

# Mitochondrial Transport of Cations: Channels, Exchangers, and Permeability Transition

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**Bernardi, Paolo.** Mitochondrial Transport of Cations: Channels, Exchangers, and Permeability Transition. *Physiol. Rev.* 79: 1127–1155, 1999.—This review provides a selective history of how studies of mitochondrial cation transport (K<sup>+</sup>, Na<sup>+</sup>, Ca<sup>2+</sup>) developed in relation to the major themes of research in bioenergetics. It then covers in some detail specific transport pathways for these cations, and it introduces and discusses open problems about their nature and physiological function, particularly in relation to volume regulation and Ca<sup>2+</sup> homeostasis. The review should provide the basic elements needed to understand both earlier mitochondrial literature and current problems associated with mitochondrial transport of cations and hopefully will foster new interest in the molecular definition of mitochondrial cation channels and exchangers as well as their roles in cell physiology.

## I. INTRODUCTION

Despite an enormous amount of literature and the importance of the problem, very little information is available about the structural features of mitochondrial cation channels and exchangers, whereas a vast amount of information is available about the functional properties of these mitochondrial transport systems. The explanation for this state of affairs is largely historical. Studies of ion transport were mostly carried out in the same laborato-

ries involved in clarifying the mechanisms of energy conservation in mitochondria and chloroplasts, and the two fields evolved in parallel. The more the debate on energy conservation became focused on the chemiosmotic hypothesis, the more studies of mitochondrial ion transport tended to become tests of the predictions of this theory, in particular about the existence of a membrane potential across the inner membrane. As a result, research on mitochondrial cation transport was shaped by the debate on chemiosmosis. Given the view, prevailing well into the

1980s, that mitochondria did not possess cation channels, it is not too surprising that research in this area did not yield results comparable to those obtained in the general field of membrane transport.

In this review, which is limited to mammalian mitochondria, I provide a selective history of how studies of mitochondrial cation transport ( $K^+$ ,  $Na^+$ , and  $Ca^{2+}$ ) developed in relation to the major themes of energy conservation. This should provide the general reader with the basic elements needed to understand earlier mitochondrial literature and current problems associated with mitochondrial transport of cations. I then cover in more detail specific transport pathways for cations and discuss open problems about their nature and physiological function. This includes the mitochondrial permeability transition and its potential role in  $Ca^{2+}$  homeostasis. Topics that are not treated in the review (mitochondrial  $Ca^{2+}$ -binding proteins,  $Mg^{2+}$  transport, electrophysiology, and mitochondrial involvement in cell death) are briefly covered in section v, where the interested reader can find essential bibliographic indications.

### A. Mechanism of Energy Conservation and Cation Transport

The history of bioenergetics came to a turning point when the late Peter Mitchell proposed his chemiosmotic hypothesis of energy conservation (223, 225). In his "Summary of the basic postulates" Mitchell stated (225)

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) The membrane-located ATPase systems of mitochondria and chloroplasts are hydro-dehydration systems with terminal specificities for water and ATP; and their normal function is to couple reversibly the translocation of protons across the membrane to the flow of anhydro-bond equivalents between water and the couple  $ATP/(ADP - P_i)$ .

2) The membrane-located oxido-reduction chain systems of mitochondria and chloroplasts catalyse the flow of reducing equivalents, such as hydrogen groups and electron pairs, between substrates of different oxido-reduction potential; and their normal function is to couple reversibly the translocation of protons across the membrane to the flow of reducing equivalents during oxido-reduction.

3) There are present in the membrane of mitochondria and chloroplasts substrate-specific exchange-diffusion carrier systems that permit the effective reversible transmembrane exchange of anions against  $OH^-$  and of cations against  $H^+$ ; and the nor-

mal function of these systems is to regulate the pH and osmotic differential across the membrane, and to permit entry and exit of essential metabolites (e.g., substrates and phosphate acceptor) without collapse of the membrane potential.

4) The systems of *postulates 1, 2, and 3* are located in a specialised coupling membrane which has a low permeability to protons and to anions and cations generally.

The 1960s and early 1970s witnessed a heated debate over chemiosmotic energy coupling (226), which was largely centered over the very existence of a mitochondrial membrane potential.

It is today universally accepted that the initial event of energy conservation is charge separation at the inner mitochondrial membrane. Electrons deriving from intermediary metabolism are funneled to oxygen through the respiratory chain in a process coupled to  $H^+$  ejection on the redox  $H^+$  pumps. Because the passive permeability to  $H^+$  (the so-called  $H^+$  leak) and to cations and anions is generally low,  $H^+$  ejection results in the establishment of a  $H^+$  electrochemical gradient ( $\Delta\tilde{\mu}_H$ ). The  $\Delta\tilde{\mu}_H$  can be written as

$$\Delta\tilde{\mu}_H = zF\Delta\psi + RT \ln [H^+]_i/[H^+]_o \quad (1)$$

where  $\Delta\psi$  denotes the membrane potential difference ( $\psi_{in} - \psi_{out}$ ) in millivolts;  $z$ ,  $F$ ,  $R$ , and  $T$  have their standard meanings; and subscripts  $i$  and  $o$  refer to intramitochondrial and extramitochondrial, respectively. The magnitude of the proton electrochemical gradient is about  $-200$  to  $220$  mV, and under physiological conditions, most of the gradient is in the form of a membrane potential (see Ref. 18 for a review).

The  $\Delta\tilde{\mu}_H$  is utilized for ATP synthesis via the  $F_0F_1$  ATPase (1, 224, 225). It must be stressed, however, that proton pumping and creation of the  $\Delta\tilde{\mu}_H$ , not ATP synthesis, is the primary event. This concept is illustrated by conditions, such as ischemia, under which mitochondria utilize glycolytic ATP to maintain the  $\Delta\tilde{\mu}_H$  and rapidly turn from the major ATP producers into the major ATP consumers of the cell, often precipitating cell death (see Ref. 99 for a review). In addition to ATP synthesis, the  $\Delta\tilde{\mu}_H$  is utilized for a variety of mitochondrial processes. Some of these processes are a prerequisite for respiration and ATP synthesis, such as import of respiratory chain and ATP synthase subunits encoded by nuclear genes (a process requiring both  $\Delta\psi$  and ATP) (238); uptake of respiratory substrates and  $P_i$  (driven by the  $\Delta pH$ ) (255); and uptake of ADP in exchange for ATP (driven by the  $\Delta\psi$ ) (187). Others serve vital regulatory functions through transport processes such as volume homeostasis ( $K^+$ ,  $Na^+$ , and anions) (129), regulation of metabolism ( $Ca^{2+}$ ) (287), and heat production ( $H^+$ ) (279) and actually compete with ADP phosphorylation. For example, when  $Ca^{2+}$

is added to energized, phosphorylating mitochondria, ATP synthesis stops, and it only restarts after  $\text{Ca}^{2+}$  has been taken up (294).

## B. Membrane Potential and Equilibrium Cation Distribution

The existence of a respiration-dependent membrane potential, negative inside, poses the major problem of mitochondrial cation distribution (15, 19, 215). Let us consider the case of  $\text{K}^+$ . The  $\text{K}^+$  electrochemical gradient can be defined as

$$\Delta\tilde{\mu}_{\text{K}} = zF\Delta\psi + RT \ln [\text{K}^+]_i/[\text{K}^+]_o \quad (2)$$

The equilibrium condition ( $\Delta\tilde{\mu}_{\text{K}} = 0$ ) is given by

$$-\Delta\psi = 60 \log [\text{K}^+]_i/[\text{K}^+]_o \quad (3)$$

Because  $[\text{K}^+]_o$  is  $\sim 150$  mM, for a membrane potential of 180 mV (negative inside)  $[\text{K}^+]_i$  at electrochemical equilibrium should be a staggering 150 M, which is obviously never achieved (a similar problem exists for  $\text{Na}^+$ , equilibrium  $[\text{Na}^+]_i$  being 5 M for a cytosolic  $[\text{Na}^+]$  of 5 mM) (15). Rather, mitochondrial  $\text{K}^+$  distribution reflects a kinetic steady state where the accumulation ratio is modulated by the relative rates of  $\text{K}^+$  uptake and efflux via separate pathways (215).

The buildup of a  $\text{K}^+$  concentration gradient is also prevented by the high permeability of the inner membrane to water so that any net uptake (or loss) of  $\text{K}^+$  salts is accompanied by osmotic swelling (or shrinkage). As noted by Garlid (127), if the average valency of transported anions is taken to be 1.5, the flux of 1 mol of  $\text{K}^+$  is accompanied by that of 1.67 osmol solute, and the change in matrix  $\text{K}^+$  (nmol/mg) corresponds to a change in matrix volume ( $\Delta V_m$ ,  $\mu\text{l/mg}$ ) according to the relation

$$\Delta V_m = 1.67\Delta\text{K}^+/\Phi \quad (4)$$

where  $\Phi$  is the osmotic strength in mosM (127). Thus, the  $\text{K}^+$  electrochemical gradient favors continuous  $\text{K}^+$  accumulation, leading in turn to matrix swelling that would result in breakdown of the outer membrane, release of cytochrome *c*, and loss of mitochondrial function.

Mitchell was aware of this problem and proposed the existence of substrate-specific exchange-diffusion carrier systems permitting the reversible exchange of cations against  $\text{H}^+$  to regulate the osmotic differential across the membrane (*postulate 3*). The very existence of these  $\text{H}^+$ -cation antiporters was a matter of debate for at least 20 years and is discussed in more detail in section II. It is important to stress here that the existence of the antiporters may in principle resolve the problem of  $\text{K}^+$  distribu-

tion and volume homeostasis but in turn creates that of energy dissipation. Indeed, electrophoretic influx of cations followed by their release via an electroneutral mechanism utilizing the proton chemical gradient would result in a futile cycle dissipating the membrane potential, as is experimentally observed by the addition of proper  $\text{K}^+$  ionophores. Respiring mitochondria in KCl media swell beyond the point of outer membrane rupture upon addition of valinomycin (which forms a charged complex with  $\text{K}^+$ , thus allowing its rapid electrophoretic uptake). Simultaneous addition of excess nigericin (which promotes the electroneutral exchange of  $\text{H}^+$  and  $\text{K}^+$ ) does prevent swelling but causes in turn permanent uncoupling. This led Mitchell to contend that the specialized coupling membrane has a low permeability to protons and to anions and cations generally (*postulate 4*).

In summary, the chemiosmotic hypothesis of energy conservation demands both the existence of electroneutral  $\text{H}^+$ -cation antiporters and a low permeability to  $\text{K}^+$  and  $\text{Na}^+$ . This allows extrusion of cations entering mitochondria down their electrochemical gradient, thus preventing osmotic swelling. The overall energy dissipation in futile cation cycling is small because of the restricted membrane permeability to cations.

With few exceptions, studies of monovalent cation transport in mitochondria were long carried out to test these predictions of chemiosmotic principles. In particular, and until very recently, the requirement for a low cation permeability has been widely implied to mean that the inner mitochondrial membrane does not possess channels for  $\text{K}^+$  (and  $\text{Na}^+$ ). This assumption was consistent with the experimental findings that electrophoretic uptake of  $\text{K}^+$  and  $\text{Na}^+$  in isolated mitochondria is extremely slow and that isolated mitochondria respiring in KCl-based media do not swell unless valinomycin is added. However, today we know that mitochondria do possess channels for cations and that energy dissipation is limited by the predictable fact that they are tightly regulated. These transport pathways are covered in more detail in section II, whereas the problem of equilibrium distribution of divalent cations is discussed in section III.

## II. TRANSPORT OF MONOVALENT CATIONS

A vast amount of information on mitochondrial transport of monovalent cations has been obtained from kinetic measurements of the matching volume changes with optical methods (see, e.g., Refs. 13, 27, 49, 56, 58, 72, 107, 112, 130, 153, 216, 217, 227, 338, 339, 356). A major asset in these studies has been the availability of ionophores and the parallel elucidation of their transport properties in both model and natural membranes (272).

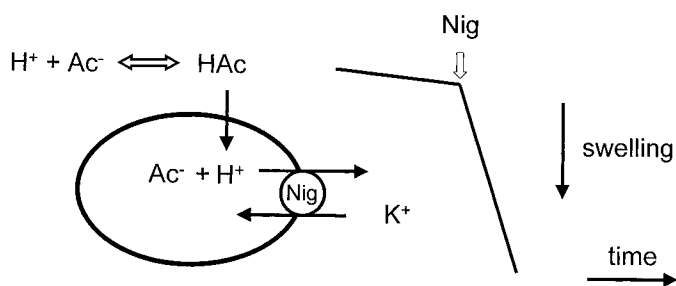


FIG. 1. Mitochondrial passive  $K^+$  fluxes in acetate ( $Ac$ )-based media: effect of nigericin. Deenergized mitochondria undergo a fast swelling process upon addition of electroneutral  $H^+/K^+$  exchanger nigericin (Nig) because  $K^+$  uptake is followed by rapid diffusion of acetic acid, resulting in net uptake of  $K^+$  acetate. Volume increase is illustrated with a downward deflection of trace because this is direction of light-scattering change that is usually followed to monitor swelling.

### A. Exchangers (Antiports)

The quest for the  $H^+/K^+$  exchanger (KHE) has characterized mitochondrial research on monovalent cation transport between the late 1960s and early 1980s. The scheme of Figure 1 introduces a powerful assay of cation transport based on changes of mitochondrial volume in the absence of respiration (passive swelling), which proved instrumental in studies of cation transport.

The mitochondrial inner membrane is highly permeable to the protonated form of acetic acid (HAc). In the presence of a  $K^+$  concentration gradient (incubation in isotonic  $K^+$  acetate medium), the KHE should catalyze  $K^+$  accumulation in exchange for  $H^+$ . The pH differential created by the electroneutral exchange would represent the driving force for accumulation of acetic acid, eventually causing matrix accumulation of  $K^+$  acetate. However, mitochondria incubated in  $K^+$  acetate only swell at a very slow rate, as indicated in Figure 1, unless the exogenous electroneutral KHE nigericin is added. The ensuing fast rate of swelling confirms the high permeability to acetic acid and indicates that these experimental conditions do allow, in principle, detection of Mitchell's postulated exchanger. Based on the low rate of swelling in the absence of nigericin, however, early critics of chemiosmosis concluded that the KHE probably did not exist.

An important point should be appreciated. Because of the low permeability of the inner membrane to charged species ( $K^+$ ,  $H^+$ , and the acetate anion in this example), swelling does not follow the addition of either the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; Fig. 2, trace *a*) or the  $K^+$ -selective ionophore valinomycin (Fig. 2, trace *b*), yet fast swelling ensues when valinomycin is added after FCCP, or when FCCP is added after valinomycin (Fig. 2, traces *a* and *b*, respectively). These examples indicate that overall electroneutral  $H^+/K^+$  exchange can either be achieved through an antiporter type of mechanism (Fig. 1) or

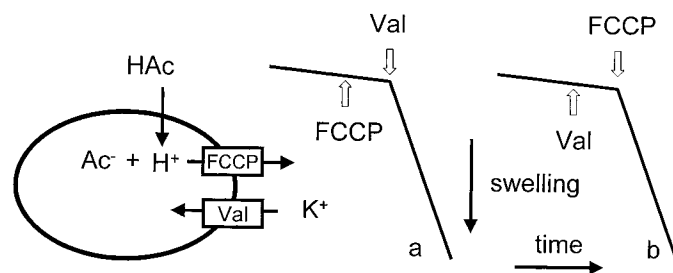


FIG. 2. Mitochondrial passive  $K^+$  fluxes in acetate-based media: effect of FCCP and valinomycin. Deenergized mitochondria do not swell upon addition of protonophore FCCP (trace *a*) or of  $K^+$  ionophore valinomycin (Val, trace *b*) alone, because membrane has a low intrinsic permeability to  $H^+$  and  $K^+$  and therefore no charge compensation is possible. Fast swelling ensues when both valinomycin and FCCP are present because coupling of electrical fluxes causes a process of overall electroneutral  $H^+/K^+$  exchange, resulting in net uptake of  $K^+$  acetate.

through coupling of independent electrical fluxes (Fig. 2). A number of studies represent the basis for the schemes depicted in Figures 1 and 2 (see in particular Refs. 16, 17, 49, 107, 227). I refer the reader to Garlid et al. (131) for the relevant transport equations and for discussion of earlier literature.

In classical studies of mitochondrial  $H^+$ /cation antiport with this method, the results depicted in Figure 3 were obtained (49, 107, 227). Rapid swelling was observed in  $Na^+$  acetate (Fig. 3, trace *a*), whereas swelling in  $K^+$  acetate was extremely slow (Fig. 3, trace *b*) yet measurable (compare with the traces typically seen in choline, Fig. 3, trace *c*). These studies suggest that mitochondria possess a very active  $H^+/Na^+$  antiporter (NHE) and a sluggish KHE (76, 227, 256).

Treatment with the electroneutral  $2H^+$ -divalent metal ion ( $Me^{2+}$ ) exchanger A-23187 is followed by relevant changes of mitochondrial  $K^+$  permeability, which were initially attributed to direct transport of  $K^+$  by A-23187 (268). Subsequent work indicated that these changes were rather due to activation of latent, endogenous pathway(s) for  $K^+$  transport following depletion of divalent cations (14, 20, 112, 356).

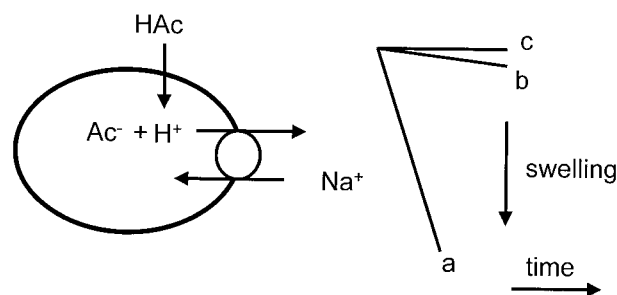


FIG. 3. Mitochondrial passive cation fluxes in acetate-based media. Deenergized mitochondria swell at a fast rate in  $Na^+$  acetate (trace *a*), at a very slow rate in  $K^+$  acetate (trace *b*), and not at all in choline acetate (trace *c*), suggesting that they have a very active  $Na^+/H^+$  exchanger and a sluggish  $K^+/H^+$  exchanger.

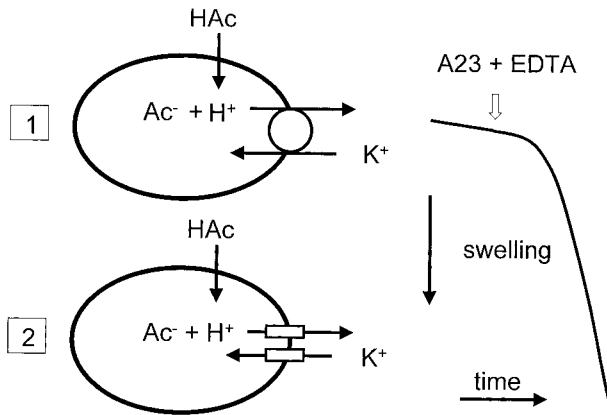


FIG. 4. Mitochondrial passive  $K^+$  fluxes in acetate-based media. Deenergized mitochondria undergo a fast swelling process after depletion of divalent cations by addition of  $2H^+$ /divalent metal ion ( $Me^{2+}$ ) exchanger A-23187 (A23) plus EDTA (lag phase reflects time required to achieve  $Me^{2+}$  depletion). Uptake of  $K^+$  acetate can be mediated by either  $H^+$ / $K^+$  electroneutral exchange (scheme 1) or by coupling of electrical  $H^+$  and  $K^+$  fluxes (scheme 2).

The swelling trace of Figure 4 depicts the typical results of a passive transport assay in  $K^+$  acetate. Mitochondria depleted of endogenous  $Me^{2+}$  (mostly  $Mg^{2+}$  and  $Ca^{2+}$ ) by treatment with A-23187 plus EDTA undergo a fast swelling process after a lag phase. The lag phase reflects the time required to achieve  $Me^{2+}$  depletion, whereas swelling indicates the activity of an overall electroneutral  $H^+$ / $K^+$  exchange. What the experiment cannot tell is whether the mechanism is intrinsically electroneutral [activation of the endogenous KHE (scheme 1)] or rather due to coupling of independent  $H^+$  and  $K^+$  conductances induced by  $Mg^{2+}$  depletion (scheme 2). This issue was addressed in experiments where  $K^+$  nitrate rather than acetate was used. Because the nitrate anion is readily permeant, addition of A-23187 should induce swelling if an increase of  $K^+$  permeability has occurred, but this was not observed (112). As shown in Figure 5, A-23187-treated mitochondria swell in  $K^+$  nitrate only in the presence of FCCP, suggesting the occurrence of obligatorily electroneutral exchange of  $H^+$  for  $K^+$  (112). Whether  $K^+$  transport is entirely due to an endogenous KHE, however, is still difficult to assess in these protocols. Indeed, after most A-23187 had been removed by an albumin wash, relevant swelling rates were recorded in the absence of FCCP (see Fig. 3B in Ref. 112), suggesting that a permeability to  $K^+$  had also developed, as confirmed by a subsequent study (36). Also, a direct contribution of A-23187 to  $K^+$  transport cannot be excluded (see discussion in Ref. 126).

The scheme of Figure 6 represents the unequivocal demonstration that isolated mitochondria do possess an endogenous, electroneutral KHE that can be activated by depletion of matrix  $Me^{2+}$  (106, 315). In this example, mitochondria are energized by substrate oxidation and

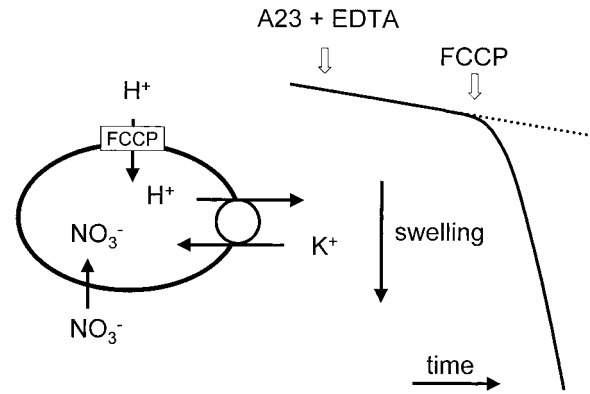


FIG. 5. Mitochondrial passive  $K^+$  fluxes in nitrate-based media. Deenergized mitochondria do not undergo a fast swelling process after depletion of divalent cations by addition of A-23187 (A23) plus EDTA (dashed trace) unless FCCP is added (solid trace). Because  $NO_3^-$  is readily permeant, any electrophoretic permeability to  $K^+$  should have caused swelling before addition of FCCP. It follows that observed swelling must be intrinsically electroneutral.

incubated in a sucrose-based, low- $K^+$  medium. Addition of a very small concentration of valinomycin induces a phase of net  $K^+$  uptake until a new steady-state extramitochondrial  $K^+$  concentration is reached. The effect of valinomycin demonstrates that under these conditions the  $K^+$  electrochemical gradient is directed inward, largely because of the transmembrane electrical potential difference. Addition of A-23187 perturbs the steady state

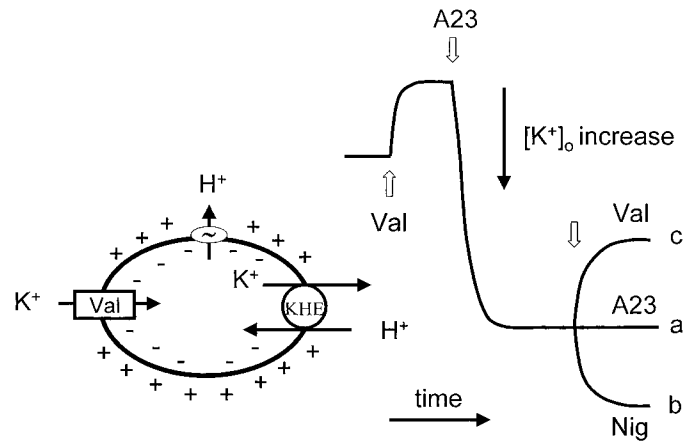


FIG. 6. Mitochondrial  $K^+$  fluxes in energized mitochondria: effects of valinomycin, A-23187, and nigericin. Mitochondria energized by substrate oxidation ( $\sim$ ) and incubated in low- $K^+$  media take up  $K^+$  electrophoretically upon addition of a nonsaturating amount of valinomycin (Val). Subsequent addition of A-23187 (A23) causes a process of net  $K^+$  efflux that levels off at a higher extramitochondrial  $K^+$  concentration ( $[K^+]_o$ ). Addition of more valinomycin (arrow, trace a) causes net  $K^+$  uptake (arrow, trace a); addition of electroneutral  $H^+$ / $K^+$  exchanger nigericin (Nig) causes further  $K^+$  release (arrow, trace b); and addition of more A-23187 does not modify steady-state  $[K^+]_o$  (arrow, trace c). Experiment indicates that A-23187 has activated an endogenous, intrinsically electroneutral  $K^+$ / $H^+$  exchanger (KHE). For further explanation, see text. [Modified from Dordick et al. (106).]

and causes a phase of net  $K^+$  efflux until a new steady state is reached that cannot be perturbed by a second addition of A-23187 (Fig. 6, *trace a*). However, further  $K^+$  efflux can be elicited by nigericin (Fig. 6, *trace b*), whereas more valinomycin causes  $K^+$  reuptake (Fig. 6, *trace c*). Thus 1) A-23187 has unmasked an endogenous pathway for  $K^+$  efflux (no further efflux can be induced by more A-23187); 2)  $K^+$  efflux has occurred against the  $K^+$  electrochemical gradient (increasing the valinomycin concentration induces more uptake, indicating the direction of the  $\Delta\tilde{\mu}_K$ ); and 3) the actual  $[K^+]_o$  represents a kinetic steady state where the rate of electrophoretic uptake (mediated by valinomycin) matches the rate of electroneutral efflux (mediated by the endogenous exchanger) (see Refs. 106, 315 for the actual experiments). This approach left little doubt that the molecular nature of the  $H^+/K^+$  exchange was intrinsically electroneutral rather than due to indirect coupling of electrical  $H^+$  and  $K^+$  fluxes. A number of studies have contributed to clarify the nature and regulation of the mitochondrial  $H^+$ /cation exchange systems (see Refs. 53, 128 for reviews). In general, the literature today agrees on the existence of two separate antiporters, i.e., the  $Na^+(Li^+)$ -selective NHE that does not transport  $K^+$  and the unselective KHE that transports  $K^+$ ,  $Na^+$ , and  $Li^+$ .

### 1. NHE

Mitochondria from all tissues tested possess a NHE that does not transport  $K^+$  and is inhibited by amiloride analogs (54), whereas it is not inhibited by  $Mg^{2+}$ . The NHE has a broad pH optimum at pH 7.0, and its activity declines linearly at increasing pH (49, 55, 107, 234). The function of the NHE is probably related to the requirements of steady-state  $Ca^{2+}$  cycling in energized mitochondria, which largely occurs through coupling of electrophoretic influx via the  $Ca^{2+}$  uniporter and efflux via the  $Na^+/Ca^{2+}$  antiporter (89), as discussed in section III.

A putative mitochondrial NHE has recently been identified (252). Yeast cells contain homologs of the human family of plasma membrane NHE (235). Screening of the *Saccharomyces cerevisiae* genome revealed open reading frames that encode protein sequences homologous to the plasma membrane  $H^+/Na^+$  antiporter NHA1. The full-length *YDR456w* gene, which contained potential  $NH_2$ -terminal signal sequences for the mitochondrial inner membrane, was isolated and used to identify a human NHE cDNA (NHE6), which encodes a 669-amino acid protein (predicted molecular mass 74,163 Da). NHE6 has high homology with both yeast and human plasma membrane NHE, particularly in the membrane-spanning region (252). A single  $\sim 5.5$ -kb mRNA is present in all adult human tissues tested, and the abundance of the transcript correlates with the relative abundance of mitochondria (252). The mitochondrial assignment is based on colo-

calization of the *YDR456w* gene product Nha2 fused to green fluorescent protein (GFP) with the stain 4',6-diamidino-2-phenylindole dihydrochloride in yeast cells and on colocalization of an overexpressed NHE-GFP chimera with Mito-Tracker Red CM-H<sub>2</sub>Xros in HeLa cells (252). Colocalization of the same *YDR456w* gene product fused to GFP with mitochondrial markers was not observed in unfixed yeast cells, however, where the NHE was exclusively found in prevacuolar compartments equivalent to the late endosome of animal cells (236), a finding that calls into question the unequivocal identification of NHE6 as a mitochondrial NHE.

A fraction highly enriched in a 59-kDa protein catalyzing  $Na^+$  transport with the properties expected of the NHE in a reconstituted system has been purified from beef heart mitochondria (134).

### 2. $H^+-K^+(Na^+)$ exchanger

Although this exchanger is usually referred to as a  $H^+-K^+$  antiporter, one of its basic features is the low selectivity for the species transported in exchange for  $H^+$ . The KHE does not discriminate between  $K^+$ ,  $Na^+$ , and  $Li^+$ , and it also transports organic cations such as tetrapropylammonium (46). Once activated, it displays a maximum velocity ( $V_{max}$ ) in excess of 300 nmol  $K^+$ ·mg protein<sup>-1</sup>·min<sup>-1</sup>. The properties of KHE can be summarized as follows: 1) the activity of the antiporter increases with the decrease of free matrix  $Mg^{2+}$  (233), and this partly explains why the exchange activity increases in hypotonic media (124) and after osmotic swelling (125). 2) Activity of the KHE increases at increasing pH, and KHE is inhibited by matrix  $H^+$  (28, 57). 3) The antiporter is fully inhibited by 0.5–1.0 mM quinine both in passive swelling assays in  $K^+$  acetate (234) and in energized mitochondria (36). 4) Although dicyclohexylcarbodiimide (DCCD) cannot be considered a specific ligand of the exchanger, this reagent only inhibits the KHE after depletion of matrix  $Mg^{2+}$  (131); this represented the basis for selective labeling with [<sup>14</sup>C]DCCD of an 82-kDa protein that, to date, still represents the best candidate for the KHE; when incorporated in liposomes, this DCCD-reactive species catalyzed  $K^+$  transport with the properties expected of the KHE (200). 5) After complete depletion of matrix  $Mg^{2+}$ , the activity of the KHE could be further stimulated by osmotic swelling of mitochondria, suggesting a direct modulation by the increased membrane tension (40).

## B. Channels (Uniports)

An assessment of the vast literature of electrophoretic transport of cations ( $K^+$  in particular) in mitochondria is complicated by two problems: 1)  $K^+$  uniports can be induced and modified by a variety of agents that

also affect the electroneutral  $H^+$ /cation exchangers (most notably  $Mg^{2+}$ ), and the relative contribution of the two transport modes is not always easy to dissect, particularly in papers published before 1980; and 2) comparison of the results obtained in different laboratories even in recent years is often difficult because of the different experimental conditions, which may affect the sensitivity to inhibitors and therefore the interpretation of the results in terms of the relevant transport pathway(s).

Although direct transport of  $Na^+$ ,  $K^+$  and  $Rb^+$  can be studied by isotopic techniques (105) or by atomic absorption spectroscopy after separation of mitochondria, most studies of mitochondrial cation uniport have taken advantage of the sensitive swelling technique described before. As depicted in Figure 7, in energized mitochondria electrophoretic cation uptake (exemplified here by  $K^+$  uptake catalyzed by valinomycin) is compensated by  $H^+$  extrusion. If buildup of a relevant  $\Delta pH$  is prevented by acetate (or phosphate), regeneration of the  $\Delta\psi$  will allow cation uptake and mitochondrial swelling, providing a convenient tool to study the transport process. An obvious problem in these studies is posed by the activity of the constitutive NHE in the case of  $Na^+$  and of the inducible KHE in the case of  $K^+$ , particularly in view of the fact that the latter is stimulated by osmotic swelling and matrix  $Mg^{2+}$  dilution. Indeed, electrophoretic uptake of  $K^+$  in energized mitochondria is followed by cyclic activation of the KHE, resulting in a train of damped volume oscillations that complicate interpretation of the transport kinetics (36). An alternative method is based on the study of the depolarizing cation currents under conditions where activity of the KHE is in excess and therefore no net cation accumulation takes place, allowing measurements of the phenomenological conductance through the channel (248).

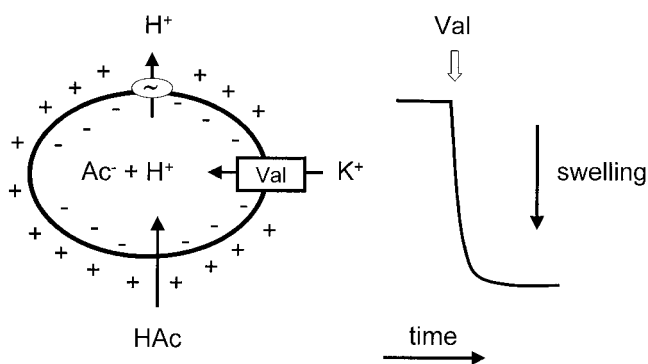


FIG. 7. Mitochondrial volume changes in  $K^+$  acetate: effect of valinomycin. Energized mitochondria ( $\sim$ ) take up  $K^+$  down  $K^+$  electrochemical gradient upon addition of valinomycin. Ensuing depolarization stimulates  $H^+$  pumping, causing in turn diffusion of highly permeant acetic acid to matrix driven by change in pH. Net result is accumulation of  $K^+$  acetate in matrix with osmotic swelling.

### 1. $Na^+$ channel

Addition of EDTA to respiring mitochondria is followed by swelling in NaCl and LiCl but not KCl media, suggesting the existence of a  $Mg^{2+}$ -modulated inner membrane channel with selectivity for  $Na^+$  and  $Li^+$  (12, 55, 314, 356). We have provided unambiguous evidence that  $Na^+$  and  $Li^+$  uptake after treatment of mitochondria with EDTA occurs via an electrophoretic mechanism (37). The fact that  $K^+$  is not transported, together with the high sensitivity of  $Na^+$  flux to nanomolar concentrations of  $Mg^{2+}$  and ruthenium red (RR), represents the best indications that flux occurs through a channel rather than through diffusive leaks.

The basic properties of the  $Na^+$  channel can be summarized as follows (37). 1) The channel is selective for  $Na^+$  and  $Li^+$  over  $K^+$ , and the  $Na^+$  ( $Li^+$ ) conductance is modulated by surface-bound  $Mg^{2+}$ . 2) Inhibition by  $Mg^{2+}$  is competitive with  $Na^+$ , and  $Na^+$  fluxes can be elicited by the addition of physiological concentrations of ATP. 3) Flux through the  $Na^+$  channel displays a broad optimum at pH 7.5–8.0, whereas it declines at both higher and lower pH values. 4) Flux increases exponentially with the  $\Delta\psi$  and displays an apparent threshold at approximately  $-140$  mV; the maximum conductance we could measure is  $0.2$  nmol  $Na^+$   $\cdot$  mg protein $^{-1}$   $\cdot$  min $^{-1}$   $\cdot$  mV $^{-1}$  (37), which is very close to the basal  $H^+$  conductance of isolated rat liver mitochondria (370). 5) The  $Na^+$  channel is inhibited by RR with an inhibitory constant ( $K_i$ ) of 40 nM (184) and by the plasma membrane ATP-sensitive  $K^+$  ( $K_{ATP}$ ) channel blocker glibenclamide with a  $K_i$  of 15  $\mu$ M (333).

### 2. Nonselective $K^+$ channel

In striking analogy with the KHE, electrophoretic flux of monovalent cations 1) is potently activated by depletion of matrix  $Mg^{2+}$  after treatment with EDTA+A-23187 and 2) displays no selectivity for  $K^+$  over  $Na^+$  and  $Li^+$  (36, 356). Flux is demonstrably electrophoretic and can support energy-dependent  $K^+$  accumulation that is potentiated by quinine (through inhibition of the KHE) and is accompanied by the expected depolarization (36). The estimated maximum conductance for  $K^+$  in  $Mg^{2+}$ -depleted mitochondria is as high as  $22.5$  nmol  $K^+$   $\cdot$  mg protein $^{-1}$   $\cdot$  min $^{-1}$   $\cdot$  mV $^{-1}$ , but these values are not likely to be ever attained in vivo, since at free  $[Mg^{2+}] > 0.3$   $\mu$ M flux became too low to be measured reliably (248). Inhibition by nanomolar concentrations of  $Mg^{2+}$  (248) and RR (184) and by micromolar concentrations of the sulfonylurea glibenclamide (333) and of the guanidine derivative U-37883A (334) strongly suggests that cation flux is mediated by a distinct but nonselective channel. Consistent with this, treatment with EDTA+A-23187 does not lead to uncoupling in sucrose-based media, indicating that the channel is not permeable to  $H^+$  (248).

$Na^+$  flux through the unselective  $K^+$  channel is addi-

tive with flux on the  $\text{Na}^+$  channel, suggesting that the two pathways are distinct (248). On the other hand, both pathways are inhibited by  $\text{Mg}^{2+}$ , RR (184), and glibenclamide (333). The problems posed by these findings and by those described in the following paragraphs are discussed at the end of this section.

### 3. Selective $\text{K}^+$ channel(s)

In 1991, Inoue et al. (176) reported a patch-clamp study of rat liver mitoplasts where a  $\text{K}^+$ -selective channel was described that could be inhibited by ATP (but not ADP) and by the plasma membrane  $\text{K}_{\text{ATP}}$  channel blockers 4-aminopyridine and glibenclamide (176). Plasma membrane  $\text{K}_{\text{ATP}}$  channels can be modulated by a variety of pharmacological compounds. Classical inhibitors are sulfonylurea derivatives such as glibenclamide and tolbutamide that have been widely employed as hypoglycemic agents (see Ref. 331 for a review). These compounds act through a specific receptor (4) that modulates the  $\text{K}_{\text{ATP}}$  channel (175). Nonsulfonylurea inhibitors are also known, such as the guanidine derivative U-37883A (138). A variety of structurally unrelated  $\text{K}_{\text{ATP}}$  channel openers have also been discovered, such as cromakalim, diazoxide, and pinacidil (113). Many of these compounds have been tested on mitochondria.

A) STUDIES WITH  $\text{K}_{\text{ATP}}$  CHANNEL OPENERS. Induction of a  $\text{K}^+$ -selective current ( $\text{K}^+$ ,  $\text{Rb}^+ > \text{Li}^+$ ,  $\text{Na}^+$ ) has been reported after treatment of isolated mitochondria with pinacidil, RP-66471, and HOE-234 but not other  $\text{K}^+$  channel openers like nicorandil and apykalim. The effects were observed both in energized mitochondria and in deenergized mitochondria incubated in thiocyanate-based media and did not require the presence of added ATP (31, 332, 336). These results suggest the existence of a mitochondrial  $\text{K}^+$ -selective channel activated by some but not all plasma membrane  $\text{K}_{\text{ATP}}$  channel openers. Other openers like diazoxide and cromakalim also affect mitochondrial  $\text{K}^+$  channels under rather specific conditions, as described in detail in section *II B 3 C*.

B) STUDIES WITH  $\text{K}_{\text{ATP}}$  CHANNEL INHIBITORS. The effect of inhibitors of  $\text{K}_{\text{ATP}}$  channels on mitochondrial cation fluxes is controversial. Glibenclamide (10  $\mu\text{M}$ ) has been shown to be ineffective on  $\text{K}^+$  uptake in isolated mitochondria in the presence of  $\text{Mg}^{2+}$  (29), whereas it inhibits the partially purified mitochondrial  $\text{K}_{\text{ATP}}$  channel in a reconstituted assay where the sensitivity to glibenclamide decreases at increasing  $\text{Mg}^{2+}$  (258). On the other hand, glibenclamide has been shown to inhibit both the  $\text{Na}^+$  channel and the nonselective  $\text{K}^+$  channel induced by treatment of isolated mitochondria with EDTA and EDTA+A-23187, respectively (333). High concentrations of glibenclamide inhibit respiration, posing the question of whether inhibition of cation fluxes by micromolar concentrations of glibenclamide is a secondary event (178).

However, 1) valinomycin catalyzed  $\text{K}^+$  uptake in energized, glibenclamide-treated mitochondria, proving that the  $\Delta\bar{\mu}_{\text{K}}$  was still directed inward (333); and 2) inhibition by glibenclamide was observed also in nonrespiring mitochondria in swelling assays based on thiocyanate diffusion (333). On balance, these findings suggest that glibenclamide is an inhibitor of mitochondrial  $\text{Na}^+$  and  $\text{K}^+$  channels but that the sensitivity critically depends on the  $\text{Mg}^{2+}$  concentration, possibly on the channel(s) involved and on other variables that depend on the incubation conditions, in particular the presence of ATP and GTP (see sect. *II B 3 C*). A binding study of isotopically labeled glibenclamide to rat liver mitochondria and submitochondrial particles has identified a single class of binding sites that are only slightly affected by  $\text{Mg}^{2+}$  and ATP and a single 28-kDa glibenclamide-binding protein that may be the sulfonylurea receptor of the mitochondrial  $\text{K}_{\text{ATP}}$  channel (335).

C) MITOCHONDRIAL  $\text{K}_{\text{ATP}}$  CHANNEL. A series of papers specifically devoted to an ATP-stimulated mitochondrial  $\text{K}^+$  conductance (mitochondrial  $\text{K}_{\text{ATP}}$  channel) has been published. A substantial concordance exists for data obtained with isolated mitochondria and with a partially purified protein of 54 kDa that exhibits the properties expected of a mitochondrial  $\text{K}_{\text{ATP}}$  channel in reconstituted systems (29, 132, 133, 178, 258, 259). Its basic properties can be summarized as follows. 1) The channel is selective for  $\text{K}^+$  over  $\text{Na}^+$ ; in the presence of  $\text{Mg}^{2+}$  it is inhibited by both ATP and ADP but not GDP (29, 258). 2) The ATP-inhibited channel can be reactivated by the plasma membrane  $\text{K}_{\text{ATP}}$  channel openers cromakalim and diazoxide, the latter being much more potent with the mitochondrial than the plasma membrane channel (133). 3) The mitochondrial  $\text{K}_{\text{ATP}}$  channel is inhibited by palmitoyl-CoA and oleyl-CoA, and the palmitoyl-CoA or ATP (ADP)-inhibited channel can be reactivated by GTP and GDP, the former being more potent (259). 4) Only after reactivation of the  $\text{Mg}^{2+}$  and ATP-inhibited channel by diazoxide is the mitochondrial  $\text{K}_{\text{ATP}}$  channel potently inhibited by glibenclamide and 5-hydroxydecanoate both in a reconstituted system (132) and in isolated heart and liver mitochondria (178). It has been shown that diazoxide exerts a marked cardioprotective effect in a rat heart global ischemia model and that cardioprotection can be reverted by glibenclamide and 5-hydroxydecanoate (132). Because 5-hydroxydecanoate has little effect on the sarcolemmal  $\text{K}_{\text{ATP}}$  channels, and because diazoxide is 2,000 times more potent at activating mitochondrial than sarcolemmal  $\text{K}_{\text{ATP}}$  channels, it has been proposed that the cardioprotective effects exerted by diazoxide are due to activation of the mitochondrial  $\text{K}_{\text{ATP}}$  channel, although the mechanism remains obscure (132). In this context, it must be mentioned that Halestrap et al. (153) reported a  $\text{K}^+$ -selective, energy-dependent swelling process elicited by treatment of energized rat liver mitochondria with EGTA- $\text{Ca}^{2+}$  buffers giv-

ing free  $[Ca^{2+}]$  in the range 0.2–1.0  $\mu M$  in the presence of 2.5 mM  $Mg^{2+}$ . Because swelling could be inhibited by MgATP and MgADP, it appears possible that the  $K_{ATP}$  channel described above was responsible for the observed  $K^+$  flux.

D) HOW MANY MITOCHONDRIAL CATION CHANNELS? The existence of an electrophoretic  $K^+$  flux in isolated, "native" mitochondria in the presence of physiological concentrations of  $Mg^{2+}$  is well established. The steady-state membrane potential of energized rat liver mitochondria decreased in a  $K^+$ -dependent manner (248), with a measured conductance of 0.11 nmol  $K^+ \cdot mg$  protein $^{-1} \cdot min^{-1} \cdot mV^{-1}$  (92), which is comparable to the basal  $H^+$  conductance of 0.2 nmol  $\cdot mg$  protein $^{-1} \cdot min^{-1} \cdot mV^{-1}$  (370). What is harder to assess at present is the relationship between this native  $K^+$  conductance and the cation channels listed above. A major problem is that the mitochondrial cation channels characterized so far in isolated mitochondria need to be activated by treatments such as  $Mg^{2+}$  depletion (37, 248, 356) or by incubation in hypotonic media (29). This, in turn, poses the question of whether inferences can be safely made on the pathways for cation transport *in vivo*. These issues cannot be resolved at present. Current ambiguities about the number of cation channels and their regulation by physiological and pharmacological ligands will hopefully be resolved by the molecular definition of the species involved.

### C. Physiological Role(s) of Mitochondrial $K^+$ Cycle

A concerted interplay between  $K^+$  uptake via one or more channels and  $K^+$  efflux via the KHE controls mitochondrial volume homeostasis *in vitro* and *in vivo*, as first suggested by Brierley more than 20 years ago (see Ref. 53 for a review). It is obvious that the existence of regulated pathways for both  $K^+$  uptake and release allows, in principle, a very fine tuning of mitochondrial volume and that the energy diverted into the  $K^+$  cycle need not be high in view of the restricted electrophoretic  $K^+$  flux. Although the role of the KHE is immediately obvious, however (preventing the osmotic burst of mitochondria in the face of  $K^+$  uptake), the requirement for  $K^+$  channel(s) mediating volume increase is less apparent. One situation where such a pathway would be clearly useful is during mitochondrial biogenesis, since  $K^+$  is the main intramitochondrial cation. The most intriguing hypothesis, however, links mitochondrial volume to metabolism through an effect on the respiratory chain (149).

Incubation in hypotonic media greatly stimulates the rate of mitochondrial pyruvate metabolism (2) and of  $\beta$ -oxidation (150), effects that can be mimicked by 1 nM valinomycin (162). Like the  $Ca^{2+}$ -mobilizing hormones phenylephrine and vasopressin, 1 nM valinomycin also

activates fatty acid oxidation and stimulates gluconeogenesis through a mechanism that does not depend on activation of carnitine palmitoyltransferase (see Ref. 149 for review). Halestrap (149) has proposed a unifying hypothesis for these observations that is based on the increased rates of substrate oxidation following an increase of matrix volume in a relatively narrow range. The effect is observed only with electron donors to the ubiquinone pool, suggesting a specific site of regulation (149). If mitochondrial  $K^+$  channels are regulated by  $Ca^{2+}$ -mobilizing hormones *in vivo*, this would account for increased oxidation of fatty acids that are not translocated by carnitine palmitoyltransferase (337). The molecular definition of the pathways involved will certainly increase our understanding of the basic mechanisms and provide tools that will eventually clarify the details of channel operation *in vivo*.

### III. TRANSPORT OF CALCIUM

Studies of  $Ca^{2+}$  transport in isolated mitochondria began in the 1960s (95, 349), and the reader can find an account of this early work in Reference 194. In this period, the prevailing view about the mechanism of transport was one where uptake was an active process linked to a high-energy intermediate of oxidative phosphorylation (71) and efflux was a passive process giving rise to steady-state  $Ca^{2+}$  cycling (108). Throughout the 1960s and early 1970s, researchers in the field mainly focused on the properties of the energy-dependent process of  $Ca^{2+}$  uptake and defined many of its basic features (67, 146, 230, 278, 305, 313, 347, 353). The parallel emergence of chemiosmotic principles (225) posed the question of the link between membrane potential and energy-dependent  $Ca^{2+}$  uptake.

#### A. Nonequilibrium $Ca^{2+}$ Distribution

Nonrespiring mitochondria take up  $Ca^{2+}$  in response to a  $K^+$  diffusion potential, 1 mol  $Ca^{2+}$  being accumulated per 2 mol  $K^+$  lost to the medium (305).  $Ca^{2+}$  is also accumulated in thiocyanate- but not acetate-based media, suggesting accumulation via an electrophoretic mechanism or via a direct  $Ca^{2+}/K^+$  exchange (313). The latter mechanism could be ruled out because  $Ca^{2+}$  uptake in energized mitochondria is not accompanied by  $K^+$  extrusion, nor is it modified by changing extramitochondrial  $[K^+]$  (274). Finally, manipulation of the membrane potential with proper ionophores in the range of  $-75$  to  $-100$  mV suggested that  $Ca^{2+}$  was equilibrating with the membrane potential through an electrophoretic process with a net charge transfer of 2 (297). These early studies established that mitochondria possess a specific  $Ca^{2+}$  transport system that has all the properties of a channel that was

and still is defined as the “Ca<sup>2+</sup> uniporter.” A major problem soon emerged, however.

The Ca<sup>2+</sup> electrochemical gradient ( $\Delta\tilde{\mu}_{\text{Ca}}$ ) can be expressed as

$$\Delta\tilde{\mu}_{\text{Ca}} = zF\Delta\psi + RT \ln [\text{Ca}^{2+}]_{\text{m}}/[\text{Ca}^{2+}]_{\text{o}} \quad (5)$$

For  $z = 2$ , the equilibrium condition ( $\Delta\tilde{\mu}_{\text{Ca}} = 0$ ) is then given by

$$-\Delta\psi = 30 \log [\text{Ca}^{2+}]_{\text{m}}/[\text{Ca}^{2+}]_{\text{o}} \quad (6)$$

Because extramitochondrial free [Ca<sup>2+</sup>] ([Ca<sup>2+</sup>]<sub>o</sub>) oscillates between ~0.1 and 1 μM, for a membrane potential of 180 mV (negative inside), equilibrium matrix free [Ca<sup>2+</sup>] ([Ca<sup>2+</sup>]<sub>m</sub>) should have been 0.1–1 M, i.e., at least 1,000-fold higher than the value of 0.1–1 mM estimated at that time as the likely range of [Ca<sup>2+</sup>]<sub>m</sub> (15).

Moyle and Mitchell (232) argued that Ca<sup>2+</sup> was not being transported with a charge of 2 but rather in symport with P<sub>i</sub> with a net positive charge of 1, which would have accounted for the observed accumulation ratio. Although a Ca<sup>2+</sup>-P<sub>i</sub> symport could conclusively be ruled out by experiments and by theoretical considerations (15, 240, 270), the issue of the net charge of Ca<sup>2+</sup> translocation in respiring mitochondria, and hence the quantitative dependence of flux on the membrane potential, is not an easy problem to address. A critical point is that Ca<sup>2+</sup> uptake depolarizes the inner membrane, and this makes measurements of transport at constant voltage problematic. Wingrove et al. (359) tackled this problem by using three levels of buffered Ca<sup>2+</sup> (0.5, 1.0, and 1.5 μM) and modified  $\Delta\psi$  by adding increasing concentrations of malonate to succinate-energized mitochondria. The low, buffered [Ca<sup>2+</sup>]<sub>o</sub> ensured that the rate of Ca<sup>2+</sup> uptake was not limited by the rate of H<sup>+</sup> pumping, and direct measurements indicated that  $\Delta\psi$  remained constant throughout the uptake process. Although these studies can only be applied to a limited range of Ca<sup>2+</sup> concentrations, the data consistently indicated transport with  $z = 2$ . Transport spanned the entire transmembrane potential region between about -80 and -180 mV, and it was offset by the effect of local fixed charges (139, 359).

The general problem of the disequilibrium between steady-state Ca<sup>2+</sup> distribution and transmembrane potential is even more dramatic in the light of subsequent determinations of [Ca<sup>2+</sup>]<sub>m</sub> by direct methods. In isolated mitochondria, values between the submicromolar and low micromolar range were consistently found (147, 218, 231, 355), whereas typical resting values between 100 and 240 nM were found in intact cells (100, 101, 228). The Ca<sup>2+</sup> concentration gradient maintained by energized mitochondria thus appears to oscillate between 0 and 10, rather than being maintained at the value of 10<sup>6</sup> that

would be predicted by thermodynamic considerations. This displacement from equilibrium is due to the fact that Ca<sup>2+</sup> distribution represents a kinetic steady state where electrophoretic Ca<sup>2+</sup> uptake is precisely matched by Ca<sup>2+</sup> efflux on at least two separate pathways. These were identified in the 1970s as a Na<sup>+</sup>-dependent Ca<sup>2+</sup> efflux pathway, most likely a Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (69, 85, 90, 91), and a Na<sup>+</sup>-independent Ca<sup>2+</sup> efflux pathway (143, 240, 274) (see Refs. 84, 139, 144, 145, 241, 302 for reviews).

An exhaustive treatment of the vast literature on mitochondrial Ca<sup>2+</sup> transport is beyond the scope of the present work, and the interested reader is referred to earlier reviews for further reference (65, 84, 139, 144, 145, 241, 302).

## B. Pathways for Ca<sup>2+</sup> Uptake

In the following paragraphs I summarize the properties of mitochondrial electrophoretic Ca<sup>2+</sup> transport systems that catalyze Ca<sup>2+</sup> uptake in energized mitochondria. In principle, the direction of Ca<sup>2+</sup> flux should only depend on the  $\Delta\tilde{\mu}_{\text{Ca}}$ . Yet, for reasons that should become clear later, Ca<sup>2+</sup> flux via the uniporter is not readily reversible upon depolarization (260), suggesting that the physiological role of the uniporter may be that of mediating Ca<sup>2+</sup> uptake.

### 1. Ca<sup>2+</sup> uniporter

A problem that was not appreciated in earlier studies of the Ca<sup>2+</sup> uniporter is that the kinetics of Ca<sup>2+</sup> uptake in respiring mitochondria become rapidly limited by the rate of H<sup>+</sup> pumping (i.e., by the rate at which the membrane potential can be regenerated) as [Ca<sup>2+</sup>]<sub>o</sub> is raised above ~10 μM. This led to a serious underestimation of both the  $V_{\text{max}}$  and the apparent Michaelis constant ( $K_{\text{m}}$ ) for Ca<sup>2+</sup> (see Refs. 51, 163 and comments therein). Azzone and co-workers (51) circumvented these problems 1) by using a K<sup>+</sup> diffusion potential induced by the addition of a vast excess of valinomycin to respiratory-inhibited mitochondria as the driving force for Ca<sup>2+</sup> uptake, so as to make Ca<sup>2+</sup> transport rate-limiting even at high [Ca<sup>2+</sup>]<sub>o</sub>; 2) by measuring the initial rate of K<sup>+</sup> efflux rather than that of Ca<sup>2+</sup> uptake, which would have required different methods for Ca<sup>2+</sup> detection in different ranges of [Ca<sup>2+</sup>]<sub>o</sub>; and 3) by using proper Ca<sup>2+</sup> buffers in the low micromolar range of [Ca<sup>2+</sup>]<sub>o</sub> (51). With this method, the  $V_{\text{max}}$  at 30°C was established to be in excess of 1,400 nmol Ca<sup>2+</sup> · mg protein<sup>-1</sup> · min<sup>-1</sup>, whereas the apparent  $K_{\text{m}}$  was <10 μM in sucrose-based media.

The Ca<sup>2+</sup> uniporter is regulated by a number of modulators (inhibitors and activators), and this partly explains the wide variation of kinetic parameters reported in earlier literature (139, 145). To add further complexity, some of these modulators behave as uniporter inhibitors

or activators depending on their concentration. I do not even attempt to provide an exhaustive compilation of the known factors, which can be found in previous reviews (65, 84, 139, 144, 145, 241, 302). I will rather discuss these effectors through selected examples.

A first class is represented by ruthenium compounds. Owing to the large electrochemical gradient for accumulation of the ruthenium cation in energized mitochondria, slow uptake may take place, but the transport rate is  $<2$  pmol  $\cdot$  mg protein<sup>-1</sup>  $\cdot$  min<sup>-1</sup> (43). In the presence of crude or partially purified RR, Ca<sup>2+</sup> flux on the uniporter is completely blocked, inhibition being of the noncompetitive type (43, 277, 348). In liver mitochondria, Ca<sup>2+</sup> flux is inhibited by 50% with  $\sim 30$  pmol RR/mg protein, but the titer is different in mitochondria from other sources. On the basis of inhibition by RR, the number of uniporter molecules has been estimated to be between 1 and 10 pmol/mg mitochondrial protein (221, 277). More recent studies show that the uniporter is inhibited by pure Ru-360 (366) and indicate that the most active inhibitor present in "RR" recrystallized from commercial sources may in fact not be the predominant red component (60).

A second class of inhibitors is represented by divalent cations that are themselves transported by the uniporter like Sr<sup>2+</sup> (67), Mn<sup>2+</sup>, Ba<sup>2+</sup> (109), and lanthanides (221). Inhibition by these cations is generally competitive in type, but not all of the effects are necessarily exerted at the transport site(s) because the uniporter is also regulated by Me<sup>2+</sup> binding sites that modulate the affinity of the uniporter for Ca<sup>2+</sup>.

This third class of effectors is best exemplified by Mg<sup>2+</sup> and Mn<sup>2+</sup>. In the low millimolar range, Mg<sup>2+</sup> transforms the relationship between rate of Ca<sup>2+</sup> transport and [Ca<sup>2+</sup>]<sub>o</sub> from hyperbolic to sigmoidal, decreases the V<sub>max</sub>, and increases the apparent K<sub>m</sub> for Ca<sup>2+</sup> from 10 to  $\sim 50$   $\mu$ M (6, 51, 353). Mg<sup>2+</sup> best represents a class of ionic modulators that affect the kinetics of Ca<sup>2+</sup> transport by binding to regulatory site(s) rather than to the transport site(s). Indeed, Mg<sup>2+</sup> is not transported by the uniporter; it does not affect the Ca<sup>2+</sup> conductance (163), and its effects can be mimicked by  $\sim 50$ -fold higher concentrations of monovalent cations like Li<sup>+</sup> (51). A second example is represented by Mn<sup>2+</sup>, which under specific conditions can stimulate rather than inhibit the kinetics of Ca<sup>2+</sup> transport (166), with an effect that counteracts that of Mg<sup>2+</sup> through a mixed-type competition (7). These experiments suggest that Mn<sup>2+</sup> can displace Mg<sup>2+</sup> from its binding site(s) and therefore that Mn<sup>2+</sup> can interact both with the Me<sup>2+</sup> regulatory site and with the transport site. This may result in either stimulation or inhibition of Ca<sup>2+</sup> flux depending on the concentration of Mn<sup>2+</sup> and on the presence or absence of Mg<sup>2+</sup> (7). Interestingly, the Ca<sup>2+</sup> uniporter is activated by external Ca<sup>2+</sup> itself so that the uniporter undergoes deactivation upon removal of extramitochondrial Ca<sup>2+</sup> (e.g., after Ca<sup>2+</sup> accumulation)

(190). Thus, at low [Ca<sup>2+</sup>]<sub>o</sub> values (comparable to those prevailing in the cytosol), the activity of the uniporter may be extremely low. It appears possible that activation of the uniporter by spermine (191, 239) is mediated by modulation of Me<sup>2+</sup> binding sites and/or by screening effects on surface fixed charges. Finally, recent work indicates that the uniporter is inhibited by adenine nucleotides in the order ATP > ADP > AMP, through an effect that is independent of ATP hydrolysis, and of changes of Ca<sup>2+</sup> and Mg<sup>2+</sup> concentrations (201).

In the presence of an outwardly directed Ca<sup>2+</sup> electrochemical gradient, the uniporter should catalyze Ca<sup>2+</sup> efflux, and Ca<sup>2+</sup> release is indeed observed upon addition of a respiratory inhibitor and/or an uncoupler after energy-dependent accumulation of Ca<sup>2+</sup>. However, Ca<sup>2+</sup> release is largely insensitive to RR (43, 183). At variance with our earlier suggestion (43), this depends on the fact that depolarization of Ca<sup>2+</sup>-loaded mitochondria causes opening of an additional pathway for Ca<sup>2+</sup> release, the voltage-dependent mitochondrial permeability transition pore (MTP) that is insensitive to RR (33, 168, 173, 183). When MTP opening is prevented by cyclosporin A (CsA), fast mitochondrial Ca<sup>2+</sup> efflux is not observed despite depolarization, indicating that flux via the uniporter is not readily reversible (260). This behavior can be explained by the low [Ca<sup>2+</sup>]<sub>o</sub> at the onset of depolarization, which deactivates the uniporter (173, 190), and by the fact that a high membrane potential may be required to maintain the uniporter in a transport-competent conformation (185).

## 2. Rapid uptake mode

Mitochondria *in vivo* are exposed to cyclic changes of cytosolic [Ca<sup>2+</sup>] ([Ca<sup>2+</sup>]<sub>c</sub>) that have the appearance of pulses, whereas the most accurate results concerning the kinetics of mitochondrial Ca<sup>2+</sup> uptake have been obtained by using Ca<sup>2+</sup> buffers (e.g., Refs. 51, 359). To study whether the amplitude and frequency of Ca<sup>2+</sup> pulses affect the properties of transport, Gunter and co-workers (322, 323) devised a pulse-generating and monitoring system that allows precise modulation of these parameters. These studies revealed that Ca<sup>2+</sup> uptake was more efficient when mitochondria were exposed to trains of Ca<sup>2+</sup> pulses of physiological height ( $\sim 400$  nM) rather than to an identical steady [Ca<sup>2+</sup>]<sub>o</sub> for the same overall time. This mechanism (dubbed the rapid uptake mode, or RaM) can be reset in  $<0.75$  s by a decrease of [Ca<sup>2+</sup>]<sub>o</sub> between 100 and 200 nM (323). Like the Ca<sup>2+</sup> uniporter, the RaM is inhibited by RR (but inhibition requires much higher concentrations) and stimulated by ATP and spermine; unlike the uniporter, it is not affected by Mg<sup>2+</sup> (323). Gunter and co-workers (322, 323) favor the idea that the RaM is mediated by a specific transport mechanism that might be responsible for mitochondrial Ca<sup>2+</sup> uptake from [Ca<sup>2+</sup>]<sub>c</sub> transients *in vivo*, as reviewed in detail elsewhere (141).

### C. Pathways for Ca<sup>2+</sup> Efflux

After the uptake of moderate Ca<sup>2+</sup> loads (typically 10–30 nmol/mg protein), respiring mitochondria retain Ca<sup>2+</sup> and maintain steady-state [Ca<sup>2+</sup>]<sub>o</sub> at a constant value of 0.5–1.0 μM. If enough RR is added to fully inhibit the Ca<sup>2+</sup> uniporter, a process of Ca<sup>2+</sup> efflux ensues that is commonly interpreted as evidence that RR-insensitive Ca<sup>2+</sup> efflux, coupled to uniporter-mediated reuptake, was also occurring before the addition of RR. Inhibition of the uniporter by RR would then allow the study of this pathway for mitochondrial Ca<sup>2+</sup> efflux in isolation. This system, the still elusive “Na<sup>+</sup>-independent pathway for Ca<sup>2+</sup> efflux” (NICE) (70, 119, 143, 270, 274), has been the subject of a vast amount of work and of considerable controversy over the years.

After the addition of RR, the rate of Ca<sup>2+</sup> efflux can be stimulated by the addition of Na<sup>+</sup>, the extent of stimulation being variable depending on the source of mitochondria (69, 89–91). This pathway, the “Na<sup>+</sup>-dependent pathway for Ca<sup>2+</sup> efflux” (NCE), has likewise been the subject of a large number of studies (e.g., Refs. 8, 9, 22, 74, 75, 93, 135, 136, 159–161, 164, 182, 283, 296, 303, 308, 318, 363) and of a recent debate about the Na<sup>+</sup>-Ca<sup>2+</sup> stoichiometry (23, 180, 361). The two pathways are distinct, since their kinetics are completely different, in that NICE is second order whereas NCE is first order in [Ca<sup>2+</sup>] (360, 361).

#### 1. NICE

Under the most simple incubation conditions NICE is slow (typically between 1 and 2 nmol Ca<sup>2+</sup> · mg protein<sup>-1</sup> · min<sup>-1</sup>) and occurs without detectable changes of the membrane potential (240). These findings suggested that maintenance of the steady-state [Ca<sup>2+</sup>]<sub>o</sub> by energized mitochondria depends on “Ca<sup>2+</sup> cycling” via separate pathways, i.e., uptake via the uniporter and efflux via NICE, possibly a H<sup>+</sup>/Ca<sup>2+</sup> antiporter (see Ref. 241 for review). The rate of RR-insensitive Ca<sup>2+</sup> efflux can be considerably increased by a variety of agents, most notably P<sub>i</sub> and oxidants of both glutathione and/or pyridine nucleotides (PN) (156, 195, 275). This led to the suggestion that mitochondrial Ca<sup>2+</sup> efflux via the putative H<sup>+</sup>/Ca<sup>2+</sup> antiporter was stimulated by oxidation (120) or hydrolysis (205) of PN.

In principle, RR-insensitive Ca<sup>2+</sup> release can occur via an entirely different mechanism. If, in the steady state, a small fraction of mitochondria undergoes reversible depolarization due to opening of the Ca<sup>2+</sup>-dependent MTP, Ca<sup>2+</sup> released from this permeabilized fraction would be taken up by the polarized mitochondria. Addition of RR would prevent this type of Ca<sup>2+</sup> redistribution among mitochondrial subpopulations and thus result in net Ca<sup>2+</sup> efflux (282). This possibility is strongly sup-

ported by the occurrence of transient, nonsynchronized depolarizations in individual isolated mitochondria (169), which matches the spontaneous oscillations of the mitochondrial membrane potential observed in living cells (204).

The nature of the effects of oxidants and other compounds that stimulate RR-insensitive Ca<sup>2+</sup> efflux (and the related problems of the nature and of the very existence of this pathway) remained controversial for a number of years (26, 30, 32, 38, 39, 41, 45, 62, 120, 140, 142, 183, 206, 254, 281, 285, 295, 301, 316, 340, 350–352, 360, 369). I think that there can be little doubt that many early studies of RR-insensitive Ca<sup>2+</sup> efflux in mitochondria have been complicated by the unrecognized contribution of the permeability transition (PT), which may be extremely difficult to detect for reasons that are addressed in a specific study of Riley and Pfeiffer (282). Unless proper measures are taken, RR-insensitive Ca<sup>2+</sup> efflux is a complex measure of both NICE and of Ca<sup>2+</sup> efflux from mitochondria undergoing PT (25, 242, 245, 273, 282, 362). It can be hardly coincidental that, with no exception, factors reported to stimulate or inhibit RR-insensitive Ca<sup>2+</sup> efflux also affect the PT in the same direction (see the specific discussion in Refs. 44, 249 and section IV).

In the light of the above concerns, Gunter and co-workers (140, 360) reassessed the problem of the Na<sup>+</sup>-independent pathways for Ca<sup>2+</sup> efflux in rat liver mitochondria under conditions where occurrence of a PT could be reasonably excluded. These studies indicated that NICE saturates at Ca<sup>2+</sup> loads of 25 nmol/mg protein, that its V<sub>max</sub> is not influenced by the concentration of P<sub>i</sub> and does not exceed a rate of 1.2 nmol Ca<sup>2+</sup> · mg protein<sup>-1</sup> · min<sup>-1</sup> (360), and that this system is able to extrude Ca<sup>2+</sup> against a gradient that is much higher than thermodynamically permissible to an electroneutral H<sup>+</sup>/Ca<sup>2+</sup> exchanger (140). Thus NICE is either a nH<sup>+</sup>/Ca<sup>2+</sup> exchanger with n > 2, or it has an active component that may be directly linked to electron flux (293). These conclusions are entirely consistent with the earlier demonstration that inverted vesicles (293) or deenergized mitochondria (38, 301) do not display a RR-insensitive H<sup>+</sup>/Ca<sup>2+</sup> exchange activity even when a large ΔpH is demonstrably provided and that NICE is inhibited by mitochondrial depolarization in the range between –180 and –140 mV (39, 41). Initiation of oxidative phosphorylation causes net Ca<sup>2+</sup> accumulation through inhibition of Ca<sup>2+</sup> efflux via this pathway (41), and this is precisely what would be expected to stimulate matrix Ca<sup>2+</sup>-dependent dehydrogenases, as discussed in section III D.

In summary, it appears safe to conclude that mitochondria possess a NICE that requires a transmembrane potential as a component of its driving force. This pathway saturates at very low Ca<sup>2+</sup> loads and is extremely slow. For practical purposes, a contribution of the PT to

RR-insensitive  $\text{Ca}^{2+}$  efflux should be suspected for rates above  $\sim 2 \text{ nmol} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$ .

## 2. NCE

The existence of a NCE mediating steady-state  $\text{Ca}^{2+}$  cycling in mitochondria has never been questioned (69, 89–91). Early work indicated its absence in mitochondria from some tissues such as liver, kidney, and lung (91), a finding that was challenged by subsequent studies (159, 164, 237). Experimental discrepancies were settled with the demonstration that NCE in liver mitochondria is inhibited by RR itself (above  $\sim 5 \text{ nmol/mg protein}$ ), by  $\text{Mg}^{2+}$  (50% inhibition at  $\sim 1 \text{ mM}$ ), and by very low concentrations (50% inhibition  $\sim 0.2 \mu\text{M}$ ) of the widely used membrane potential probe triphenylmethylphosphonium (361). Complications arising from the PT (see sect. III C1) are generally negligible in studies of NCE because the efflux rate via the  $\text{Na}^+$ -independent pathways (NICE and PT) is always subtracted, and conditions are normally selected so that their contribution is minimal. A considerable amount of information exists about this pathway, which is widely considered as a  $\text{Na}^+/\text{Ca}^{2+}$  exchanger that mediates physiological  $\text{Ca}^{2+}$  cycling through a concerted interplay with the NHE (89).

The kinetic parameters for  $\text{Ca}^{2+}$  efflux are somewhat variable for mitochondria from different sources. The  $V_{\text{max}}$  value varies between a minimum of 2.6 and a maximum of  $18 \text{ nmol Ca}^{2+} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$  in liver and heart mitochondria, respectively (91, 361). The dependence on  $\text{Na}^+$  is sigmoidal, with typical  $K_m$  values centered at  $\sim 8\text{--}10 \text{ mM Na}^+$ , and  $\text{Na}^+$  can be substituted by  $\text{Li}^+$  (85).  $\text{Ca}^{2+}$  efflux is inhibited by  $\text{Sr}^{2+}$  (303),  $\text{Ba}^{2+}$  (207, 208),  $\text{Mg}^{2+}$  (361),  $\text{Mn}^{2+}$  (135), and by a variety of compounds of pharmacological interest such as amiloride (182, 308, 318), trifluoperazine (160), diltiazem (74, 75, 283), verapamil (363), clonazepam, and bepridil (74), whereas it is stimulated by short-chain alcohols (296). A prominent modulatory effect by matrix pH has been reported, with a sharp optimum at pH 7.6 (22), and NCE is stimulated by treatment with glucagon and  $\beta$ -adrenergic agonists (136).

The rate of  $\text{Ca}^{2+}$  efflux via NCE is significantly inhibited by both antimycin A and protonophores, suggesting that it may be favored by the transmembrane potential (85, 90). The related issue of the  $\text{Na}^+/\text{Ca}^{2+}$  stoichiometry is complex, and data in the literature are contradictory. The dependence on external  $\text{Na}^+$  has been reported to fit both the cube (85) and the square of  $[\text{Na}^+]_o$  (90), and an overall electroneutral exchange has been initially suggested on both kinetic (3) and thermodynamic grounds (52). This issue has been recently reexamined in studies where matrix pH and  $[\text{Ca}^{2+}]_m$  were directly monitored with fluorescent probes, a technique that was not available in earlier studies. The results indicate that NCE is not elec-

troneutral and that a stoichiometry of  $3\text{Na}^+:\text{Ca}^{2+}$  appears more plausible (23, 180). A 110-kDa protein able to catalyze electroneutral, diltiazem-sensitive  $\text{Na}^+/\text{Ca}^{2+}$  exchange has been purified from beef heart mitochondria (199), and this is currently the only candidate as the protein mediating NCE.

## 3. $\text{Ca}^{2+}$ cycling and the problem of net $\text{Ca}^{2+}$ release

Coupling of electrophoretic  $\text{Ca}^{2+}$  uptake with  $\text{Ca}^{2+}$  efflux on separate pathways in energized mitochondria allows fine regulation of  $[\text{Ca}^{2+}]_o$  and  $[\text{Ca}^{2+}]_m$  and is illustrated in Figure 8. Note that energy in the form of a  $\Delta\tilde{\mu}_H$  is required both for  $\text{Ca}^{2+}$  uptake and for  $\text{Ca}^{2+}$  release, owing to 1) the electrophoretic nature of transport on both the uniporter and RaM; 2) the  $3\text{Na}^+:\text{Ca}^{2+}$  stoichiometry of NCE and energy dependence of NICE, tentatively depicted here as a  $3\text{H}^+/\text{Ca}^{2+}$  exchanger; and 3) the requirement for  $\text{H}^+$  extrusion posed by operation of NHE and possibly NICE itself. Steady-state  $[\text{Ca}^{2+}]_o$  can be increased (and  $[\text{Ca}^{2+}]_m$  decreased to an extent that also depends on matrix  $\text{Ca}^{2+}$  buffering) by increasing the activity of the efflux systems, or by decreasing the activity of the uptake systems, or both (241, 244). The energetic consequences are quite different depending on whether the former or the latter mechanism prevails, however. If net efflux results from stimulation of the efflux pathways, the increase of  $[\text{Ca}^{2+}]_o$  will stimulate the kinetics of the uniporter and RaM. In turn, this will 1) limit the increase of  $[\text{Ca}^{2+}]_o$  and 2) cause an increased rate of  $\text{Ca}^{2+}$  cycling, and therefore of energy drain, which is a steep function of  $[\text{Ca}^{2+}]_o$ . This is experimentally observed by adding the electroneutral  $2\text{H}^+/\text{Ca}^{2+}$  ionophore A-23187 to respiring mitochondria that have accumulated  $\text{Ca}^{2+}$ , a condition

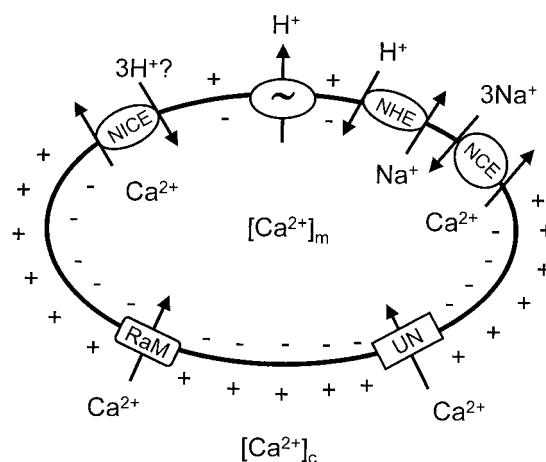


FIG. 8. Pathways for  $\text{Ca}^{2+}$  transport in energized mitochondria.  $\sim$ , Redox-linked  $\text{H}^+$  pumps; UN,  $\text{Ca}^{2+}$  uniporter; NICE,  $\text{Na}^+$ -independent pathway for  $\text{Ca}^{2+}$  efflux; NCE,  $\text{Na}^+$ -dependent pathway for  $\text{Ca}^{2+}$  efflux; NHE,  $\text{H}^+/\text{Na}^+$  exchanger; RaM, rapid uptake mode;  $[\text{Ca}^{2+}]_c$ , cytosolic  $\text{Ca}^{2+}$  concentration;  $[\text{Ca}^{2+}]_m$ , mitochondrial  $\text{Ca}^{2+}$  concentration. For explanation, see text.

where all the respiratory capacity can be diverted into  $\text{Ca}^{2+}$  cycling (see, e.g., Refs. 45, 163). Thus, and as long as the membrane potential is high, net  $\text{Ca}^{2+}$  efflux through stimulation of the efflux pathways has both a low efficiency and a high energetic cost. The low  $V_{\text{max}}$  and early saturation of the efflux pathways by  $[\text{Ca}^{2+}]_{\text{m}}$  are probably designed to pose an upper limit to the energy that can be spent in regulation of  $[\text{Ca}^{2+}]_{\text{m}}$  and  $[\text{Ca}^{2+}]_{\text{o}}$  through  $\text{Ca}^{2+}$  cycling. On the other hand, because the rate of uptake is extremely fast when  $[\text{Ca}^{2+}]_{\text{o}}$  increases, this situation exposes mitochondria to the risks of  $\text{Ca}^{2+}$  overload. This may be prevented by opening of the MTP, which mediates mitochondrial depolarization and fast  $\text{Ca}^{2+}$  release *in vitro* (44) and perhaps *in vivo*. This aspect is discussed more in detail in section IV.

#### D. Mitochondria in $\text{Ca}^{2+}$ Homeostasis

$\text{Ca}^{2+}$  modulates three intramitochondrial enzymes involved in energy metabolism, i.e., the pyruvate dehydrogenase complex (97), the  $\text{NAD}^+$ -linked isocitrate dehydrogenase (98), and the 2-oxoglutarate dehydrogenase (219). This led to the hypothesis that mitochondrial  $\text{Ca}^{2+}$  uptake upregulates the activity of these enzymes, hence energy production, following an increase of  $[\text{Ca}^{2+}]_{\text{c}}$  (96). Convincing evidence has accumulated that this is the case in different systems (e.g., Refs. 110, 118, 148, 299), and today it can be hardly doubted that one of the main functions of mitochondrial  $\text{Ca}^{2+}$  transport is to relay an increased metabolic demand through a  $\text{Ca}^{2+}$  signal (see Refs. 155, 287 for reviews).

On the other hand, the role of mitochondria in intracellular  $\text{Ca}^{2+}$  homeostasis has been more controversial, with consensus following a cyclic pattern. The  $[\text{Ca}^{2+}]_{\text{c}}$  transients visualized by means of aequorin luminescence showed that diffusion of  $\text{Ca}^{2+}$  through the cytosol is so constrained that a rise in  $[\text{Ca}^{2+}]_{\text{c}}$ , produced by local  $\text{Ca}^{2+}$  entry through cell membrane or by local  $\text{Ca}^{2+}$  injection, was confined to the immediate vicinity of these sites. The diffusion constraints were lifted by treatment with cyanide or RR, suggesting that energized  $\text{Ca}^{2+}$  sequestering by mitochondria was the dominant factor in the constraints (292). Throughout the late 1970s and early 1980s, measurements with intracellular microelectrodes and microinjected probes gave values for  $[\text{Ca}^{2+}]_{\text{c}}$  after stimulation that were mostly compatible with a role of mitochondria in regulation of  $[\text{Ca}^{2+}]_{\text{c}}$  homeostasis (see Ref. 346 for an excellent review of the resting and stimulated values).

In the early 1980s, fluorescent  $\text{Ca}^{2+}$  indicators became available that could be loaded into cells without disruption of the plasma membrane (342–345). This powerful technique allowed measurements of  $[\text{Ca}^{2+}]_{\text{c}}$  in virtually any cell and revealed that resting values are typically centered at or below  $\sim 100$  nM (271). Cytosolic

$[\text{Ca}^{2+}]_{\text{c}}$  does increase upon activation of specific signaling pathways and gives rise to  $\text{Ca}^{2+}$  transients the amplitude and frequency of which depends on the type of stimulus and on the type of cell (341). The maximum amplitude of the  $[\text{Ca}^{2+}]_{\text{c}}$  rise rarely exceeds  $1 \mu\text{M}$ , however, whereas the frequency can vary widely (see Ref. 144 for representative examples). The range of variations of  $[\text{Ca}^{2+}]_{\text{c}}$  measured with this approach is well below the  $K_{\text{m}}$  of the mitochondrial  $\text{Ca}^{2+}$  uniporter, and this has been used as an argument against a role of mitochondrial  $\text{Ca}^{2+}$  transport in cellular  $\text{Ca}^{2+}$  homeostasis under physiological conditions (68). As a result, throughout the 1980s and early 1990s, mitochondria were mostly presumed to come into play only after sustained elevations of  $[\text{Ca}^{2+}]_{\text{c}}$ , such as those observed in pathological conditions (117, 253). More recent results have completely changed this picture and indicated that mitochondria play a key role in cellular  $\text{Ca}^{2+}$  homeostasis.

In 1990, bright fluorescent spots were observed in fluorescent ratio images of fura 2-loaded quiescent cardiac myocytes. The fluorescence intensity of these  $\text{Ca}^{2+}$  “hot spots” could be increased by agents that deplete  $\text{Ca}^{2+}$  in the sarcoplasmic reticulum and decreased by uncouplers of oxidative phosphorylation and respiratory inhibitors, suggesting  $\text{Ca}^{2+}$  compartmentation inside mitochondria (358). In the first quantitative measurement of  $[\text{Ca}^{2+}]_{\text{m}}$ , Miyata et al. (228) reported a resting level of  $<100$  nM in cardiac myocytes that was increased to 600 nM, over the course of many contractions, as the pacing frequency was increased to 4 Hz (228). A strong indication that mitochondrial  $\text{Ca}^{2+}$  fluxes are fast enough *in vivo* to change  $[\text{Ca}^{2+}]_{\text{m}}$  and  $[\text{Ca}^{2+}]_{\text{c}}$  during the cardiac contraction cycle came from a study based on shock-freezing of ventricular myocytes at defined stages of systole and diastole, followed by electron-probe microanalysis. This study revealed that the mitochondrial  $\text{Ca}^{2+}$  content changed during each individual contraction cycle and that a substantial amount of  $\text{Ca}^{2+}$  was taken up during the systole and released during later systole and diastole (177). Finally, mitochondrially targeted aequorin was introduced as a tool to monitor changes of  $[\text{Ca}^{2+}]_{\text{m}}$  *in situ* (286, 300). Inositol 1,4,5-trisphosphate ( $\text{IP}_3$ )-induced  $\text{Ca}^{2+}$  mobilization from intracellular stores caused increases of intramitochondrial aequorin fluorescence the speed and amplitude of which could not be accounted for by the relatively small increases in mean  $[\text{Ca}^{2+}]_{\text{c}}$  (284). This indicated a direct link between mitochondria and intracellular stores and suggested that mitochondria sense microdomains of high  $[\text{Ca}^{2+}]_{\text{c}}$  located close to the  $\text{IP}_3$ -sensitive channels (284). These findings perfectly match the premonition that “in cell regions where the sequestering machinery is sufficiently dense, different  $\text{Ca}^{2+}$  message functions inside a cell may be effectively segregated, permitting private-line intracellular communication” (292).

Ample evidence that mitochondria play a key role in physiological intracellular  $\text{Ca}^{2+}$  homeostasis is today available, as demonstrated by an ever-increasing number of studies in different cell types (see, e.g., Refs. 21, 64, 165, 170, 179, 203, 357). These findings will hopefully attract novel interest in the molecular mechanisms that mediate mitochondrial  $\text{Ca}^{2+}$  transport.

#### IV. PERMEABILITY TRANSITION

As a result of  $\text{Ca}^{2+}$  accumulation (or simply as a consequence of *in vitro* aging), energized mitochondria may undergo a sudden permeability increase of the inner membrane to solutes of molecular mass up to  $\sim 1,500$  Da (214), the PT. In the past, the apparent lack of solute selectivity has been widely taken to imply that the permeability pathway lacked specificity and that it was likely to represent a form of "unspecific membrane damage," as also suggested from the protective effects of phospholipase  $\text{A}_2$  inhibitors (306). A series of more recent findings indicates that the PT may in fact be mediated by opening of a specific, nonselective high-conductance inner membrane channel, the MTP.

1) The PT is inhibited by nanomolar concentrations of the cyclic immunosuppressant peptide CsA (59, 88, 123). Because CsA does not inhibit phospholipase  $\text{A}_2$ , the popular hypothesis that the PT could be formed by acyllysophospholids after  $\text{Ca}^{2+}$ -dependent activation of phospholipase  $\text{A}_2$  (25, 173, 269) was largely dismissed.

2) The inner mitochondrial membrane possesses ion channels that can be studied by electrophysiology (186, 266, 319; see Refs. 211, 320 for reviews). Among these, the high-conductance ( $\sim 1$  nS) "mitochondrial megachannel" (MMC) (186, 266) is inhibited by CsA (328) and responds to inducers and inhibitors in the same way as does the PT (47, 327, 329). This left little doubt that the MMC and MTP coincide and that the PT is due to opening of proteinaceous pores. This hypothesis had already been proposed by Haworth and Hunter (157, 158, 167, 168), who carried out a thorough characterization of inducers and inhibitors of the PT that stood the test of time. I think that the early dismissal of the pore theory of the PT was an indirect consequence of the Nobel Prize award to Peter Mitchell in 1978 and of the widespread conviction that mitochondria did not possess ion channels at all.

The MTP is not cation selective, yet it exhibits a prominent  $\text{Ca}^{2+}$  dependence that explains its traditional coverage in reviews of mitochondrial  $\text{Ca}^{2+}$  transport (144, 145). I think that its discussion here is fully justified by a plausible role in mitochondrial  $\text{Ca}^{2+}$  homeostasis that may be of relevance in a variety of forms of cell death. Space limitations prevent an extensive coverage of the huge literature on the PT, which can be found in a specific review (371).

#### A. Regulation

The MTP can be defined as a voltage-dependent, CsA-sensitive, high-conductance inner membrane channel of unknown molecular structure. The voltage dependence appears to be an intrinsic property of the pore. It has been demonstrated in isolated mitochondria with depolarizing  $\text{H}^+$  (33) or  $\text{K}^+$  currents (312) and in single-channel measurements by modulating the applied voltage (330). In the fully open state, the apparent pore diameter is  $\sim 3$  nm, based on both solute exclusion (157, 214) and conductance measurements (328). The pore open-closed transitions are highly regulated by multiple effectors at discrete sites (see Refs. 34, 42, 144, 145, 371 for reviews). Factors that affect the PT can be conveniently classified into matrix and membrane effectors as schematically illustrated in Figure 9.

##### 1. Matrix effectors

Pore opening is favored by increases of  $[\text{Ca}^{2+}]_m$ , an effect that can be counteracted by other  $\text{Me}^{2+}$  like  $\text{Mg}^{2+}$ ,  $\text{Sr}^{2+}$ , and  $\text{Mn}^{2+}$  (157). Inhibition is competitive, suggesting  $\text{Me}^{2+}$  interactions at a common site (47).

Pore opening is strongly promoted by an oxidized state of PN and of critical dithiols at discrete sites, and both effects can be separately reversed by proper reductants (73, 81). The dithiol-disulfide interconversions correlate with the redox state of glutathione and can be blocked by 1-chloro-2,4-dinitrobenzene, suggesting that the dithiol is in redox equilibrium with matrix glutathione (73). This finding easily accounts for the PT-inducing effects of both peroxides and redox cycling agents (83, 188, 262) and for the corresponding inhibition with monofunctional thiol reagents like *N*-ethylmaleimide (189, 262) and monobromobimane (80).

Opening of the MTP is inhibited by CsA (59, 88, 123)

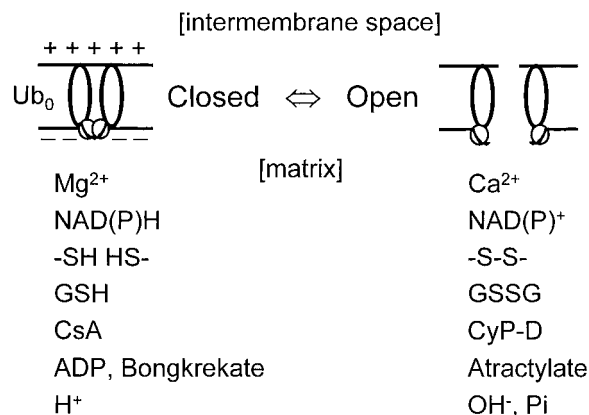


FIG. 9. Modulation of permeability transition by matrix and membrane effectors. Signs denote transmembrane electrical potential.  $\text{Ub}_0$ , ubiquinone 0; CsA, cyclosporin A.

most likely after binding to cyclophilin D (CyP-D), a matrix peptidyl-prolyl *cis,trans*-isomerase (PPIase) (78, 364) which would be the endogenous MTP modulator (79, 246). As a result of CsA binding, the PPIase activity of CyP-D is inhibited, but this may not be relevant for MTP inhibition (311). Likewise (and at variance from immunosuppression), the effect of CsA does not involve inhibition of calcineurin (246). Cyclosporin A is not a true pore blocker, however, and inhibition is transient in longer time-frame experiments (61). Inhibition by CsA can be largely relieved by increasing  $[Ca^{2+}]$  (47, 48), and it depends on matrix ADP (251) and  $Mg^{2+}$  (250), which both are pore inhibitors per se (167).

Inhibition of MTP can also be observed with bongkrekate (198), whereas atractylate opens the pore (167, 198). Because atractylate and bongkrekate are selective inhibitors of the adenine nucleotide translocase (ANT), these experiments led to the suggestion that the MTP may be directly formed by the ANT (151), an issue that is discussed in section IV A 3.

Finally, the PT is potently inhibited at matrix pH below  $\sim 7.0$  (47) via reversible protonation of critical histidyl residues that can be blocked by diethylpyrocarbonate (247). This finding represented the first indication that the pore can be modulated by proton pumping and paved the way for the demonstration that the MTP is voltage dependent (33). Inorganic phosphate is a powerful MTP inducer whose effect can be only partly explained by buffering of matrix pH (193).

## 2. Membrane effectors

A high (inside-negative) membrane potential tends to stabilize the MTP in the closed conformation (33, 260, 261). We have postulated the existence of a voltage sensor that decodes the changes of both the transmembrane voltage (261) and of the surface potential (42, 61) into changes of the MTP open probability. This would easily account for pore opening after depolarization as such (33, 312, 330) and for the effects of a large variety of membrane-perturbing agents that can either inhibit or promote the PT. In general, amphipathic anions (like fatty acids produced by phospholipase  $A_2$ ) favor the PT, with an effect that cannot be explained by depolarization (61, 261). Conversely, polycations like spermine (192), amphipathic cations like sphingosine and trifluoperazine (61), and positively charged peptides (280) inhibit pore opening (see Ref. 42 for review). The putative voltage sensor may comprise critical arginine residues, as suggested by the recent finding that mitochondria treated with the arginine-selective reagents 2,3-butanedione or phenylglyoxal became resistant to pore opening by  $Ca^{2+}$  plus depolarization (114).

We recently described direct MTP regulation by electron flux within respiratory chain complex I, with an

increased open probability when flux increases (121). This led to the discovery that the PT is regulated by quinones, most likely through a specific binding site whose occupancy profoundly affects the open-closed transitions depending on the bound species. Binding of ubiquinone 0 ( $Ub_0$ ) or decylubiquinone prevents  $Ca^{2+}$ -dependent pore opening irrespective of the inducing agent, and the inhibitory effect of  $Ub_0$  and decylubiquinone (but not that of CsA) can be relieved by pore-inactive quinones like ubiquinone 5 (122). I have tentatively assigned a membrane site of action to  $Ub_0$  in the scheme of Figure 9, which might correspond to a binding site on respiratory chain complex I (122).

## 3. Molecular nature

Largely because of the effects of bongkrekate and atractylate on the PT, the early suggestion had been made that the pore may be formed by the ANT (151), possibly after oxidant-dependent dimerization (154). The ANT reconstituted in giant liposomes exhibits a striking high-conductance (but CsA-insensitive) channel activity that is stimulated by  $Ca^{2+}$  and displays a marked voltage dependence with prominent gating effects (63) that are consistent with the reported voltage dependence of the pore in intact mitochondria (261, 262, 312). Furthermore, after reconstitution in liposomes, the ANT can catalyze  $Ca^{2+}$ -dependent (but CsA-insensitive) malate transport that is inhibited by ADP and favored by atractylate (298). Although these observations certainly demonstrate that the ANT can form large channels in reconstituted systems, their relevance to the PT is less obvious. Most pore inducers and inhibitors, including CsA, do not affect the activity and the conformation of the translocase; MTP opening by a variety of oxidants (with the possible exception of diamide) is not accompanied by dimerization of the ANT (82); and it should be considered that the transition of the very abundant ANT from the “m” (bongkrekate-bound) to the “c” conformation (atractylate-bound) (310) is accompanied by major structural rearrangements (210) that may profoundly affect general membrane properties like the surface potential and thus modulate the PT indirectly (see discussion in Ref. 42). Our recent demonstration that specific quinones inhibit the PT (121) irrespective of the mode of induction (122) adds further questions because the only known mitochondrial ligands of quinones are the respiratory complexes involved in the Q cycle (115).

Because no single candidate protein can currently account for the complexity of PT modulation in isolated mitochondria, there has been a tendency to increase the number of the MTP putative components to include matrix CyP-D, the inner membrane ANT, the outer membrane porin, peripheral benzodiazepine receptor and Bcl-2, hexokinase bound to porin, and intermembrane

creatine kinase. I think that we should recognize that the structural basis for the PT remains undefined at present and that the related issue of whether the PT can be fully accounted for by a single channel modulated by multiple effectors remains an open question. Although it is conceivable that the MTP is a supramolecular structure involving (or regulated by) more than one protein, its molecular nature remains a matter for future work.

## B. Channel Kinetics and Population Dynamics

The MMC (MTP) flickers between closed and open states (186, 266, 328–330), but transient, reversible MTP openings would be missed in a population of isolated mitochondria unless these are synchronized, a condition that has so far been achieved only under rather selective incubation conditions in hypotonic media (170, 171) and in digitonin-treated Ehrlich ascites cells (116).

Changes in  $\Delta\psi$  resolved in individual mitochondria loaded with a fluorescent probe demonstrated transient, asynchronous cycles of depolarization-repolarization in individual mitochondria. After a variable length of time, depolarizations became long lasting and were accompanied by permeabilization to calcein (molecular mass  $\sim 620$  Da) (169). Pharmacological evidence suggests that both the reversible depolarizations (which are not accompanied by calcein diffusion) and the long-lasting, irreversible depolarizations are due to MTP opening (169). It appears conceivable that the MTP can open for times that are too short for diffusion of calcein (which is well below the MTP cutoff of 1,500 Da) and/or that the MTP can open in a form that displays a lower cutoff, which would correspond to a lower conductance state. The latter possibility is supported by the finding that during the synchronized cycles of MTP opening-closure in mitochondrial suspensions, the MTP is permeable to ions ( $H^+$ ,  $Ca^{2+}$ ,  $K^+$ ) but not to sucrose (170). This might correspond to a lower conductance state of the pore (172), as discussed more in detail below.

Synchronized MTP opening in isolated mitochondria in suspension can be achieved in protocols that exploit the pore voltage dependence. Graded depolarizations recruit increasing fractions of mitochondria to open the MTP (261), suggesting that either 1) the threshold potential for pore opening (the “gating potential”) is heterogeneous, and represents a continuum in a population of mitochondria in suspension, or 2) the resting  $\Delta\psi$  in a population of energized mitochondria is heterogeneous, and therefore a smaller depolarization is required for pore opening in the subfractions with lower  $\Delta\psi$  (261). The two explanations are not mutually exclusive. In Figure 10A, I follow the second interpretation for simplicity, in an example where the threshold is arbitrarily set at  $-160$  mV and mitochondria initially maintain a range of membrane

potentials above the threshold and therefore a closed pore (*left*). A moderate depolarization will recruit to the PT only mitochondria that are close enough to the threshold (Fig. 10A, *middle*), a condition where mixed populations of mitochondria with closed and open pores coexist (261); a deeper depolarization will then open the MTP in the whole population (Fig. 10A, *right*). A second situation can be envisioned in which the threshold rather than the resting  $\Delta\psi$  is changed and brought closer to the resting potential for example by an increased  $Ca^{2+}$  load (Fig. 10B). In this case, mitochondria with the lowest  $\Delta\psi$  (or the highest gating potential) will open the pore first and undergo collapse of  $\Delta\psi$  with  $Ca^{2+}$  release (Fig. 10B, *middle*).  $Ca^{2+}$  will be taken up by polarized mitochondria, raise their gating potential, and cause further spreading of the PT until all the population undergoes a PT (Fig. 10B, *right*). Finally, inhibitors (like ADP or  $H^+$ ) can move the gating potential in the opposite direction and further away from the resting  $\Delta\psi$  (Fig. 10C, *left*). In this case, depolarization may not be followed by a PT at all (Fig. 10C, *middle and right*).

Thus a PT may be either the consequence of depolarization (Fig. 10A) or its cause (Fig. 10B). Although a PT is always followed by depolarization (Fig. 10B), depolarization is not always followed by a PT (Fig. 10C), and whether a PT will occur or not under a given in vivo condition is not easy to predict. The MTP open-closed transitions are combinatorial events reflecting 1) the conflicting inputs of inducing an inhibitory factors acting at the same time and 2) the relative control strength exerted by each level of modulation.

## C. Consequences and Reversibility

The consequences of the PT and the issue of its reversibility can only be addressed in studies in vitro and largely depend on the experimental conditions. In the following discussion I distinguish primary consequences of the PT, i.e., those that are intrinsically linked to opening of the MTP, and secondary consequences, i.e., those depending on additional variables that can be manipulated in vitro but are much less predictable in vivo. The issue of reversibility, and its very definition, largely depends on understanding these variables.

The only primary consequence of MTP opening is mitochondrial depolarization, followed by equilibration of the (electro)chemical gradients of ions and of solutes with molecular masses below the pore cutoff. It must be stressed that collapse of ion gradients occurs irrespective of whether the MTP opens in a lower conductance state, whereas diffusion of larger solutes can be critically affected by either the pore size or the open time (169, 263, 264). Onset of the PT in vitro is followed by loss of matrix  $Mg^{2+}$  and of adenine and pyridine nucleotides (e.g., Ref.

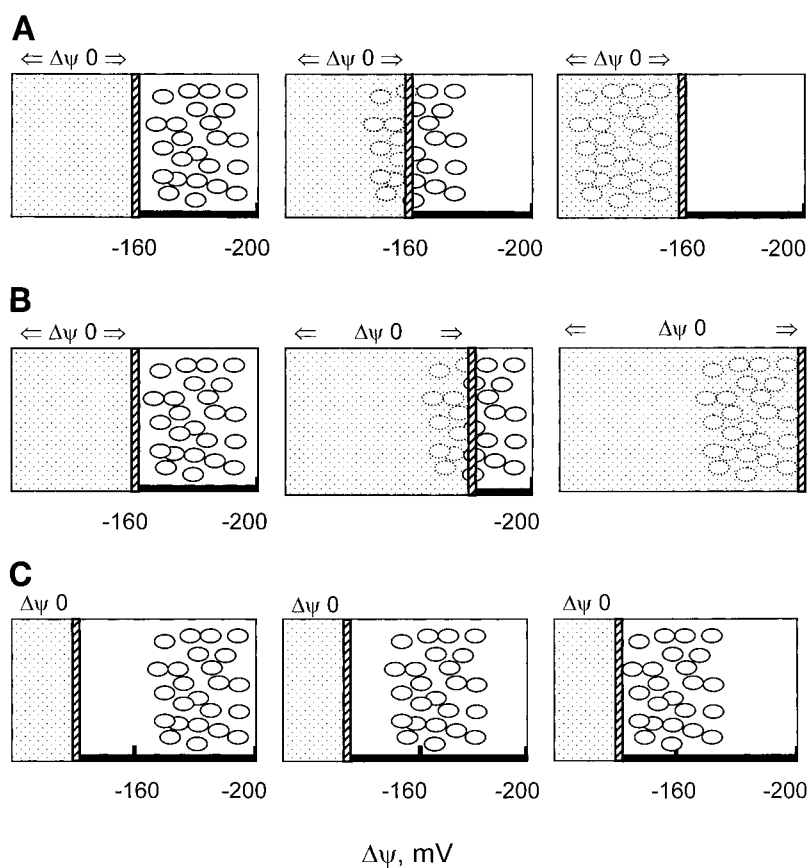


FIG. 10. Dynamics of permeability transition in a mitochondrial population. Indicated membrane potential difference ( $\Delta\Psi$ ) values only apply where abscissa is represented with a thick line. Shaded areas denote regions where  $\Delta\Psi = 0$  because of mitochondrial permeability transition pore (MTP) opening, while hatched bar denotes threshold potential at which MTP opening occurs. Individual mitochondria are depicted as ovals, and solid or broken line identifies mitochondria with a closed or open MTP, respectively. For explanation, see text.

282), which will tend to stabilize the MTP in the open conformation and convey the impression that opening is essentially an “irreversible” phenomenon. This is not the case, however. Closure of MTP is followed by mitochondrial resealing (86, 87) and repolarization (265), indicating that no permanent change of inner membrane permeability occurs as a consequence of a PT.

Although common PT assays *in vitro* are based on swelling, swelling is not a necessary consequence of pore opening. Swelling results from solute and water flux from the intermembrane/intercrystal spaces to the matrix only if there is an osmotic imbalance, but it does not occur for very short open times and/or for lower conductance states of the MTP (61, 169, 170). Permeability transition-dependent swelling *in vitro* can be completely prevented if the incubation medium contains low concentrations [e.g., 25 mM polyethylene glycol 3,400 (267)] of pore-impermeant solutes (267, 350). Thus whether swelling and outer membrane rupture occur after a PT *in vivo* is extremely hard to predict because of the high content of macromolecules of the cytosol and of the highly structured nature of the mitochondria themselves (212). In any case, it must be stressed that mitochondria are able to shrink and fully recover energy-linked functions even after PT-dependent large-amplitude swelling with outer membrane rupture in saline media, provided that cyto-

chrome *c* is added back (265). This indicates that no permanent damage to mitochondria occurs even after large-amplitude swelling, which remains one of the most useful tools to analyze the kinetics of MTP opening in individual mitochondria *in vitro* (213).

The PT causes secondary consequences on *in vitro* respiration that depend on the substrate. With PN-linked substrates (like pyruvate, glutamate, and malate), MTP opening is followed by respiratory inhibition because matrix PN are readily lost at the onset of the PT (354), and respiration can be at least partially restored by the addition of  $\text{NAD}^+$  (121). With complex II-linked substrates (like succinate), the PT is rather followed by uncoupling (e.g., Ref. 121). The consequences of a PT on respiration *in vivo* will therefore depend on whether, and to what extent, PN depletion and subsequent hydrolysis by intermembrane glycohydrolase take place (50). Irrespective of whether respiration is inhibited or stimulated, the collapse of the  $\Delta\tilde{\mu}_H$  caused by the PT will curtail ATP synthesis as long as the pore is open.

#### D. Potential Role in $\text{Ca}^{2+}$ Release

If a  $\text{Ca}^{2+}$  concentration gradient exists between the matrix and the external medium (or the cytosol), onset of

the PT will lead to  $\text{Ca}^{2+}$  release. The possibility that the PT might serve as a mitochondrial  $\text{Ca}^{2+}$  release channel has been discussed in the field (145), but until very recently, major conceptual obstacles prevented serious consideration and experimental testing of this idea. As discussed above, many of these hurdles (like pore reversibility and onset of structural damage) have probably been overestimated. The lack of channel selectivity and its large size deserve a separate comment (see also Ref. 44).

1) Emergence of channels with different ion selectivity has been an evolutionary process, which is reflected by the high degree of homology among the main channel subfamilies. For example, an ancestral  $\text{K}^+$  channel may have given rise to both the voltage-gated and the cyclic nucleotide-gated  $\text{K}^+$  channels, followed by further evolution of the latter into the more complex voltage-gated  $\text{Ca}^{2+}$  and  $\text{Na}^+$  channels by small mutations coupled to gene duplication events (304). However, only the existence of  $\text{Ca}^{2+}$  and  $\text{Na}^+$  gradients across the plasma membrane, which could be exploited for signal transduction, conferred an evolutionary advantage to the independent emergence of different ion selectivities. As discussed in section II, no  $\text{K}^+$  and  $\text{Na}^+$  concentration gradients exist across the inner membrane. Thus MTP opening may lead to selective  $\text{Ca}^{2+}$  release without major consequences on  $\text{K}^+$  and  $\text{Na}^+$  homeostasis, whereas no evolutionary pressure existed for the development of cation selectivity. In this respect, the MTP is similar to the  $\text{Ca}^{2+}$  release channel of the sarcoplasmic reticulum, which operates as a selective channel despite the large size ( $>38 \text{ \AA}$ ), the high conductance for monovalent cations ( $\sim 1 \text{ nS}$  at saturating  $\text{K}^+$ ), the permeability to solutes like glucose (220), and the low permeability ratio when both  $\text{K}^+$  and  $\text{Ca}^{2+}$  are present ( $P_{\text{Ca}}/P_{\text{K}} \sim 6$ ) (317).

2) Even after complete depolarization,  $\text{Ca}^{2+}$  efflux down its concentration gradient via a selective channel (like the uniporter) is opposed by the buildup of a  $\text{Ca}^{2+}$  diffusion potential (5). According to the Nernst equation, the maximal magnitude of the  $\text{Ca}^{2+}$  diffusion potential is  $-30 \text{ mV/decade}$ , which will be reduced (with a proportional increase of the  $\text{Ca}^{2+}$  efflux rate) by the sum of all charge-compensating currents (influx of positive charges, efflux of negative charges, or both). Because the inner membrane has a very low permeability to charged species in general, the basal rate of  $\text{Ca}^{2+}$  efflux would be extremely low and could only be increased by independent modulation of the membrane ion permeability as can be achieved *in vitro*, e.g., by increasing the  $\text{H}^+$  conductance (43). A nonselective pore of large size confers the clear advantage of providing charge compensation within the channel itself, thus allowing maximal  $\text{Ca}^{2+}$  flux (i.e., at zero potential). This would guarantee fast  $\text{Ca}^{2+}$  release even for vanishingly small  $[\text{Ca}^{2+}]$  gradients, with regula-

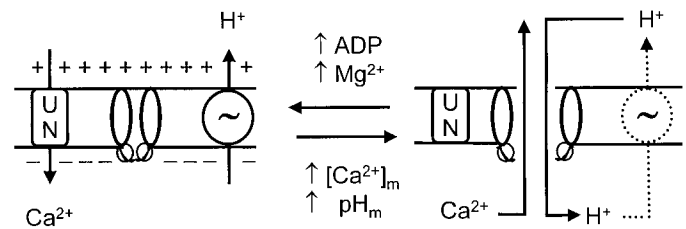


FIG. 11. MTP as a  $\text{Ca}^{2+}$  release channel. ~ within circle, redox-driven  $\text{H}^+$  pumps; twin barrels, MTP; UN,  $\text{Ca}^{2+}$  uniporter.

tion being directly achieved through modulation of the pore open time.

These concepts are summarized in Figure 11. As a consequence of a  $\text{Ca}^{2+}$  signal (and/or of the accompanying depolarization and matrix pH increase), MTP opening occurs (Fig. 11, right). Fast  $\text{Ca}^{2+}$  efflux ensues that is compensated by  $\text{H}^+$  flux within the channel (the same result could be achieved by  $\text{K}^+$  or  $\text{Na}^+$  influx, or by anion efflux). The drop of  $\Delta\tilde{\mu}_{\text{H}}$  would be followed by ATP hydrolysis, with increase of matrix  $[\text{ADP}]$  and  $[\text{Mg}^{2+}]$ . The combination of these events would result in MTP closure and repolarization (Fig. 11, left). This could be the basis for transient MTP openings *in vivo*, a possibility that is supported by recent studies based on a calcein entrapment technique that may reveal pore openings even when the  $\Delta\psi$  is apparently constant (263, 264), and by the finding that the mitochondrial  $\Delta\psi$  may oscillate *in vivo* (204, 291). It is hopeful that the increased understanding of pore regulation *in vitro*, and the development of better methods of detection (264) and of more selective inhibitors (122, 246), will allow rigorous testing of this working hypothesis in intact cells.

## V. APPENDIX

### A. $\text{Ca}^{2+}$ -Binding Proteins

Several  $\text{Ca}^{2+}$ -binding proteins have been purified from mitochondrial fractions that facilitate electrical  $\text{Ca}^{2+}$  fluxes in reconstituted systems, but their molecular structure remains to be elucidated (11, 222, 321, 368). A new member of the mitochondrial metabolite carrier superfamily (Aralar), which binds  $\text{Ca}^{2+}$  through EF-hand binding domains, has been recently cloned (94). Finally, an association with mitochondria has been reported for synexin (annexin VII), a widely distributed member of the annexin gene family that forms  $\text{Ca}^{2+}$  channels and drives  $\text{Ca}^{2+}$ -dependent membrane fusion (324).

### B. Transport of $\text{Mg}^{2+}$

Mitochondria can take up and extrude  $\text{Mg}^{2+}$  through pathways that remain largely undefined (104, 181). A

cAMP-dependent pathway for fast release of mitochondrial  $Mg^{2+}$  that may be hormonally regulated has been described (288–290). This issue has been the subject of some controversy (10), and the interested reader is referred to References 104, 181 for general reviews.

### C. Electrophysiology

In addition to the already-mentioned MMC-MTP (186, 266) and the  $K_{ATP}$  channel (176), other inner membrane channels have been characterized by electrophysiology, but none could be assigned to the cation transport pathways described in this review. For these, the reader can consult Reference 320 and the *Journal of Bioenergetics and Biomembranes Special Issue on Mitochondrial Channels* (volume 28, number 2, 1996). For the outer membrane voltage-dependent anion channel, see also the specific review by Colombini et al. (77), while an account of the channel activity of members of the Bcl2 protein family, which largely localize to the outer membrane, can be found in Reference 309.

### D. Mitochondria and Cell Death

Largely because of the PT, mitochondria became the focus of intense research in toxic, hypoxic, and ischemic cell injury (111, 174, 257). This was followed by studies addressing the role of the PT in glucocorticoid-induced apoptosis (367) and of its potential role in the release of an intermembrane apoptogenic protein (325). The discovery that cytochrome *c* is required for apoptosis in a cell-free system (202, 372), and that its release regulates apoptosis (209, 365), led to an explosion of the interest in mitochondria and cell death. Coverage of this fast-moving field is outside the scope of this review but is specifically addressed in Reference 46a. The interested reader can find a detailed account of the many facets of mitochondrial involvement in cell death in the *Biochimica et Biophysica Acta Bioenergetics Special Issue Mitochondria in Cell Death* (35), which includes  $Ca^{2+}$  signaling (141, 172, 287), oxidative stress and ischemia-reperfusion injury (102, 152, 197), excitotoxicity (229, 243), Bcl-2 proteins (276), cytochrome *c* (66), the PT (137, 196, 326), and neuromuscular and neurodegenerative diseases (24, 103, 307).

#### NOTE ADDED IN PROOF

Two additional papers on the PT should be considered. Crompton and co-workers (*Eur. J. Biochem.* 258: 729–735, 1998) have described a CsA-sensitive permeabilization of liposomes reconstituted with CyP-D together with the ANT and porin that copurified on a CyP-D affinity column. On the other hand, Kinnally and co-workers (*J. Biol. Chem.* 271: 4846–4849,

1996) have recorded a MMC in yeast mitochondria where expression of all ANT isoforms had demonstrably been knocked out. These findings add to the open questions about the nature of the PT and its relationships with the ANT.

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