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INVITED REVIEW

Regulation of the mitochondrial proton gradient by cytosolic Ca^{2+} signals

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Abstract Mitochondria convert the energy stored in carbohydrate and fat into ATP molecules that power enzymatic reactions within cells, and this process influences cellular calcium signals in several ways. By providing ATP to calcium pumps at the plasma and intracellular membranes, mitochondria power the calcium gradients that drive the release of Ca^{2+} from stores and the entry of Ca^{2+} across plasma membrane channels. By taking up and subsequently releasing calcium ions, mitochondria determine the spatiotemporal profile of cellular Ca^{2+} signals and the activity of Ca^{2+}-regulated proteins, including Ca^{2+} entry channels that are themselves part of the Ca^{2+} circuitry. Ca^{2+} elevations in the mitochondrial matrix, in turn, activate Ca^{2+}-dependent enzymes that boost the respiratory chain, increasing the ability of mitochondria to buffer calcium ions. Mitochondria are able to encode and decode Ca^{2+} signals because the respiratory chain generates an electrochemical gradient for protons across the inner mitochondrial membrane. This proton motive force (Δp) drives the activity of the ATP synthase and has both an electrical component, the mitochondrial membrane potential ($\Delta\Psi_m$), and a chemical component, the mitochondrial proton gradient (ΔpH_m). $\Delta\Psi_m$ contributes about 190 mV to Δp and drives the entry of Ca^{2+} across a recently identified Ca^{2+}-selective channel known as the mitochondrial Ca^{2+} uniporter. ΔpH_m contributes ~30 mV to Δp and is usually ignored or considered a minor component of mitochondria respiratory state. However, the mitochondrial proton gradient is an essential component of the chemiosmotic theory formulated by Peter Mitchell in 1961 as ΔpH_m sustains the entry of substrates and metabolites required for the activity of the respiratory chain and drives the activity of electroneutral ion exchangers that allow mitochondria to maintain their osmolarity and volume. In this review, we summarize the mechanisms that regulate the mitochondrial proton gradient and discuss how thermodynamic concepts derived from measurements in purified mitochondria can be reconciled with our recent findings that mitochondria have high proton permeability in situ and that ΔpH_m decreases during mitochondrial Ca^{2+} elevations.

Keywords Bioenergetics · Cell biology · Mitochondria

Introduction

Mitochondria are multitalented organelles whose functions extend far beyond energy conversion and the control of lipid metabolism. Mitochondria act as signalling platforms to amplify antiviral responses [8], control cell fate decisions by releasing proapoptotic factors [27, 30], and shape the spatiotemporal patterns of finely encoded calcium signals by taking up, sequestering, and releasing calcium ions at strategic locations inside cells [55]. This last aspect has received much attention because cells rely on spatially and temporally restricted elevations in their intracellular calcium concentration to precisely regulate multiple specific functions, such as the secretion of granules, the contraction of muscle fibers,

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and the transcription of genes. The preservation of the information to be encoded as calcium signals implies a tight control of the mechanisms that generate, propagate, and decode the unique spatial and temporal pattern associated with a specific cellular response. By taking up and subsequently releasing Ca^{2+} ions, mitochondria can alter both the spatial extent and the duration of cytosolic calcium signals. The molecules that mediate the uptake of calcium into the mitochondrial matrix have been recently identified and comprise a Ca^{2+}-selective channel known as the mitochondrial Ca^{2+} uniporter (MCU) [6, 19] and a regulatory EF hand-containing protein (MICU1) [43]. A mitochondrial Na^+/Ca^{2+} exchanger (NCLX) and a H^+/Ca^{2+} exchanger whose identity remains controversial catalyze the extrusion of Ca^{2+} from the matrix in exchange for sodium and proton, respectively [26, 42]. The mitochondrial modulation of cellular Ca^{2+} signals can be a significant determinant of the final cellular responses: in pancreatic acinar cells, mitochondria form a diffusion barrier that prevents the propagation of calcium waves, thereby spatially restricting the calcium signals to the apical pole, where a calcium elevation induces the secretion of granules containing digestive enzymes [53]. In T lymphocytes, mitochondria sustain the entry of calcium ions across the plasma membrane by removing the calcium-dependent inactivation of store-operated calcium channels, thereby prolonging the duration of the calcium signals that induce the proliferation and differentiation of T cells [24]. The accumulation of mitochondria at the sites of contact between T cells and antigen-presenting cells sustains the local activity of store-operated Ca^{2+} entry channels at the immunological synapse [51]. Mitochondrial Ca^{2+} buffering therefore constitutes an efficient and versatile mechanism for the spatial and temporal control of cellular Ca^{2+} signals, provided that cells can control the specific location of their mitochondria [18]. The entry of Ca^{2+} ions into the mitochondrial matrix, on the other hand, directly impacts the metabolism of mitochondria and their energy state because elevations in the mitochondrial matrix Ca^{2+} concentration, $[Ca^{2+}]_{mit}$, activate three dehydrogenases of the citric acid cycle within the matrix, thus increasing the availability of reducing equivalents that fuel the respiratory chain complexes [23, 46]. The increased respiratory rates tend to make the mitochondrial membrane potential more negative, which in turn favors the entry of additional Ca^{2+} ions across the uniporter. The energetic state of mitochondria therefore determines the ability of these organelles to take up Ca^{2+} ions and at the same time is increased by the uptake of Ca^{2+}, a positive feedback loop that amplifies the Ca^{2+} buffering capacity of mitochondria.

The capacity of mitochondria to shape calcium signals strictly depends on the ability of these organelles to move protons across their inner membrane (IMM) during oxidative phosphorylation, the process that converts the energy stored in reducing equivalents into high energy phosphates contained within newly generated ATP molecules. Mitochondria have two membranes, an outer membrane permeable to large molecules and an inner membrane impermeable to ions that contain the respiratory chain complexes. Proton translocation across the IMM is a key energy-conserving event that couples the oxidation of highly reduced matrix equivalents (NADH, $FADH_2$, and NADPH) to the synthesis of ATP. For each pair of electrons entering the respiratory chain, ten protons are extruded from the matrix to the intermembrane space by the respiratory complexes I, III, and IV (Fig. 1, brown), while two protons are simultaneously released from NADH in the matrix, generating an electrochemical gradient for protons of more than 200 mV across the IMM [49]. This proton motive force is then used to drive the catalytic activity of complex V, an F_1F_0 ATP synthase comprising a proton channel linked to a rotating catalytic subunit hanging in the matrix and driven by the flux of protons, which adds a new phosphate to an ADP molecule when protons enter the matrix [29]. Proton translocation is so central to the ability of mitochondria to convert reducing equivalents into useful energy that the only protein-coding mitochondrial genes that have not been transferred from the mitochondrial genome to the nuclear genome encode subunits of the respiratory complexes that move protons (complexes I, III, IV, and V). retention of these genes in the maternally transmitted mitochondrial genome can be explained by the need to balance precisely the fluxes of protons in and out of the mitochondrial matrix as their bi-allelic transmission could potentially disrupt the stoichiometry of proton transport [54].

The mitochondrial proton circuit

The concept that the energy released during the oxidation of energetic substrates is stored in a proton gradient across the IMM was formulated in 1961 by the British biochemist Peter Mitchell (1920–1992), who was awarded the Nobel prize in chemistry in 1978 for "his contribution to the understanding of biological energy transfer through the formulation of the chemiosmotic theory" [37]. In his Nobel speech, Mitchell listed the four postulates of the chemiosmotic theory, which can be summarized in modern-day terms as follows:

1. The respiratory chain complexes couple the fluxes of electrons to the ejection of protons across the inner mitochondrial membrane. This system converts the energy of substrate oxidation into a proton electrochemical potential gradient, known as the proton motive force (Δp).

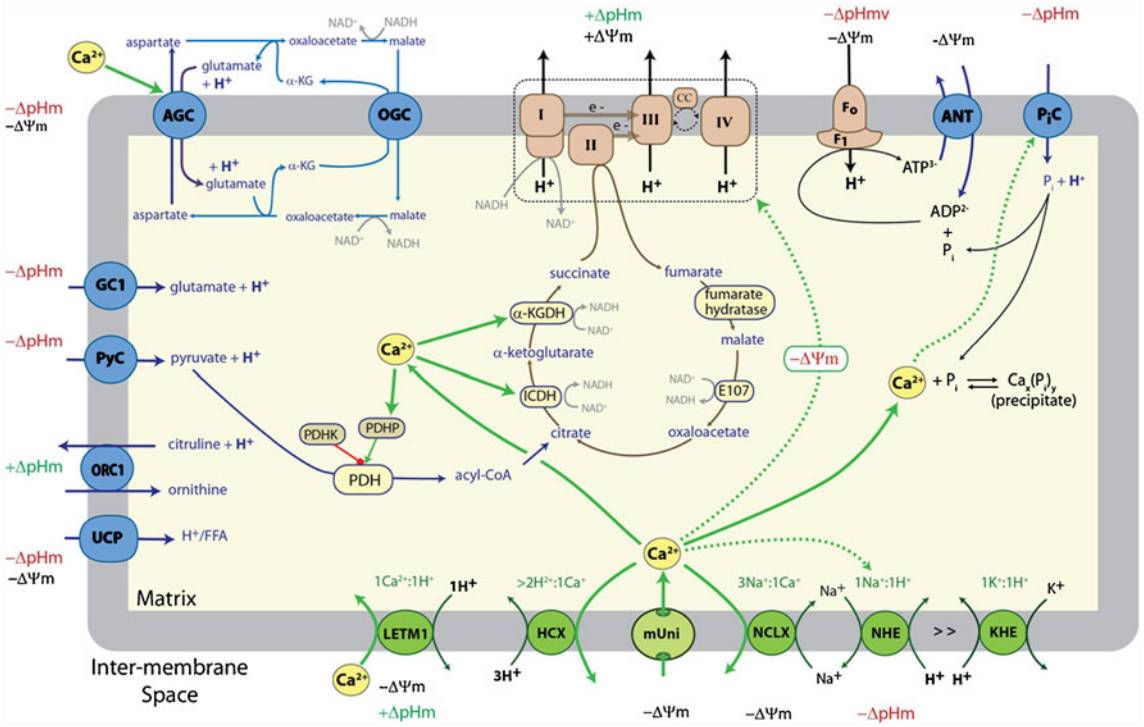

Fig. 1 Ca^{2+} and mitochondrial H^+ transport and ΔpH_m. Mitochondrial transporters are grouped as the respiratory chain (*brown*), mitochondrial carrier proteins (*blue*), and cation channels and exchangers (*green*). The respiratory chain extrudes H^+ at complexes I, III, and IV. Complex II shuttles electrons from succinate/$FADH_2$ to complex III and IV. Increased matrix Ca^{2+} stimulates Kreb's cycle enzymes (pyruvate dehydrogenase (*PDH*) via pyruvate dehydrogenase phosphatase (*PDHP*), α-ketoglutarate dehydrogenase (*α-KGDH*), and isocitrate dehydrogenase (*ICDH*)) to increase the supply of reducing equivalents to the respiratory chain. Increased pyruvate metabolism should stimulate the pyruvate carrier (*PyC*). ATP synthesis by the F1F0-ATP synthase requires phosphate from the P_i/H^+ carrier (*P_iC*) and ADP from the adenine nucleotide translocase (*ANT*). High matrix $[Ca^{2+}]$ can lead to Ca^{2+}-P_i precipitation, reducing matrix $[P_i]$ and driving P_iC. Calcium in the intermembrane space activates electrophoretic aspartate/glutamate–(H^+) carriers (*ACG*). Other H+-coupled carriers include the electrogenic ornithine/citruline carrier (*ORC1*), oxoglutarate carrier (*OGC*), and uncoupling proteins (*UCP*). Ca^{2+} enters mitochondria via the Ca^{2+} uniporter (*mUni*) and LETM1, the latter being subject to debate. Ca^{2+} extrusion is mediated by an electrogenic Na^+/Ca^{2+} exchanger (*NCXL*) that depends on a Na^+ gradient created by the electroneutral Na^+/H^+ exchanger (*NHE*) and a Ca^{2+}/H^+ exchange system (*HCX*). Osmotic matrix swelling can also activate a slower K^+/H^+ exchanger (*KHE*). $+\Delta\Psi_m$ indicates hyperpolarization, $-\Delta\Psi_m$ indicates depolarization

2. A proton-translocating ATPase reversibly couples the translocation of protons across the inner mitochondrial membrane to the hydrolysis and synthesis of ATP. The enzyme is vectorially orientated so that ATP hydrolysis ejects protons from the matrix, whereas proton influx powers ATP synthesis.
3. The inner membrane contains exchangers coupling anion entry and cation extrusion to proton entry. Otherwise, cations would accumulate in the matrix driven by the negative membrane potential and mitochondria would not be able to maintain their osmolarity and volume. The exchangers also permit the entry and extrusion of substrates and of metabolites without collapsing the membrane potential.
4. The inner membrane has a low permeability to ions in general and to protons in particular. Otherwise, ion leaks would dissipate the redox-generated proton motive force and ATP would not be synthesized.

Postulates 1 and 2 have been established beyond doubts by measurements in isolated mitochondria, and the path of protons across the respiratory chain has been mapped by structural studies and molecular dynamics simulations [2, 25]. Postulates 3 and 4 have also been validated by functional measurements in suspended mitochondria [9], but only a handful of candidate proteins have been conclusively shown to catalyze mitochondrial ion/H^+ exchange [40, 57], and the molecular identities of most anion and cation exchangers are not known.

The proton motive force defined in postulate 1 represents the work done by the respiratory chain at thermodynamic equilibrium to actively extrude positively charged protons across a membrane capacitor. Δp corresponds to the sum of the electrical and proton concentration gradients that oppose proton extrusion, i.e., the sum of the mitochondrial potential ($\Delta\Psi_m$, negative inside) and of the pH difference (ΔpH_m, alkaline inside) between the matrix and the intermembrane space (IMS). Both $\Delta\Psi_m$ and ΔpH_m are actively generated by

the respiratory chain during proton translocation. Thus, unlike the membrane potential that electrophysiologists measure at the plasma membrane of cells, the membrane potential of mitochondria is not a diffusion potential but reflects the active charge separation process. Δp is the driving force that energizes the back-flux of protons into the matrix via the ATPase described in postulate 2, and in respiring mitochondria both $\Delta\Psi_m$ and ΔpH_m promote proton entry into the negatively charged and alkaline matrix. $\Delta\Psi_m$ was estimated from the equilibrium distribution of potassium ions in suspended liver mitochondria treated with valinomycin, a potassium ionophore that allows K^+ ions to equilibrate according to the membrane potential, and ΔpH_m by measuring the distribution of radiolabelled weak acids or bases. These measurements yielded values of 150–190 mV for $\Delta\Psi_m$ and 0.5–1.4 pH units for ΔpH_m, adding up to a proton motive force $\Delta p = \Delta\Psi + (60\ mV \times \Delta pH)$ of ~220 mV [38]. The relative contributions of $\Delta\Psi_m$ and ΔpH_m depended on the availability of electrophoretically translocatable species such as potassium and phosphate in the incubation medium, but in no occasion was the pH component greater than the electrical component. At physiological potassium and phosphate concentrations, ΔpH_m was found to contribute only ~15 % to the proton motive force driving the ATP synthase, implying that the main determinant of the ability of mitochondria to convert metabolic substrates into ATP is $\Delta\Psi_m$. The negative potential also provides the driving force for the entry of Ca^{2+} ions into the mitochondrial matrix across the MCU. However, as formulated in Postulate 3, the generation and maintenance of a ΔpH_m is essential to drive the fluxes of anions and cations in and out of mitochondria, respectively.

The mitochondrial proton gradient

ΔpH_m is determined by the moment-to-moment balance of proton fluxes across the IMM, and by the concurrent pH changes occurring in the matrix and the extramitochondrial compartment, which are limited by the proton buffering capacity of each compartment. The buffering power (β) is a measure of the ability of a weak acid or base to minimize the change in pH upon a flux of protons into or out of a solution, or cellular compartment. A difference in β between the cytosol (β_{cyto}) and mitochondria (β_{mito}) will amplify ($\beta_{mito} < \beta_{cyto}$) or dampen ($\beta_{cyto} < \beta_{mito}$) the change in ΔpH_m resulting from a proton flux across the IMM depending on the sign of the difference in β. Further, the power of a buffer varies with pH and is greatest at a buffer's pKa [48]. Numerous studies have characterized the intrinsic β_{cyto} in intact cells and tissues, its temperature dependence, and the contribution of bicarbonate to total cell β [13]. Such measures were facilitated by pH-sensitive fluorescent dyes (for example [32]). In contrast, we are only aware of two reports that characterize β_{mito} in intact cells or tissue. Durand et al., using ^{32}P NMR to monitor mitochondrial and cytosolic pH in perfused liver at 4°C, reported β_{mito} to be greater that β_{cyto} from pH 6.6–7.6, with a maximum of ~150 mM at pH 7.2 [20]. In contrast, we employed a ratiometric, pH-sensitive circularly permuted YFP combined with the cytosolic pH-sensitive dye SNARF to monitor ΔpH_m in intact HeLa cells at 37°C [44]. With this approach, we found intrinsic β_{mito} and β_{cyto} to be remarkably similar, with maxima near 18 (β_{mito}) and 22 (β_{cyto})mM at pH 7.2. Despite the significant discrepancies in the absolute buffering powers measured in these two studies, in both reports β_{mito} was markedly reduced at alkaline pH (>~7.5). Thus, it would appear that a given proton flux would generate a larger steady-state ΔpH_m in energized mitochondria with an alkaline pH than in de-energized mitochondria, in which the matrix pH approaches that of the cytosol.

As described above, the generation of a mitochondrial proton gradient relies on proton ejection from the matrix by respiratory complexes I, III, and IV, a process that increases both $\Delta\Psi_m$ and ΔpH_m. In 2009, Letm1 was reported to mediate ruthenium red-sensitive, mitochondrial Ca^{2+} uptake via 1:1 Ca^{2+}/H^+ exchange, an electrogenic mode of proton extrusion that could enhance ΔpH_m at the expense of $\Delta\Psi_m$ [26] (Fig. 1, labelled "$+\Delta pH_m$" in green). However, it remains to be seen how this observation fits with reports of Letm1 mediating K^+/H^+ exchange [40, 57] and with numerous observations that mitochondrial Ca^{2+}/H^+ exchange is insensitive to ruthenium red [9]. The instantaneous proton gradient depends on the extent to which proton extrusion is countered by proton entry via the F_1F_0-ATPase (complex V), cation/H^+ exchangers, and several members of the mitochondrial family of carrier proteins (Fig. 1, labelled "$-\Delta pH_m$" in red). In all tissues, mitochondria possess a fast-acting, electroneutral Na^+/H^+ exchanger (NHE) that sets the mitochondrial Na^+ gradient equal to ΔpH_m and should mediate a proton flux sufficient to support basal Ca^{2+} extrusion via the mitochondrial Na^+/Ca^{2+} exchanger, NCLX [9]. The slower K^+/H^+ exchanger (KHE) is involved in the maintenance of mitochondrial volume and is activated by matrix swelling and increased ΔpH_m [7, 21, 22]. A family of ~20 mitochondrial carriers mediates the transport across the IMM of solutes that are required for oxidative phosphorylation and related metabolic reactions (for an extensive review refer to [41]). The carriers coupled to proton flux comprise: (1) the phosphate carrier (PiC, SLC25A3) that mediates electroneutral phosphate/proton (P_i/H^+) symport (or P_i/OH^- antiport) to supply the matrix with phosphate for the generation of ATP at the expense of ΔpH_m, (2) the electroneutral glutamate carrier (GC1, SLC25A18) that mediates glutamate entry together with a proton [41], and the electrogenic, Ca^{2+}-sensitive, aspartate/glutamate–H^+ carriers (AGC1/2, aralar/citrin, SLC25A12/

13) [17, 50]. A pyruvate carrier, that remains to be molecularly identified, also mediates pyruvate/H^+ symport (PyC). Finally, protons can enter the matrix via uncoupling proteins (UCPs). UCP1 (SLC25A8), expressed in brown adipose fat, acts as a mitochondrial proton channel to mediate adaptive thermogenesis [12]. Most non-adipose tissues express UCP2 (SLC25A8) or UCP3 (SLC25A9) that mediate H^+ transport upon stimulation by fatty acids and purine nucleotides [41], but these isoforms do not appear to contribute to basal H^+ flux [11, 15]. Experiments in isolated mitochondria indicate that the F_1F_0-ATPase, NHE, and Pi/H^+ symport are the main mediators of H^+ back-flux, but the relative contributions of these transporters to changes in ΔpH_m that occur in intact cells have not been quantified. It is noteworthy that cytosolic or extramitochondrial pH was historically assumed to equilibrate with intermembrane space pH (pH_{IMS}) due to proton flux through the voltage-dependent anion channels in the outer membrane [45]. Combined with experimental difficulties in selectively measuring pH_{IMS}, this assumption accounts for the fact that ΔpH_m is calculated as the difference between matrix pH and extra-mitochondria pH in the vast majority of existing literature. However, one study in isolated mitochondria and one in intact cells demonstrated that pH_{IMS} can be up to 0.7 units more acidic than the cytosol dependent on matrix volume and possibly on $\Delta\Psi_m$ [14, 45]. Consequently, it is very possible that most published measurements of ΔpH_m, largely in isolated mitochondria, underestimate ΔpH_m.

Regulation of ΔpH_m in intact cells: is Ca^{2+} boosting or slowing mitochondrial metabolism?

The regulation of ΔpH_m in intact, living cells has been less studied than the regulation of $\Delta\Psi_m$ because ΔpH_m contributes only a minor fraction of the proton motive force and is more difficult to measure than $\Delta\Psi_m$. To determine ΔpH_m, one must measure concomitantly the absolute pH levels on the two sides of the IMM, i.e., within the mitochondrial matrix and in the cytosol or IMS. This is difficult to achieve with synthetic pH indicators, which cannot be specifically targeted to a specific cellular compartment. Despite these limitations, measurements with pH-sensitive fluorescent dyes reported ΔpH_m values of 0.9 pH units in cardiac myocytes [33], of 0.3–0.43 in resting and stimulated hepatocytes, respectively [47], and of 0.3 in MDCK cells [5]. More recent measurements with genetically encoded pH-sensitive indicators targeted to the matrix and the outer surface of the IMM reported a ΔpH_m of 0.8 pH units [45], but did not provide insight as to its dynamic regulation. Using a matrix-targeted pH-sensitive cpYFP combined with a cytosolic fluorescent pH indicator, we recorded dynamic ΔpH_m changes during cellular activation [44]. Surprisingly, we observed that ΔpH_m decreases during activation of HeLa cells with Ca^{2+}-mobilizing agonists. Earlier studies had reported a matrix alkalinization in pancreatic beta cells stimulated with glucose [56] and in a minority of HeLa cells stimulated with histamine, the majority of HeLa cells showing no change in matrix pH [1]. In our hands, both the cytosolic and matrix pH decreased during cytosolic Ca^{2+} elevations (Fig. 2), but the acidification was more pronounced in the mitochondrial compartment, causing ΔpH_m to decrease. The parallel acidification of the cytosol and matrix compartments reflected the activity of plasma membrane Ca^{2+} pumps (PMCA), and a similar phenomenon is thought to occur in glutamate-stimulated cortical neurons [10]. The PMCA releases large quantities of acid (or protons equivalents) into the cytosol during calcium extrusion, and the resulting increase in cytosolic proton concentration is

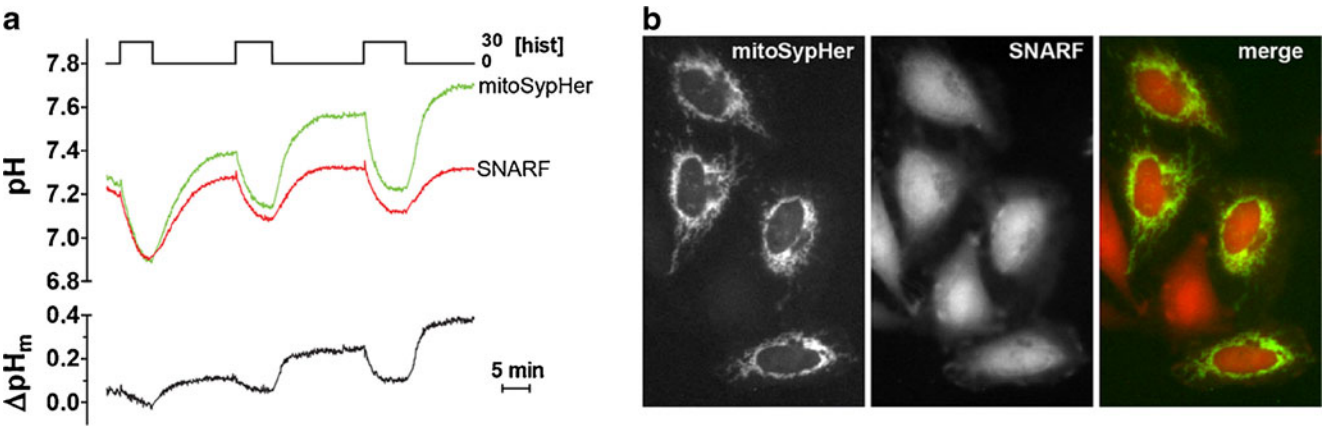

Fig. 2 Dynamic recordings of ΔpH_m. Mitochondrial pH was monitored at 37°C in HeLa cells transfected with mitoSypher and loaded with 5-(and 6)-carboxy-SNARF-1 to monitor cytosolic pH simultaneously on an epifluorescence microscope. Traces in **a** are the averaged pH values of transfected cells in **b**. Cells were stimulated with the Ca^{2+}-mobilizing agonist histamine (30 μM) as indicated. ΔpH_m was calculated as the cell-wise difference between mitochondrial and cytosolic pH. These data are a subset of research originally published in the *Journal of Biological Chemistry* [44]

rapidly transmitted to the mitochondrial matrix, as was verified in permeabilized cells equilibrated with solutions of different pH [44]. The rapid equilibration of the matrix pH with the environmental pH was not due to electrophoretic entry of protons but was mediated predominantly by electroneutral ion/H^+ exchangers, and thus remains consistent with the third and fourth postulates of the chemiosmotic theory. The larger decrease in mitochondrial pH that caused the ΔpH_m dissipation reflected the lower buffering power of the alkaline matrix compared to the near-neutral cytosol [44], as discussed above. The observed ΔpH_m dissipation appears at first glance at odds with a wealth of studies showing that cytosolic Ca^{2+} elevations boost mitochondrial metabolism [23, 46]. However, this loss of ΔpH_m occurred with a time course and amplitude that match histamine-induced increases in $\Delta\Psi_m$ in HeLa cells [28]. We observed, as well, that the acidifying drag exerted by the cytosolic Ca^{2+} elevations was followed by a slow matrix alkalinization as the cytosolic Ca^{2+} signal subsided in intact cells (Fig. 2), consistent with the idea of Ca^{2+}-dependent metabolic priming [28]. Concordantly, mitochondria slowly alkalinized when exogenous Ca^{2+} was added to permeabilized cells, as expected from Ca^{2+}-dependent activation of mitochondrial matrix enzymes. Cytosolic Ca^{2+} elevations thus appear to exert opposing effects on ΔpH_m as they stimulate mitochondrial respiration while acidifying a matrix that does not buffer protons efficiently. In cells that are essentially glycolytic, the later mechanism dominates and ΔpH_m decreases during Ca^{2+} elevations, as was observed in astrocytes exposed to glutamate, where Ca^{2+}-induced ΔpH_m drops were proposed to facilitate neurotransmission by decreasing the oxygen consumption of astrocytes during synaptic transmission [4]. In pancreatic beta cells whose mitochondria are tuned to track nutrient changes, the former mechanism dominates and mitochondria alkalinize during Ca^{2+} elevations to boost ATP production and stimulate the secretion of insulin [3, 56].

The interplay between Ca^{2+} signals and ΔpH_m regulation depends on the nature and specific sequence of activation of the transporters that move ions and metabolites in the different cell types. Calcium uptake into mitochondria, whether through the MCU or the proposed Letm1-mediated Ca^{2+}/H^+ exchange, depolarizes energized mitochondria [34, 47]. Within seconds, mitochondrial depolarization reduces the electrical component of Δp, which favors increased proton extrusion by the respiratory chain and increases ΔpH_m (for a detailed description see [52]). Within less than 1 min [28], increased matrix Ca^{2+} activates dehydrogenases of the Szent-Györgyi and Krebs cycle and increases the supply of reducing equivalents to respiratory chain, which further increase ΔpH_m [35, 36]. This latter mechanism can be sustained for several tens of minutes and is proposed to account for the gradual increase in ΔpH_m in Fig. 2. Cytosolic Ca^{2+} elevations also activate the aspartate/glutamate–H^+ carriers (AGC1/2) via EF hand motifs facing the intermembrane space. The ACGs are essential components of the malate/aspartate shuttle that transports glycolytic NADH equivalents into the matrix (Fig. 1). While AGC1/2 co-transport a proton with glutamate into the matrix, the NADH that they supply to the respiratory chain results in the subsequent extrusion of 2.5–3 protons and in an increased ATP production [31]. Based on this, the activation of AGC1/2 by Ca^{2+} should elicit a net increase in ΔpH_m, but this effect has not been directly observed. While mitochondrial Ca^{2+} elevations are generally reported to stimulate respiration and ATP synthesis, mitochondrial Ca^{2+} also consumes a portion of ΔpH_m by two indirect mechanisms. First, the elevated matrix Ca^{2+} is extruded primarily by electrophoretic $3Na^+/1Ca^{2+}$ exchange via the NCLX [16], which tends to dissipate ΔpH_m as the incoming sodium must be subsequently extruded by the electroneutral $1Na^+/1H^+$ mitochondrial NHE. In permeabilized HeLa cells, inhibition of the NCLX with CGP-37157 enhanced the rate of matrix alkalinization by approximately threefold and increased the peak mitochondrial pH by ~60 % in response to Ca^{2+} [44], illustrating a clear impact of the serial $Ca^{2+}/Na^+/H^+$ exchange on ΔpH_m. Second, mitochondrial Ca^{2+} uptake can result in the formation of insoluble Ca^{2+}–phosphate complexes, hydroxyapatite and tricalcium phosphate, which allow mitochondria to accumulate large Ca^{2+} loads while maintaining their free matrix Ca^{2+} concentration at low micromolar levels [39]. The formation of Ca^{2+}–phosphate complexes in the matrix promotes phosphate uptake, and since phosphate primarily enters the matrix via the phosphate/H^+ symport large Ca^{2+} loads may impose a significant loss of ΔpH_m. Consistent with this established aspect of mitochondrial physiology, we found that mersalyl-sensitive phosphate/H^+ symport reduced steady-state ΔpH_m by 0.2–0.3 units in permeabilized HeLa cells [44]. However, the extent to which phosphate/H^+ symport counteracts Ca^{2+}-dependent, ΔpH_m increasing mechanisms in intact, stimulated cells remains to be quantified. Whether Ca^{2+} elevations will predominantly alter the activity of the citric acid cycle via the MCU or the AGC or cause indirect effects via the NCLX or the PiC will therefore depend (1) on the bioenergetics status of the cell, (2) the expression levels of the different transporters, and (3) the availability of ions and of metabolic substrates to drive the activity of these transporters. From an experimenter's standpoint, whether Ca^{2+} elevations will alkalinize or acidify the mitochondrial matrix to increase or dissipate ΔpH_m is largely determined by the concentration of the charged and electroneutral species that are present in the recording medium. This important bioenergetic parameter therefore not only reflects the energetic status and transporter display of the different cells and species but is also largely determined by the imposed experimental conditions.

Conclusion

In this brief review, we have attempted to reconcile the thermodynamic concepts developed largely from experiments in isolated mitochondria with recent observations that mitochondria exhibit a relatively high proton permeability and exist in a rapidly adapting dynamic equilibrium with the cytosolic pH. This dynamic equilibrium of ΔpH_m presumably results from the transport of multiple permeant ions and metabolites across the inner mitochondrial membrane that cannot be fully mimicked by a simplified experimental solution. While we have discussed the framework for the in situ regulation of ΔpH_m, many questions remain to be investigated. For example, how will specific regulatory factors like Na^+/H^+ exchange impact ΔpH_m in highly glycolytic cells that might consume glycolytic ATP to maintain a modest $\Delta\Psi_m$ compared to cells mediating robust oxidative phosphorylation. Moreover, the ability now to measure ΔpH_m in intact cells, combined with established methods to measure $\Delta\Psi$, will allow us to examine more directly the physiological regulation of Δp in intact cells by cytosolic Ca^{2+} signalling.

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