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Review

# Pathophysiology of mitochondrial volume homeostasis: Potassium transport and permeability transition

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ABSTRACT

Regulation of mitochondrial volume is a key issue in cellular pathophysiology. Mitochondrial volume and shape changes can occur following regulated fission–fusion events, which are modulated by a complex network of cytosolic and mitochondrial proteins; and through regulation of ion transport across the inner membrane. In this review we will cover mitochondrial volume homeostasis that depends on (i) monovalent cation transport across the inner membrane, a regulated process that couples electrophoretic $K^+$ influx on $K^+$ channels to $K^+$ extrusion through the $K^+$–$H^+$ exchanger; (ii) the permeability transition, a loss of inner membrane permeability that may be instrumental in triggering cell death. Specific emphasis will be placed on molecular advances on the nature of the transport protein(s) involved, and/or on diseases that depend on mitochondrial volume dysregulation.

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## 1. Introduction

Chemiosmotic energy conservation demands both a "low permeability to protons and to anions and cations generally" (4th postulate) and "substrate-specific exchange-diffusion carrier systems that permit the effective reversible trans-membrane exchange of anions against $OH^-$ ions and of cations against $H^+$ ions" (3rd postulate) [1,2]. After a long debate, a general consensus has been reached on the following points: (i) the inner membrane has an intrinsically low permeability to charged species; (ii) inner membrane channels exist, which mediate cation uptake across the inner membrane and whose conductance is tightly controlled; (iii) cation uptake is counterbalanced by cation efflux via pathways that are designed to prevent excessive osmotic swelling ($K^+$–$H^+$ and $Na^+$–$H^+$ exchangers) and calcification of mitochondria ($Ca^{2+}$ efflux systems); and (iv) the inner membrane permeability can increase because of a "permeability transition" [3] due to opening of the permeability transition pore (PTP), a regulated high conductance channel [4]. Mitochondrial volume and shape changes can also occur following regulated fission–fusion events, which are modulated by a complex network of cytosolic and mitochondrial proteins [5]. In this short review we will only cover mitochondrial volume homeostasis that depends on $K^+$ transport across the inner membrane, a regulated process that couples electrophoretic $K^+$ influx on $K^+$ channels to $K^+$ extrusion through the $K^+$–$H^+$ exchanger (KHE); and the permeability transition. Specific emphasis will be placed on recent advances on the molecular nature of the transport protein(s) involved, and/or on diseases that depend on mitochondrial volume deregulation through these systems.

An increase of mitochondrial volume can occur via two distinct mechanisms, which were clearly identified as early as in 1965 [6]. The first type of swelling (the high-energy swelling of Azzone and Azzi) occurs in energized mitochondria (i.e., in mitochondria that maintain a low passive permeability to protons, and therefore also maintain a transmembrane electrical potential difference, which is the main driving force for $K^+$ accumulation). Swelling occurs when electrophoretic $K^+$ influx rate (via leaks and/or opening of $K^+$ channels) exceeds the rate of $K^+$ release through the KHE. $K^+$ uptake is compensated by $H^+$ extrusion, driving in turn the accumulation of Pi so that the accumulated species is eventually K-Pi, which is responsible for the osmotic swelling of the matrix. The second type of swelling (the low-energy swelling of Azzone and Azzi) instead occurs in deenergized mitochondria (i.e. in mitochondria that are depolarized because of opening of the PTP, which causes a generalized increase of permeability to solutes with molecular masses up to about 1500 Da). In this case no ion gradients can be maintained, and swelling occurs because of the osmotic pressure exerted by non diffusible matrix proteins and macromolecular complexes. PTP opening requires matrix $Ca^{2+}$ and Pi, and is regulated by the $H^+$ electrochemical gradient in the sense that depolarization favors PTP opening while an acidic matrix pH inhibits it [7].

*Abbreviations:* Cs, Cyclosporin; CyP, cyclophilin; DCCD, N,N′-dicyclohexylcarbodiimide; KHE, $K^+$–$H^+$ exchanger; PTP, permeability transition pore; ROS, reactive oxygen species; UCMD, Ullrich congenital muscular dystrophy; WHS, Wolf–Hirschhorn Syndrome

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Irrespective of the initiating mechanism, swelling causes matrix expansion and unfolding of the cristae, which in turn increases the fraction of intermembrane cytochrome *c* [8]; if matrix expansion exceeds the viscoelastic resistance of the outer membrane, rupture of the latter causes release of intermembrane proteins, including cytochrome *c* and apoptosis-inducing factor. Apoptosis or necrosis may follow, depending on whether ATP levels remain high enough to allow caspase 9 activation [9,10].

## 2. $K^+$ channels

The inner mitochondrial membrane displays low but measurable electrophoretic permeability to $K^+$, with an estimated conductance of 0.11 nmol $K^+ \times$ mg protein$^{-1} \times$ min$^{-1} \times$ mV$^{-1}$ [11], which is comparable to the basal $H^+$ conductance of 0.2 nmol $\times$ mg protein$^{-1} \times$ min$^{-1} \times$ mV$^{-1}$ [12]. The $K^+$ (and $Na^+$) conductance can be increased dramatically after depletion of divalent cations with EDTA and A23187 [13,14] through channel (s) that are inhibited by ruthenium red [15]. The nature of these channels is still unknown, but it appears possible that divalent cation depletion allows monovalent cation flux through the mitochondrial $Ca^{2+}$ uniporter [15], as shown by patch-clamp experiments for $Na^+$ [16]. A variety of $K^+$ channels have been described in the inner mitochondrial membrane.

*$K_{ATP}$ channels*, selective $K^+$ channels inhibited by ATP, were first identified in liver mitochondria by patch-clamp experiments [17] and then extensively characterized by pharmacology, particularly in relation to their role in cardioprotection [18]. A *Shaker-type Kv1.3 channel* has been identified in lymphocytes [19] and macrophages [20]. Its interaction with Bax, which behaves like $K^+$ channel-blocking toxins, is an important step in the process of mitochondrial apoptosis in lymphocytes [21]. *$Ca^{2+}$-activated Big Conductance $K^+$ (BK) channels* were originally described in a human glioma cell line, as a cationic conductance that opens when matrix $Ca^{2+}$ increases [22]. Cytoprotective BK channels are also present in brain [23–25], skeletal muscle [26] and heart [27] mitochondria, and their expression may be regulated by oxygen availability [28]. Finally, the mitochondrial expression of *two-pore domain TASK-3 channels* has been recently described in both normal and transformed human cells [29].

## 3. $K^+$–$H^+$ exchanger

As mentioned above, electrophoretic $K^+$ entry must be compensated by $K^+$ efflux. The relative activity of these systems regulates mitochondrial $K^+$ and volume homeostasis, and contributes to regulation of matrix pH. While the existence of the KHE has been demonstrated beyond reasonable doubt by a number of experimental approaches (see [4] for review) its molecular nature has remained an open question.

### 3.1. Molecular nature of $K^+$–$H^+$ exchanger

Based on the discovery that N,N′-dicyclohexylcarbodiimide (DCCD) irreversibly inhibits the KHE under very selective conditions, Martin et al. used [$^{14}$C]DCCD labeling of mitochondria to identify a 82 kDa protein as the putative KHE [30]. The purified protein reconstituted into lipid vesicles catalyzed DCCD- and $Mg^{2+}$-sensitive electroneutral $K^+(Na^+)$–$H^+$ exchange [31]. As of today the identity of the 82 kDa species is not known.

While characterizing a collection of yeast genes putatively encoding mitochondrial cation transporters, Nowikovsky et al. [32] identified Mdm38p (Yol027cp) as essential for mitochondrial $K^+$–$H^+$ exchange activity and for respiratory growth of yeast cells. Passive swelling experiments with isolated yeast mitochondria in $K^+$ acetate (a sensitive method that allows detection of electroneutral $K^+$–$H^+$ exchange activity, see [4] for review) and direct $K^+$–$H^+$ exchange assays in yeast submitochondrial particles demonstrated that Mdm38p/Yol027cp is essential for growth and for mitochondrial $K^+$–$H^+$ exchange, which was totally lacking in *mdm38* knock-out mutants [32,33]. These results are compelling evidence that Mdm38p/Yol027cp is an essential component of the mitochondrial KHE [32,33].

Strong support for this hypothesis came from the finding that nigericin, which catalyzes electroneutral $K^+$–$H^+$ exchange, restored aerobic growth of Mdm38p/Yol027cp-null yeast strains and reverted mitochondrial swelling in situ [34]. Reversion to normal mitochondrial $K^+$ homeostasis and growth features was also achieved by expression of the Mdm38p yeast homologues Ypr125wp/Mrs7p and of the human homolog of Mdm38p/Yol027cp, LETM1. Since all these proteins are functionally conserved from yeast to man, and are essential components of the KHE, Mdm38p was renamed Mkh1p.

Mkh1p family proteins including human LETM1 bear only one transmembrane domain, suggesting that they may not constitute the KHE itself (at least as a monomer) but rather an essential component that allows the exchange activity to take place. Indeed, biochemical data indicate that these proteins are part of a higher molecular weight complex [35,36] suggesting that the KHE might consist of discrete subunits.

### 3.2. $K^+$–$H^+$ exchanger and Wolf–Hirschhorn Syndrome

The Wolf–Hirschhorn Syndrome (WHS, MIM 194190) is a complex disease involving the central nervous system [37,38] caused by partial, heterozygous deletion of the terminal portion of the short arm of one chromosome 4 involving the 4p16.3 region [39,40]. A critical region of 165 kb including several genes has been defined (WHSCR-1) that causes the clinical hallmarks of the disease, i.e. severe growth and mental retardation, hypotonia, midline fusion defects and typical facial dysmorphism [41,42]. A subset of cases of WHS is also characterized by seizures, which start during the first year of life and are a frequent cause of death [43]. Interestingly, the *LETM1* gene has been localized less than 80 kb distal to the WHSCR-1 region. It is invariably deleted in WHS patients with seizures and is preserved in those without epilepsy [44] irrespective of the WHSCR-1 deletion. These findings render the LETM1 haploinsufficiency a potential causative event for seizures associated with WHS and possibly for other forms of epilepsy. Given the key role of LETM1 in mitochondrial $K^+$ homeostasis, it may be predicted that reduced activity of the KHE is followed by osmotic matrix swelling, outer membrane rupture, cytochrome *c* release and impaired ATP production. In fact, overexpression of Mkh1p resulted in mitochondrial matrix contraction, while Mkh1 protein depletion in wild-type cells caused matrix swelling [34,36]; and, like in the yeast model, mitochondrial swelling and fragmentation of Mkh1p-depleted cells could be rescued by proper amounts of nigericin [35], findings that further underscore the role of the mitochondrial $K^+$ cycle in pathophysiological swelling–contraction events of the organelles. If our working hypothesis on the role of LETM1 in WHS is correct, impaired mitochondrial ATP production following mitochondrial swelling may affect the neurons' ability to maintain the plasma membrane potential, and possibly increase excitability to the threshold required to trigger seizures, a hypothesis that has not been tested so far. Fibroblastoid cells from one WHS patient showed a detectable decrease in LETM1 protein content, but there was no obvious phenotypic effect on mitochondria. Only stronger reduction in LETM1 expression caused mitochondrial changes and finally cell death [35]. Yet it is well possible that in other types of cells, e.g. neurons, WHS patients have LETM1 protein contents below a threshold sufficient for maintaining mitochondrial cation homeostasis. Thus, the basis for cell dysfunction in WHS may be lowered $K^+$–$H^+$ exchange activity, which could be treated with pharmacological agents. To summarize, molecular and physiological studies on yeast and human cell cultures underscore the prominent

role of the KHE in maintaining mitochondrial $K^+$ and volume homeostasis, and genetic studies on WHS patients identify the deletion of the human KHE gene LETM1 as most likely responsible for seizures associated with the disease. Understanding the pathophysiology of LETM1 deficiency in vivo may thus lead to important advances toward the therapy of WHS and possibly of other forms of CNS diseases with seizures, which are often linked to mitochondrial defects [45] and may also involve the PTP [46].

## 4. Permeability transition pore

The PTP is a high-conductance, $Ca^{2+}$-activated channel modulated by a variety of pathophysiological effectors [47–49] (see [50,51] for recent reviews). Despite intensive studies, the molecular nature of the PTP remains unsolved, and popular candidates such as the adenine nucleotide translocator and VDAC did not stand testing in mitochondria where the relevant protein(s) had been ablated by genetic methods [52–54] (see [55] for review). The only molecular regulator of the PTP identified so far is cyclophilin (CyP)-D, a matrix peptidyl–prolyl-*cis*–*trans*-isomerase whose enzymatic activity can be inhibited by cyclosporin (Cs)A [56], with parallel desensitization of the PTP to opening [57–59]. At variance from immunosuppression, desensitization of the PTP by CsA does not involve calcineurin, and can be achieved with CyP inhibitors devoid of immunosuppressive activity [60–62].

Since the discovery of its desensitizing properties on the PTP, CsA has become the standard tool to test the role of the PTP in disease models. Its widespread utilization is largely responsible for our increased understanding of the role of the PTP in pathophysiology of diseases (see [50] for an extensive review). A key advance is the recent demonstration that acute administration of a single intravenous bolus of 2.5 mg CsA$\times kg^{-1}$ body weight immediately before percutaneous coronary intervention decreased the infarcted area by about 40% in treated vs untreated patients, a finding that holds great promise for the treatment of coronary infarction [63].

The role of CyP-D as an endogenous regulator of the PTP has been unequivocally demonstrated in mitochondria from mice with genetic inactivation of the *Ppif* gene, which encodes CyP-D in the mouse [64–67]. Mitochondria from $Ppif^{-/-}$ individuals display a higher $Ca^{2+}$ threshold for PTP opening which matches that of CsA-treated mitochondria from wild-type, strain-matched individuals. The $Ppif^{-/-}$ mice are strikingly resistant to ischemia–reperfusion injury of the heart [64,66] and brain [67], and to experimental autoimmune encephalomyelitis [68].

Through the use of CsA the unexpected mitochondrial pathogenesis of collagen VI myopathies, diseases due to mutations of the collagen VI genes, has recently been established. Myopathic mice with genetic inactivation of the *Col6a1* gene (which completely lack the collagen VI protein) [69] undergo muscle fiber death due to increased opening of the PTP [70]. In the first example of a genetic muscle disease that could be cured with a drug, CsA rescued the mice from fiber cell death through a demonstrably mitochondrial effect [70]. These promising results were extended to cultures from patients with collagen VI muscular dystrophies, where both CsA and Debio 025 (a non immunosuppressive CyP inhibitor) repaired the mitochondrial functional defect [71]; and to 4 patients with Ullrich Congenital Muscular Dystrophy and 1 patient with Bethlem Myopathy, who all underwent amelioration of the mitochondrial phenotype and apoptotic rate, with increased muscle regeneration, after short-term treatment with CsA [72]. Recent studies on cultured myoblasts from patients with Ullrich Congenital Muscular Dystrophy suggest that the PTP voltage threshold is shifted toward the resting potential by chronic $Ca^{2+}$ overload of the sarcoplasmic reticulum–mitochondrial functional complex, which in turn makes pore opening more likely to occur [73].

Recent results suggest that inappropriate PTP opening may also play a role in other forms of muscular dystrophy. Indeed, crossing $Ppif^{-/-}$ mice with dystrophic mice lacking delta-sarcoglycan markedly decreased the disease in both skeletal muscle and heart; and crossing with mice bearing the deletion of Lama2 (which encodes the alpha-2 chain of laminin-2) cured the premature lethality and other indices of this dystrophic disease model [74]. Results are also available on treatment of *mdx* mice (a model of Duchenne Muscular Dystrophy) with the CyP inhibitor Debio 025, a derivative of CsA that maintains the PTP desensitizing properties but is devoid of immunosuppressive activity [71]. High dose (50 mg/kg/d) treatment with Debio 025 yielded only partial protection from fibrosis and minimal reversal of central nucleation [74], and similar results have been obtained with lower doses given orally [75]. The latter authors also performed

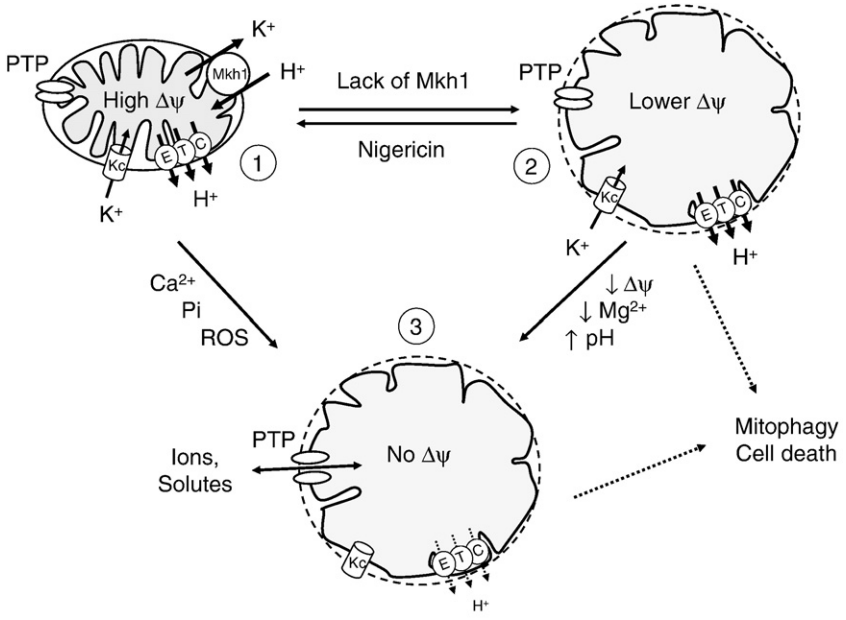


**Fig. 1.** Mitochondrial volume homeostasis mediated by $K^+$ transport and the PTP. The scheme summarizes the role of $K^+$ fluxes and PTP opening in mitochondrial volume homeostasis. Kc, potassium channels; ETC, electron transfer chain; Mkh1, essential mitochondrial KHE factor; Δψ, mitochondrial membrane potential difference; PTP, permeability transition pore. For explanation see text.

functional measurements in *mdx* mice, and found that resistance to mechanical stress was improved and relaxation time was reduced by Debio 025 indicating improved $Ca^{2+}$ handling, an effect that was not seen with CsA [75]. Based on these results, it appears likely that PTP opening plays a role in Duchenne Muscular Dystrophy as well, but that either the effects of CyP-D inhibition on the PTP are less effective (e.g. because of the high $Ca^{2+}$ levels) or because additional pathways, such as activation of the ubiquitin–proteasome pathway, are the determinant factors in this disease [76].

## 5. $K^+$ fluxes and the permeability transition

Since the PTP is modulated by the membrane voltage [7], opening of $K^+$ channels should favor PTP opening. Consistent with this prediction, Scorrano et al. have demonstrated that treatment of isolated mitochondria with valinomycin in $K^+$-based media caused PTP opening, with increasing fractions of mitochondria opening their pores as the depolarizing $K^+$ current increased [77]. In these experiments enough nigericin (which catalyzes electroneutral $K^+$–$H^+$ exchange) was added to prevent $K^+$ accumulation. Since valinomycin forms a charged complex with $K^+$ allowing the electrophoretic transport of $K^+$ across the inner membrane, this experimental setup should be equivalent to opening of selective inner membrane $K^+$ channels [77].

Rather than causing increased damage through the PTP, however, opening of mitochondrial $K_{ATP}$ channels has instead been reported to have a cardioprotective role (reviewed in [18]) through an *inhibitory* effect on the PTP [78–80]. According to Facundo et al. the protective effect is due to a decreased production of ROS [79], while Garlid and coworkers contend that opening of $K^+$ channels (or addition of valinomycin) causes *increased* production of ROS, followed by activation of PKC$\varepsilon$ which would eventually lead to PTP inhibition [80]. The basis for these discrepancies is not immediately obvious, but the intriguing possibility exists that increased $K^+$ conductance through endogenous channels may affect the probability of opening depending on the status of the PTP at the onset of the $K^+$ current, in particular the presence of a permissive matrix load of $Ca^{2+}$ and phosphate [81], and on the complex balance of all factors (both inducing and inhibiting) which modulate the PTP open time [4].

In this context the recent work of Szabó et al. on mitochondrial Kv1.3 channels and Bax-induced apoptosis is extremely relevant [21]. This work demonstrated that in lymphocytes Bax physically interacts with and inhibits Kv1.3 channels causing hyperpolarization and then overproduction of ROS, which eventually causes PTP opening, stable depolarization, cytochrome *c* release and apoptosis [21]. Thus, the consequences of $K^+$ channel opening or closing on ROS production, PTP opening and cell survival may also depend on the cell type and on additional factors, such as Bax for Kv1.3 channels. While it is perhaps too early to draw general conclusions on the mechanistic links between $K^+$ fluxes and PTP regulation, however, a general consensus is emerging that the PTP is the target for cytoprotection downstream of $K^{+\cdot}$ channels.

## 6. Mitochondrial swelling and autophagy

Changes of mitochondrial volume may strongly modulate mitochondrial physiology. Moreover, osmotic swelling is one of the fundamental features of pathological states of mitochondria, which results in the activation of downstream cascades, most notably life-or-death decisions that also include the switch between necrosis and apoptosis [9]. An emerging hypothesis is that swelling may trigger autophagy, a process through which aging or damaged organelles are degraded via the lysosomal pathway, a process also called "mitophagy" [34,82]. This phenomenon has been observed following both opening of the PTP [82] and lack of the KHE [34], with nigericin reverting both swelling and mitophagy in the latter case [34]. These observations suggests that swelling itself rather than the underlying cause (dysregulation of $K^+$ homeostasis or opening of the PTP) triggers mitophagy, possibly as the result of exposure of the inner membrane following outer membrane rupture. It cannot be excluded, however, that lack of the KHE may contribute to PTP opening because of the ensuing matrix swelling, which causes dilution of pore inhibitory factors like $Mg^{2+}$ and adenine nucleotides.

## 7. Summary

Fig. 1 summarizes the topics discussed in the review, and highlights the consequences of deregulation of $K^+$ fluxes and PTP opening on mitochondrial volume homeostasis. Under physiological conditions (scheme 1) owing to the low passive permeability of the inner membrane (and to the closed state of the PTP) $H^+$ pumping by the electron transfer chain (ETC) creates a proton electrochemical gradient, which is largely in the form of a membrane potential difference ($\Delta\psi$). Influx of $K^+$ via $K^+$ channels (Kc) causes a transient depolarization which is compensated by increased electron flux and increased $H^+$ pumping. The buildup of a $K^+$ gradient (and matrix swelling) is prevented by operation of the KHE (Mkh1) (the matching Pi fluxes driven by oscillation of the $\Delta$pH are omitted for clarity, see [4] for review). In the absence of the Mkh1 protein (or for a lower activity of the exchange process), $K^+$ influx is not compensated and the matrix expands (scheme 2), yet the permeability barrier of the inner membrane is maintained, the membrane potential is lowered (probably by increased $H^+$ leaks) and swelling can be reverted by the artificial KHE nigericin. PTP opening could occur because of the lowered $\Delta\psi$, $Mg^{2+}$ dilution and alkalinization (scheme 3), leading to complete collapse of the $\Delta\psi$, ion/solute equilibration and secondary impairment of respiration due to release of pyridine nucleotides and cytochrome *c*, and to substrate depletion. PTP opening could also occur in KHE-competent mitochondria because of $Ca^{2+}$/Pi overload and increased ROS balance. Swollen mitochondria undergo mitophagy and/or contribute to cell death.

## 8. Conclusions

Over 50 years after its formulation, the chemiosmotic theory of energy conservation maintains all its vitality, and provides the framework that allows to understand mitochondrial bioenergetics in the cell. In his summary of the basic postulates, Mitchell stated that "It will now be useful to summarise the basis of the chemiosmotic coupling hypothesis in the form of four essential postulates; for, these postulates can be used, on the one hand, for the further development of the theory of chemiosmotic coupling, and on the other hand, as the target for critical experiments designed to show that the chemiosmotic hypothesis may be untenable" [1]. We are confident that the continuing work on the molecular definition of mitochondrial cation transport and of the PTP will contribute to our understanding of mitochondrial volume homeostasis; and add to the enormous amount of critical experiments that show that the chemiosmotic hypothesis is not untenable.

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## Note added in proof

A manuscript relevant to the relationship between activity of the KHE and PTP proposed here was recently published. A simulation based

on measured values of relevant parameters shows that the balance between the mitochondrial membrane potential difference ($\Delta\psi$) and the $\Delta$ pH is affected by the ratio of the rate constants of K+ uniport (i.e., the sum of the rate constants for all K+ channels) and of KHE, with a sharp decrease of $\Delta\psi$ and increase of $\Delta$ pH as the ratio increases, as it occurs when the activity of KHE decreases [Dzbek and Korzerniewski (2008) J Biol Chem in press 10.1074/jbc.M802404200]. The decrease of Dy after switching off Mkh1p has actually been measured [34], and the combination of depolarization and matrix alkalinization is a powerful trigger for PTP opening [4].

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