THE CALCIUM CYCLE OF MITOCHONDRIA

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1. Introduction

Historically, the study of Ca^{2+} transport by mitochondria may be divided into three phases. During the first phase, characterized by the discovery of the process [1], and by the establishment of its fundamental properties, emphasis was placed on the massive accumulation of Ca^{2+} and phosphate, the so-called 'matrix-loading' process. It soon became evident, however, that more information could be gained by studying the uptake of limited, and structurally inoffensive, amounts of Ca^{2+}. The study of the so-called 'membrane-loading' process thus characterized the second phase. With very few exceptions, however, these studies have concentrated on the uptake of Ca^{2+} (requirements, inhibition, mechanism, physiological implications), almost as if the opposite process, the release of Ca^{2+}, did not occur. Yet, release of Ca^{2+} from mitochondria, at a rate comparable to that of uptake, must necessarily occur to prevent calcification of the organelle. The third phase is rather recent, and has seen increasing emphasis on ways and means to release Ca^{2+} from mitochondria. Recent data, and concepts gradually emerging, now permit us to look at the process from a new perspective, which is synthesized here in the proposal of a mitochondrial Ca^{2+} cycle.

2. The energy linked uptake of Ca^{2+} by mitochondria

Comprehensive reviews [2–4] have summarized its most important properties. For the purpose of this article, however, only some of them are of interest, and will be discussed. The first is the nature of the process, which is now being recognized as purely electrophoretic, i.e., driven by the electrical component of the total protonmotive force generated by coupled respiration [5–7] and occurring with a transfer of two charges. Alternative mechanisms, implying partial charge compensation by H^+ antiport [8,9], or by symport of phosphate [10], appear to be negated by recent experimental evidence [11]. The possibility of a partial charge compensation by co-transport of β-hydroxy butyric acid [12], however, has not yet been fully explored.

Another relevant point is the affinity of the electrophoretic uniporter for Ca^{2+}. This point has been the topic of a vigorous controversy, since in the past various, but mostly indirect, methods of measurement have yielded K_m values as low as 1 μM, and as high as 100 μM (discussed in [3]). Recent measurements using direct methods have now established a K_m, at least for heart mitochondria [13], of ~10 μM. The K_m may be somewhat lower (~5 μM) in other mitochondrial types [14,15] but is probably the same in liver (unpublished observations). Of interest is the fact that the kinetics of the uptake reaction, which are hyperbolic in the absence of Mg^{2+} [13] become markedly sigmoidal in its presence [13,16–18]. In effect, Mg^{2+} is an efficient inhibitor of the electrophoretic uniporter, and more so in heart than in liver mitochondria [19,20]. In heart, 1 mM Mg^{2+} shifts the K_m for Ca^{2+} to ~30 μM [13].

A last important point is the maximal rate of energy-linked uptake, which varies from 3–10 nmol Ca^{2+} .mg protein^{-1} .s^{-1} in the presence of phosphate [13,21]. These values refer to heart mitochondria, where measurements using direct methods have recently been carried out, but are probably of the same order of magnitude in liver and other mitochondria.

3. The problem of the equilibrium of Ca^{2+} uptake via the electrophoretic uniporter

Assuming a membrane potential, negative inside, of 180 mV in energized mitochondria [22], and knowing that the electrophoretic uniporter transfers 2 charges/cycle, one would expect a gradient of Ca^{2+} activities across the inner membrane of 10^6, if the process would reach Nernstian equilibrium. The direct measurement of the activity gradient of Ca^{2+} between mitochondria and medium is at the moment not possible. However, indirect estimates of its magnitude can be derived from assays of enzymes which are known to be Ca^{2+}-modulated in the mitochondrial matrix and in the cytosol: phosphorylase *b* kinase, α-glycerophosphate dehydrogenase, pyruvate dehydrogenase phosphate phosphatase and isotonic dehydrogenase [23–26]. These estimates indicate that the gradient may have any value from $0–10^3$, but not higher. That the gradient is only a fraction of 10^6 is on the other hand also shown implicitly by the following simple considerations: since the activity of matrix Ca^{2+} probably oscillates from $10^{-5}–10^{-6}$ M [25,26] if Nernstian equilibrium would be reached against −180 mV, the cytosolic Ca^{2+} would be lowered to the intolerably low activity of $10^{-11}–10^{-12}$ M. In principle, lower gradients of Ca^{2+} activity between mitochondria and medium could be obtained by equilibration with potentials lower than −180 mV. However, the membrane potential is an essential factor in the various aspects of the energy-coupling operations in mitochondria, and it is thus extremely unlikely that it is permitted to fluctuate very widely. Thus, the fact that the Ca^{2+} activity gradient between mitochondria and medium is only a fraction of 10^6, leads to the conclusion that the electrophoretic Ca^{2+} uniporter operates essentially as a one-way system and does not mediate the efflux of Ca^{2+}. Efflux must be mediated by a separate system, which operates independently of the membrane potential, or is even driven by it in the direction of Ca^{2+} release.

4. The release of Ca^{2+} from mitochondria

The concept that mitochondria, in addition to accumulating Ca^{2+}, discharge it continuously, carrying thus out what in effect is a Ca^{2+} cycle, was first indicated by early experiments of Drahota et al. [27], supported more recently by results of Stucki and Ineichen, [28]. It is important to realize that the concept of separate pathways for Ca^{2+} uptake and release is an essential component of the proposal of a Ca^{2+} cycle: indeed, if Ca^{2+} would enter and leave mitochondria via the electrophoretic uniporter only [29] no 'cycle' would be established. It follows from this that it is essential to demonstrate unequivocally the existence of separate pathways for Ca^{2+} influx and efflux. In this, an essential tool has proven to be ruthenium red. This inhibitor, which blocks the electrophoretic uniporter completely [30,31], was first shown by Rossi et al. [32], to permit, and indeed to promote, the efflux of the Ca^{2+} accumulated by mitochondria under certain experimental conditions. Clearly, in this case Ca^{2+} left mitochondria by a way different from the uptake uniporter, which was blocked by the inhibitor. The experiments in [32] offered no clues as to the mechanism of the release reaction, however. The first indication as to the possible nature of the release pathway came from experiments by Carafoli et al. [33] in which Na^+ was shown to specifically promote efflux of Ca^{2+} from ruthenium red-inhibited mitochondria, thus providing the release leg of the Ca^{2+} cycle. One point must be stressed in this context. Experimental proof for the existence of separate Ca^{2+} influx and efflux pathways can only be assumed when a releasing agent is applied, and shown effective, in the presence of ruthenium red. In its absence, release may largely occur via reversal of the unoccupied electrophoretic uniporter. This is the case, for example, for agents that collapse the membrane potential, like uncouplers, respiratory inhibitors, or even phosphoenolpyruvate, which has been frequently mentioned as a possible 'natural' release-inducer [34,35], but which has recently been shown to act by lowering substantially the membrane potential [36]. Objections against the assumption that Ca^{2+} release in the presence of ruthenium red implies the operation of a separate release pathway have been raised [28], based on the proposal that the binding of ruthenium red to the electrophoretic uniporter would be dependent on the electrical potential difference across the inner membrane. In this suggestion, membrane domains of lower electrical difference would permit slow release of Ca^{2+} via the electrophoretic uniporter even in the presence of ruthenium red. Complete

de-energization of the membrane (e.g., by uncouplers), would lead to a rapid, ruthenium red-insensitive, Ca^{2+} release, due to the complete reversal of the inhibitor-free uniporter. However, experiments based on Ca^{2+}/Ca^{2+} exchanges in the presence and in the absence of ruthenium red, in energized and de-energized liver mitochondria [37] have shown that the proposal is untenable: the electrophoretic uniporter, operating in these experiments as a Ca^{2+}/Ca^{2+} exchanger, was still inhibited by ruthenium red in completely de-energized mitochondria.

The previously mentioned Na^+-induced Ca^{2+} release was first observed in heart mitochondria, and later shown to be operating in the majority of mitochondrial types [38–40], but not in mitochondria from liver, kidney and lung. The Na^+-promoted pathway has now been characterized in detail [39,41,42] and properties relevant to this article will be mentioned. It is half-maximally activated by 6–8 mM external Na^+, and exhibits a markedly sigmoidal dependence of the Ca^{2+}-efflux rate on the Na^+ concentration. Hill coefficients of ~3 indicate that the route is at least electroneutral, but possibly even electrogenic, i.e., it may exchange > 2 Na^+/Ca^{2+}. However, the measurement of the precise Ca^{2+}/Na^+ stoichiometry is prevented by the operation of the fast Na^+/H^+ antiporter, [43,44], which returns to the external medium the Na^+ that has entered mitochondria in exchange for the lost Ca^{2+}. The maximal rate of Ca^{2+} release is ~0.25 nmol Ca^{2+} .mg protein^{-1} .s^{-1} in heart mitochondria [41] and 0.07–0.3 nmol Ca^{2+} .mg protein^{-1} .s^{-1} in the other Na^+-sensitive mitochondria investigated [38,39]. It is, therefore, $>$ 10-times slower than the maximal rate of uptake on the electrophoretic uniporter. Experiments on heart mitochondria [42] have shown that the Na^+/Ca^{2+} antiporter operates also as a Ca^{2+}/Ca^{2+} exchanger. In this case, the kinetics of the reaction is hyperbolic, the stoichiometry is 1:1, and the K_m for (external) Ca^{2+} is 13 μM.

The Na^+-promoted release of Ca^{2+} could be due to the combined operation of the Na^+/H^+ antiporter with either a Na^+/Ca^{2+} or a H^+/Ca^{2+}, antiporter. In the former case, the Na^+/H^+ antiporter would be required to dissipate the excess matrix Na^+, in the latter, to dissipate the excess matrix H^+. Compelling evidence against the existence of a (fast) H^+/Ca^{2+} exchange has been provided [39]. Substitution of the natural Na^+/H^+ antiporter with an artificial (i.e., nigericin) K^+/H^+ antiporter in a K^+ medium failed to induce release of Ca^{2+}, showing that the Na^+ requirement is not due to the necessity of dissipating the excess matrix H^+ [39].

Na^+-insensitive mitochondria (e.g., liver) release Ca^{2+} by a pathway that has not yet been characterized, but which is also independent of the uptake route [37,45–47]. Recent experiments have indicated that it may be linked to the redox state of pyridine nucleotides [48]. Other experiments, carried out in ruthenium red-inhibited, inverted liver submitochondrial vesicles [49] have shown that it may be linked to the extrusion of inorganic phosphate. Fatty acids [50,51], have also been suggested to play a role in releasing Ca^{2+} from liver and kidney mitochondria. Their effect may be more general, and possibly important in other mitochondria as well.

5. Integration of the influx and efflux pathways: the Ca^{2+} cycle

The concepts discussed above are synthesized in the scheme of fig.1. A logical question at this point

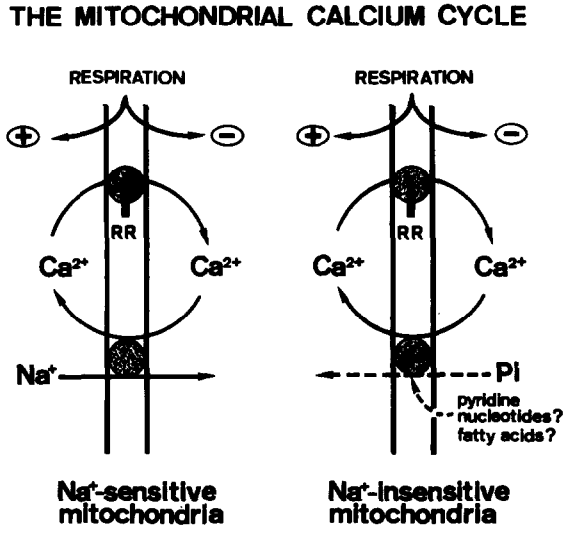

Fig.1. In the scheme, stoichiometries are not indicated. The dashed arrows indicate that the participation of phosphate in the release of Ca^{2+} is only a suggestion, and that the mechanisms of the effects of pyridine nucleotides and fatty acids on the release of Ca^{2+} are still unclear.

is whether, given the existence of both an uptake and a release process, mitochondria ever accumulate substantial amounts of Ca^{2+} under the normal life conditions of the cell. When isolated, there is little doubt that they can do it, since the experimental conditions routinely employed in vitro tend to optimize the uptake process. It is also amply demonstrated that they are able to accumulate large amounts of Ca^{2+} in tissues which presumably have elevated intracellular Ca^{2+} concentrations, either due to special physiological demands, or to pathological deviations [52]. In these cases, it is likely that the uptake route predominates over the release route, due to the higher velocity of the former in response to the increased cytosolic Ca^{2+} activity.

Normally, however, mitochondria operate in an ambient Ca^{2+} activity which can be assumed to be < 1 μM outside, and 1–10 μM inside (see above). Moreover, they are presumably surrounded by enough Mg^{2+} to slow down the uptake route substantially [53,54], and by ~ 5 mM ionized Na^+ [55]. Putting together these pieces of information, and considering the known kinetic parameters of the influx and efflux routes [42], an extrapolation to the probable in vivo situation can now be attempted, at least for the case of Na^+-sensitive mitochondria.

At the presumed maximal Ca^{2+} activity of the cytosol (1 μM), the uptake route (K_m, ~10 μM), would operate at a rate corresponding to the uptake of ~0.25 nmol Ca^{2+} .mg protein^{-1} .s^{-1}, (from [13,20]). In the presence of Mg^{2+} (1 mM) this velocity would be decreased $\geqslant 4$ times, to ~0.06 nmol .mg protein^{-1} .s^{-1} [13]. On the other hand, the presumed matrix Ca^{2+} activity of 1–10 μM would be expected to slow down substantially the rate of Na^+-induced Ca^{2+} efflux. From a maximum of ~0.25 nmol .mg protein^{-1}.s^{-1} this rate could drop to ~0.04–0.06 nmol (extrapolated from [56]). This is based on the assumption that the K_m of the Na^+/Ca^{2+} antiporter for Ca^{2+}, which is 13 μM on the outside of mitochondria, is the same at the matrix side. As a result, then, in the conditions which are likely to prevail in vivo, the two opposite Ca^{2+} transport routes would operate at approximately the same rate, rationalizing both the observation that the gradient of Ca^{2+} activities across the inner membrane of energized mitochondria is only a fraction of that predicted on the basis of the Nernstian equilibrium of the electrophoretic uniporter, and the observation that mitochondrial calcification never occurs under normal in vivo conditions. This reasoning can in all likelihood be extended also to the case of Na^+-insensitive mitochondria, except that in this case essential pieces of information are still missing on the mechanism and the kinetic parameters of the independent Ca^{2+} efflux pathway. In addition, it must be remembered that Mg^{2+} is less efficient as an inhibitor in liver mitochondria [19].

It follows from these considerations that mitochondria must be regarded (apart from the cases of Ca^{2+}-transporting cells, and of cells under pathological conditions) essentially as short-term intracellular Ca^{2+} regulators. It is easy to see how the manipulation of the rates of the influx of efflux legs of the Ca^{2+} cycle may result in substantial Ca^{2+} shifts in the cytosol, if one considers that mitochondria represent $\leqslant 90\%$ of the total Ca^{2+}-transporting membrane area of cells [57].

One evident feature of the proposed Ca^{2+} cycle is that it would be energy dissipating. Na^+, indeed, induces uncoupling when added to Ca^{2+}-loaded, Na^+-sensitive mitochondria [39]. The level of energy dissipation, however, need not be large, if it is assumed that the Ca^{2+} uptake route normally operates at only a fraction of its maximal possible velocity. It has already been calculated that Ca^{2+} cycling accounts for only ~20% of the state 4 respiration measurable in isolated liver mitochondria [28].

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