Topical Review

Mitochondria as all-round players of the calcium game

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Although it has been known for over three decades that mitochondria are endowed with a complex array of Ca^{2+} transporters and that key enzymes of mitochondrial metabolism are regulated by Ca^{2+}, the possibility that physiological stimuli that raise the $[Ca^{2+}]$ of the cytoplasm could trigger major mitochondrial Ca^{2+} uptake has long been considered unlikely, based on the low affinity of the mitochondrial transporters and the limited amplitude of the cytoplasmic $[Ca^{2+}]$ rises. The direct measurement of mitochondrial $[Ca^{2+}]$ with highly selective probes has led to a complete reversion of this view, by demonstrating that, after cell stimulation, the cytoplasmic Ca^{2+} signal is always paralleled by a much larger rise in $[Ca^{2+}]$ in the mitochondrial matrix. This observation has rejuvenated the study of mitochondrial Ca^{2+} transport and novel, unexpected results have altered long-standing dogmas in the field of calcium signalling. Here we focus on four main topics: (i) the current knowledge of the functional properties of the Ca^{2+} transporters and of the thermodynamic constraints under which they operate; (ii) the occurrence of mitochondrial Ca^{2+} uptake in living cells and the key role of local signalling routes between the mitochondria and the Ca^{2+} sources; (iii) the physiological consequences of Ca^{2+} transport for both mitochondrial function and the modulation of the cytoplasmic Ca^{2+} signal; and (iv) evidence that alterations of mitochondrial Ca^{2+} signalling may occur in pathophysiological conditions.

Setting the frame for mitochondrial Ca^{2+} transport: mechanism of energy conservation and non-equilibrium distribution

Energy conservation is achieved through charge separation at the inner mitochondrial membrane, whereby electrons derived from intermediary metabolism are transferred to oxygen through the respiratory chain in a process coupled to H^+ pumping. Since the passive permeability to H^+ is low, this process results in the establishment of a H^+ electrochemical gradient ($\Delta\mu H$). $\Delta\mu H$ is approximately 200 mV, and under physiological conditions most of the gradient is in the form of a membrane potential difference ($\Delta\Psi$) (Mitchell, 1966). This has major implications for Ca^{2+} transport and distribution, because for a divalent cation ($z = 2$ in the Nernst equation) the equilibrium condition ($\Delta\mu Ca = 0$) is given by:

$$-\Delta\Psi = 30 \log ([Ca^{2+}]_m/[Ca^{2+}]_c).$$

Since the inner mitochondrial membrane possesses electrophoretic systems for Ca^{2+} transport, and since typical values of cytosolic free $[Ca^{2+}]$ ($[Ca^{2+}]_c$) oscillate between about 0·1 and 1 μM, for a membrane potential of −180 mV (negative inside) equilibrium matrix free $[Ca^{2+}]$ ($[Ca^{2+}]_m$) should be 0·1–1 M, which is 100000- to 1000000-fold higher than the measured range of $[Ca^{2+}]_m$. This displacement from equilibrium is due to the fact that Ca^{2+} distribution is modulated by kinetic rather than thermodynamic parameters (Azzone *et al.* 1977), in the sense that it represents a steady state where electrophoretic Ca^{2+} uptake is precisely matched by Ca^{2+} efflux via at least two separate pathways: (i) a Na^+-independent Ca^{2+} efflux pathway, possibly a H^+–Ca^{2+} exchanger, and (ii) a Na^+-dependent Ca^{2+} efflux pathway, most probably a Na^+–Ca^{2+} exchanger. An additional pathway for Ca^{2+} release may be represented by the permeability transition pore (PTP), a non-selective high-conductance channel, which is under intense scrutiny as both a potential target and an effector mechanism in cell death. The following paragraphs summarise the functional properties of these mitochondrial Ca^{2+} transport pathways (schematically represented in Fig. 1), whose molecular nature is still unsolved (see Bernardi, 1999, for a detailed review and original references).

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Pathways for Ca^{2+} uptake

Calcium uniporter. The Ca^{2+} uniporter is a mitochondrial channel that transports Ca^{2+} and Sr^{2+} but not Mg^{2+}. In respiring mitochondria the kinetics of Ca^{2+} uptake become rapidly limited by the rate of H^+ pumping as $[Ca^{2+}]_o$ is raised above about 10 μM. This may lead to a serious underestimation of both the V_{max} and the apparent K_m for Ca^{2+}. The true kinetic properties of the uniporter have been defined through the use of K^+ diffusion potentials induced by valinomycin in respiratory-inhibited mitochondria. These studies established a V_{max} in excess of 1400 nmol Ca^{2+} (mg protein)$^{-1}$ min^{-1} and an apparent K_m lower than 10 μM in sucrose-based media (Bragadin *et al.* 1979). The Ca^{2+} uniporter is regulated by a number of modulators (inhibitors and activators). Ruthenium compounds (typically ruthenium red, RR), represent a class of non-competitive inhibitors. A second class of inhibitors is divalent cations that are themselves transported by the uniporter (e.g. Sr^{2+}, Mn^{2+}, Ba^{2+} and lanthanides). Inhibition is generally competitive, but not all of the effects are exerted at the transport site(s) because the uniporter is regulated by metal ion binding sites that modulate the affinity for Ca^{2+}. Mg^{2+}, Mn^{2+} and Ca^{2+} itself can exemplify this class of effectors. In the millimolar range, Mg^{2+} changes the relationship between the rate of Ca^{2+} transport and $[Ca^{2+}]_o$ from hyperbolic to sigmoidal, decreases the V_{max} and increases the apparent K_m for Ca^{2+} from 10 to about 50 μM (Bragadin *et al.* 1979). Mg^{2+} affects the uniporter by binding to a regulatory site(s) rather than to the transport site(s). Accordingly, Mg^{2+} is not transported by the uniporter, it does not affect the Ca^{2+} conductance (Heaton & Nicholls, 1976), and its effects can be mimicked by approximately 50-fold higher concentrations of monovalent cations like Li^+ (Bragadin *et al.* 1979). Under specific conditions Mn^{2+} can stimulate rather than inhibit the kinetics of Ca^{2+} transport by counteracting the effects of Mg^{2+} through a mixed-type competition (Allshire *et al.* 1985). The uniporter is activated by external Ca^{2+} itself, and it undergoes deactivation upon removal of extramitochondrial Ca^{2+} (Kroner, 1986). Thus, at the low $[Ca^{2+}]$ values prevailing in the cytosol the activity of the uniporter could be extremely low, due also to inhibition by adenine nucleotides (ATP > ADP > AMP) (Litsky & Pfeiffer, 1997). Depolarisation after energy-dependent accumulation of Ca^{2+} is readily followed by Ca^{2+} release that is enhanced by RR (Igbavboa & Pfeiffer, 1988). Thus, under these conditions Ca^{2+} release is not occurring through the uniporter but rather via the voltage-dependent mitochondrial PTP, and it is accordingly inhibited by cyclosporin A (CsA). The uniporter is therefore not readily reversible, possibly because of the low $[Ca^{2+}]_o$ at the onset of depolarisation, which deactivates the uniporter, and because a high membrane potential may be required to maintain it in a transport-competent conformation (Kapus *et al.* 1991).

Rapid uptake mode (RaM). Ca^{2+} uptake is more efficient when energised mitochondria are exposed to trains of Ca^{2+} pulses of physiological concentration (about 400 nM) rather than to an identical steady $[Ca^{2+}]_o$ for the same overall time. This mechanism can be reset in less than 0·75 s by a decrease in $[Ca^{2+}]_o$ to between 100 and 200 nM. Like the Ca^{2+} uniporter, the RaM is inhibited by RR albeit at higher concentrations; unlike the uniporter, Mg^{2+} does not affect it. Gunter and co-workers favour the idea that it is mediated by a specific transport mechanism that might be responsible for mitochondrial Ca^{2+} uptake from $[Ca^{2+}]_c$ transients *in vivo* (Sparagna *et al.* 1995). Up to now, however, no direct evidence for this type of mechanism in intact cells has been obtained.

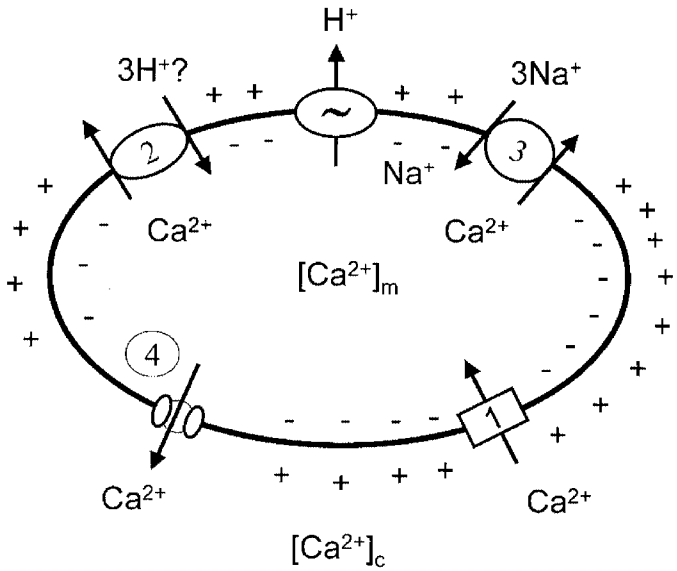

Figure 1. Schematic representation of the mitochondrial Ca^{2+} transport pathways in energised mitochondria

1, uptake pathways (uniporter, RaM). 2–4, efflux pathways: H^+–Ca^{2+} (2) and Na^+–Ca^{2+} (3) exchangers, and the permeability transition pore (4).

Pathways for Ca^{2+} efflux

Na^+-independent Ca^{2+} efflux. Respiring mitochondria maintain steady-state $[Ca^{2+}]_o$ at a constant value of 0·25–1·0 μM. If RR is added to block the uniporter, a process of Ca^{2+} efflux ensues that is taken as evidence that RR-insensitive Ca^{2+} efflux (coupled to uniporter-mediated reuptake) was also occurring prior to the addition of RR via a specific pathway (Vasington *et al.* 1972). Many early studies of RR-insensitive Ca^{2+} efflux in mitochondria, however, have been complicated by the unrecognised contribution of the permeability transition. In the steady state, a small fraction of mitochondria may indeed undergo reversible depolarisations due to opening of the Ca^{2+}-dependent PTP. Ca^{2+} released from this permeabilised fraction would be taken up by the polarised mitochondria, and addition of RR would result in what appears to be net Ca^{2+} 'efflux' (Riley & Pfeiffer, 1985). A reassessment of the Na^+-independent pathway for Ca^{2+} efflux in rat liver mitochondria under conditions in which the occurrence of a permeability transition could be excluded established that: (i) it saturates at Ca^{2+} loads of 25 nmol (mg protein)$^{-1}$; (ii) its V_{max} is not influenced by the concentration of inorganic phosphate and does not exceed a rate of 1·2 nmol Ca^{2+} (mg protein)$^{-1}$ min^{-1}; and (iii) it extrudes Ca^{2+} against a gradient that is much higher than thermodynamically permissible for an electroneutral H^+–Ca^{2+} exchanger (Wingrove & Gunter, 1986*a*). Thus, either Ca^{2+} efflux occurs via a $n$$H^+$–$Ca^{2+}$ exchanger with $n > 2$, or it has an active component. Accordingly, Ca^{2+} efflux is inhibited rather than stimulated by small depolarisations (Bernardi & Azzone, 1983).

Na^+-dependent Ca^{2+} efflux. After the addition of RR, the rate of Ca^{2+} efflux can be stimulated by the addition of Na^+ (Crompton *et al.* 1978). Ca^{2+} efflux is likely to occur via a Na^+–Ca^{2+} exchanger that mediates physiological Ca^{2+} cycling. The V_{max} ranges between 2·6 (liver) and 18 nmol Ca^{2+} (mg protein)$^{-1}$ min^{-1} (heart). The dependence on Na^+ is sigmoidal, with typical K_m values of about 8–10 mM Na^+. Ca^{2+} efflux is inhibited by Sr^{2+}, Ba^{2+}, Mg^{2+} or Mn^{2+}, by RR above 5 nmol (mg protein)$^{-1}$, by submicromolar concentrations of the membrane potential probe triphenylmethylphosphonium, and by a variety of compounds of pharmacological interest such as amiloride, trifluoperazine, diltiazem, verapamil, clonazepam, bepridil and CGP37157, a recently developed specific blocker (Cox & Matlib, 1993), and is stimulated by short-chain alcohols. It is modulated by matrix pH (optimum at pH 7·6) and by *in vivo* treatment with glucagon and β-adrenergic agonists. Ca^{2+} efflux is inhibited by antimycin A and protonophores, indicating that the transmembrane potential favours the exchange (Crompton *et al.* 1976). This is consistent with studies indicating that the likely stoichiometry is $3Na^+$:Ca^{2+} (Wingrove & Gunter, 1986*b*; Jung *et al.* 1995).

Permeability transition pore. The PTP is a high conductance, non-selective channel that exhibits a prominent dependence on matrix Ca^{2+} and is inhibited by CsA. Reversible PTP openings have been resolved both in individual isolated mitochondria (Huser *et al.* 1998) and in intact cells (Petronilli *et al.* 1999), but whether these are accompanied by Ca^{2+} release *in situ* remains unclear because evidence based on the effects of CsA alone may be ambiguous. On the one hand, PTP inhibition by CsA is transient in long time-frame experiments, and depends strictly on variables that cannot be controlled *in vivo* like matrix ADP and Mg^{2+} (which are required for inhibition) and Ca^{2+} (which counteracts it). On the other hand, CsA may also inhibit cyclophilin B-modulated Ca^{2+} transport in the endoplasmic reticulum (ER) (Bram & Crabtree, 1994), which complicates the interpretation of results at the cellular level. It should be noted that coupling of high V_{max} uptake transport systems with slow and easily saturable release systems exposes mitochondria to the hazards of Ca^{2+} overload and that fast mitochondrial Ca^{2+} release demands charge compensation, which cannot be easily achieved through the impermeable inner membrane. In this respect the large size and lack of selectivity of the PTP confer the clear advantage of providing charge compensation within the channel itself, thus allowing maximal Ca^{2+} flux (i.e. at zero potential). This would guarantee fast Ca^{2+} release even for vanishingly small $[Ca^{2+}]$ gradients, and regulation would be achieved directly through modulation of the pore open time. Whether the PTP can function as a physiological mitochondrial Ca^{2+} release channel *in vivo* remains an intriguing possibility (Bernardi & Petronilli, 1996) that deserves further testing.

Mitochondrial Ca^{2+} transport in living cells

Based on the thermodynamic and kinetic properties of the transport pathways (see above) and the $[Ca^{2+}]$ measured in the cytoplasm of resting (~0·1–0·2 μM) or stimulated (0·5–3 μM) cells, the general prediction was that, under physiological conditions, mitochondrial Ca^{2+} accumulation would be marginal (while significant uptake could possibly be achieved in pathological Ca^{2+} overload). Direct measurements of mitochondrial $[Ca^{2+}]$ with a highly specific probe, a targeted chimera of the Ca^{2+}-sensitive photoprotein aequorin, proved the exact opposite (Rizzuto *et al.* 1992). In HeLa cells, stimulation with physiological stimuli acting on G_q-coupled receptors (and thus causing the release of Ca^{2+} from the ER) caused a large, rapid rise in $[Ca^{2+}]$ in the mitochondrial matrix ($[Ca^{2+}]_m$) (Fig. 2). Through the work of various laboratories, using widely different probes (recombinant aequorin or mitochondrially accumulated fluorescent probes), this observation has been extended to a large variety of cell types, including fibroblasts, endothelial and epithelial cells, cardiac and skeletal muscle cells, neurones and pancreatic β-cells, to name but a few (for a review, see Duchen, 1999). It is thus now accepted that in virtually every cell type, mitochondrial Ca^{2+} uptake invariably follows stimulation with an agonist causing a cytoplasmic $[Ca^{2+}]$ rise.

The accumulation of Ca^{2+} occurs via the expected route for Ca^{2+} uptake, i.e. the Ca^{2+} uniporter, as highlighted by two

simple observations: (i) collapse of the proton-motive force with a protonophore virtually abolishes mitochondrial Ca^{2+} uptake (Fig. 2); and (ii) injection of RR fully inhibits the uptake of Ca^{2+}. As to the amplitude of the $[Ca^{2+}]_m$ peak, values $> 10\ \mu M$ (and see below) are reached in < 5 s, thus revealing a striking discrepancy between a rapid rate of mitochondrial Ca^{2+} uptake in intact cells and the biochemical properties characterised *in vitro* (Rizzuto *et al.* 1993).

An easy way of rationalising conflicting observations is to invoke the existence of cellular microscopic environments where the concentration of the molecules participating in a given reaction is different from that measurable on a macroscopic cellular scale, the so called 'bulk phase'. Though in many cases this may be an elegant way of concealing ignorance, ample theoretical and experimental evidence has accumulated demonstrating not only the existence but also the physiological importance of 'microdomains'. This is particularly true for Ca^{2+}, for which probes and measuring systems are far more accurate than for any other cellular parameter.

We followed the hypothesis that mitochondrial Ca^{2+} accumulation in living cells depended on the existence of a microheterogeneity in cellular Ca^{2+} concentration and in particular on localised hot spots of high Ca^{2+} concentration in the vicinity of the organelles. At the time this initial unexpected observation was made, alternative possibilities were considered, such as: (i) the existence within the cytoplasm of 'factors' that modify the kinetic parameters of the Ca^{2+} uniporter; (ii) underestimation by the classical probes of the real changes in bulk cytoplasmic Ca^{2+}; and (iii) artefacts of the technique, at the time in its infancy.

Perhaps the most convincing evidence that only the microdomain hypothesis could explain the experimental findings was obtained in digitonin-permeabilised cells. Under these conditions the rate and extent of mitochondrial Ca^{2+} accumulation were almost perfectly reproduced by perfusing the cells with $InsP_3$, the physiological agonist that mobilises Ca^{2+} from the ER (Rizzuto *et al.* 1993). In contrast, perfusion of buffered Ca^{2+} medium, with an up to 3- to 4-fold higher $[Ca^{2+}]$ than that measured in the bulk cytoplasm, resulted in sluggish mitochondrial Ca^{2+} uptake. Only when the buffered $[Ca^{2+}]$ was increased to 5–10 μM (10- to 20-fold greater than the level measured in the bulk cytosol) did the rate of mitochondrial Ca^{2+} accumulation approach that observed in intact cells (Rizzuto *et al.* 1993). In other words, if the functional interactions between the ER and mitochondria are kept intact (as in permeabilised cells), even in the absence of any soluble cytoplasmic factor the localised release of Ca^{2+} through the physiologically relevant channels results in efficient mitochondrial Ca^{2+} accumulation.

This initial observation was then reproduced in different cell types and using different probes to measure mitochondrial

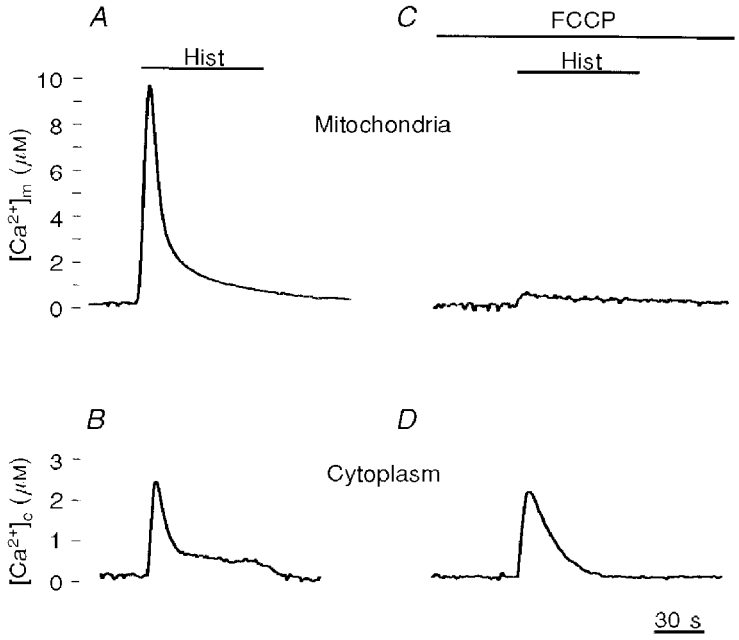

Figure 2. Agonist-dependent mitochondrial and cytoplasmic $[Ca^{2+}]$ increases in the presence and absence of the protonophore FCCP

The traces show calibrated $[Ca^{2+}]$ values obtained in HeLa cells transiently expressing an aequorin chimera localised in the mitochondria (*A* and *C*) or in the cytoplasm (*B* and *D*). Detection and calibration of the luminescence signals were carried out as previously described (Brini *et al.* 1995). Where indicated, HeLa cells were challenged with histamine (100 μM; Hist), an agonist coupled to the generation of inositol 1,4,5-trisphosphate and thus the release of Ca^{2+} from intracellular stores. In *C* and *D*, the cells were treated with 5 μM FCCP (carbonyl cyanide *p*-(trifluoro-methoxy)-phenylhydrazone), which was added 1 min before the agonist and maintained throughout the stimulation.

and cytoplasmic [Ca^{2+}]. The vast majority of authors now agree that in living cells mitochondrial Ca^{2+} accumulation results from the close apposition of the organelles to either ER/SR Ca^{2+} release channels or to plasma membrane Ca^{2+} channels. An obvious prerequisite for such a hypothesis is the existence of morphologically identifiable close appositions between mitochondria and Ca^{2+} channels. A retrospective analysis of standard electron micrographs of various tissues reveals that indeed close apposition of mitochondria to ER cisternae occurs frequently. In some cell types, for example Purkinje neurones, the proximity of mitochondria to InsP_3 receptor-rich ER cisternae has been reported repeatedly (Satoh *et al.* 1990). Similarly, in cardiac myocytes the distance between mitochondria and SR terminal cisternae, which contain the ryanodine receptors, has been reported to be as small as 5 nm (Ramesh *et al.* 1998). The possibility that these close appositions between the ER and mitochondria are an artefact of fixation is unlikely: (i) close interactions of ER and mitochondria can be also observed in rapidly frozen samples, where the cellular architecture is known to be highly preserved (Pezzati *et al.* 1997); and (ii) in intact cells, apposition of mitochondria to the ER (at a distance of less than 100 nm) is a common finding and involves a significant portion of the mitochondrial surface (Rizzuto *et al.* 1998) (Fig. 3).

A corollary of the concepts outlined above is that only a subfraction of the mitochondrial network should experience the Ca^{2+} hot spots. In fact, not only are the channels non-homogeneously distributed along the ER–SR and the plasma membrane, but also the morphological data indicate that the physical proximity of mitochondria to other membranes involves only part of their surface. Evidence for the heterogeneity of Ca^{2+} uptake within mitochondria upon physiological stimulation is supported by a number of indirect observations. For example, similar cytoplasmic Ca^{2+} increases result in different average accumulations of Ca^{2+} in the mitochondria depending on the source of Ca^{2+}. In HeLa cells, for example, Ca^{2+} release from the stores is more efficient at inducing mitochondrial Ca^{2+} accumulation than Ca^{2+} influx through the plasma membrane (Rizzuto *et al.* 1993), while the opposite is true for rat pituitary GH3 cells (Pizzo *et al.* 1997). Furthermore, the apparent increases in [Ca^{2+}], measured with targeted recombinant aequorin, are reduced upon repetitive activation of the same Ca^{2+} channels (e.g. in HeLa cells and in adrenal medullary cells) despite identical elevations in cytoplasmic [Ca^{2+}] (Rizzuto *et al.* 1994). It has been argued that, since aequorin is irreversibly discharged upon binding of Ca^{2+} and emission of the photon, a selective consumption of the photoprotein occurs only in the domains of the highly interconnected mitochondrial network (Rizzuto *et al.* 1998) and/or in subpopulations of mitochondria exposed to high Ca^{2+} microdomains, where no probe remains available for a subsequent stimulation. We think that final proof for such heterogeneity still awaits conclusive evidence from the direct measurement of the [Ca^{2+}] in single organelles, which is beyond the reach of currently available indicators like rhod-2 and similar dyes.

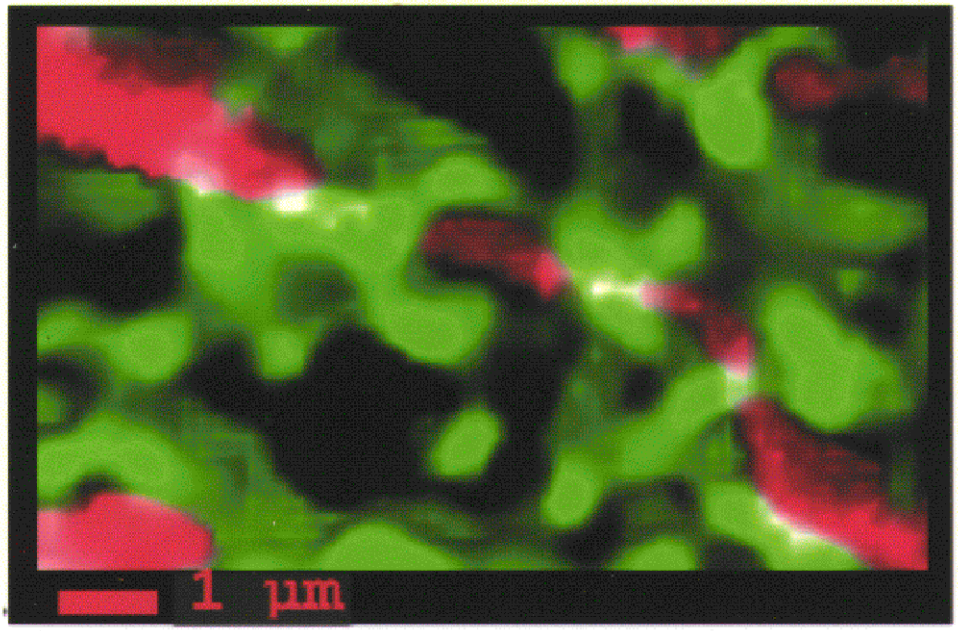

Figure 3. 3-D imaging of mitochondrial and ER structure in living HeLa cells

The two organelles were labelled with appropriately targeted chimeras of green fluorescent protein variants with different spectral properties. The images acquired at each focal plane were computationally deblurred and 3-D reconstructed. The mitochondrial and ER images are displayed in red and green, respectively, while the white areas are those in which overlap between the two images was detected. Reproduced with permission from Rizzuto *et al.* 1998.

A key question concerns the Ca^{2+} levels that are reached within the matrix when the organelles are exposed *in situ* to the high [Ca^{2+}] microdomains. Targeted recombinant aequorin should be instrumental in their determination, given its unsurpassed selectivity of localisation. Furthermore, thanks to the use of mutated aequorin isoforms (Kendall *et al.* 1992) and of different coenzymes (Barrero *et al.* 1997), aequorin allows us to explore a very ample range of Ca^{2+} concentrations, from 0·1 μM up to 1–2 mM. Our initial estimates of the average peak levels of matrix Ca^{2+} concentrations in HeLa cells stimulated by histamine (4–5 μM; Rizzuto *et al.* 1993) were probably underestimated by a factor of 2–3, and a recalculation of the algorithm used to transform the crude luminescence signal into Ca^{2+} levels (Brini *et al.* 1995) rather suggests values of about 10–12 μM. A much more important cause of underestimation of the peak levels is due, however, to the heterogeneity of the responding mitochondrial population (see above). Due to the intrinsic calibration properties of aequorin, this underestimation can be larger than one order of magnitude. Using a low-affinity aequorin, Montero *et al.* (2000) have shown that in adrenal medullary cells the peak $[Ca^{2+}]_m$ of the highly responding mitochondria can be as high as 500–700 μM. The peak levels of Ca^{2+} calculated with rhod-2 are much lower than those revealed by aequorin, and hardly different from those of the cytoplasm, i.e. 1–2 μM (Babcock *et al.* 1997). It is difficult to explain this discrepancy, particularly for estimates obtained in the same cell type. In our opinion, the intrinsic physicochemical characteristics of the different probes and the heterogeneity of the response within the organelle population may be at the basis of these very different results. In particular, the average aequorin signal is intrinsically biased towards the Ca^{2+} levels reached in the subpopulation of highly responding cells. For example, if 50% of mitochondria respond (as has been suggested for the adrenal cells) with a Ca^{2+} rise of 500 μM, while the rest of the population reaches 1–2 μM, the average signal calculated with a low Ca^{2+} affinity aequorin would give an apparent mean peak of around 200 μM. The average signal of fluorescent dyes of the BAPTA family, such as rhod-2, in contrast, is biased towards the lower responding population. Assuming a situation such as that outlined above, a mitochondria-trapped dye with a K_d for Ca^{2+} of 1 μM would produce a signal that, on average, would be calibrated as an apparent increase of about 3 μM. The absolute levels of [Ca^{2+}] reached within the mitochondrial matrix are of major relevance in coping with the activation of metabolic reactions occurring within the matrix (see below). If the high values indicated by aequorin are indeed correct, it can be predicted that most of the Ca^{2+}-dependent enzymes located in the organelle matrix (that have a K_d for Ca^{2+} of around 1–10 μM) would not only be maximally activated, but also would remain so for prolonged periods of time. Increases within the 1–2 μM range, as suggested by the rhod-2 data, would lead to only partial activation and only for a short time (see below).

The mechanism and role of Ca^{2+} efflux from mitochondria in intact cells have been less extensively investigated. The lack of effect of CsA on Ca^{2+} efflux and the reduction in the rate of $[Ca^{2+}]_m$ decrease upon treatment with the specific inhibitor CGP37157 (Brini *et al.* 1999; Montero *et al.* 2000; Colegrove *et al.* 2000; T. Pozzan & R. Rizzuto, unpublished data) suggest that under physiological conditions the Na^+–Ca^{2+} exchange mechanism may be the predominant efflux pathway.

The physiological role of mitochondrial Ca^{2+} uptake

Mitochondrial metabolism

A first, obvious possibility stems from the fine biochemical work of Denton, McCormack, Hansford and co-workers, who, in the 1970s, demonstrated that the main function of mitochondria, oxidative phosphorylation, could be a Ca^{2+}-regulated process, because three dehydrogenases of the Krebs cycle (pyruvate-, isocitrate and oxoglutarate dehydrogenase) are activated, with different mechanisms, by an increase in the [Ca^{2+}] to which they are exposed (McCormack *et al.* 1990; Hansford, 1994). In the latter two cases, Ca^{2+} acts as an allosteric regulator, while in the case of pyruvate dehydrogenase a Ca^{2+}-dependent phosphatase converts the enzyme complex into the active form. As a consequence, a [Ca^{2+}] rise to micromolar levels in the mitochondrial matrix could increase the availability of reducing equivalents to the respiratory chain. This appears to actually occur in living cells, where the agonist-dependent Ca^{2+} rise is paralleled by a rise in the reduced form of NADH levels (Rizzuto *et al.* 1994).

When the phenomenon was investigated at the single cell level, a more sophisticated scenario emerged that shed further light not only on mitochondrial function but also on the significance of the spatio-temporal complexity of intracellular Ca^{2+} signalling. Indeed, much is known about the mechanisms that allow a variety of agonists to cause the periodic spiking of cytoplasmic Ca^{2+} concentration (the phenomenon commonly referred to as 'Ca^{2+} oscillations'), but little is known about how these oscillations are perceived and decoded by a living cell. Pralong and co-workers showed that agonist-dependent Ca^{2+} spiking causes oscillatory increases in NADH levels (Pralong *et al.* 1994). Hajnoczky and co-workers went further and demonstrated that cytoplasmic Ca^{2+} oscillations, by causing parallel rises in the mitochondrial matrix, induce a rise in NADH levels that lasts longer that the Ca^{2+} rise itself (e.g. for a kinetic delay imposed by the rephosphorylation of pyruvate dehydrogenase) (Hajnoczky *et al.* 1995). As a consequence, a rapid pace of cytoplasmic, and hence, mitochondrial Ca^{2+} spiking causes a nearly sustained rise in NADH levels. In other words, cytoplasmic Ca^{2+} oscillations, through the effect on mitochondrial Ca^{2+} uptake, are decoded inside the mitochondria into a long-term activation of mitochondrial metabolism. Ca^{2+} oscillations proved to be the most efficient route for achieving such an effect, because they allow a

repetitive, long-term activation of the dehydrogenases. Conversely, given that mitochondrial Ca^{2+} uptake is mediated by the transient generation of Ca^{2+} microdomains in the proximity of mitochondria and then rapidly slows down, a sustained rise in the cytoplasm causes a short-lived rise in mitochondrial [Ca^{2+}] and hence in NADH levels (Hajnoczky *et al.* 1995).

The increased availability of NADH in the stimulated cell (i.e. a cell where Ca^{2+} has triggered energy-consuming processes such as secretion, motion or simply Ca^{2+} re-extrusion) will in turn allow the respiratory chain to match the increased energy demand with an increased rate of respiration, which rebuilds the proton-motive force used for ATP phosphorylation. If this is the case, the net effect should be that of increasing mitochondrial and eventually cytoplasmic ATP levels. To test this hypothesis, a mitochondrially targeted ATP probe based on the luminescent protein luciferase was constructed (Jouaville *et al.* 1999). The starting point in the development of this new technique was the observation that in a 'cytoplasmic' environment (which includes competing anions and proteins) the affinity of luciferase for ATP is markedly lower than that measured in saline solution (Allue *et al.* 1996). Thus, the *in situ* light emission of the photoprotein can be used to report variations of ATP levels in the physiological range (1–10 mM). Using this novel probe a set of interesting observations was made. The first is that agonists causing an intracellular Ca^{2+} signal increase mitochondrial, and subsequently cytosolic, ATP levels (Jouaville *et al.* 1999; Fig. 4). This is demonstrably due to an increased activity of the mitochondrial ATP synthase, because it is abolished by oligomycin (Fig. 4), and it strictly depends on the Ca^{2+} rise. Indeed, when the latter is dampened (e.g. by depleting Ca^{2+} from the ER or by chelating Ca^{2+} in the cytoplasm), the ATP rise is reduced proportionally (Jouaville *et al.* 1999). Finally, this 'Ca^{2+} effect' appears to constitute a long-lasting activation mechanism. In HeLa cells maintained in glycolytic conditions stimulation with Ca^{2+} does not cause a rise in ATP levels, but enhances the rise in ATP levels caused by the subsequent readdition of oxidisable substrates tens of minutes after the removal of the agonist (and hence well after the return of mitochondrial [Ca^{2+}] to basal values) (Jouaville *et al.* 1999). As to the mechanisms allowing this long-term activation, they could include prolonged activation of pyruvate dehydrogenase activity (e.g. for a delay in the phosphorylation step), a direct effect on the respiratory chain, possibly dependent on a Ca^{2+}-dependent increase in matrix volume (Robb-Gaspers *et al.* 1998), or other as yet unidentified mitochondrial targets.

In keeping with the complex spatio-temporal patterns in Ca^{2+} signalling, modulation of ATP production could also occur with a defined, functionally significant micro-heterogeneity. This concept is supported by emerging evidence. Landolfi and co-workers showed that ATP microdomains may exist in the proximity of ER Ca^{2+} pumps, based on the effect on ER Ca^{2+} refilling (Landolfi *et al.* 1998). Kennedy and co-workers showed that the agonist-induced ATP rises are more sustained in the sub-plasmalemmal space and in mitochondria than in the bulk cytosol of insulin-secreting cells, as indicated by luciferase chimeras specifically targeted to these compartments (Kennedy *et al.* 1999). Finally, we would like to mention that the role of mitochondrial Ca^{2+} in modulating ATP levels may also play a direct signalling role in specialised cell systems such as the pancreatic β-cell, where control is exerted directly on insulin secretion. We refer the reader to the work of Wollheim's group for a detailed account of these important observations (Maechler & Wollheim, 2000, this issue).

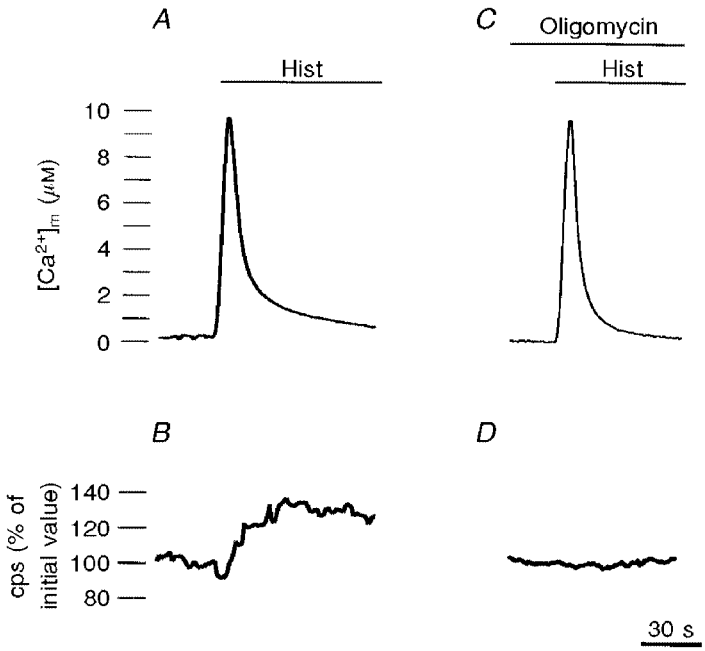

Figure 4. Agonist-dependent mitochondrial [Ca^{2+}] (*A* and *C*) and [ATP] (*B* and *D*) increases in the presence and absence of the ATP synthase inhibitor oligomycin

The calibrated [Ca^{2+}] values were obtained as in Fig. 2, while ATP levels were inferred from light emission of a luciferase chimera targeted to the mitochondria (Jouaville *et al.* 1999). cps, counts per second. Where indicated, the cells were treated with 100 μM histamine. In *C* and *D*, the cells were treated with 2 μM oligomycin, which was added before the agonist and maintained throughout the stimulation.

Mitochondria and the determination of the spatio-temporal pattern of Ca^{2+} signalling

The obvious relevance of mitochondrial Ca^{2+} uptake for the control of organelle function, as well as the general consensus that only a small fraction of Ca^{2+} released from the ER (or entering from the plasma membrane channels) actually ends up in the mitochondria, has led to an underestimation of the role of mitochondria in controlling the cytosolic Ca^{2+} rise. In other words, mitochondrial Ca^{2+} uptake was considered relevant for conveying an activatory signal to these organelles, but not significant in the overall balance of cellular Ca^{2+} homeostasis.

In the past few years, converging evidence that this is not the case has emerged from the work of various groups and there is now an established consensus for the concept that mitochondria participate in shaping the spatio-temporal complexity of calcium signalling in at least three different ways.

(i) Mitochondria, clustered in a defined portion of a polarised cell, could act as an 'immobile buffer' preventing, or delaying, the spread of the Ca^{2+} signal to the rest of the cell. For example, in the highly polarised pancreatic acinar cells, in some cases the Ca^{2+} signal is restricted to the apical pole, thus inducing the release of enzyme-containing granules, while in other cases it also spreads to the rest of the cell, thus reaching the nucleus and causing long-term effects dependent on the activation of gene transcription. Petersen and co-workers have recently demonstrated that a layer of mitochondria localised at the base of the granule-rich area plays a key role in restricting the effect of weaker agonists to the secretory pole. If the ability of mitochondria to accumulate Ca^{2+} is abolished, the signal conveyed by the same agonists is converted into a global signal extending to the basolateral portion of the cell (Tinel *et al.* 1999).

(ii) Cross-talk between the intracellular stores and the mitochondria could 'bypass' the bulk cytosol and communication could be restricted to regions of close contact with the ER. Indeed, it can be envisaged that mitochondrial Ca^{2+} uptake participates in dissipating the microdomains of high $[Ca^{2+}]$ at the mouth of the ER channels, which, in turn, exert a feedback control on the channel. The first demonstration of this possibility came from the work of Lechleiter and co-workers in *Xenopus* oocytes. They analysed the propagation pattern of cytosolic Ca^{2+} waves and demonstrated that mitochondrial energisation enhances Ca^{2+} release through the InsP_3-gated channel by increasing Ca^{2+} uptake into mitochondria and thereby dissipating the Ca^{2+} microdomain at the mouth of the channel (Jouaville *et al.* 1995). More recently, similar evidence was obtained in mammalian cells. In primary cultures of hepatocytes, Hajnoczky and co-workers demonstrated that mitochondrial Ca^{2+} uptake suppressed the positive effects of Ca^{2+} on the InsP_3 receptor, thus reducing Ca^{2+} release at submaximal doses of agonists (Csordas *et al.* 1999; Hajnoczky *et al.* 1999). In astrocytes, Boitier and co-workers showed that mitochondrial Ca^{2+} uptake reduces the diffusion of Ca^{2+} waves, thus exerting a negative feedback control on the explosive recruitment of InsP_3 receptors (Boiter *et al.* 1999). In BHK-1 fibroblasts, Landolfi and co-workers showed that local Ca^{2+} buffering by mitochondria (as well as ATP production) exerts the opposite effect on the InsP_3 receptor, as inhibition of mitochondrial Ca^{2+} uptake and/or of ATP production reduces the rate of Ca^{2+} release (Landolfi *et al.* 1998). These results are not surprising, nor contradictory, given the known bell-shaped Ca^{2+} response curve of the InsP_3 receptor, with significant differences among the various isoforms (e.g. type 2 lacks the inhibition at high $[Ca^{2+}]$; Ramos-Franco *et al.* 1998). Rather, they highlight how mitochondria can finely tune the microenvironment of the ER Ca^{2+} release sites, which in turn is the real determinant of the amplitude and spatio-temporal pattern of the Ca^{2+} increase.

(iii) Transient openings of the PTP could induce organellar Ca^{2+}-induced Ca^{2+} release, and the ensuing depolarisation and/or Ca^{2+} rise could rapidly travel inside the cell via the mitochondrial network providing further complexity to the influence of these organelles in shaping the global Ca^{2+} signal of a cell (Ichas *et al.* 1997).

The concept that mitochondrial Ca^{2+} homeostasis plays a fundamental role in controlling the cytosolic Ca^{2+} response should by no means be limited to the prevailing route of the Ca^{2+} rise of non-excitable cells (Ca^{2+} release from the ER via the InsP_3-gated channel). Work from numerous laboratories has now demonstrated that mitochondria also participate in modulating $[Ca^{2+}]_c$ when the source of Ca^{2+} is the extracellular medium and the rise is mediated by the opening of plasma membrane channels. In neurones, mitochondria placed in the proximity of the plasma membrane channels determine the kinetic profile of the $[Ca^{2+}]_c$ rise, acting probably as fixed buffers that first limit the peak amplitude, and then contribute to the long-lasting plateau phase of the $[Ca^{2+}]_c$ rise by gradually releasing Ca^{2+} via the Na^+–Ca^{2+} exchanger (Werth & Thayer, 1994; White & Reynolds, 1995; Colegrove *et al.* 2000). The role of mitochondria in regulating Ca^{2+} influx is not limited to neuronal channels. In T lymphocytes, Hoth and co-workers showed that Ca^{2+} uptake by a resident mitochondrial pool enhances the ubiquitous current activated by the release of ER Ca^{2+} (commonly referred to as 'capacitative calcium entry', CCE) by relieving the Ca^{2+} inhibition in the microdomain at the mouth of the channel (Hoth *et al.* 1997).

In conclusion, although the physiological effects and molecular mechanisms responsible for the functional interactions are different in the various cell systems, it can be concluded that mitochondria experience a two-sided, close relationship with the cellular Ca^{2+} pools that allows them to invariably undergo a major rise in matrix $[Ca^{2+}]$ when a Ca^{2+} signal is elicited in a cell. On the one hand, this allows the stimulation of mitochondrial metabolism to

match the increased cell needs while on the other, it places a fixed buffer of high capacity in the proximity of Ca^{2+}-sensitive release pathways, thus allowing different modes of regulation of Ca^{2+} release. How these interactions are formed and modified, and how they in turn affect the long-term cellular responses, are interesting biological questions that need to be addressed.

Pathophysiological alterations of mitochondrial Ca^{2+} signalling

As discussed, mitochondrial Ca^{2+} uptake depends on a large thermodynamic force for accumulation and a kinetic limitation that allows high rates of accumulation only transiently, i.e. when the organelles are exposed to high $[Ca^{2+}]$ microdomains generated at the mouth of the opening channels. Such an arrangement restricts the timing of Ca^{2+} uptake to the earliest phases of the cytosolic Ca^{2+} rise, and thus prevents the cell from dissipating the $\Delta\Psi$ generated by the respiratory chain in futile Ca^{2+} cycling across the mitochondrial inner membrane throughout agonist stimulation. However, at least in principle, it could be negatively affected in a number of pathophysiological conditions that affect mitochondrial structure and function. We discuss here only a few examples where an alteration of mitochondrial Ca^{2+} handling could be shown and discuss the possible implication of these alterations in generating the cellular phenotype.

Mitochondrial diseases

Mitochondrial diseases are a heterogeneous group of disorders sharing a genetic defect involving mitochondrial proteins and the common morphological hallmark of altered mitochondrial number and/or shape. As to the molecular defect, numerous pathogenic mutations have been identified in mitochondrial DNA (mtDNA), the 16·5 kb circular DNA encoding 13 polypeptides that are either components of the respiratory chain complexes or of the mitochondrial ATPase. The defects range from large scale deletions, such as those found in Kearns-Sayre syndrome and progressive external ophtalmoplegia (PEO), to point mutations in transfer RNAs (tRNAs), such as those found in MELAS (myopathy, encephalopathy, lactic acidosis and stroke-like episodes) and in MERRF (myoclonic epilepsy with ragged red fibres), and to point mutations in structural genes, such as those found in Leber's optic atrophy or NARP (neuropathy, ataxia and retinitis pigmentosa) (Wallace, 1994; Schon *et al.* 1997). While the molecular identity of the mutations is in many cases known, the pathway(s) that leads to the functional cell defect is often obscure. Indeed, in many cases biochemical analysis of the various disorders, which may reveal comparable, partial deficiencies of mitochondrial function, does not clarify the cellular and clinical specificity of the various disorders. We investigated the possibility that genetic defects affecting the generation of the proton gradient across the mitochondrial membrane (and thus the driving force for Ca^{2+} accumulation) could impair mitochondrial Ca^{2+} homeostasis. We thus carried out experiments in transmitochondrial cell lines ('cybrids') harbouring in the same nuclear background either normal mtDNAs, mtDNAs harbouring the tRNAlys mutation of MERRF, or mtDNAs harbouring the ATPase6 mutation of NARP. We observed that the mitochondrial Ca^{2+} response to agonists was drastically reduced in MERRF cybrids, while the response in NARP cells was normal, the cytoplasmic response being normal in all cases. The impairment of the Ca^{2+} response caused a smaller increase in ATP levels upon agonist stimulation, as monitored by recombinant luciferase. Interestingly, an inhibitor of the mitochondrial efflux pathways caused a significant restoration of both the Ca^{2+} and the ATP response, thus implying that the defect in mitochondrial Ca^{2+} homeostasis is a relevant event in the cellular pathogenesis of this disorder and, potentially, a therapeutic target (Brini *et al.* 1999).

The oncoprotein Bcl-2

A second, interesting example is the effect of the oncoprotein Bcl-2 on subcellular Ca^{2+} homeostasis. While the role of Bcl-2 and related proteins in the control of apoptotic cell death is widely accepted, its mechanism of action is still unsolved. Bcl-2 can form ion channels in lipid bilayers, and it is widely distributed in intracellular membranes including the outer mitochondrial membrane. Among other possibilities, it has been proposed that Bcl-2 could act on Ca^{2+} signalling by affecting ion fluxes across the organelle membranes (Minn *et al.* 1997). We utilised targeted aequorins to specifically monitor $[Ca^{2+}]$ in the cytosol, in the mitochondrial matrix and in the lumen of intracellular Ca^{2+} stores (the ER and the Golgi apparatus) and revealed that Bcl-2 overexpression alters Ca^{2+} handling in all these compartments (Pinton *et al.* 2000). The key event was an increase in the Ca^{2+} leak from the intracellular stores, which consequently attained a lower state of filling. Thus, upon agonist stimulation the amount of Ca^{2+} released from the ER (and that accumulated in the mitochondria) was significantly reduced. Also, in this case the mitochondrial Ca^{2+} response was reduced, but this was caused by a global reduction of the cellular Ca^{2+} signal rather than an intrinsic limitation of the capacity of mitochondria to accumulate Ca^{2+}. The relevance for cell survival of the alteration in Ca^{2+} signalling in the mitochondria (in which Ca^{2+} overload has been proposed to be a major apoptotic signal) and in the other cell compartments is the aim of studies currently underway in our laboratories.

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