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Electrophysiology of the Inner Mitochondrial Membrane

Mario Zoratti[1] and Ildikó Szabó[1]

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The application of electrophysiological techniques to mitochondrial membranes has allowed the observation and partial characterization of several ion channels, including an ATP-sensitive K^+-selective one, a high-conductance "megachannel", a 107 pS anionic channel and three others studied at alkaline pH's. A reliable correlation with the results of non-electrophysiological studies has been obtained so far only for the first two cases. Activities presumed to be associated with the Ca^{2+} uniporter and with the adenine nucleotide translocator, as well as the presence of various other conductances have also been reported. The review summarizes the main properties of these pores and their possible relationship to permeation pathways identified in biochemical studies.

KEY WORDS: Ion channels, patch-clamp, mitochondrial megachannel, permeability transition, mitochondria.

INTRODUCTION

In the bioenergetics literature, ion transport across the inner mitochondrial membrane is often discussed with reference to "uniporters" rather than to "channels". The preeminence of the more general term stemmed in part from the lack of clear evidence on the mechanism underlying the observed electrophoretic ion fluxes. In recent years, however, the application of electrophysiological techniques has provided indisputable evidence that *bona fide* channels exist in the inner mitochondrial membrane. The job of matching the known channels of electrophysiological studies with the uniporters of bioenergetics is now under way, and much remains to be done. In this review we summarize the results of patch-clamp (see Fig. 1 for a brief description of the method) and bilayer studies, and attempt a correlation with the information obtained by other techniques (Table I). Since even moderately anion-selective channels carry a large flux of cations, and for the sake of completeness, anion-selective channels are also considered. We have devoted particular attention to our main area of interest, the permeability transition pore, which is often discussed in the context of Ca^{2+} transport studies. Related reviews have appeared (Kinnally *et al.*, 1992; Moran and Sorgato, 1992; Sorgato and Moran, 1993; Mannella, 1992)

[1] CNR Unit for the Physiology of Mitochondria, Department of Biomedical Sciences, University of Padova, Padova, Italy.

CATIONIC CHANNELS

The ATP-Sensitive K^+ Channel

Inoue *et al.* (1991) have characterized a small (about 10 pS in 100/33.3 mM K^+) channel in the membrane formed by fusion of rat liver mitoplasts obtained by digitonin treatment. The channels exhibited a degree of cooperative gating, resulting in conductance variations corresponding to multiples of the unitary conductance. The dependence of the conductance value on salt concentration was not reported. The channel was strongly selective for K^+, even over Na^+, as determined from reversal potential measurements. ATP, added to the matrix side of excised inside-out patches, inhibited channel activity in a Mg^{2+}-independent, reversible manner. ADP and GTP had no effect. A half-maximal decrease of the channel open probability was obtained with 0.8 mM ATP, and near complete inhibition with 2 mM.

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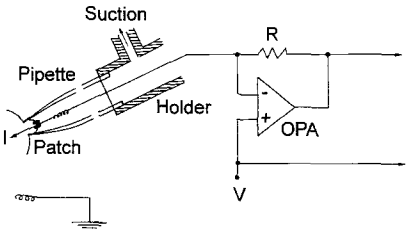

Fig. 1. Schematic illustration of the patch-clamp technique. A glass pipette, tapered to a tip diameter of 0.5–2 μm and fire-polished, is partially filled with an electrolyte solution bathing a wire electrode connected to a low-noise electronic device comprising a current-to-voltage converter and an operational amplifier (OPA). The electronics allow the precise control of the voltage applied between the wire electrode and a reference (ground) electrode in a chamber positioned above the objective of an inverted microscope, which also contains the cells (mitoplasts in our case) to be studied. The pipette holder is fixed to a micromanipulator which allows the tip of the pipette to be pressed against the target cell. Suction is applied to the inside of the pipette through the side-arm of the holder, so that a patch of cell membrane is pulled slightly inside the pipette, resulting in the formation of a high-resistance "seal" between the membrane and the glass. Current can then flow between the pipette electrode and the ground only through any open channel that may be present in the membrane patch. The opening or closing of a single channel produces measurable discrete variations in the current level. The patch may be excised (as depicted), thus exposing the inside surface of the membrane to the bath solution. Channel activity may be controlled by the transmembrane voltage applied or by ligand binding. Full details may be found, for example, in Hamill *et al.* (1991) and Sakmann and Neher (1983). The drawing is highly schematic and its scale varies.

Whether the presence of ATP on the cytoplasmic side of the patch affected channel operation was not reported. The authors also showed that channels partially inhibited by ATP were further inhibited by 4-aminopyridine and glibenclamide, thus identifying the channel as related to the ATP- and glibenclamide-sensitive K^+ channels observed in plasma membranes. The presence of this channel might account for the nearly perfect cationic patch selectivity observed by Antonenko *et al.* (1991a) in the presence of propranolol.

These properties strongly suggest that the channel may correspond to the K^+-selective, ATP-dependent, glibenclamide-sensitive channel (Paucek *et al.*, 1992; Manon and Guerin, 1993). Garlid's group associates the activity observed upon reconstitution in planar bilayers of a protein fraction containing mainly a 54-kDa polypeptide (Mironova *et al.*, 1981; Paucek *et al.*, 1992) with this channel. This activity is characterized by a conductance of some 30 pS in symmetrical 1 M KCl (Paucek *et al.*, 1992), or 24 pS in 0.1 M KCl (Mironova *et al.*, 1981), and by a tendency to close at high potentials of either sign (Mironova *et al.*, 1981). The channel appears to form cooperating oligomers in the bilayer (Mironova *et al.*, 1981). It should be noted, however, that the channel studied by Mironova, while strongly preferring cations over anions, did not select for K^+ over Na^+. This property might cast doubt over its identification with the one described by Inoue and with the K^+-selective channel of biochemical studies (Paucek *et al.*, 1992). It might, instead, correspond to the unselective channel observed by Diwan's group (see below). Interestingly, Mironova *et al.* did not observe channels in the their planar bilayer experiments if Ca^{2+} was present.

Table I. A Tentative Match Between Some Inner Mitochondrial Membrane Channels Identified by Suspension Studies and by Electrophysiology*

Suspension studies		Electrophysiology
ATP-sensitive K^+ channel[a,b]		10-pS ATP-sensitive K^+ channel[c]
Ca^{2+} uniport[d]	?	20-pS Ca^{2+}-selective channel[e]
Unselective M^+ uniport[f]	?	40-pS unselective channel[g]
Na^+ (Li^+) uniport[h]		?
PTP[d]		MMC[i,j]
IMAC[k]	?	15-pS pH-dependent channel[l,m]
?		107-pS anionic channel[n]
?		SMAC and INMAC[o]

* Question marks denote uncertainty or lack of information.
[a] Beavis *et al.*, 1993. [b] Paucek *et al.*, 1992. [c] Inoue *et al.*, 1991. [d] Gunter and Pfeiffer, 1990. [e] Mironova *et al.*, 1982. [f] Nicolli *et al.*, 1991. [g] Paliwal *et al.*, 1992. [h] Bernardi *et al.*, 1990; [i] Kinnally *et al.*, 1989. [j] Petronilli *et al.*, 1989. [k] Beavis, 1992. [l] Antonenko *et al.*, 1991a. [m] Kinnally *et al.*, 1992. [n] Sorgato *et al.*, 1987. [o] Hayman *et al.*, 1993.

In proteoliposomes containing the protein fraction mentioned above, Paucek *et al.* (1992) observed selective (over Na^+) K^+ uptake exhibiting a saturation behavior with a K_m of 32 mM. The authors reported that K^+ uptake by the vesicles was inhibited by both ATP and ADP (added externally) in the presence of Mg^{2+}, with K_m's of 45 and 280 μM, respectively. Inhibition required both Mg^{2+} and ATP. These results apparently differ from those of Inoue and coworkers, a discrepancy which might be attributed to the different concentration ranges employed, or to the presence of regulatory sites with different properties on the matrix and cytoplasmic sides.

The Ca^{2+} Uniporter

The V_{max} for Ca^{2+} transport driven by valinomycin-mediated K^+ diffusion from RLM has been estimated

as 1400 nmol (mg protein)$^{-1}$ min^{-1} at 30°C (Bragadin *et al.*, 1979). Coupled with an estimate of 1 pmol × (mg protein)$^{-1}$ for the density of uniporter molecules (Reed and Bygrave, 1974), this would yield a time-averaged minimum "turnover number" of 15,000 ions × (carrier molecule)$^{-1}$ × sec^{-1}, hardly compatible with anything but a channel. Mironova *et al.* (1982, 1994) have reported the purification from heart mitochondria of a peptide capable of inducing Ca^{2+}-selective electrophoretic transport across black lipid membranes. The channel apparently could give rise to many conductances corresponding to multiples of a unitary 20 pS one (in 12 mM $CaCl_2$, 20 mM Tris · Cl), presumably by aggregation and cooperativity phenomena. The peptide copurifies with a 40 kDa glycoprotein (orosomucoid). Antibodies raised against this preparation, but not antibodies raised against the pure orosomucoid, block Ca^{2+} uptake by mitoplasts (Saris *et al.*, 1993; Mironova *et al.*, 1994).

The Peptide-Sensitive Channel

The French group of Thieffry and Henry has investigated, mainly by the tip-dip and planar bilayer techniques, a cationic mitochondrial channel which these researchers have assigned to the outer membrane (Thieffry *et al.*, 1988, 1992; Henry *et al.*, 1989; Fèvre *et al.*, 1990, 1993, 1994, Chich *et al.*, 1991; Vallette *et al.*, 1994). This assignment was based on the fact that the channel properties were modified by trypsinization of whole mitochondria (Chich *et al.*, 1991). We have recently studied this channel in patch-clamp experiments on VDAC-less[2] yeast mitochondria suspended in a hyposmotic medium, which do not show any EM evidence for the presence of outer membrane (Zoratti *et al.*, 1994). In the course of most experiments, what appears to be a transition to the whole-cell configuration took place spontaneously, resulting in (1) the apparent change of polarity of the voltage dependence of the channels, (2) an increase in the number of observable channels, and (3) a marked change in the aspect of the mitoplast observed by phase contrast optics, which essentially disappeared, presumably because of the washout of the matrix space by the experimental medium (Szabó *et al.*, submitted). These observations suggest that at least some copies of the channel are present in the inner envelope as well; the topic is therefore included in this review.

The selectivity sequence of the channel is $K^+ > Na^+ > TEA^+ > Cl^- >$ acetate, with $P_{Na}/P_{Cl} = 3$ (Thieffry *et al.*, 1988, 1992). The mammalian channel apparently exists in two forms, exhibiting transitions between four conductance states, differing by 100, 220, and 220 pS (150 mM NaCl) in one case (type I), and by 220 pS intervals in the other (type II) (Thieffry *et al.*, 1988). In the case of yeast several substate levels were observed, the main ones being separated by 330 pS (Fevre *et al.*, 1990; Thieffry *et al.*, 1992). In both cases, the occupancy of the conductance states was markedly voltage-dependent. In tip-dip experiments negative bath potentials favored the lowest (type I) or the intermediate (type II) conductance state (Thieffry *et al.*, 1988). A residual conductance was always present in tip-dip and bilayer experiments, suggesting that the channels may never close completely. A more complicated voltage dependence was reported for the yeast channel: in bilayer experiments, voltages below −30 mV (*cis* side) were reported to drive the channel to the lowest conductance state, while around zero mV the fully open state was preferred. Positive potentials induced fast transitions between substates and eventually led to long-lasting closures (Fevre *et al.*, 1990; Thieffry *et al.*, 1992). In our experiments on VDAC-less yeast mitoplasts the channel inactivated rapidly at potentials negative on the matrix side of the patched membrane, and it activated at positive (unphysiological) potentials. Negative potentials also resulted in the preferential occupation of lower-conductance states (and vice versa). When mainly outside-out membrane vesicles were fused to planar bilayers, the same behavior was observed, or, in some cases, the channels inactivated at both positive and negative voltages. Complete closures were routinely observed (Szabó *et al.*, submitted).

The channel is referred to as "peptide sensitive channel" (PSC) because the presence of μM concentrations of leader peptides induces a remarkable change of its kinetic properties; the frequency of transitions to low-conductance states increases dramatically, i.e., a voltage-dependent channel fast

[2] Abbreviations used: AdNT: adenine nucleotide translocator; CCCP: carbonyl cyanide *m*-chlorophenylhydrazone; EM: electron microscopy; FCCP: carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; IMAC: inner membrane anion channel; IMM: inner mitochondrial membrane; INMAC: intermediate anion channel; mBzR: mitochondrial benzodiazepine receptor; MMC: mitochondrial megachannel; P_0: open probability; PSC: peptide-sensitive channel; PPIX: protoporphyrin IX; PTP: permeability transition pore; SMAC: small anion channel; UCP: uncoupling protein; VDAC: voltage-dependent anion channel (mitochondrial porin).

block develops (Henry *et al.*, 1989; Févre *et al.*, 1994). Thieffry and coworkers have presented evidence suggesting that the phenomenon may be due to the translocation of the peptide through the channel (Thieffry *et al.*, 1992; Vallette *et al.*, 1994).

The Adenine Nucleotide Translocator

In intact mitochondria, Beavis *et al.* (1993) reported that a K^+ permeation pathway was inhibited by ATP, ADP, and AMP with IC_{50} values of 0.5, 2.3, and 8 μM, respectively, while GDP was ineffective. The uptake was glibenclamide-insensitive, but it was inhibited by carboxyatractyloside, a well-known inhibitor of the adenine nucleotide translocator (AdNT). Other AdNt inhibitors also affected K^+ fluxes. The authors nonetheless concluded, on the basis of results showing differential inhibition of the AdNT and of K^+ fluxes, that K^+ uptake probably was not mediated by the AdNT.

An opposite conclusion had previously been reached by Halestrap and coworkers, who observed that swelling of liver mitochondria in KCl media was stimulated by low (0.1–1 μM) Ca^{2+} concentrations (Halestrap *et al.*, 1986; Halestrap, 1989). They reported that the effect required phosphate, and exhibited a preference for K^+ over other cations. Swelling was also induced by butyrate and hormones (Halestrap, 1989). Atractylate and carboxyatractylate were found to enhance swelling and ADP to inhibit it, as did ATP and quinine. Cyclosporin had no effect (Davidson and Halestrap, 1990). The suggestion was made that K^+ influx might be mediated by the AdNT in its C conformation, as a consequence of binding by PPi, whose matrix levels were found to increase under the experimental conditions. Dierks *et al.* (1990a,b) have presented evidence suggesting a channel-like behavior of inner mitochondrial membrane carriers treated with SH reagents. The possible involvement of the AdNT in the formation of the permeability transition pore is mentioned elsewhere in this mini-review series (Bernardi *et al.*, 1994), and below. Tikhonova *et al.* (1994) have recently reported that the incorporation of AdNT into planar bilayers gives rise to high-conductance, mersalyl-activated channels.

ANIONIC CHANNELS

The "107 pS" ("IMM") Channel

The first mitochondrial channel to be observed by patch-clamp, in the landmark study by Sorgato *et al.* (1987), this is a slightly anionic (P_{Cl}/P_K = 4.5), strongly voltage-sensitive, approximately 107 pS (150 mM KCl) channel which is driven to (the) closed state(s) by negative (physiological) transmembrane potentials. The channel displays infrequent substates, the main one with a conductance about 1/2 of the fully open state (Klitsch and Siemen, 1991). The pore has been observed in mitochondria from the liver of cuprizone-treated mice, from rat liver and heart, ox heart (Sorgato *et al.*, 1987, 1988, 1989), rat brain (Moran *et al.*, 1990), and probably in rat brown adipocytes (Klitsch and Siemen 1991). A protein capable of forming channels with the same properties (planar lipid bilayer experiments) was found to be present in preparations of beef heart F_0F_1 ATPase and of the F_0 sector (Sorgato *et al.*, 1988), but its molecular identity has not yet been reported. The channel most likely resides in the inner mitochondrial membrane. The considerations on which this assignment was initially based concerned mainly the presence of a "cap" on the mitoplasts, which were presumed to be outer membrane remnants, and which were avoided. While other hypotheses are possible on the nature of the "caps" (Munn, 1974), all observations to date, including reconstitution studies (Moran *et al.*, 1990), are consistent with the "inner membrane channel" being indeed located there.

Attempts to inhibit the channel with any one of 12 known channel blockers (Sorgato *et al.*, 1989) failed. Among the compounds tested were carboxyatractylate and bongkrekate, ruthenium red, oligomycin, and quinine. Protons also had no effect (Sorgato *et al.*, 1987). The channel is also insensitive to cyclosporin A (Szabo and Zoratti, 1991). The channel described by Klitsch and Siemen (1991) in brown adipose tissue mitochondria, which most likely is the same described by Sorgato and coworkers, is inhibited by adenosine- and guanoside di- and triphosphates, as well as by GMP.

Kinnally and coworkers have also devoted considerable attention to the pharmacology of this channel. The authors reported that relatively high (1–2 μM) antimycin concentrations reversibly decreased the open probability of the channel (Campo *et al.*, 1992). The presence of antimycin resulted in a shift to more positive potentials of the curves relating open probability to voltage. Increasing concentrations of the mitochondrial poison caused a progressive decrease in mean open time and an increase in mean closed time, as well as a decrease in

mean burst length. These effects on the channel parameters mimicked those observed as a consequence of shifting the transmembrane voltage in the negative direction (Campo *et al.*, 1992; Klitsch and Siemen, 1991). Similar effects were induced by nanomolar concentrations of protoporphyrin IX (PPIX) and of the drugs PK11195 and Ro5-4864, all high-affinity ligands of the mitochondrial benzodiazepine receptor (mBzR) (Kinnally *et al.*, 1993). These results suggested to the authors that a connection might exist between the 107 pS channel and the mBzR and between the 107 pS channel and the mitochondrial megachannel (see below). The amphipatic drugs amiodaraone and propranolol were also reported to inhibit the channel (Antonenko *et al.*, 1991b). Interestingly, these compounds reportedly cause an approximately 40% increase in the channel conductance. The protonophores FCCP and CCCP have recently been reported to inhibit the channel (Campo *et al.*, 1994).

According to Kinnally *et al.* (1991), the 107 pS channel is observed only rarely if Ca^{2+} is not chelated during the isolation of mitochondria. Conversely, if the mitoplasts are washed with EGTA, the activity is present in 65–70% of patches. However, the addition of Ca^{2+} to active channels does not inhibit them.

The 107 pS activity has yet to find a counterpart among the mitochondrial permeation pathways studied by biochemical means. An identification with either the uncoupling protein (UCP) (Nicholls, 1974, 1979; Jezek *et al.*, 1990; Jezek and Garlid, 1990; Klingenberg, 1990, 1993) or the inner membrane anion channel (IMAC) studied chiefly by Garlid's group (reviews: Garlid and Beavis, 1986; Beavis, 1992) seems doubtful. Klitsch and Siemen (1991) concluded against an identification of the channel with the UCP for the following reasons: 107 pS seemed too high a conductance for the UCP; the channel has been found in several tissues instead of just brown fat; the nucleotide concentrations required to inhibit the channel did not match those needed to block the UCP; the voltage dependence of the channel makes it an unlikely candidate for a thermogenic role. The IMAC is activated at high pH's, while the 107 pS channel is essentially pH-insensitive (Sorgato *et al.*, 1987). Furthermore, the IMAC is inhibited by quinine, while the IMM channel is not (both, however, are inhibited by amiodarone and propranolol).

The Alkali-Activated Anionic Channel

Antonenko *et al.* (1991a) and Kinally *et al.* (1992) have reported that the ion selectivity of mitoplast patches is cationic at pH 6.8, and becomes somewhat less so at pH 8.3. The pH shift appeared to cause the activation of a class of channels, which could be reversed by returning to neutral pH or by the addition of Mg^{2+} to the bath. The channels in question were identified as approximately 15 pS (150 mM KCl) conductances, which could be inhibited by amiodarone, propranolol, and quinine, as well as by tributyltin.

The pH- and Mg^{2+}-dependence and the pharmacological properties suggested that the channel might be identifiable as the IMAC, an anion-selective, pH- and Mg^{2+}-controlled permeation pathway (on IMAC see, e.g., Selwyn and Walker, 1977; Selwyn *et al.*, 1979; Garlid and Beavis, 1986; Beavis and Garlid, 1987, 1988; Beavis 1989, 1992; Beavis and Powers, 1989; Powers and Beavis, 1991). The IMAC has been reported to be inhibited by amphiphilic local anaesthetics, as well as by the benzodiazepines Ro5-4864 and clonazepam (Beavis, 1989).

The INMAC and SMAC

Ashley and coworkers (Hayman *et al.*, 1993; Hayman and Ashley, 1993) have incorporated purified inner mitochondrial membranes into planar lipid bilayers. In experiments conducted at pH 8.8, two probably related channels, dubbed intermediate and small mitoplast anion channels (INMAC and SMAC), were observed. The SMAC seemed to derive from the INMAC upon more vigorous sonication of the membrane vesicles. The two channels had conductances of about 57 (INMAC) and 26 (SMAC) pS in asymmetric 300 : 50 mM choline chloride; SMAC measured 97 pS in symmetric 300 mM KCl. Both exhibited anionic selectivity, with P_{Cl}/P_K ratios of 8.8 and 7.1, respectively ($P_{Cl}/P_{choline} = 13$ and 11). The SMAC discriminated poorly among several anions. The INMAC exhibited a series of substates, including levels coincident with the two states observed for the SMAC. The authors suggested that the observed activities might arise from a four-subunit aggregate of cooperating channels, which would become disrupted upon sonication.

The channels were not inhibited by pH shifts to 5.5, and they were insensitive to ATP, quinine, propranolol, amitriptyline, hydrogen peroxide, and SITS. INMAC was also not affected by Ca^{2+} and Mg^{2+} while in the case of SMAC these cations induced rectification, probably by changing the membrane

surface potential. INMAC was voltage-insensitive. In the case of SMAC, positive (*cis*) voltages drove the channel into lower conductance substates (Hayman and Ashley, 1993). These various properties seem to set these channels apart from the other conductances described to date, although the possibility remains that they may coincide with the alkali-activated anionic channel (see above). It should be mentioned that the properties of channels in native membranes are not necessarily strictly maintained in reconstituted systems.

A point of some interest is that the authors of these studies chose to work at high pH (and in choline-based media) to minimize the appearance of other, mainly cationic, activities. This would seem to imply the existence of cationic channels whose activity is reduced at alkaline pH. No such channels have been reported; a shift to less cationic selectivity upon alkalinization in the experiments of Antonenko *et al.* (1991a) has been attributed instead to the activation of anionic conductances (see above).

THE MITOCHONDRIAL MEGACHANNEL

Properties

In 1989 Kinnally and coworkers reported that the *I–V* relationship of patches of mitoplast membrane often changed in time in a manner consistent with the appearance, in a previously silent patch, of high-conductance channels. The current was conducted by an array of conductances ranging from 20 pS to about 1 nS (150 mM KCl), displaying weak selectivity (Kinnally *et al.*, 1989). Somewhat later, and independently, our group (Petronilli *et al.*, 1989) obtained similar results, and recognized that most of the observed conductances represented substates of a huge, 1.3 nS (150 mM KCl) "mitochondrial megachannel" (MMC) [nicknamed instead "multi-conductance channel" (MCC) by Kinnally and coworkers]. The most relevant of the many substates was identified as one having a conductance approximately 1/2 of the full 1.3 nS value. This level often appears as an intermediate step during closing or opening events, and it is characterized by rapid flickering to and from the fully closed state (Szabó *et al.*, 1992; Szabo and Zoratti, 1993). Its occupancy increases with medium salt concentration (Szabó and Zoratti, unpublished results). The maximum conductance of the channel may actually vary from one observation to the other (Szabó and Zoratti, 1991), and values as high as 1.5 nS have been reported (Zorov *et al.*, 1992a).

Convincing evidence leads to the conclusion that the channel forms an ion-conducting pathway across the matrix-enclosing envelope. The most compelling argument is probably the identification (see below) of the MMC with the permeability transition pore, the operation of which results in osmotic swelling. Furthermore, the MMC is often observed together with the 107 pS IMM channel (see above), in patch-clamp experiments on mitoplasts possessing only remnants of the outer membrane, according to EM photographs. The possibility exists that the channels may be localized at contact sites between the outer and inner membranes, since Moran *et al.* (1990) have observed high conductances in patch-clamp experiments on proteoliposomes containing a contact site fraction from rat brain mitochondria. These authors reported, in particular, the presence of 475 and 550 pS channels, in clusters of 2–4 units. These conductances most likely correspond to the MMC-composing units ("half-conductance substates") envisioned by us (see below). Mitoplasts, at least those prepared by osmotic shock, like ours, are likely to contain some residual contact sites. It should be mentioned that Sorgato, Morans and collaborators only rarely observe MMC-like conductances in their experiments on mitoplasts (Sorgato, personal communication). The reasons for this discrepancy between these authors' results and those of Kinnally and coworkers as well as ours are not clear.

According to our observations (Petronilli *et al.*, 1989; Szabó and Zoratti, 1993), the channel is driven to reside in long-lasting ("absorbing") closed state(s) by negative (i.e., physiological) voltages. However, as long as it is open, in the negative voltage range, the channel shows a strong preference for the highest conductance state. Increasing positive potentials increase instead the occupancy of lower-than-maximal conductance states, with frequent transitions between the various conductance levels. The response to negative voltage pulses is "slow": seconds are required, on average, before the channel closes. This property was therefore missed in earlier studies by our group, in which the preferred experimental protocol consisted of trains of equal but opposite voltage pulses, generally lasting only 1 sec, separated by short intervals at zero mV (Petronilli *et al.*, 1989). Kinnally's group has observed the same voltage dependence pattern, but reported that the channel also frequently behaved differently, showing little

tendency to close at any voltage, or opening at negative and closing at positive voltages, with "slow" kinetics (Kinnally *et al.*, 1989; Zorov *et al.*, 1992a; Kinnally *et al.*, 1992), or exhibiting lower open probability and lower conductance levels in the approximately 0 to +40 mV range, with a return to high P_0's and conductances above +40 (Campo *et al.*, 1992). The gating events often took place in stepwise fashion (Zorov *et al.*, 1992a,b; Kinnally *et al.*, 1992). These authors also reported that the application of voltages above ±60 mV, of either sign, could elicit megachannel activity from silent patches (Zorov *et al.*, 1992a; Kinnally *et al.*, 1992).

The major positive modulator of the MMC is Ca^{2+}. Kinnally *et al.* (1991) reported that megachannel activity was initially present only in a minority of patches if the mitochondria has been isolated in the presence of EGTA, vs. 96% if the chelator had been omitted. The effect of Ca^{2+} was not reversible, in the sense that an EGTA wash of the isolated organelles did not result in the disappearance of the activity. According to these authors, a $[Ca^{2+}]$ greater than approximately 10 μM favors lower MMC conductance levels (Kinnally *et al.*, 1991, 1992). We observe MMC activity in about 2/3 of patches, even though 1 mM EGTA is always present during the isolation (but our standard incubation medium contains 0.1 mM $CaCl_2$). Activity can be elicited in most silent patches by a further increase in $[Ca^{2+}]$ (Szabó and Zoratti, 1992; Szabó *et al.*, 1992). In this case we have observed reversibility by chelation (Szabó *et al.*, 1992). The site involved in activation is most likely located on the matrix side of the membrane. The Ca^{2+}-activation patch-clamp experiments indicate that Ca^{2+} has a direct effect on the MMC open probability. The possibility should not be discounted however, that Ca^{2+} plays a role in the permeability transition also by favoring the formation of contact sites between the outer and inner membrane (Kinnally *et al.*, 1991; Szabó *et al.*, 1993; references therein).

Identification as the Permeability Transition Pore

Activation by Ca^{2+} is one of the characteristics the MMC has in common with the pore responsible for the permeability transition (PTP) (reviewed elsewhere in this issue). We have compared some properties of the PTP and of the MMC, and the effects of several agents on both, finding a perfect qualitative match, and have concluded that the megachannel coincides with the PTP (Szabó and Zoratti, 1991, 1992; Szabó *et al.*, 1992; Bernardi *et al.*, 1992). These results are summarized in Table II. The single most compelling piece of evidence is probably the strong inhibitory effect of cyclosporin A (Szabó and Zoratti, 1991). Cyclosporin acts in an apparent Ca^{2+}-competitive fashion: in patch-clamp experiments, MMC activity can be inhibited by cyclosporin, reinstated by an increase in $[Ca^{2+}]$, inhibited again by more cyclosporin, reactivated by more Ca^{2+}, and so forth (Szabó *et al.*, 1992). The same characteristics are displayed by the permeability transition in swelling experiments (Bernardi *et al.*, 1992). An analogous competitive interaction exists between Ca^{2+}, the positive effector, and some other divalent cations or protons, which instead inhibit the pore (Szabó *et al.*, 1992; Bernardi *et al.*, 1992; Haworth and Hunter, 1979). This behaviour ought to be accounted for by molecular models of the PTP and its workings.

While the evidence in favor of the identification of the MMC and PTP seems satisfactory, notice should be taken of the similarity in the reported

Table II. A Comparison of the Properties of the MMC (Electrophysiological Experiments) and of the PTP (Swelling Experiments)

MMC	PTP
Activation by sub-mM Ca^{2+} at matrix sites[a,b]	Activation by Ca^{2+} at matrix sites[c,d]
Ca^{2+}-competitive inhibition by Mg^{2+}, Mn^{2+}, Sr^{2+}, and Ba^{2+} [b]	Ca^{2+}-competitive inhibition by the same cations, with similar parameters[c,d,e]
Inhibited by lanthanides[f]	Inhibited by lanthanides[e]
Ca^{2+}-competitive inhibition by sub-μM Cyclosporin A[g]	Ca^{2+}-competitive inhibition by Cyclosporin A[c,h,i]
Ca^{2+}-reversible inhibition by H^+ on matrix side[b]	Inhibition by H^+ on matrix side[c,e,j,k]
Inhibition by sub-mM ADP on matrix side[a]	Inhibition by ADP on matrix side[l]
Inhibition by Amiodarone in the μM range[m]	Inhibition by Amiodarone in the μM range[n]
Fully open at voltages close to zero[o]	Activated by depolarization[p,q]
Data consistent with two cooperating channels[b,o]	Ca^{2+} an ADP have two sites per channel, with cooperative effects[e,l]

[a] Szabó and Zoratti, 1992. [b] Szabó *et al.*, 1992. [c] Bernardi *et al.*, 1992. [d] Hunter and Haworth, 1979. [e] Haworth and Hunter, 1979. [f] Szabó *et al.*, unpublished. [g] Szabó and Zoratti, 1991. [h] Crompton *et al.*, 1988. [i] Broekemeier *et al.*, 1989. [j] Nicolli *et al.*, 1993. [k] Halestrap, 1991. [l] Haworth and Hunter, 1980. [m] Antonenko *et al.*, 1991b. [n] Bernardi *et al.*, unpublished. [o] Szabó and Zoratti, 1993. [p] Bernardi, 1992. [q] Petronilli *et al.*, 1993.

pharmacological properties of the IMAC (see above) and of the PTP (see below). Ca^{2+} was reported to inhibit the IMAC with an IC_{50} of 17 μm (Beavis and Powers, 1989), but Selwyn *et al.* (1990) concluded for an activation in the low μM range, in a remarkable parallelism with the effects of Ca^{2+} on MMC activity reported by Kinnally and coworkers (see above). While a detailed discussion of the issue is outside the scope of this review, a scrutiny of the relationship between entities would seem warranted.

Pharmacology

Besides cyclosporin A (see above), a few other organic compounds have been found to affect MMC activity. Antimycin A has been reported to have complex effects: at 1–2 μM, it inhibited the MMC, but its effect was overcome by potentials higher than ±40 to 60 mV, especially in the negative range. At concentrations higher than 2–4 μM, antimycin favored the open state at both positive and negative potentials (Campo *et al.*, 1992). The authors proposed that the drug action involves alteration of voltage sensing by the channel. The cationic, amphiphilic compounds amiodarone, propranolol, and quinine were also found to inhibit MMC activity, at concentrations in the range of 0.004, 0.7, and 1.4 mM, respectively (Antonenko *et al.*, 1991b; Kinnally *et al.*, 1992). Interestingly, the action by these drugs often involved a stepwise conductance decrease (Kinnally *et al.*, 1992), i.e., the progressive involvement of subconductance levels, in analogy to the observations by the same group on voltage-induced MMC activation (Zorov *et al.*, 1992a,b). As with antimycin, the inhibitory action of the amphiphilic drugs is counteracted by relatively high voltages of either sign, again with negative voltages being more effective than positive ones (Kinnally *et al.*, 1992). Campo *et al.* (1994) have also reported that the mitocondrial protonophoric uncouplers FCCP and CCCP increase the open probability of the megachannel.

The hypothesis that the MMC might coincide with the mitochondrial benzodiazepine receptor (mBzR) (see below) prompted tests on the effects of mBzR ligands. We reported that alpidem, which binds with nanomolar affinity to the mBzR (Langer *et al.*, 1990) elicited high, flickering currents from a high percentage of silent mitoplast patches (Zoratti *et al.*, 1992; Szabó *et al.*, 1993a,b) when used in the 15 nM to 2 μM concentration range. The size of the conductance steps was compatible with the hypothesis that they might arise from MMC activation. Kinnally *et al.* (1993) reported that the benzodiazepine Ro5-4864, which binds specifically to the mitochondrial mBzR, inhibited MMC activity with an IC_{50} of about 70 nM, while clonazepam and Ro15-1788, which bind specifically to the central BzR, were at least 500 times less effective. Interestingly, these compounds also affected the 107 pS channel as mentioned above. Protoporphyrin IX, a potent inhibitor of mBzR ligand binding (Verma and Synder, 1988), was also found to inhibit MMC activity at low (8–40 nM) concentrations. Higher (200–400 nM) concentrations were found to reverse the effect, eliciting renewed, Ro5-4864-sensitive MMC activity. The reversal of the inhibitory effect by higher concentrations of the inhibitor, observed also for antimycin, points to the presence of two interacting sites.

Toward an Identification at the Molecular Level

While the molecular identification of the PTP remains to be achieved, electrophysiological tools are effectively being used for this purpose. "Some swelling and biochemical studies suggest that the adenine nucleotide translocator might be a constituent, perhaps the only one, of the PTP (reviewed in Gunter and Pfeiffer, 1990)." This hypothesis recently received support from the observation that egg lipid proteoliposomes containing isolated AdNT exhibited high-conductance channel activity (see above) (Tikhonova *et al.*, 1994). However, caution must still be exercised in evaluating these results, since rather similar activity is sometimes exhibited by egg lipid liposomes themselves (our unpublished observations).

An alternative hypothesis, already mentioned in the PT literature (LeQuoc and LeQuoc, 1985; Crompton and Costi, 1988; McGuiness *et al.*, 1990), is that the MMC might comprise VDAC as the channel-forming constituent. Since the most often encountered full conductance size of the MMC (1.3 nS) corresponds to twice the full size of VDAC, since, as mentioned above, MMC activity displays a prominent 1/2 size substate, and since channel behavior is consistent with a binary structure comprising two cooperating units, we proposed that the MMC might actually contain two cooperating VDAC molecules (Zoratti *et al.*, 1992; Szabó and Zoratti, 1993; Szabó *et al.*,1992, 1993b) plus other components. This proposal found support in the literature and in experimental data, as summarized in Table III. Wunder and Colombini (1991) have attributed to VDAC the MMC-like activity they observed

Table III. A List of Observations in Favor of the "Dimeric mBzR" Identity of the MMC/PTP

The pore sizes of VDAC and of the PTP are similar[a,b]
The characteristics of the MMC suggest it is formed by two cooperating VDAC-size channels.[c,d]
VDAC can form cooperating dimers in membranes.[e,f,g]
The AdNT functions as a dimer.[h]
The PTP contains couples of cooperative binding sites for Ca^{2+} and ADP.[i,j]
Both VDAC and the MMC display a variety of conductance substates, and have low and variable selectivity.[k,l,m]
The voltage dependences of the VDAC and of the MMC are compatible.[d,e]
VDAC can display fast kinetics, reminescent of the behavior of the "half-conductance substate" of the MMC.[e,n]
The mitochondrial membranes contain supramolecular complexes containing VDAC and AdN.[o,p]
BzR ligands affect MMC operation[d,q] Ro5-4864 binds to VDAC with nM affinity.[r]
NADH facilitates VDAC closure[s] and inhibits the PT[j].

[a] Massari and Azzone, 1972. [b] Mannella *et al.*, 1992. [c] Szabó *et al.*, 1992. [d] Szabó and Zoratti, 1993. [e] Szabó *et al.*, 1993b. [f] Krause *et al.*, 1986. [g] Zoratti *et al.*, unpublished results. [h] Klingenberg, 1981. [i,j] Haworth and Hunter, 1979, 1980. [k] Colombini, 1989. [l] Petronilli *et al.*, 1989. [m] Zoratti *et al.*, 1992. [n] Mirzabekov *et al.*, 1993. [o] Brdiczka, 1991. [p] McEnery *et al.*, 1992. [q] Kinnally *et al.*, 1993. [r] Snyder *et al.*, 1987. [s] Zizi *et al.*, 1994.

in proteoliposomes containing mitochondrial membranes. Furthermore, after the report (McEnery, 1992; McEnery *et al.*, 1992) that the mBzR is a supramolecular complex containing VDAC, the AdNT, and a well-investigated (Alexander and Klotz, 1993) 18-kDa peptide, evidence has been obtained that mBzR ligands affect MMC function (see above), and the identification of the PTP with the mBzR has been proposed (Zoratti *et al.*, 1992; Kinnally *et al.*, 1993; Szabó and Zoratti, 1993; Szabó *et al.*, 1993b).

Electron micrographs recently obtained by our group suggest, however, that yeast mitochondria isolated from a mutant, VDAC-less strain (as well as the wild type organelles) might undergo permeabilization in a cyclosporin A-sensitive process (Szabó *et al.*, unpublished data). It seems therefore that VDAC might not be necessarily a component of the permeability transition pore, and that it might be substituted by another channel. The only large channel of the VDAC-less strain is a moderately cationic, substate-rich, voltage-dependent pore which we have identified as the PSC (see above). The range of conductances it displays overlaps that of VDAC. In bilayer experiments utilizing purified VDAC-less inner and outer membrane and contact site fractions, the PSC has been observed in a high percentage of experiments in all three preparations (Szabó *et al.*, unpublished data). The channel and its properties are sufficiently similar to VDAC to justify doubt that the MMC might, at least in some cases, comprise the PSC. Work is under way in our laboratory to clarify this point. Since the PSC has been proposed to have a role in peptide translocation, the possibility of its participation in the MMC raises interesting perspectives.

OTHER CONDUCTANCES

Inoue *et al.* (1991) also mentioned a 105 pS, unselective channel, observable only at positive (unphysiological) potentials, which they tentatively identified as the channel first described by Sorgato *et al.* (1987). They also reported the presence of two other unselective, voltage-independent channels, with conductances of about 55 and 200 pS under their experimental conditions.

Moran *et al.* (1990) reported the observation of various conductances in patch-clamp experiments on proteoliposomes containing a preparation of contact sites from rat brain. In addition to the 107 S channel, the authors identified two sets of low-conductance, voltage-insensitive channels (6, 12 and 21, 28, 33 pS; 150 mM KCl), plus, rarely, 90 pS events. The main activity in the system arose from a 475 pS (with substates of 245 and 373) and a 550 pS channel. These activities were voltage-independent. Higher conductances, up to 970 pS, were also observed. It is likely that the large conductances represent activity by (components of) the mitochondrial megachannel (see above).

In the absence of an obviously difficult characterization, the small conductances cannot be placed in the context of other observations. Two mitochondrial cation uniports, a rather unselective one (Brierley *et al.*, 1978; Bernardi *et al.*, 1989; Nicolli *et al.*, 1991) and one preferring Na^+ and Li^+ over K^+ (Wehrle *et al.*, 1976; Brierley *et al.*, 1978; Bernardi *et al.*, 1990), might be mentioned at this point. The former might possibly be equated with the 40 pS channel mentioned below. The latter does not have even a tentative electrophysiological counterpart. According to Kapus *et al.* (1990) both these pathways are inhibited by ruthenium red, suggesting that they might be manifestations of the Ca^{2+} uniporter.

Diwan's group (Diwan *et al.*, 1990; Costa *et al.*,

1991; Paliwal *et al.*, 1992) has achieved the partial purification, by quinine affinity chromatography, of two channels. Incorporation of a protein fraction into liposomes allowed the detection of activity by approximately 40 and 130 pS conductances (150 mM KCl) by patch-clamp. The former were tentatively associated with a 57-kDa protein, the latter with a 53-kDa component. The 40 pS activity was not modulated by voltage, and did not significantly discriminate between K^+ and Na^+ (Paliwal *et al.*, 1992). Other data on its selectivity properties, or on those of the voltage-dependent 130 pS channel, and whether quinine affects either of these channels, have not been reported.

PERSPECTIVES

Establishing reliable correlations between the channels observed in electrophysiological experiments and those identified by biochemical techniques clearly is a major and urgent task for researchers in this field. If any one channel were to be singled out as attention-worthy, it would probably be the Ca^{2+} uniporter. Patch-clamp studies seem to have produced, so far, no information relevant for this specific case. However, the experimental conditions used clearly have not been the most suited for such an investigation. As the channel-forming proteins become known through biochemical studies, electrophysiologists trying to identify their activity in the native membrane should benefit from the results of work on reconstituted systems and the availability of antibodies.

Progress in the pharmacology of the various channels is also of great relevance: the ability to control channel activity is a prerequisite for the development of mitochondrial "electro-bioenergetics", i.e., the study of transmembrane potential generation and utilization by the machinery of the IMM by electrophysiological means. Some excellent work on non-mitochondrial "pumps" has already been done, mostly utilizing reconstituted systems. The application of patch-clamp technology to reconstituted mitochondrial proton pumps is presumably already feasible, and might provide important information on such topics as, for example, the thermodynamic control of pump (and electrogenic carrier) activity.

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