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Minireview

High-Conductance Pathways in Mitochondrial Membranes

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The outer and inner membranes of mitochondria have recently been studied with the patch clamp technique. What has emerged is still an ill-defined picture for either membrane, primarily for the wide range of conductances found. Interestingly, however, a few conductances (in the range of 10–80 pS) seem to be ubiquitously distributed. Parallel studies *in situ* and in reconstituted systems have allowed the assignment to distinct membrane locations of some conductances, whose physiological role is, however, not yet elucidated.

KEY WORDS: Channels; intracellular membrane channels; mitochondrial channels; patch clamp.

INTRODUCTION

The voltage-dependent anion channel (VDAC) of the outer membrane (OM) was the first high-conductance ion channel described in mitochondria (Schein *et al.*, 1976; Colombini, 1979). At the time, and for several more years, this discovery did not receive great attention in the context of mitochondrial physiology because of the undeclared dogma that the *raison d'etre* of mitochondria lay at the level of the inner membrane, site of energy coupling processes. In addition, uphill transport across the inner membrane was found to occur through carriers with mechanisms adhering strictly to the tenets of the chemiosmotic hypothesis (LaNoue and Schoolwerth, 1979; Skulachev, 1988). These circumstances thus lent credence to the idea that mitochondrial permeability could not be due to high-conductance pores, or, if these existed as VDAC in the OM, that they were probably mere leftovers of the eusymbiotic origin of the organelle.

However, since the discovery of a second highly conducting element, this time belonging to the inner membrane (IM) (Sorgato *et al.*, 1987), a real abundance of other conductances have been reported to exist in mitochondria (Kinnally *et al.*, 1987, 1989; Thieffry *et al.*, 1988; Dihanich *et al.*, 1989; Petronilli *et al.*, 1989; Sorgato *et al.*, 1989; Moran *et al.*, 1990; Moran *et al.*, 1991; Costa *et al.*, 1991; Zorov *et al.*, 1991). It is fair to say that a clear role for these channels has not yet been found. Nonetheless, in the light of these new findings, the concept of transport mechanisms at the basis of mitochondrial permeability needs to be extended, and the role of the OM reevaluated.

This paper will deal mainly with the electrophysiological description of the outer and inner membranes of mitochondria, resulting from our experience with patch clamping mitochondrial membranes, either *in situ* or incorporated in liposomes.

CONDUCTANCES OF THE INNER MITOCHONDRIAL MEMBRANE

Studies *in situ*

Methods

The patch clamp technique (Hamill *et al.*, 1981) has been applied directly to the mitochondrial IM (mitoplasts), following removal of the outer one. This was accomplished by subjecting mitochondria, derived mostly from mouse liver, to steps of osmotic shock of different duration, depending on whether the animal

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had been supplied (Sorgato *et al.*, 1987), or not (Sorgato *et al.*, 1989), with cuprizone, a copper chelating agent which induces the formation of mitochondria of larger size (Tandler and Hoppel, 1973). This particular diet, however, did not seem to influence the electrophysiology of the membrane (Sorgato *et al.*, 1989).

As apparent from morphological investigations with the electron microscope, the swelling–shrinking treatment, though severe and prolonged, was unable to remove completely the OM (see Fig. 1a in Sorgato *et al.*, 1987). OM patches of different extension remain firmly bound to the inner one through the contact sites, zones where the two membranes are closely and tightly apposed. Fortunately, under the light microscope of the patch clamp setup, these "cap" areas can be distinguished fairly easily (see Fig. 1c in Sorgato *et al.*, 1987) and thus be avoided.

Results

The first high-conductance channel described in the IM was of 107 pS (in 150 mM KCl), found first in liver mitoplasts from cuprizone-fed animals (Sorgato *et al.*, 1987) and soon after also found in mitoplasts of untreated animals (Sorgato *et al.*, 1989). As mentioned above, this finding was quite unexpected and the electrical features of this channel did not help in understanding of its function. For instance, its voltage sensitivity was such as to allow the opening of the pore at positive voltages or at small negative voltages (> -100 mV), inside the organelle. As it turns out, this range of values is considered unphysiological for mitochondria since under these conditions no synthesis of ATP can occur (Sorgato *et al.*, 1985; Zoratti *et al.*, 1986). Finally, the channel ion permeability was rather poorly specific.

The sharpness of the voltage dependence of the 107-pS channel is so peculiar as to enable its easy identification in a patch, as in fact was done in other laboratories (Petronilli *et al.*, 1989; Antonenko *et al.*, 1991; Kinnally *et al.*, 1991). Yet other conductances were also found in the IM (Thieffry *et al.*, 1988; Kinnally *et al.*, 1989; Petronilli *et al.*, 1989; Costa *et al.*, 1991; Zorov *et al.*, 1991). In our hands, the patch clamping of the bare membrane (i.e., when the pipette is not covering the "cap" region) shows the frequent presence of conductance values ranging from approximately 10–80 pS (Sorgato *et al.*, 1989; Moran *et al.*, 1991). Apparently these conductances are found irrespective of the sign of the imposed potential, which strongly argues for a lack of voltage dependence. However, as they are almost always associated with each other and with larger conductances, their detailed analysis in term of selectivity and voltage gating has been precluded.

Conductances up to approximately 180–200 pS have also been found in the IM (Sorgato *et al.*, 1989), with a more frequent peak around 120–140 pS. However, these conductances generally appear less frequently than those described above.

In Table I, a summary of these results is presented.

Table I. Summary of Conductances Present in Mitochondrial Membranes[a]

Membrane fraction	Conductance [pS]	Voltage dependence	Method
Inner membrane	10–80	no	1,2
	107	yes	1,2
	120–200	no	1,2
Outer membrane	10–50	no	3,4
	99	yes	3,4
	156	yes	3,4
	220	yes	3,4
	307	yes	3,4
	535	no	4
Contact sites	10–80	no	5
	475*	no	5
	550	no	5
	up to 1 nS	no	5

[a]Data were collected from patch clamping, in symmetrical 150 mM KCl solution, of: 1, mouse liver mitoplasts; 2, proteoliposomes containing an IM fraction or mitoplast or mitochondrial membranes; 3, integral mouse liver mitochondria; 4, proteoliposomes containing the isolated OM or mitochondrial membranes; 5, proteoliposomes containing a mitochondrial fraction enriched in contact sites. In the Table, 1–5 refer to the method used, as outlined above. The asterisk indicates that the 475 pS conductance had two distinct substates, of 245 and 373 pS, respectively. For all experimental details, appropriate references are given in the text.

Studies with Proteoliposomes

Methods

The use of proteoliposomes, enlarged sufficiently to be amenable to the patch pipette, was initiated to check whether a partially purified mitochondrial IM fraction contained ion channels (Sorgato *et al.*, 1989). This method, which involves careful dehydration–rehydration of the protein–lipid mixture, proved to be successful for the 107-pS channel (see below). From thereon mitochondria, mitoplasts, the isolated OM, or the contact site fraction have all been incor-

porated into liposomes and electrophysiologically characterized.

When a comparison was possible with data obtained *in situ* (as for the outer and inner membranes), the reconstituted systems seemed to reproduce native conductances with good fidelity (Sorgato *et al.*, 1989; Moran *et al.*, 1990, Moran *et al.*, 1991). The method described by Criado and Keller (1987) thus has turned out to be a useful tool for the study of mitochondrial electrophysiology (see also Costa *et al.*, 1991). Additionally, equivalent results are obtained with proteoliposomes containing either mitochondria or mitoplast membranes enriched in lipids by freezing and thawing. Thus it appears that freezing and thawing *per se*, or in combination with the procedure for proteoliposomes formation, is not harmful to mitochondrial channels (Moran *et al.*, 1991).

Results

Proteoliposomes containing mitoplasts or mitochondria showed all the conductances found in integral mitoplasts (Moran *et al.*, 1991) (Table I). However, when a particular IM fraction is used (Galante *et al.*, 1981), the 107 pS and lower conductances are the most frequently observed (Moran *et al.*, 1990) (Table I). Importantly, the voltage dependence of the 107-pS conductance in reconstituted liposomes is comparable to (albeit less pronounced than) that of the same conductance in mitoplasts (Sorgato *et al.*, 1987, 1989).

CONDUCTANCES OF THE OUTER MITOCHONDRIAL MEMBRANE

Studies *in situ*

Methods

The conductances of the OM of mitochondria have been studied for many years but only in planar bilayers (for reviews see Colombini, 1986 and Benz, 1990). The patch clamp technique was first applied by Tedeschi *et al.* (1987), and more recently by us (Moran *et al.*, 1991), to integral liver mitochondria. We found that, because of their small dimension and particular texture, the OM of mitochondria was not easily amenable to the formation of high-resistance seals. In our experience, seals of maximally 10 GΩ resistance formed in approximately 10% of the attempts. Electron microscopy of liver mouse mitochondria, suspended in the experimental medium (containing 150 mM KCl), is shown in Fig. 1.

Results

In the OM of intact mitochondria, conductances with values of approximately 100, 150, 220, and 300 pS are found (Moran *et al.*, 1991) (Table I). In all cases, the probability of opening is higher at positive potentials. (Here, the voltage sign refers to that of the pipette in a mitochondria-attached patch.) These features clearly depart from those reported for VDAC in planar membranes, where the conductance (around 600 pS in 150 mM KCl) drops to approximately half when applied voltages exceed ± 30 mV (Colombini, 1979, 1986; Benz, 1990).

As in the IM, conductances ranging from approximately 10 to 50 pS were also present in the OM patches (Moran *et al.*, 1991) (Table I).

Studies with Proteoliposomes

Methods and Results

Nonsynaptosomal mitochondrial OM, obtained from rat brain (Sandri *et al.*, 1988), or whole mouse liver mitochondrial membranes, isolated as in Sorgato *et al.* (1987), have been incorporated in liposomes as outlined above. Electron microscopy of the isolated OM is shown in Fig. 2.

High-resistance patch seals (greater than, or equal to, 20 GΩ) can be routinely formed with these proteoliposomes (Moran *et al.*, 1991). The electrical behavior of the reconstituted OM is superimposable on patterns evidenced *in situ*, with the addition of a more frequent appearance of a voltage-independent 530-pS conductance (Table I), already detected in contact site fractions (Moran *et al.*, 1990) (see below). The ionic selectivity of a proteoliposome patch, containing several conductances, was found to be slightly cationic (Moran *et al.*, 1991).

CONDUCTANCES OF THE CONTACT SITE FRACTION

Studies with Proteoliposomes

Methods

A membrane fraction, enriched in contact sites (according to the biochemical criteria of Kottke *et al.*,

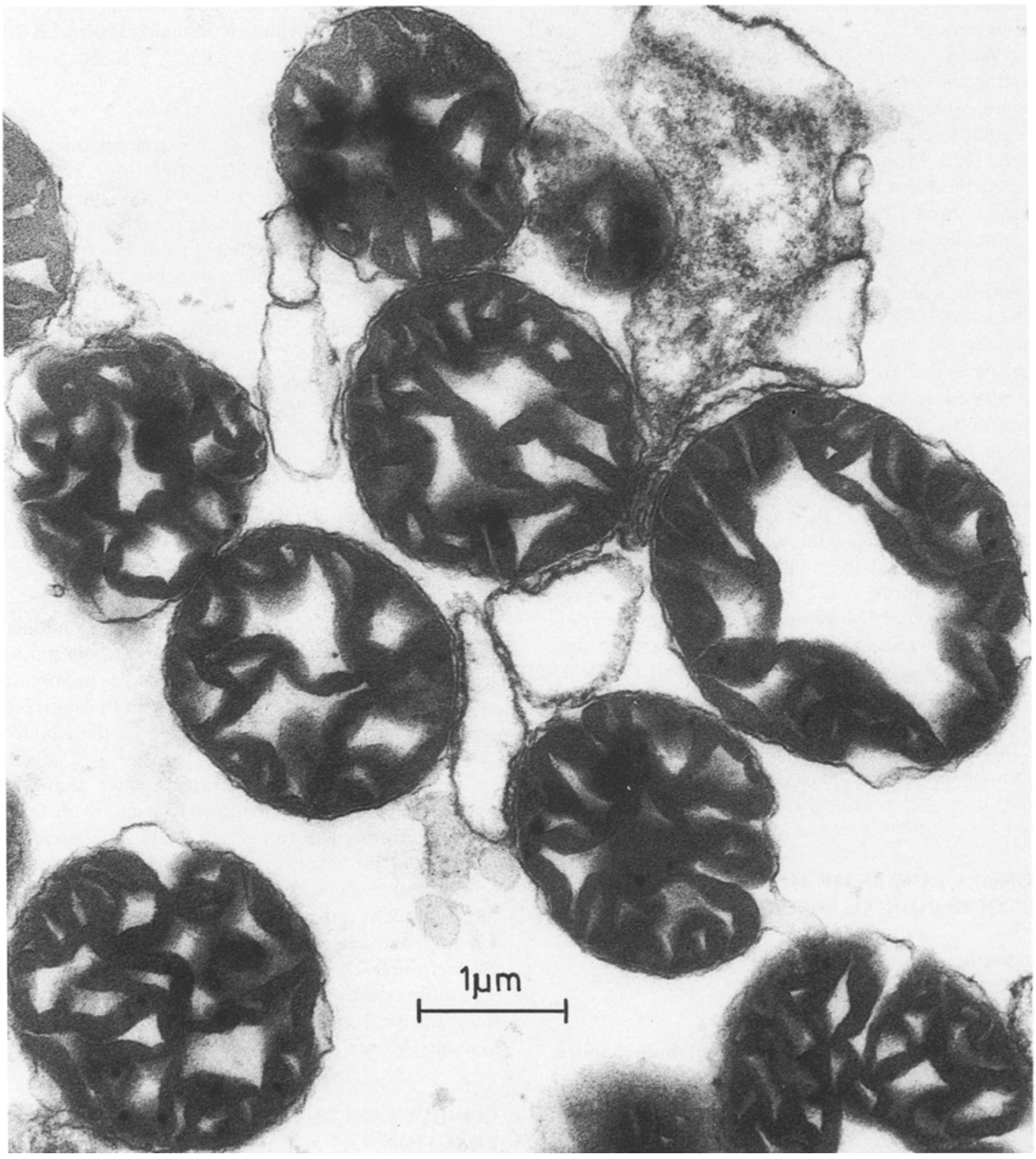

Fig. 1. Electron micrograph of mitochondria in 150 mM KCl (courtesy of Prof. C. Milanesi and Mr. V. Miolo of the Dip. di Biologia, Universitá di Padova). An aliquot of mouse liver mitochondria, isolated according to Sorgato *et al.* (1987) and suspended in 150 mM KCl, 20 mM Hepes-KOH (pH 7.2), and 0.1 mM $CaCl_2$, was fixed with four volumes of 1.25% glutaraldehyde and postfixed with 1% osmium tetroxide (in 0.12 sodium cacodylate, pH 7.2). The sample was dehydrated in ethanol and embedded in EPON. Thin sections were counterstained with 1% uranylacetate and lead citrate. This picture shows that the 150 mM KCl-containing medium is not harmful to the integrity of the OM.

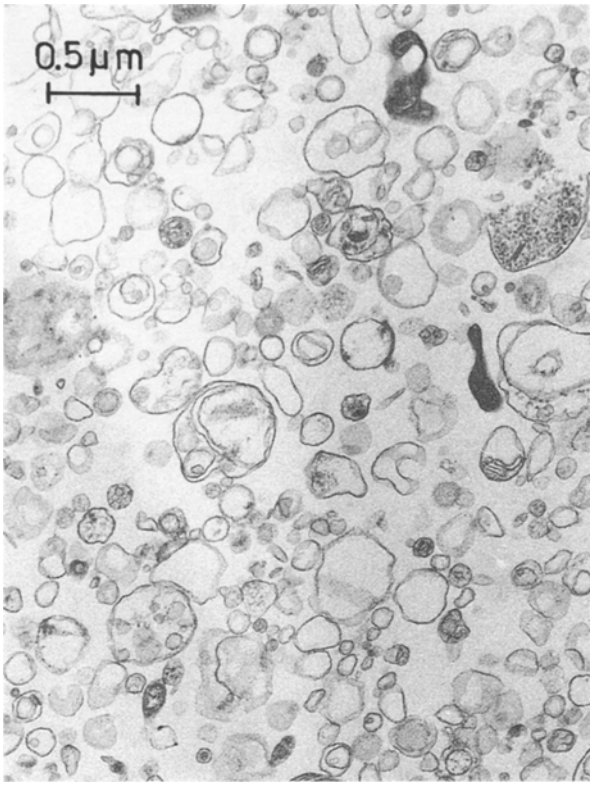

Fig. 2. Electron micrograph of the isolated OM (courtesy of Dr. G. Fox of the Max-Planck-Institut für biophysikalische Chemie, Goettingen). Nonsynaptosomal rat brain outer mitochondrial membrane was isolated and characterized as detailed in Moran *et al.* (1990). An aliquot was suspended in 250 mM mannitol and 50 mM Hepes-KOH (pH 7.2) and fixed and postfixed as detailed in the legend to Fig. 1. The sample was then stained *en bloc* with 1% uranyl acetate, dehydrated in ethanol, and embedded in EPON.

1988) was obtained from nonsynaptosomal rat brain mitochondria (Sandri *et al.*, 1988) and incorporated in liposomes, as detailed above.

Electron microscopy of this fraction showed at least three types of structures to be present (Fig. 3a): unilamellar vesicles like those in the OM fractions but with a larger diameter (cf Fig. 2), double-membrane vesicles with a point of adhesion (marked with an arrow) as in Kottke *et al.* (1988), and double-membrane vesicles with evident retainment of cristae. In this latter case (Fig. 3b), the inner and outer membranes were intimately apposed through extended contacts.

Results

Contact sites between the two mitochondrial membranes have long been known to exist (Hacken-

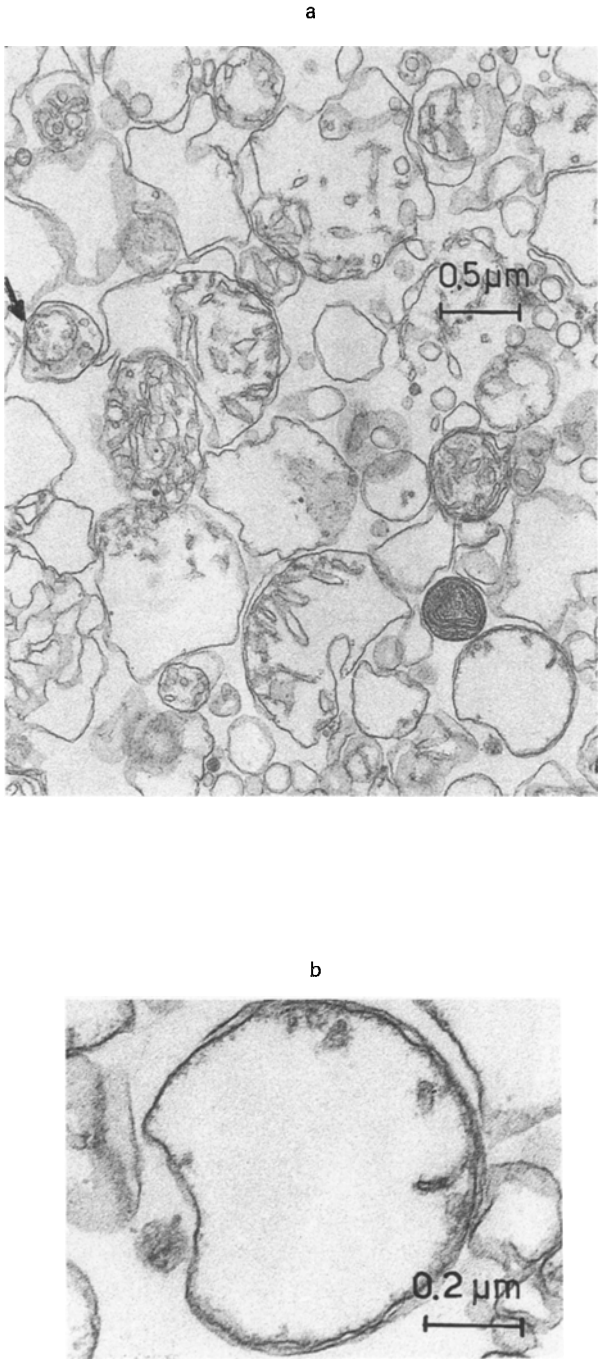

Fig. 3. Electron microscopy of the isolated contact site fraction (courtesy of Dr. G. Fox of the Max-Planck-Institut für biophysikalische Chemie, Goettingen). Nonsynaptosomal rat brain mitochondrial contact site fraction was isolated and characterized as in Moran *et al.* (1990). For electron microscopy, an aliqout was treated as detailed in the legend to Fig. 2. In Fig. 3b, a vesicle with retainment of cristae, present in the bottom right of Fig. 3a, is shown at higher magnification.

brock, 1968). However, only recently have they been implicated in such physiological roles as the import of precursors (Schleyer and Neupert, 1985), the formation of mitochondrial networks (Amchenkova *et al.*, 1988), or the function of VDAC (Benz *et al.*, 1990).

Except for the presence of small currents (some of which correspond to conductances as low as 6 pS, Moran *et al.*, 1990), the electrical pattern of the contact site fraction departed markedly from that of the outer or inner membrane. Indeed, its characteristic feature is the presence of very large currents apparently not sensitive to voltage. The most frequently observed currents correspond to conductances of 475 and 550 pS, the less frequent ones to a whole range of even higher values, up to approximately 1 nS. Another distinctive feature of this fraction is the partial closure of the 475-pS channel to levels of 245 and 373 pS (Moran *et al.*, 1990). These results are summarized in Table I.

By definition, contact sites include regions of both the outer and inner membrane of mitochondria. An unresolved question is whether the entire junction or hemijunctions are reconstituted in the patched proteoliposome membrane. Isolated liver gap junctions seem to incorporate as a whole in single bilayers (Spray *et al.*, 1986). However, the situation is not yet defined for the supposed analogous mitochondrial structure, and so we do not know to which connecting part the detected currents are to be attributed.

DISCUSSION

The *in vivo* ionic permeability of the IM of mitochondria must be finely controlled in order to allow the formation and maintenance of the protonic potential difference necessary for energy-demanding reactions, the most important being the synthesis of ATP. This necessarily implies that most of the conductances detected by patch clamping the IM and found operative at potential values close to the physiological mitochondrial membrane potential (Kinnally *et al.*, 1989; Petronilli *et al.*, 1989; Moran *et al.*, 1990; Zorov *et al.*, 1991) cannot occur as such *in vivo*. In order to make sense of these pores, one may postulate that, under physiological conditions, they probably transport molecules different from the inorganic ions used in these experiments. In this respect, macromolecules are the best candidates (Pfanner and Neupert, 1990). Alternatively, one may postulate that the channels operate *in vivo* under the strict control of factors or

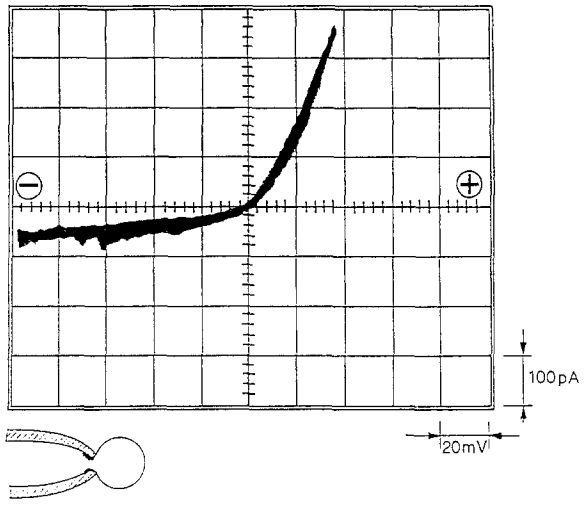

Fig. 4. Current–voltage relationship of the IM. This was obtained by patch clamping mitoplasts in the whole cell configuration, under steady-state conditions. Symmetrical 150 mM KCl solutions were used. Potential values refer to those found inside the vesicle.

modulators which are lost or inactivated in the *in vitro* experiments.

A different consideration applies to the 107-pS channel. As it turns out from its electrical analysis, the probability for this pore to be open at physiological potentials in minimal (Sorgato *et al.*, 1987, 1989; Moran *et al.*, 1990). Importantly, the same behavior was detected for the entire IM in a whole-mitoplast patch (Sorgato *et al.*, 1987, 1989, and Fig. 4). Thus, the question that naturally springs to one's mind is what might be the sense in harboring an apparently inactive protein in the inner membrane, given also that mitochondria need to transport this protein from the cytoplasm.

This paradox probably finds its explanation in the outcome of our analysis of the contact site fraction, where the 107-pS conductance was rarely, if ever, found (Moran *et al.*, 1990). In that context it was suggested that the voltage-induced closure of the 107-pS channel was a safeguard mechanism to protect against collapse of the mitochondrial potential. This voltage-induced closure might become operative whenever this particular protein was in places other than contact sites, or when the junction connecting the two membranes was damaged. On the other hand, when in the contact sites, the 107-pS channel might behave differently, either because of the particular lipid composition or because of its contiguity with other proteins.

Another important question, related to the wide

range of conductances observed in the IM, regards the number of proteins involved and their location (i.e., in the contact sites or not). So far, from kinetic studies (Petronilli *et al.*, 1989) and by use of drugs (Szabo and Zoratti, 1991; Antonenko *et al.*, 1991) or different calcium levels (Kinnally *et al.*, 1991), it seems that at least two proteins can be distinguished: one responsible for the conductance around 100 pS, and another for the conductance range 0.4–1.3 nS. In our opinion, attention should be particularly addressed to the so-called low conductances since they are present in both mitochondrial membranes as well as in the contact site fraction. Although obviously hypothetical, one may envisage the existence of a unique protein having the same role in the different membranes.

Except for the work of Tedeschi *et al.* (1987) and Kinnally *et al.* (1987), the mitochondrial OM has been studied previously only in planar membranes. The results of our recent investigation (Moran *et al.*, 1991), carried out with the patch clamp technique, differ substantially from those reported earlier. In essence, we detect single channels with electrical properties different from VDAC's in planar membranes. Such single-channel events were not reported in the earlier patch-clamp studies, involving membrane currents from low-resistance patches. Contrary to the results of the latter studies, the current intensities measured in our high-resistance integral mitochondrial membrane patches were not comparable with that expected by the supposed density of VDAC proteins in the OM (Freitag *et al.*, 1982; De Pinto *et al.*, 1987).

Recently, Wunder and Colombini (1991), applying the patch clamp technique to liposomes containing *Neurospora crassa* mitochondrial membranes, showed conductance behavior that could be attributed to VDAC. However, they also found that VDAC deactivates or leaves the patch rapidly. This fact, or the voltage-pulse protocol used by us (perhaps too rapid for the slow kinetics of VDAC), or a combination of the two factors, could explain the lack of detection of VDAC in our experiments. Whatever the real cause, the reproducibility of our data from *in situ* and reconstitution studies indicates that the OM of animal mitochondria is more dynamic and complex than anticipated.

CONCLUSIONS

As it stands, it is evident that mitochondrial electrophysiology raises more questions than it provides answers, probably because such investigation have started relatively recently. On the other hand, this same fact makes one confident that the combined effort of many laboratories will soon provide some explanation as to the physiology of this newly described mitochondrial permeability.

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