detected. Mitochondrial glycerol 3-phosphate dehydrogenase (G3PDH), which is highly active in fast-twitch muscle fibers and pancreatic β-cells, is also activated by Ca^{2+} through an increased affinity for glycerol 3-phosphate but no change in Vmax(54). Ca^{2+} binding to two EF-hand motifs close to the carboxy terminus of the enzyme is thought to be responsible for the Ca^{2+} activation of G3PDH ((55;56)).

Another important element in the interaction of Ca^{2+} with metabolic events is the impact on substrate transport (see review (56). Of the Ca^{2+} sensitive transporters, the aspartate/glutamate exchangers, citrin and aralar, are the best characterized (57-59) along with the ATP-Mg/Pi carrier (sCaMC)(60;61). These transporters belong to the carrier family with EF-hand Ca^{2+}-binding motifs on the external membrane N-terminal domains. Through these mechanisms, Ca^{2+}_c can directly impact the transport of redox elements, the net ATP content of the matrix, as well as potentially impact cellular signaling through cytosolic amino acid levels (62;63). It has been proposed that the activation of sCaMC may be involved in the regulation of ATP synthesis with work in the heart (56), however, how an increase in ATP influx into the matrix would support ATP production is unclear other than increasing the net concentration of adenylates and perhaps improving the kinetic driving force for Complex V.

As discussed above, the actual mechanisms for Ca^{2+} modulation of the mitochondrial enzymes of energy conversion are not extensively illuminated as discussed over a decade ago by McCormack et al (12). The lone exception is the well characterized PDH system, where the tightly associated kinase and weakly associated Ca^{2+} phosphatase result in an effective Vmax regulatory mechanism, *in vitro*. A V_{max} regulatory mechanism is much more likely to have an impact in the intact cell since it will generally not be dependent on substrate concentrations and is the result of an amplification of the Ca^{2+} signal through a post-translational modification (PTM) system. In contrast, the substrate affinity effects of Ca^{2+} on ICDH and αKDH are not well characterized, and if these effects exist in the matrix, they will require the substrate concentrations to be well below the Vmax values to have any impact. Secondly, these affinity effects are not persistent and only occur with direct association of Ca^{2+}, again limiting the amplification of the Ca^{2+} signal when compared to a PTM. An excellent review on the current understanding of the mechanisms and phenomenology of Ca^{2+} activation of these enzymes was recently provided by Denton (64).

The direct Ca^{2+} effects on PDH, NAD-IDH, αKDH and even G3PDH suggest that Ca^{2+} provides a large stimulus for carbohydrate oxidation. However, there is little evidence for direct Ca^{2+} activation of enzymes from the ketone and fatty acid oxidation pathways which supports the energy conversion of many tissues, most notably the heart. As will be seen in the discussion of intact systems, the stimulation of NAD(P)H generation in intact systems such as heart and substrate dependent effects on isolated mitochondria suggests that Ca^{2+} dependent mechanisms of NADH generation from fatty acid oxidation are also likely in play but not elucidated at this time.

It is interesting to consider that if the PDP-1 phosphatase had been a bit weaker associated with the PDH complex, the effect of Ca^{2+} on PDH *in vitro* would have been missed completely. In addition, the phosphorylation of PDH is persistent (65) through isolation also permitting *in vitro* analysis of events occurring *in vivo*. Due to the prevalence of protein complexes in the mitochondria matrix and the potential association of other PTM generating systems in these complexes, it is possible that many interactions have been missed due to weak associations or ill defined PTM. In addition, the matrix conformation of proteins may result in different Ca^{2+} interactions, *in vivo*, than in isolated enzymes. Thus, the evaluation of the effects of Ca^{2+} on intact mitochondrion might yield more interaction sites than a screen of isolated enzymes.

Isolated Mitochondria

Is PDH regulated by phosphorylation in intact mitochondria and does Ca^{2+} maintain its regulatory effect? For monitoring mitochondrial protein phosphorylation turnover, inorganic ^{32}P can be added to energized mitochondria and converted into $\gamma^{32}P$-ATP via oxidative phosphorylation as well as $\beta^{32}P$-ADP by adenylate kinase (for methods overview see (66)). The initial ^{32}P incorporation studies from Randle's lab (37;67) in intact

mitochondria demonstrated that the PDH E1 phosphorylation sites are dynamically modulated, responded to pyruvate and correlated with extracted PDH activity. They also demonstrated that the PDH phosphatase was under Ca^{2+} control in intact adipose mitochondria in 1974(68) These results have been repeated using ^{32}P(69) and proteomic approaches(70) as well as using quantitative mass spectrometry(39) on the E1 active sites in isolated heart and liver mitochondria. In an important series of papers by McCormack and Denton (10;71;72), the observations of Ca^{2+} activity in isolated proteins were validated in intact heart and adipose mitochondria. These studies demonstrated that ~50 nM free Ca^{2+} in the extra-mitochondrial space of heart mitochondria enhanced the apparent affinity of α-ketoglutarate that was sensitive to Na^{+}, Mg^{2+} and ruthenium red (RuRed), an inhibitor of Ca^{2+} entry across the inner membrane (73). No effect of Ca^{2+} was detected on the maximum coupled respiratory rate in these studies. These observations led to the notion that Ca^{2+} could serve as a regulator of oxidative phosphorylation independent of the classical role of ADP, ATP, Pi and NADH. This was summarized by Denton and McCormack in 1980 as "*….changes in cytoplasmic and intramitochondrial* Ca^{2+} *may be a rather general means whereby stimulation of the supply of reducing equivalents for respiration can be achieved with minimum increases in the NAD+/NADH and ADP/ATP ratios*." (10). This was one of the first discussions of the role of $Ca^{2+}{}_{m}$ signaling in the regulation of metabolic homeostasis in the cell independent of the substrates of energy conversion.

Building on this work, Moreno-Sanchez (74;75) and Moreno-Sanchez and Hansford ((76-78) determined the effects of extra-mitochondrial Ca^{2+} on oxidative phosphorylation using a variety of methods. Figure 1:Panel A shows the dose dependent increase in the maximum velocity of liver mitochondria oxidizing succinate despite the lack of a previously identified Ca^{2+}-sensitive dehydrogenase linked to succinate oxidation. Indeed, the effect was not diminished in the presence of rotenone where NAD(P)H oxidation is completely. In this discussion we will refer to fluorescence studies on NADPH and NADH as NAD(P)H where the origins of the fluorescence signal has not been fully characterized (liver and most other tissues) but as NADH in the heart where the NAD(P)H contribution is believed to be minimal(79). These results were expanded by Murphy et al(80) showing that the oxidation of durohydroquinone was activated by Ca^{2+} suggesting an effect within Complex III. No effect on Complex IV was detected by Murphy et al. It should be noted that Johnston and Brand (81)found no effect of Ca^{2+} on succinate oxidation in rat liver mitochondria, however, the incubation and preparation conditions were highly varied amongst these early studies. Another series of studies follows the strategy of Koretsky and Balaban (82) in using the slope of the linear mitochondrial NADH versus respiratory rate relationship to isolate the effects of [NADH] on oxidative phosphorylation. In these studies, the effect of increasing NADH alone, via dehydrogenases, can be determined by simply titrating the concentration of carbon substrates for a given mitochondrial dehydrogenase (82). Intriguingly, Moreno-Sanchez et al. found that the [NADH] to respiration relationship had a steeper slope and was shifted to the left (i.e. higher rates at lower [NADH]) with the addition of Ca^{2+} (Figure 1:Panle B) (83). These data implied that the effect of Ca^{2+} cannot be ascribed to changes in NADH from alterations in the original Ca^{2+} dehydrogenases alone and other elements should be considered.

These initial results and subsequent reports suggested that Ca^{2+} might be doing much more in the regulation of oxidative phosphorylation than just modifying dehydrogenase activity including the alteration of the adenine nucleotide translocase (ANT)(75), the F_1-F_0-ATPase (Complex V)(84), the cytochrome chain (80)or other intermediary metabolism enzymes. It is also important to note that the concentration range of these studies represents the range we believe that normal physiological Ca^{2+} signaling occurs (0.01 to 1 μM). Many earlier studies on isolated mitochondria focused on concentrations exceeding 50 μM such as the convincing demonstration of the inhibition of Complex V ATPase activity (85) as well as the early

demonstrations of an activation of ANT by external Ca^{2+} that was shown not to occur at physiological [Ca^{2+}](86). Many of these early studies with high μM Ca concentrations may provide important information on Ca^{2+} effects during patho-physiological conditions but likely play little role under normal physiological conditions.

Territo et al (87) expanded the approach of titrating substrates and Ca^{2+} (82;83) and applied it to both [NADH] as well as the mitochondrial membrane potential (ΔΨ) in porcine heart mitochondria. ΔΨ is of primary interest since it is the major driving force for ATP production by Complex V and the relationship between the free energy from NADH (ΔG_{NADH}) and ΔΨ reveals information on the efficiency of the cytochrome chain in converting ΔG_{NADH} to ΔΨ. An example from this study is presented in Figure 1 Panel C for a glutamate + malate dose response curve in the presence and absence of Ca^{2+}. What is shown here is a current (i.e. oxygen consumption) versus voltage (ΔΨ) plot of oxidative phosphorylation. The slope of this relationship is proportional to the resistance of the system for converting a given voltage into a current flux down the cytochrome chain. In the absence of a considerable leak pathway, this must also reflect the flux of protons across Complex V, as this is the major source of proton re-entry into mitochondria under State 3 conditions. As seen in Figure 1 Panel C, the effective resistance of Complex V in the membrane was remarkably dependent on [Ca^{2+}]. Under low Ca^{2+} conditions, mitochondria were capable of generating very high State 3 ΔΨ, however; ΔΨ could not be used to generate ATP. These data suggest that dehydrogenase reducing equivalent supply to the cytochrome chain was more than adequate to generate ΔΨ; only this energy could not be used by Complex V in the absence of Ca^{2+}. Similar observations were made with all substrates used, including succinate where NADH is not utilized. Thus, Ca^{2+} activated oxidative phosphorylation independent of dehydrogenase activity as suggested by the earlier studies. Data with arsenate as a substrate to isolate ANT(75) suggested that the majority of this non-dehydrogenase effect in heart mitochondria was due to an activation of Complex V by Ca^{2+} (87), and, as previously demonstrated by Beis and Newshome (86), there was no Ca^{2+} effect on ANT.

Another important finding from this study was that the linear dependence of respiration on ΔΨ had a maximum slope (in the presence of Ca^{2+}) of only ~9 nmoles O_2 /min/nmole cyto a/mV ΔΨ. This is an interesting number since it provides the relationship of ΔΨ versus ATP production by Complex V at Vmax (maximum [ADP] and [Pi]). Note that without calcium (87) or with non-saturating ADP(88), this number is even lower. Taking the maximum oxygen consumption per nmole cyto a in the heart approaching 700 nmoles O_2/nmole cyto a/min (89), the normalized rate of respiration only changes ~1.3% /mV ΔΨ. Thus, ΔΨ alone is not a powerful modulator of ATP production rate and will be discussed further below.

Panov and Scaduto (90) also observed an increase in NADH, ΔΨ and respiration rate by adding Ca^{2+} during the titration of ADP in the presence of saturating carbon substrate. Similar results were also obtained using ATPase additions by Territo et al. (91) in the presence and absence of Ca^{2+}, where the activation of oxidative phosphorylation by Ca^{2+} was associated with an increase in NADH but a decrease in ΔΨ polarization consistent with an increase in both NADH generation and Complex V activity. Panov and Scaduto also confirmed that Ca^{2+} increased the Vmax of respiration for the many fuels tested, while the apparent affinity for ADP was unchanged.

Another issue that could be tested in isolated mitochondria is whether the affinity of Ca^{2+} to stimulate ATPase activity matches the affinity for the generalized activation of oxidative phosphorylation discussed above. This was tested by Balaban et al. (92) for the limited case of a reconstituted system of the Ca^{2+} ATPase of the sarcoplasmic reticulum (SERCA) and cardiac mitochondria. By reintroducing the isolated sarcoplasmic reticulum (SR) to a

suspension of isolated mitochondria of the porcine heart, the effects of Ca^{2+} on SERCA and mitochondrial oxidative phosphorylation simultaneously can be determined. An example from this study is shown in Figure 1 Panel D with the SR reconstituted system and a control experiment with a non-Ca^{2+} dependent ATPase. In Figure 1 Panel D, the resistance of the system is examined by plotting the rate of respiration versus the normalized change in [NADH]. Regrettably, mitochondrial $\Delta\Psi$ could not be measured due the interference from the membrane potential in the active SR vesicles in the system. As seen with the addition of apyrase, a Ca^{2+} independent ATPase, an increase in respiratory rate was associated with a net decrease in NADH as the rate of utilization increased beyond the capacity for NADH production. However, simply adding Ca^{2+} to the SR-mitochondria reconstituted system resulted in a larger increase in respiration, >5 fold, with a slight increase in [NADH]. Thus, the affinity for Ca^{2+} of SERCA and mitochondrial respiration is appropriate to increase respiration over 5 fold with little or no change in driving force evaluated as [NADH]. These data offer strong support that Ca^{2+} could provide the balanced activation of the mitochondria and ATPase activity in the cell to result in metabolic homeostasis, at least at the level of NADH, observed during increases in cardiac workload.

What is the alteration in Complex V resulting in the change *in vitro* ATPase activity and increased V_{max} of ATP synthesis with Ca^{2+} in the intact mitochondria? Hopper et al. (70) found a Ca^{2+} sensitive de-phosphorylation of the γ subunit of Complex V using a phosphoprotein sensitive dye. In Saris' lab, evidence was found for a proportional increase in the phosphorylation of the c subunit with Ca^{2+} stimulation of liver mitochondria (93). However, there are no clear mechanisms linking Ca^{2+} effects in the intact mitochondria and subsequent *in vitro* activity. What is clear is that, with regard to protein phosphorylation of Complex V, there are many more potential sites that could influence enzymatic activity (39;94-100)however, none have been shown to be Ca^{2+} sensitive as of yet. It is interesting to note that many of these sites are not detected in different mass spectrometry studies, suggesting tissue, species, tissue preparation, or technology driven differences in the detection. Also possible is that the mole fraction of protein that is phosphorylated is very small making the detection difficult and very dependent on the peptide enhancement schemes (100;101). Due to the variability in detection and continuing growth of the discovered sites, it is unlikely that the full phosphorylation scheme of Complex V, as well as other mitochondrial proteins, is fully described. As such, the precise role of Ca^{2+} in Complex V phosphorylation, or for other PTMs, is still to be determined.

Cellular Studies

To study the interplay of mitochondrial energy metabolism and Ca^{2+} it would be ideal to monitor the mitochondrial metabolic status and the Ca^{2+} concentrations in the matrix and adjacent cytosol during cellular work transitions. Using the enhanced mitochondrial NADH fluorescence (79;102;103) ($NADH_m$), likely originating from binding in Complex I(103), fluorescent probes of mitochondrial membrane potential (104-106) as well as $Ca^{2+}{}_c$ and $Ca^{2+}{}_m$ using various fluorescent indicators (107-109), this ideal situation is now nearly realized and has provided many new insights. Measurement limitations in most microscopy studies include the direct determination of ΔG_{ATP} or, more importantly, free ADP and P_i, and the net energetic "current", oxygen consumption, as these are usually limited to larger samples using ^{31}P NMR and oxygen electrodes, respectively. Both of these parameters are critical in the analysis of energy metabolic state. Another limitation of cellular systems is the lack of true work when compared to *in vivo* tissues or isolated organ systems, especially muscle cells, which cannot be easily mechanically loaded in culture or isolated cell systems. Indeed, most cells in established or primary culture are likely working at least an order of magnitude lower than *in vivo*. Thus, interpretation of these experiments needs to be tempered by these limitations especially when dealing with the regulation of energy

conversion in the mitochondria which may be working close to resting conditions in many preparations. Exceptions to this work level limitation likely includes neuronal preparations where the metabolic work associated with ion transport in re-polarization may be appropriately simulated or even over-stimulated (see (15)) as well as secretory systems like β cell preparations.

With many cellular systems the addition of Ca^{2+}, via a wide variety of methods, has convincingly demonstrated an activation of NAD(P)H generation by the entry of Ca^{2+} into the mitochondrial matrix (12). One of the earliest demonstrations of a Ca^{2+} activation of NAD(P)H generation in suspensions of rat liver cells (110) demonstrated that the respiratory stimulation by glucagon, vasoactive intestinal peptide and antidiuretic hormone is proportional to the increase in NAD(P)H fluorescence and was dependent on extracellular Ca^{2+} (Figure 2: Panel A). Since respiration, or NAD(P)H oxidation, increased with these hormones, there is no question that the increase in [NAD(P)H] was the result of increased generation of NAD(P)H and not a decrease in oxidation rate. Using a much more sophisticated and integrative approach, Robb-Gaspers et al. (111) measured Ca^{2+}_c, Ca^{2+}_m, NAD(P)H, ΔΨ, and PDH activity and were able establish some of the cellular mechanisms involved in this phenomenon. The hormone induced increase in [NAD(P)H] was associated with an increase in Ca^{2+}_c, but also Ca^{2+}_m. Also unique to this study was the demonstration that a small increase in ΔΨ was dependent upon Ca^{2+}_m (Figure 2: Panel B). These studies demonstrated that the hormone induced increase in respiration, and ATP hydrolysis is supported, in part, by an increase in ΔΨ as a result of net increase in NAD(P)H, implying that, in liver, the potential energy for performing work was actually increased with these hormones. This group also showed that the activation of PDH alone did not result in the changes in NAD(P)H, suggesting other mechanisms must be in play. The lack of PDH involvement in liver could be due to the dominance of the non-Ca^{2+} sensitive PDP-2 isoform in liver (36) reducing the sensitivity of PDH activity to Ca^{2+}. Related to this point, it is important to point out that the overall dynamic range of ATP production in the liver is very low, that is only 50% (112) while the heart, skeletal muscle, kidney and brain could have alterations in ATP production of 10 fold or more. Thus, the liver may not have the same dynamic modulation of energy metabolism as other tissues possibly explaining the presence of the Ca^{2+} insensitive PDP.

Pralong et al. (113) and Hajnóczky et al. (114)made important observations that the frequency of changes in $[Ca^{2+}_c]$ may encode information for activating metabolism in liver and pancreatic β cells. Hajnóczky et al. showed remarkable temporal fidelity between Ca^{2+}_c and Ca^{2+}_m during the transient increases in Ca^{2+} observed upon exposure to hormones (Figure 2: Panel C). However, the NAD(P)H responses were clearly initiated by Ca^{2+} but had a much longer time constant of recovery when compared to Ca^{2+}_c or Ca^{2+}_m (Figure 2:Panel D). Is the persistent increase in NAD(P)H observed due to the slow metabolic rate to dissipate a transient increase in NAD(P)H production level, or is it due to a persistent increase in NAD(P)H production after the Ca^{2+} pulse? We can estimate the NAD(P)H turnover from the initial rate of reduction of NAD(P)H with anoxia or with a metabolic inhibitor. That is, the initial rate of reduction of NAD(P)H should reflect the rate of oxidation during the steady state when the production and oxidation are matched. In these series of experiments, the rate of NAD(P)H reduction at anoxia or with inhibitors in these cells far exceeds the delay in NAD(P)H oxidation after Ca^{2+} stimulation suggesting that the NAD(P)H turnover is very high in these tissues. Thus, the high turnover of NAD(P)H predict a rapid oxidation of NAD(P)H when Ca^{2+}_m is reduced if its production tracked Ca^{2+}_m. Clearly, this does not occur and the NAD(P)H levels remain elevated for a long times after the Ca^{2+}_m pulse. These data imply that some-type of persistent modification of the NAD(P)H generation system was occurring that could not be explained by the rapid reversible binding of Ca^{2+} to ICDH and αKDH. This persistent effect suggests that a

persistent PTM was generated by Ca^{2+}_m in the NAD(P)H generation system. Since liver PDH was ruled out in previous studies, it is likely that other, un-described, persistent enzymatic perturbation associated with NAD(P)H generation are modulated by Ca^{2+}_m. One of these PTM in liver could be mitochondrial matrix volume which has been shown to be modified by Ca^{2+} and alter several aspects of energy metabolism (115). However the source of this persistent NAD(P)H production stimulation effect in the liver is unknown.

As discussed above, numerous single cell studies have demonstrated similar Ca^{2+} dependent activation of NAD(P)H generation (29;116). Duchen (117) showed that Ca^{2+} influx during a depolarization in neuronal cells increased $NAD(P)H_m$ and was blocked with RuRed (Figure 3: Panel A). This result was replicated in cardiac cells by Liu and O'Rourke (118) in a comprehensive study where the previously discussed metabolic homeostasis was demonstrated for $NADH_m$ in single paced myocytes (Figure 3: Panel B). ATP hydrolysis was increased by 4 Hz pacing that increased Ca^{2+}_m but was associated with no change in $NADH_m$. This result suggests that the delivery of reducing equivalents was matched to consumption despite maintaining ΔG_{NADH} implying a matched increase in $NADH_m$ generating capacity during the work transition. This effect was dependent on Ca^{2+}_m since RuRed disrupted the $NADH_m$ homeostasis with pacing. These data confirm the notion that the activation of $NADH_m$ generation by Ca^{2+}_m is required for the maintenance of metabolic homeostasis.

Das and Harris in a series of papers (119-121) found that the extracted ATPase activity of cardiac Complex V was increased with pacing and adrenergic stimulation suggesting that work may also impact the downstream elements in oxidative phosphorylation and not just NADH generation. Similar observations have also been made in the canine heart with dobutamine, *in vivo* (122). These studies were accomplished with a rapid extraction of sub-mitochondrial particles to assay Complex V ATPase activity; that is Complex V working in "reverse" hydrolyzing ATP. Our own experience confirms the necessity to perform these studies immediately after collecting tissue or after perturbations in mitochondria (122). It is also important to note that these *in vitro* Complex V assays are done in the presence of high EGTA concentrations making the direct contribution of Ca^{2+} on the Complex V ATPase activity unlikely(84), despite the fact that a Ca^{2+} binding site has been found in the Complex V β subunit (123). Indeed, we have found no effect of added Ca^{2+} on the isolated Complex V ATPase activity in blue native gel assays. Thus, the effect on Complex V by Ca^{2+} is apparently occurring from indirect effects within the tissue or mitochondria. These studies demonstrate the persistent Ca^{2+} effect on *in vitro* ATPase activity, much like what has been observed for PDH activity with increases in workload in heart and muscle (124-126), consistent with a PTM alteration of the F_1-ATPase interfering with the normal substrate and product binding, regulatory protein interactions or possible rotation of the gamma subunit occurring in the ATPase reaction, *in vitro* (84;122). The extrapolation of these ATPase activity measures to the ATP synthetic reaction in the intact mitochondria, *in vitro* or *in vivo*, is also tenuous, but clearly reflects persistent changes in the enzyme that are responsive to tissue workload. If Complex V maximum activity is altered by Ca^{2+} as suggested by these extraction studies and in intact mitochondria, this would provide a direct mechanism for altering ATP production with little or no change in its reactants, ADP and Pi, due to a matched increase in the Vmax of ATP production capacity.

Recently, evidence has been generated that suggest the effect of Ca^{2+} on NADH generation and mitochondrial function may also be important in the steady state and not just during workload transitions. In several cell lines, Cardenas et al showed that inability to release Ca^{2+} from the endoplasmic reticulum via inositol trisphosphate receptor Ca^{2+} release channel revealed a phenotype characteristic of an overall compromised mitochondrial function(14). These novel data are consistent with the notion that Ca^{2+} modulation of

mitochondrial function might be critically important in the maintenance of mitochondrial function in the steady state.

Intact tissues and *in vivo* measures

The gold standard in establishing a signaling network associated with physiological events is a demonstration of its presence *in vivo* or in intact tissues performing normal functions. Indeed, it was critical to demonstrate early on that PDH activation occurs in tissues during changes in workload or increases in Ca^{2+}_c. This was demonstrated in several intact systems in the early 1980's including heart (125;127), skeletal muscle (128), brain (129) and liver (130). The activation of PDH with exercise in human muscle has also been well established (131). Again, the effects in liver might be due to parameters other than Ca^{2+}_c due to the lack of a Ca^{2+} sensitive pyruvate dehydrogenase phosphatase isozyme (36) and low energy conversion dynamic range. These early observations were consistent with the activation of PDH in maintenance of energy metabolic homeostasis in these tissues, matching the rate of NADH generation with demand.

The heart has been the major model for these studies due to its large metabolic dynamic range, the ease of measuring work both in intact systems *in vitro* and *in vivo*, and its ability to maintain metabolic homeostasis in the face of large alterations in ATP hydrolysis rates. This has been illustrated in numerous studies over the last 50 years with the non-destructive ^{31}P NMR studies (132-135), including novel human data (136;137), providing important confirmation of several decades earlier tissue extractions (138-140). An example from one of these studies is presented in Figure 3, Panel C where the steady state ^{31}P NMR detected ATP metabolites were monitored in a canine heart *in vivo* using a directly applied surface coil(141). As seen in this example, large increases in ATP turnover with work transitions were not, even transiently(141), associated with a change in the ATP hydrolysis products. Portman et al. (142) demonstrated that the capacity to maintain cardiac metabolic homeostasis may not be fully developed in neonatal hearts, while the hypertrophied heart (143) or post-ischemic heart may also have a disrupted metabolic response to work (144).

The best evidence that the metabolic homeostasis is partially dependent on Ca^{2+}_m signaling is, again the effect of RuRed on this process. RuRed disrupts the ability of the perfused heart to maintain ΔG_{ATP} with work transitions (145;146). An example is shown in Figure 3, Panel D where the heart is shown not to maintain [CrP] and [Pi] during a pacing experiment in the presence of RuRed. Thus, as for the cellular studies shown in Figure 2 for $NADH_m$ levels, RuRed disrupts the ability to a metabolic homeostasis in a perfused heart work transition. As discussed earlier, McCormack et al. (124) demonstrated that RuRed also blocks the activation of PDH during hormonal stimulation of intact rat hearts, again consistent with a role of Ca^{2+} entry in dehydrogenase activation with work. We have attempted to use RuRed on the heart *in vivo* using a local infusion into the coronary arteries (for method see (147)) however, we found that the heart could not tolerate the normal doses of RuRed when directly infused *in vivo*. Thus, a definitive study demonstrating a role for Ca^{2+} in regulating the metabolic homeostasis *in vivo* is still lacking.

These studies suggested that metabolic homeostasis exists within the cytosolic ΔG_{ATP} with changes in work load, but what is occurring with regard to the free energy within the mitochondria? Several studies have demonstrated that with physiological increases in workload that $NADH_m$ remains near nearly constant (140;148;149) as demonstrated in cells under some conditions. In a series of studies, Brandes and Bers (150-153) evaluated the role of Ca^{2+}_c and Ca^{2+}_m in the regulation of $NADH_m$ isolated intact trabeculae during pacing transitions. An excellent temporal correlation between Ca^{2+}_m and $NADH_m$ was observed (153). However, a clear initial mismatch of NADH generation and production was observed during a work transition both immediately after a pacing increase or decrease. Thus, in this

preparation the temporal fidelity of workload and metabolic compensation is poor but the metabolic compensation observed was dependent on alterations in Ca^{2+}_m.

It is important to note that if the cardiac workload is extended over normal physiological ranges, the metabolic homeostasis breaks down. Examples include observation of a net NADH oxidation as the ATPase activity is increased from moving an unloaded vented heart to a normal loaded perfused heart *in vitro* (154) and a net reduction in NADH in the over-paced working heart due to demand ischemia(148). In addition to NADH levels, the cytosolic ΔG_{ATP} is also not maintained in these extremes, increasing with KCl arrest (155;156) and decreasing as maximum workloads (133;135). Thus, not surprisingly, metabolic homeostasis only operates effectively over physiological workloads. It is also important to point out that Arai et al. (157) also demonstrated that the redox state of cytochrome c was unchanged *in vivo* with dobutamine infusions using reflection absorption spectrophotometry consistent with the notion that the redox state of the respiratory chain is also maintained nearly constant, *in vivo*. Regrettably, fluorescence interference from the visceral pericardium makes $NADH_m$ fluorescence measurements from large animal hearts, *in vivo*, difficult to perform (158).

The measurement of mitochondrial $\Delta\Psi$ in working intact tissues is difficult to accomplish. However, several investigators have made estimates using a variety of approaches. Wan et al.(159) in the perfused rat heart using ^3H tetraphenylphosphonium to estimate $\Delta\Psi$ found a slight depolarization with increasing workload with unchanged ADP, Pi and $NADH_m$. The authors suggested that the only way to accomplish this is modification of Complex V kinetics since $\Delta\Psi$ depolarized and its substrates remained constant. This observation and conclusion is similar to the isolated mitochondria studies with Ca^{2+} and ATPase additions by Territo et al (91). Kauppinen and Hassinen(160) using safranin and Kauppinen(161) using ^3H triphenylmethylphosphonium also found small depolarization with pacing in the perfused heart. Piwnica-Worms et al (162) demonstrated that the retention of Tc sestamibi in heart cells is critically dependent on the $\Delta\Psi$ to the extent that $\Delta\Psi$ can be estimated using this distribution. Nuclear imaging of Tc-sestamibi has been used extensively in clinical studies to determine cardiac perfusion and viability. In these studies, it is common to collect resting and maximum exercise Tc-sestamibi images. In general, the clinician is looking for the same signal distribution in both conditions, implying that in the normal heart, $\Delta\Psi$ only slightly decreases with exercise (for example see Verzijlbergen et al. (163)) Thus, the best evidence available suggests that the mitochondrial $\Delta\Psi$ depolarizes modestly during increases in workload and associated increases in net Ca^{2+}_c and no evidence exists in the heart that $\Delta\Psi$ is hyperpolarizing and could drive ATP production higher during increases in workload in the heat.

So how is cardiac ATP synthesis balanced with ATP hydrolysis during these dynamic changes in cardiac workload without significant changes in [ADP] and [Pi], the substrates for ATP synthesis? As discussed above, the current consensus is that Ca^{2+} dependent activation of NADH generation via known and likely unknown, activation of NADH generation pathways is important. Indeed, the demonstrated activation of PDH with workload is consistent with this notion (125;127). However, if NADH alone was driving ATP production higher, then the driving force for Complex V, $\Delta\Psi$, would have to be increased sufficiently to drive ATP synthesis at a fixed concentration of ADP, Pi and ΔG_{ATP}. How big a change in $\Delta\Psi$ would have to occur to increase ATP production 3 to 4 fold under these conditions? As discussed above, the relationship between $\Delta\Psi$ and ATP production is linear over the physiological rates with a slope ~1.3% change in ATP production/mV $\Delta\Psi$ (87). This suggests that increasing the heart rate by 3 fold would require an additional ~150mV over the resting value of 180 mV taking $\Delta\Psi$ to an unreasonable value >300 mV. Indeed, most of the current evidence suggests that the $\Delta\Psi$ decreases with

increasing workload, decreasing the driving force for ATP production, not increasing it. Thus, a model that just uses $\Delta\Psi$ or an activation of the dehydrogenases, to attain a metabolic homeostasis would not work if this linear observation between $\Delta\Psi$ and ATP production in isolated mitochondria is correct. Clearly the activation of NADH generation by Ca^{2+} must be coupled to other "downstream" events in oxidative phosphorylation to be effective in maintaining the metabolic homeostasis.

Alternatively, activation of the Complex V V_{max} by mitochondrial Ca^{2+} would permit a higher rate of ATP production at a fixed or reduced $\Delta\Psi$ and a constant ΔG_{ATP} as suggested by several investigators. This is consistent with isolated mitochondria data where Ca^{2+} increases the maximum rate of ATP production of Complex V and the observation that the maximum velocity of Complex V ATPase activity is nearly proportional to the workload of the hearts it was extracted from(122). Therefore, it is reasonable to propose that activation of the maximum velocity of Complex V for ATP synthesis is part of the Ca^{2+} activation scheme toward maintenance of metabolic homeostasis. Although this activation of Complex V ATP synthetic activity has been demonstrated in isolated mitochondria with appropriate sensitivity to Ca^{2+} and has been demonstrated to be persistent in the isolated Complex V, implying a PTM, the precise mechanism of this modification remains unknown. Phillips et al. has suggested this might be due the labile nature of many PTM's in the bacterial system that might be still in play in the mitochondrial matrix relying on autophosphorylation mechanisms(164).

An activation of NADH generation and ATP synthetic capacity at Complex V by Ca^{2+} provides a balanced activation of mitochondrial metabolism at the entry of reducing equivalents to the respiratory chain with the re-entry of protons via actual ATP production at Complex V. Both of these effects are generated by apparent changes in the actual kinetics of these reaction systems. However, this simple model still leaves some important gaps in how the respiratory rate is matched to the ATP hydrolysis rate. The classical mechanism for the regulation of the rate of oxygen consumption in the mitochondria is the redox state of Complex IV or cytochrome a, a_3 (cyto a). Though still somewhat controversial, the general consensus is that the redox state of cyto a must become more reduced for an increase in respiration to occur and that this process is roughly linear. Simply viewed, the only reactants in the terminal reduction of oxygen are reduced cyto a and oxygen. With saturating oxygen, the only parameter that can regulate the rate of reduction of oxygen, or oxygen consumption, is the redox state of Complex IV or a modification of its kinetics. It has been appreciated since the earliest studies that with increased ATP production in isolated mitochondria via the activation of Complex V, or even increased substrate delivery, Complex IV becomes more reduced, permitting the higher rate of oxygen reduction and removal of reducing equivalents(165;166). Since cyto a is believed to be highly oxidized (~98%) under resting conditions, even the linear relationship between redox state and oxygen consumption results in the very large dynamic range required in many tissues. With cyto a becoming just 10% reduced, a 5-fold or greater increase in respiration can be realized. Thus, as stated above, the fact that respiration increases with workload implies that the redox elements in Complex IV are becoming more reduced or the kinetics of the reaction are modified. Currently, the best hypothesis for how the respiratory rate is modulated is through a net small depolarization of $\Delta\Psi$, as observed in most studies to date, increasing the net delivery of reducing equivalents to Complex IV. Recent evidence is suggesting that the kinetics of Complex IV can be altered by PTM as well as different allosteric regulators (167-171), and even though a Ca^{2+} binding site has been identified (172), no direct evidence is available that Ca^{2+} alters the kinetics of this enzyme between its redox state and oxygen. Bender and Kadenbach (167) reported that Ca^{2+} attenuated the inhibition of Complex IV caused by incubating mitochondria with cAMP, though Ca^{2+} had no effect on Complex IV when added without cAMP or to the isolated enzyme. Due to the importance of Complex IV in the determination

of oxidative phosphorylation rate as discussed above, the further study of both direct and indirect effects of Ca^{2+}on the kinetics of this reaction, as well as the relationship between $\Delta\Psi$ and oxidative phosphorylation in intact systems are warranted.

Another possibility for the observed metabolic homeostasis in intact tissues is the compartmentation of metabolic intermediates in the cytosol much like that demonstrated for Ca^{2+} (173-175). The basic concept is that regional changes in ADP, Pi and creatine in the regions around the mitochondria are major factors in driving mitochondrial ATP production. While the maintenance of ΔG_{NADH} with workloads is inconsistent with a local delivery of ADP and Pi(8), the slight depolarization of $\Delta\Psi$ however could be reflecting local increases in these metabolites activating Complex V directly. Currently, direct imaging of the cellular distribution of these metabolites, so important in the analysis of Ca^{2+} compartmentation, is not feasible. Hopefully, the development of imaging tools capable of determining the local metabolite concentrations within working cells will contribute to a better understanding of the role of cellular metabolite compartmentation in this process.

General Summary

The strong link between ΔG_{Ca} and ΔG_{ATP} makes Ca^{2+} a primary candidate as the molecular signal in the maintenance of the cellular metabolic homeostasis. Isolated studies identified several dehydrogenases activated by Ca^{2+} along with substrate transport mechanisms. Studies on intact mitochondria and cells suggest that Ca^{2+} stimulates the production of reducing equivalents through these mechanisms as well as other processes in addition to these of these classical sites. The activation of metabolism by Ca^{2+} in cells is apparently distributed over many elements of oxidative phosphorylation as witnessed by near homeostatic regulation of NADH, $\Delta\Psi$ and ΔG_{ATP} in active tissues that is disrupted by altering mitochondrial Ca^{2+} transport. We have summarized these processes in a schematic diagram (Figure 4) to emphasize the distribution of Ca^{2+} regulated sites of oxidative phosphorylation. The best evidence for Ca^{2+} activation downstream of the dehydrogenases is a V_{max} activation of Complex V, but the specific mechanisms remain elusive. Thus, it is evident that Ca^{2+} is playing a much more integrative role in the regulation of mitochondrial energy metabolism that exceeds a simple activation of dehydrogenases and may include a systemic activation of the metabolic network contributing to the overall metabolic homeostasis of the cell. Fortunately, continuing advances in the proteomics field have already begun to provide specific information on how Ca^{2+} may alter several types of PTM in oxidative phosphorylation Complexes. It is anticipated that the molecular mechanisms of Ca^{2+} interaction with these distributed sites in mitochondrial energy metabolism will be forthcoming in the very near future.

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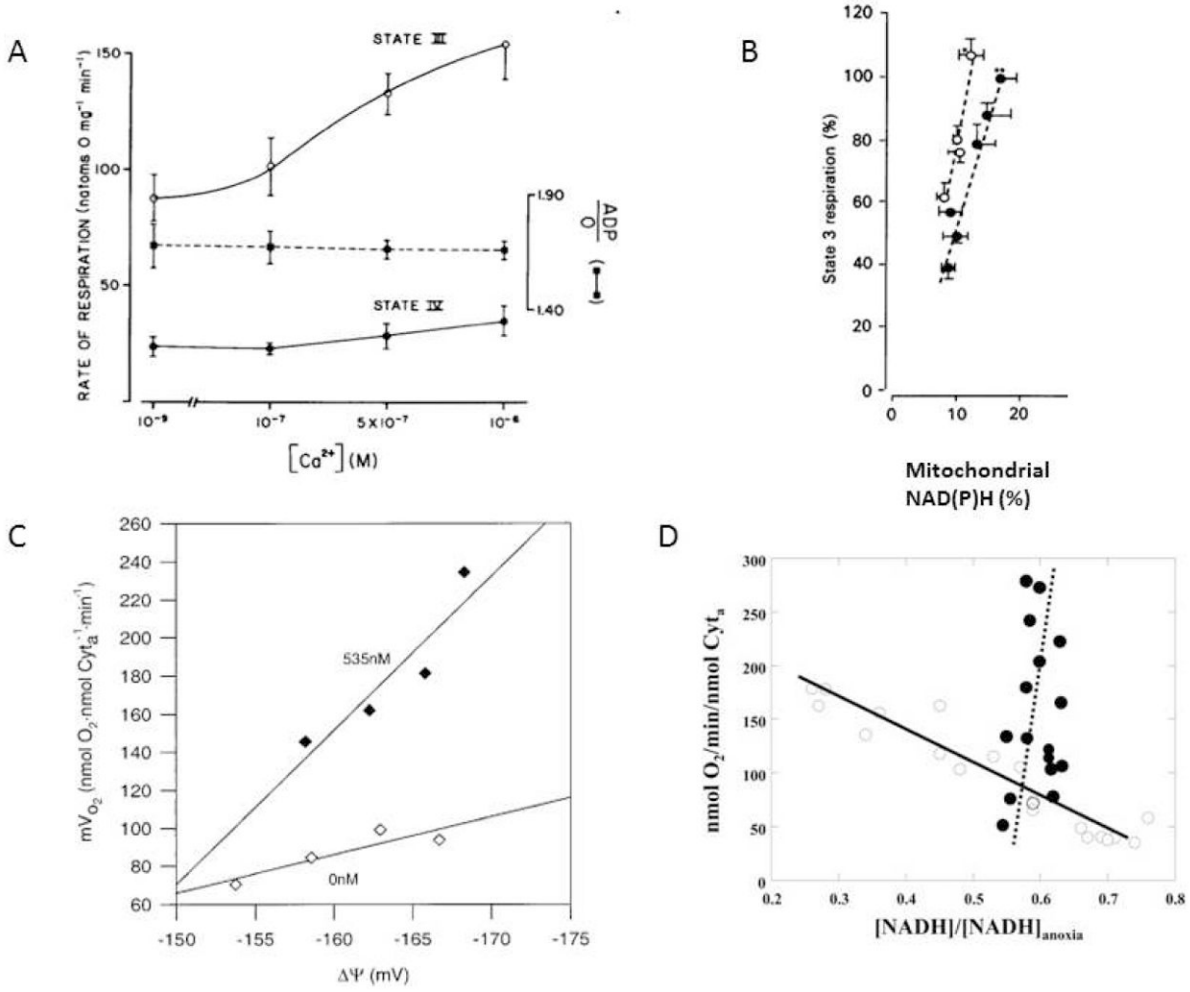

Figure 1.

A) Effect of extra-mitochondrial Ca^{2+} on the rate of respiration in isolated rat liver mitochondria respiring on 5 mM succinate. A 5 minute pre-incubation with Ca^{2+} was used before driving State 3 respiration with 250 μM ADP(74). Reproduced with permission. B) Effect of extramitochondrial Ca^{2+} on the relationship between [NAD(P)H] and respiration in isolated liver mitochondria. All experiments were conducted with 5 mM malate. Solid points represent the control study with effectively no Ca^{2+} and mitochondrial [NAD(P)] increased with increasing glutamate (0.25, 0.5, 1, 2, 5 and 10 mM). The open circles are with fixed substrates 1 mM glutamate and 5 mM malate with the extramitochondrial Ca^{2+} varied from 5 nM (initial value), 66, 130, 225 and 400 nM (adapted from(83) reproduced with permission) C) Mitochondrial membrane potential versus oxygen consumption of State-3 respiration with variable substrates in the presence and absence of Ca^{2+}in porcine heart mitochondria. In all experiments only the glutamate /malate total concentration was varied from 0.5-5.0 mM to generate a difference in driving force from the citric acid cycle. The open symbols are in the nominal absence of Ca^{2+}. The filled symbols are in the presence of the previously determined optimal Ca^{2+} concentration of 535 nM. Data from (87), reproduced with permission. D) Relationship between [NADH] and oxygen consumption with apyrase or a sarcoplasmic reticulum(SR)-mitochondria reconstitution system Open symbols are data collected by varying [Apyrase]. Increasing [Apyrase] increased respiration but decreased [NADH]. Closed Signals are fixed [SR] and [mitochondria] (ratio of SR to

mitochondria 0.5) with varying [Ca^{2+}] over low physiological levels of 0 to 492 nM (calculated free concentration). Increasing [Ca^{2+}] increased respiration over 5 fold with a slight increase in [NADH]. Lines are the linear regression of the data points. Data from (92), reproduced with permission.

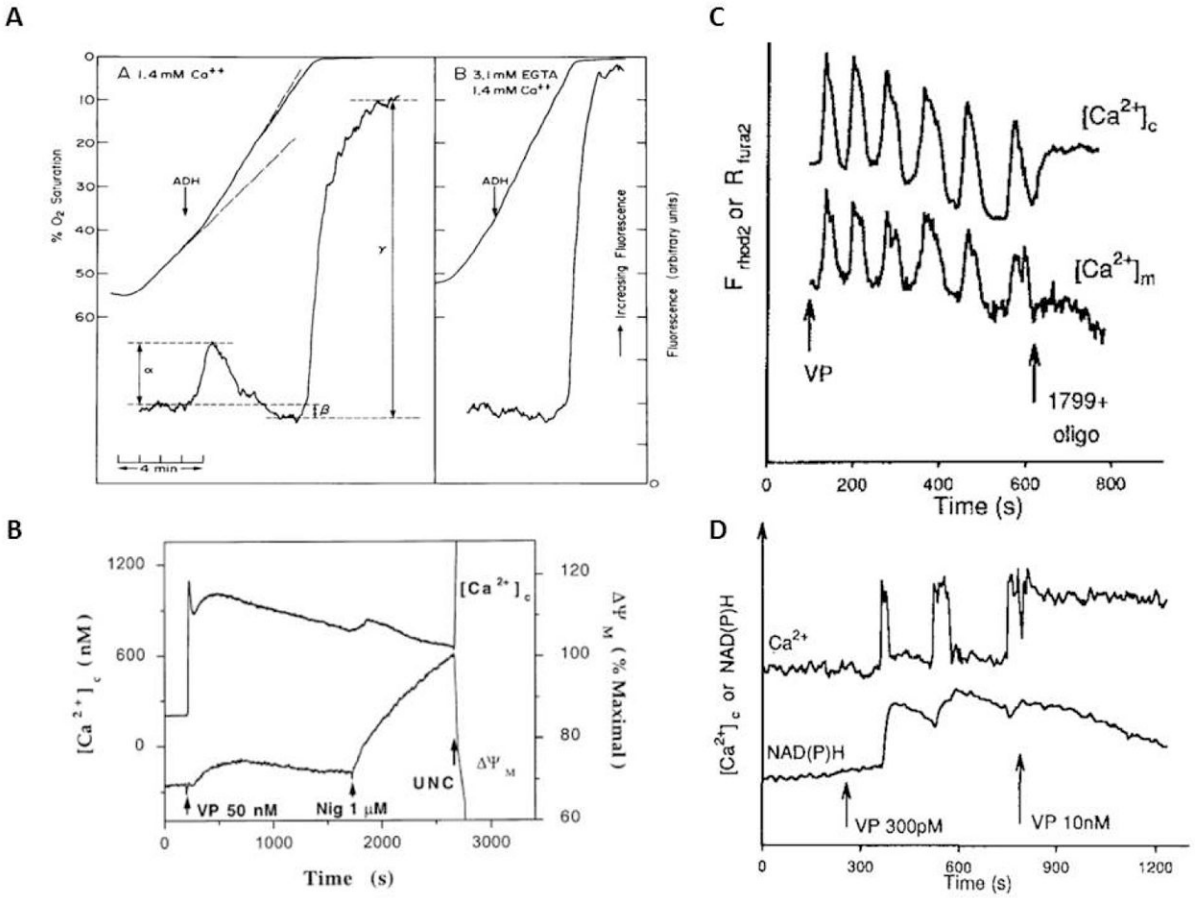

Figure 2.

A) Effect of ADH on NADH and oxygen consumption of isolated rat hepatocytes. In both panels 100 nM ADH was added to the chambers at the arrow in the absence (A) and presence (B) of 3.1 mM EGTA. Data from (110), reproduced with permission. B) Measurements of $Ca^{2+}{}_c$ and ΔΨ in cultured hepatocytes with vasopressin (50 nM). $[Ca^{2+}]_c$ was measured using fura-2 and ΔΨ was monitored with TMREE. Data from (111), reproduced with permission. C) Temporal correlation of $Ca^{2+}{}_c$ and $Ca^{2+}{}_m$ during hormonal stimulation of hepatocytes. $[Ca^{2+}]_m$ was monitored in hepatocytes loaded with dihydro-Rhod 2-AM, and $[Ca^{2+}]_c$ was measured in cells loaded with Fura 2-AM. Data from (114), reproduced with permission. D) 4. Relationship between the frequency of $Ca^{2+}{}_c$ and $NAD(P)H_m$ fluorescence. NAD(P)H and $[Ca^{2+}]_c$ were measured simultaneously during the addition of vasopressin (VP). Data from (114), reproduced with permission

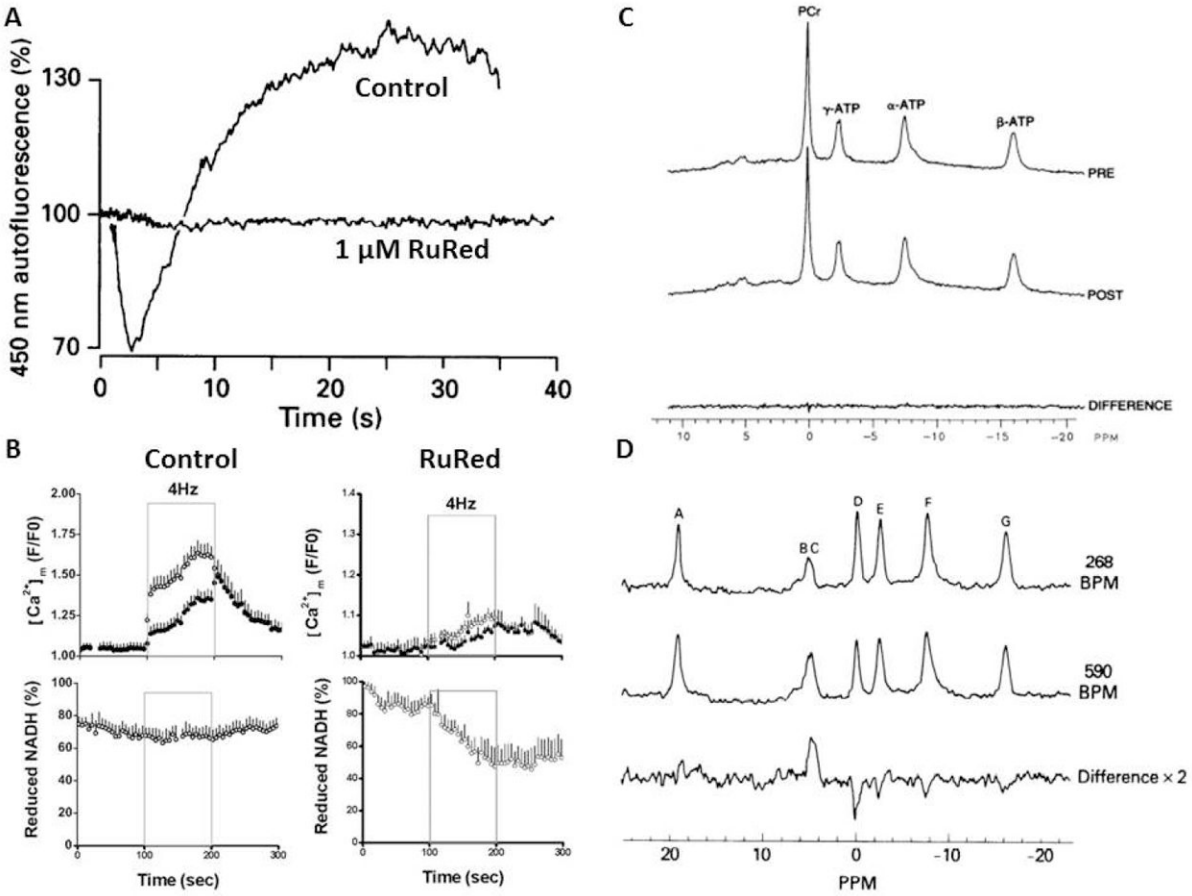

Figure 3.

A) Effect of patch clamp depolarization (from -70 mV holding potential to 0 mV) of freshly isolated neurons NAD(P)H fluorescence in the absence and presence of RuRed. RuRed did not block the cellular membrane Ca^{2+} current associated with the depolarization but blocked the NAD(P)H fluorescence response. Data adapted from(117), reproduced with permission. B) Effect of 4 Hz pacing on isolated cardiac myocytes NADH fluorescence and $Ca^{2+}{}_m$ in the absence (control) and presence RuRed. The pacing of the myocytes occurred at the times indicated. Data from (118) reproduced with permission. C) Effect of pacing work on the ^{31}P NMR spectrum of the intact canine heart. Top spectrum represents the control spectrum while the post spectrum represents the average ^{31}P NMR spectrum during pacing the heart to maximum pacing induced coronary blood flow and oxygen consumption. The bottom trace is the difference of the control and maximum pacing protocol. Data from(141), reproduced with permission. D) Effect of RuRed on the ^{31}P NMR spectrum of the perfused rat heart undergoing an increase in pacing rate. The top spectrum is during the control pacing rate, middle spectrum was collected during the increase in pacing rate to 580 beats/min, bottom spectrum represents the difference between these two conditions with a large increase in Pi and decrease in CrP. The peak assignments are A: external reference, B-C: Pi, D: CrP, E: γP-ATP, F: αP-ATP/NAD and G: βP-ATP. Data from (146), reproduced with permission.

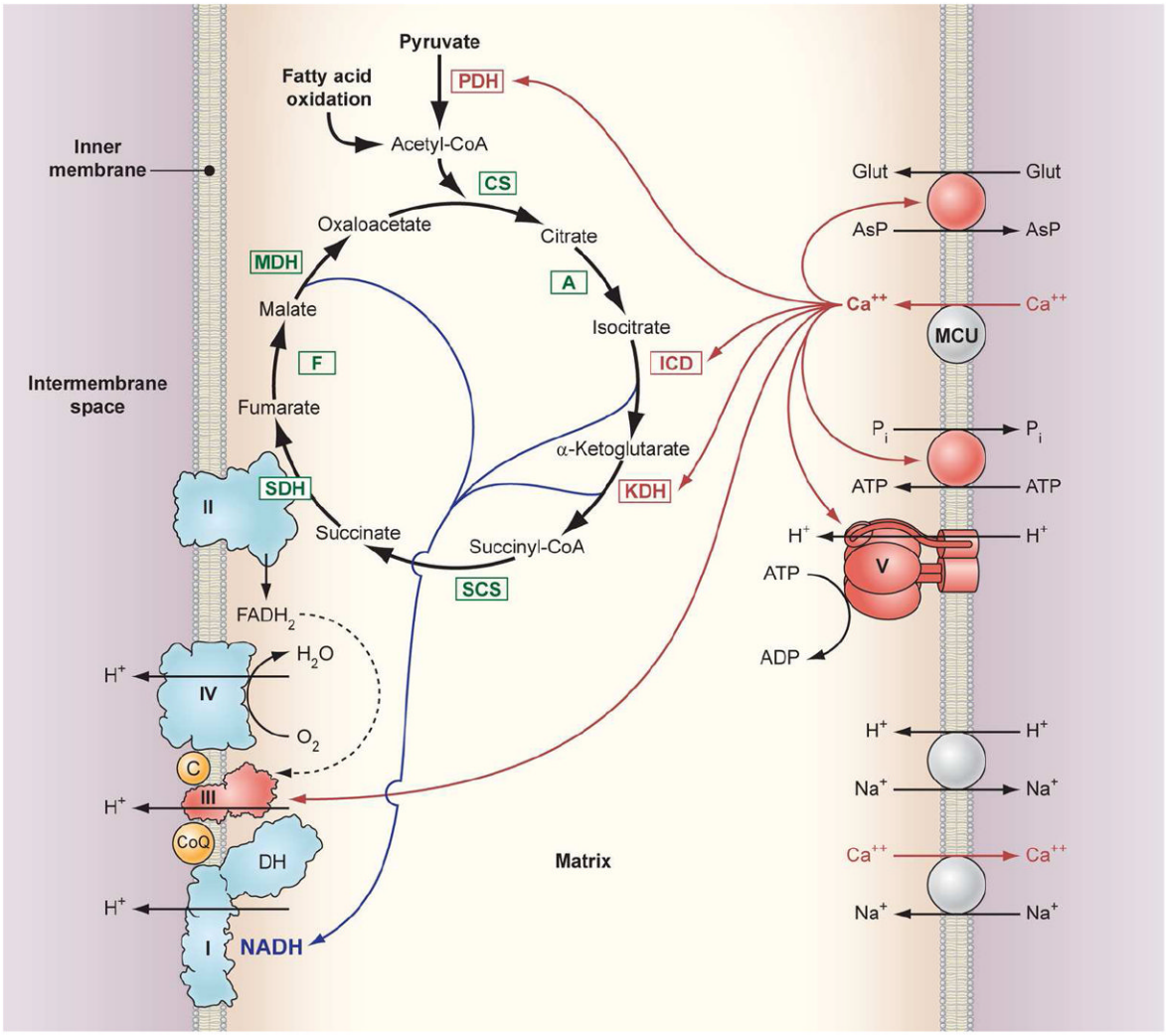

Figure 4.

Schematic diagram of the interaction of matrix Ca^{2+} with processes involved in oxidative phosphorylation. Enzyme abbreviations: PDH: Pyruvate dehydrogenase, CS: Citrate synthase, A: Aconitase, ICD: Isocitrate dehydrogenase, KDH: α ketoglutarate dehydrogenase, SCS: Succinyl CoA synthetase, SDH: Succinate dehydrogenase (also Complex II), F: Fumarase, MDH: Malate dehydrogenase, MCU: Mitochondria calcium uniporter. The Complexes of oxidative phosphorylation are labeled as roman numerals from I-V. The DH on Complex 1 refers to the intrinsic and possibly associated NADH dehydrogenase activity. The red arrows from Ca^{2+} to the different interaction sites imply either a direct or indirection modulation of the transport or enzymatic activity.