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Channels in Mitochondrial Membranes: Knowns, Unknowns, and Prospects for the Future

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ABSTRACT: Rapid diffusion of hydrophilic molecules across the outer membrane of mitochondria has been related to the presence of a protein of 29 to 37 kDa, called voltage-dependent anion channel (VDAC), able to generate large aqueous pores when integrated in planar lipid bilayers. Functional properties of VDAC from different origins appear highly conserved in artificial membranes: at low transmembrane potentials, the channel is in a highly conducting state, but a raise of the potential (both positive and negative) reduces drastically the current and changes the ionic selectivity from slightly anionic to cationic. It has thus been suggested that VDAC is not a mere molecular sieve but that it may control mitochondrial physiology by restricting the access of metabolites of different valence in response to voltage and/or by interacting with a soluble protein of the intermembrane space. The latest application of the patch clamp and tip-dip techniques, however, has indicated both a different electric behavior of the outer membrane and that other proteins may play a role in the permeation of molecules. Biochemical studies, use of site-directed mutants, and electron microscopy of two-dimensional crystal arrays of VDAC have contributed to propose a monomeric β barrel as the structural model of the channel. An important insight into the physiology of the inner membrane of mammalian mitochondria has come from the direct observation of the membrane with the patch clamp. A slightly anionic, voltage-dependent conductance of 107 pS and one of 9.7 pS, K^+-selective and ATP-sensitive, are the best characterized at the single channel level. Under certain conditions, however, the inner membrane can also show unselective nS peak transitions, possibly arising from a cooperative assembly of multiple substates.

KEY WORDS: mitochondria, outer membrane channels, VDAC, inner membrane channels, mCS channel, planar lipid bilayer, patch clamp.

I. INTRODUCTION

Mitochondria are the cellular organelles responsible for the major production of ATP, necessary for the life of the cell. For years, a great deal of effort was devoted to the understanding of their metabolic routes and to the unraveling of the molecular mechanism of energy transduction mediated by the inner membrane proteins. After the general agreement on the principles dictated by the chemiosmotic hypothesis, other topics have replaced mitochondrial bioenergetics as the general interest. However, the fact that of all mammalian intracellular channels, those of mitochondria were the first to be observed directly with the patch clamp technique (Sorgato, Keller, and Stühmer, 1987) has undoubtedly acted to revitalize attention on this organelle and to open

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new perspectives in the knowledge of its physiology.

In the outer mitochondrial membrane, a few conductances characteristic of channels have been described. The voltage-dependent anion channel (known with the acronym VDAC but also as mitochondrial porin) was the first discovered by Schein, Colombini, and Finkelstein (1976). It is present in mitochondria of the entire eukaryotic kingdoms; it has been isolated, sequenced, and characterized extensively structurally and electrophysiologically. It thus deserves a wider analysis also with respect to its presumed implication in the control of mitochondrial physiology. A poor correspondence with the functional features of VDAC integrated in planar bilayers has however emerged from more recent analyses of the outer membrane (Tedeschi, Mannela, and Bowman, 1987; Thieffry et al., 1988; Moran et al., 1992), some of which are indicative of the presence of channels distinguishable from VDAC.

The electrophysiological study of the inner membrane started much later (Sorgato, Keller, and Stühmer, 1987) and the ill-defined picture of many of the observed conductances appear commensurate with the immaturity of the field. Similar uncertainties apply also to channels described by patch clamping other intracellular membranes (Rahamimoff et al., 1988; Lemmens et al., 1989; Mazzanti et al., 1990), which makes it desirable that this common effort also accelerates the pace of identification of the precise function and mechanism of all these channels.

Isolation of proteins involved in channel activity of the inner membrane has just begun; this article therefore focuses on data related to their functional aspects.

The electric properties of mitochondrial membranes have been investigated with the planar bilayer and the patch clamp techniques. The first technique has been applied more extensively to the outer membrane, the second to the inner one. Because conflicting data have been obtained with the two techniques, a brief introduction to the methodologies will be given to put in immediate context the appreciation of the discrepant results.

II. ELECTROPHYSIOLOGICAL TECHNIQUES

Ion channels are membrane proteins that form water-filled pores through which ions can flow passively down their electrochemical gradient (Figure 1). The ability to conduct ions is expressed in electric terms (siemen = 1/Øhm) by the conductance value, which, according to Ohm's law, is the ratio of the current flowing through the channel and the potential difference across the membrane.

Properties of channels are best studied by measuring the current flowing through a channel-containing membrane. In the presence of ionic solutions of known concentration and of a controlled electric field (voltage-clamp condition), the estimated current (I) is defined as

$$I = \gamma N p_o (V-V_i)$$

where γ is the channel conductance, N the number of channels present in the membrane under investigation, p_o the probability of the channel to be open under those particular conditions, V the imposed potential difference, and V_i the equilibrium potential of the flowing ion. The value of this latter parameter can be approximated to that of the concentration gradient of the ion (expressed in logarithmic terms, as in the Nernst equation). The measured current can be microscopic or macroscopic, depending on whether N is ≈ 1 or much greater than 1. With microscopic currents, the conductance of a single channel can thus be directly evaluated together with the mean open probability and kinetics (in terms of time constants of the open and closed states). On the other hand, by looking at macroscopic currents, the γ N p_o term is obtained, which is useful especially for studying channels' p_o in response to external parameters such as membrane potential difference or effector concentration. Additionally, analysis of the decay time of macroscopic currents can provide clues to the single channel kinetics.

The potential difference at zero current is usually measured to determine the ion selectivity of a channel. Solutions containing different concentrations of the same salt are placed at the two sides bathing the membrane. Depending on the valency of the permeable ions, a different potential value (reversal potential) will be found to equilibrate with the logarithmic ratio of the concentrations (strictly speaking of the activities) so that no net current can be detected. With this value and the known ion concentrations, the

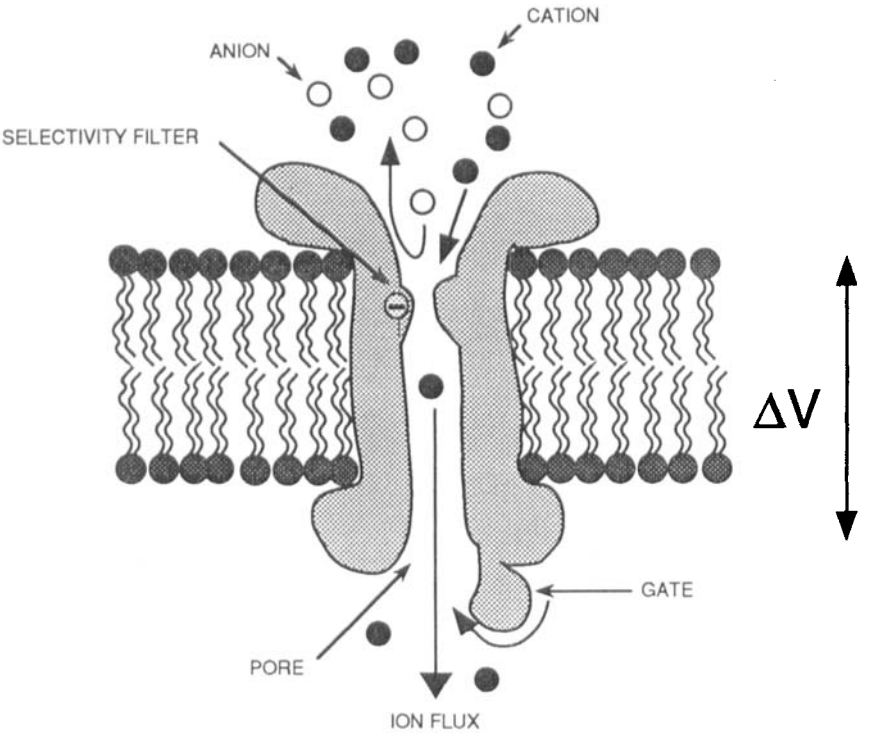

FIGURE 1. Schematic representation of an ion channel. A channel is a macromolecule embedded in the bilayer, limiting a pore through which ions flow down their electrochemical gradient. Constrictions in the pore due to the presence of specific charges or of steric hindrance confer the ionic selectivity. The properties of a channel to pass from the open (conducting) to the closed (nonconducting) state are provided by the gating mechanism. In the figure, one of the several operational modes of such mechanism (Hille, 1984) is represented, that is, a movement of a domain (gate) causing occlusion of the pore. ΔV is the potential difference across the membrane.

Goldman-Hodgkin-Katz constant field equation is then applied to determine permeability ratios.

The patch clamp and the planar bilayer methods have generally been used to obtain the above measurements. The patch clamp technique (Hamill et al., 1981) provides the possibility of recording currents through an electrically isolated, small piece of a cellular membrane or of a liposome (Figure 2). A clean, fire-polished pipette, connected to the current measuring circuit, is driven to the membrane surface and when the two are in contact, the application of a slight negative hydrostatic pressure through the pipette allows the formation of a tight seal between the membrane and the glass of the pipette (cell-attached configuration; see Figure 2A). Ideally, the seal has a very high electric resistance (gigaseal), a prerequisite for measuring small currents flowing through one or few channels present in the patch. Starting from this configuration, by various manipulations, it is possible to obtain other configurations each of which has several experimental advantages. One is the excised inside-out patch, obtained by pulling away the pipette from the cell or organelle, that will allow direct access of added molecules to the inner face of the membrane (Figure 2B). Otherwise, by breaking the patch either by high negative pressure or by voltage pulses, determination of the macroscopic current of the whole membrane is possible (whole cell configuration; see Figure 2C).

In contrast to the patch clamp, which permits the behavior of channels to be followed in their native environment, the planar lipid bilayer technique relies on artificial membranes (Figure 3). Two methods are most frequently used to form bilayers separating two aqueous compartments. In one, the bilayer is obtained from phospholipids dissolved in organic solvents (usually aliphatic hydrocarbons) painted onto a hole of a hydro-

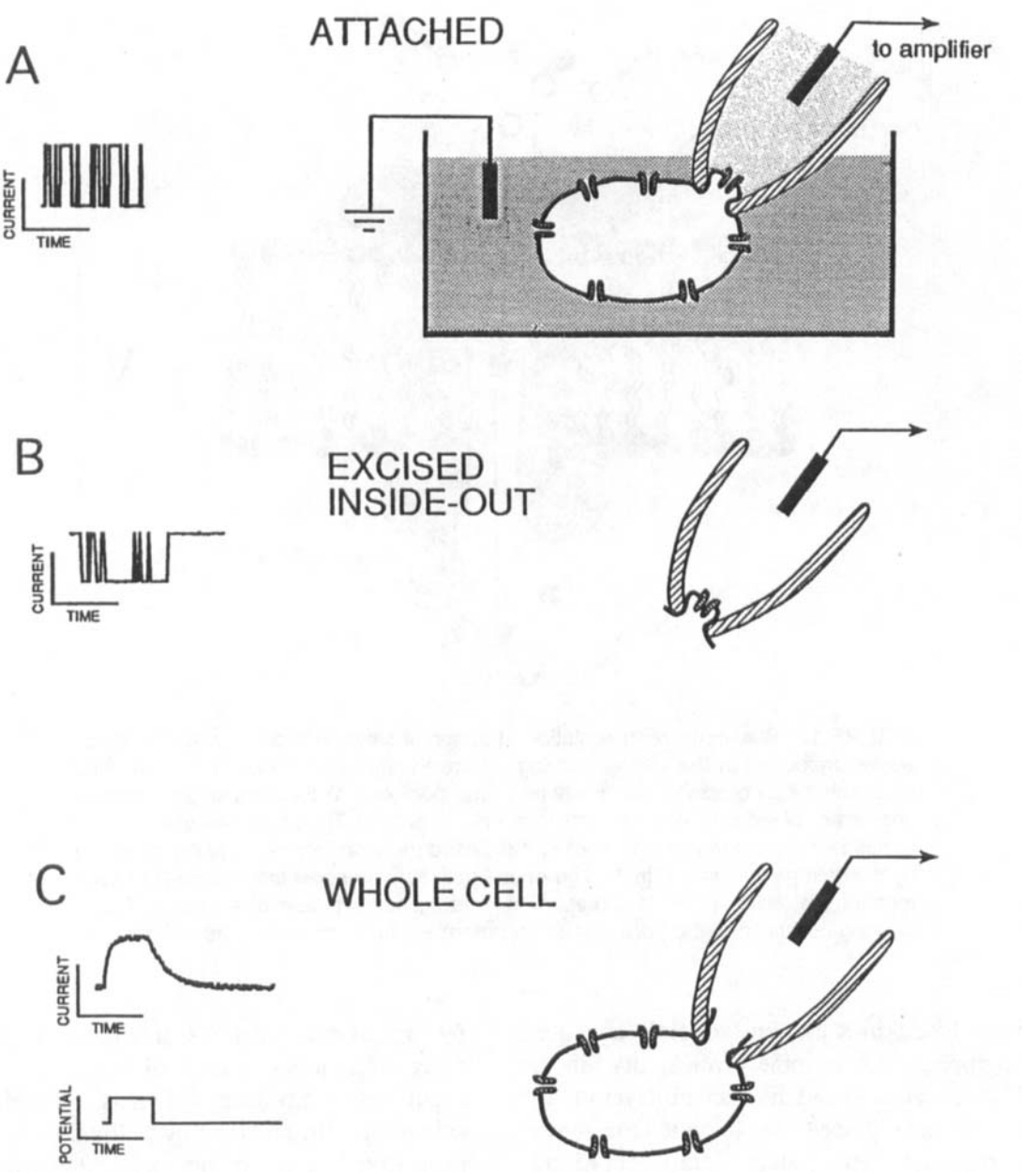

FIGURE 2. Schematic representation of the patch clamp technique. (A) By applying a slight negative pressure to the tip of a pipette attached to a membrane (cell-attached configuration), a high-resistance seal can form between the glass and the membrane. Current flowing through the small membrane patch can therefore be measured by a current-voltage converter amplifier under controlled potential difference. (B) If the patch in A is detached from the rest of the membrane, then the excised inside-out configuration is obtained. In either A and B patches, unitary current events can be resolved. (C) Vice versa, if the patch in A is broken, then access to the whole membrane is gained (whole cell configuration). This allows measurement of macroscopic currents, for example, in response to a voltage step, as shown. For other details, see text.

phobic septum separating the two compartments (called *cis* and *trans* chamber) (Mueller et al., 1963). In the other, lipid monolayers are first formed at the air-water interface of each compartment and then made to meet at the hole by raising the water level (Montal and Mueller, 1972). Channels are incorporated into the bilayer by fusion of proteoliposomes or channel-containing detergent micelles, usually added to only one side. Successful incorporations are easily de-

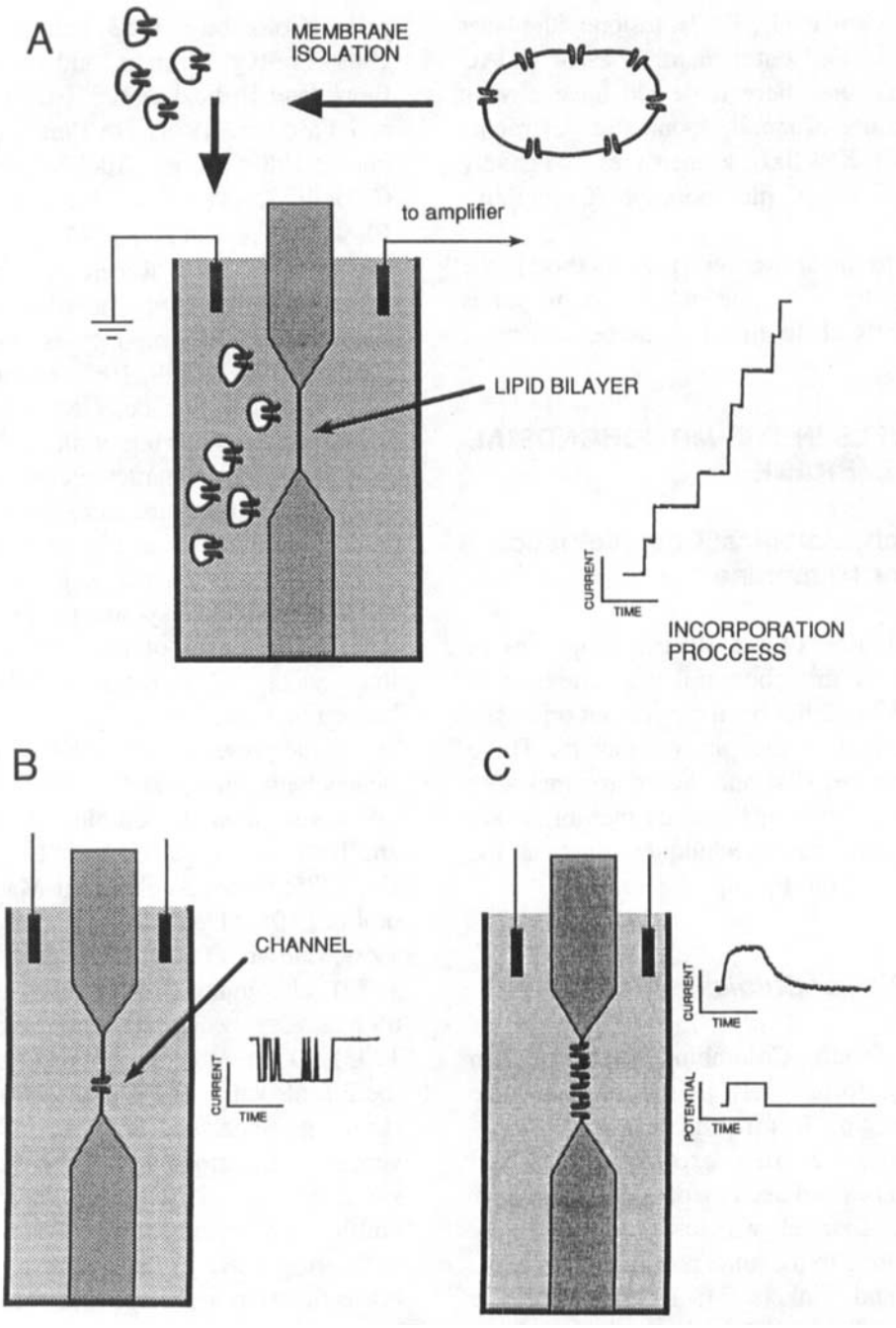

FIGURE 3. Schematic representation of the planar bilayer technique. (A) Channel-containing vesicles can be incorporated in a lipid bilayer formed at the hole of a septum separating two aqueous compartments (see text). This process can be followed by the stepwise current increase at a given applied voltage. Single channel (B) or multichannel (C) currents can thus be measured.

tected under voltage clamp by step increases of the bilayer current (Figure 3A). Hence, by controlling the amount of channel incorporation (i.e., by choosing the right size of the hole and/or the amount of material to add or by dialyzing the channel-containing side after occurrence of the desired number of fusions), it is possible to study microscopic (Figure 3B) or macroscopic (Figure 3C) currents. To our knowledge, except for two studies (Schein, Colombini, and Finkelstein,

1976; Mirzabekov et al., 1993), fusion with planar bilayers of isolated outer membranes or VDAC in the experiments here reviewed have always involved the use of small amounts of detergents (usually Triton X®-100), as their presence greatly enhances the rate of incorporation (Colombini, 1983).

Similar to the above described method is the so-called tip-dip technique, where the bilayer is formed directly at the tip of a microelectrode.

III. CHANNELS IN THE MITOCHONDRIAL OUTER MEMBRANE

A. Electrophysiological Characteristics of the Outer Membrane

The definition of the electric properties of either the outer mitochondrial membrane or of the isolated VDAC has been carried out primarily with the method of the planar bilayers. These data are presented first and then compared with more recent analyses of the outer membrane accomplished with other techniques, such as the patch clamp and the tip-dip.

1. Planar Bilayer Studies

In 1976, Schein, Colombini, and Finkelstein found almost fortuitously a high conductance channel belonging to mitochondria of *Paramecium aurelia*. As a crude extract of inner and outer membranes had been incorporated in planar bilayers, the channel was at first tentatively thought to belong to the inner membrane (Schein, Colombini, and Finkelstein, 1976). Later, on fractionation of mitochondrial membranes from rat liver, Colombini (1979) conclusively attributed it to the outer membrane. The channel was termed voltage-dependent anion channel (VDAC) (Schein, Colombini, and Finkelstein, 1976) because of both its ability to discriminate anions over cations under certain conditions and its strong voltage dependence. These features were found to be retained by other proteins of apparent molecular mass 29 to 37 kDa, extracted by different means from mitochondria of primitive and advanced eukaryotic cells (fungal, yeast, protozoa, plant, insect, fish, and a variety of mammalian cells) (Colombini, 1983; Palmieri and De Pinto, 1989; Freitag, Neupert, and Benz, 1982; Roos, Benz, and Brdiczka, 1982; De Pinto, Prezioso, and Palmieri, 1987; De Pinto, Benz, and Palmieri, 1989; Forte, Adelsberger-Mangan, and Colombini, 1987; Ludwig, Benz, and Schultz, 1989; De Pinto et al., 1985, 1987, 1991a; Ludwig et al., 1988). Recently, a 31-kDa protein with identical electric properties has been isolated from human B lymphocytes total membranes (Thinnes et al., 1989, 1990) and a mitochondrial 34-kDa protein was detected in human heart by Western blots (Towbin et al., 1989).

The electric characteristics of VDAC from all species studied are amazingly similar in artificial membranes and hardly resemble features of other known channels: these are large conductance, high and symmetrical sensitivity to potential differences of opposite sign, change of ionic selectivity as a function of the applied membrane potential.

In the presence of 1 *M* KCl, the conductance ranges between 4 and 4.5 nS (Colombini, 1979, 1983; Smack and Colombini, 1985; Roos, Benz, and Brdiczka, 1982; Fiek et al., 1982; Pfaller et al., 1985; Forte, Adelsberger-Mangan, and Colombini, 1987; De Pinto et al., 1985, 1987, 1989, 1991; Ludwig et al., 1986, 1988; Thinnes et al., 1989), although a slightly lower estimate of 3.7 nS was recently demonstrated (Mirzabekov et al., 1993). Other more pronounced differences are the 2.5 nS value of *Paramecium aurelia* VDAC (Ludwig, Benz, and Schultz, 1989) and the diverging estimations of 2.8 and 5 nS for *Neurospora crassa* VDAC (Dill, Holden, and Colombini, 1987, and Freitag, Neupert, and Benz, 1982, respectively). The pore conductance is a linear function of the specific conductance of the bulk aqueous phase and does not show saturation with increasing salt (at least for KCl, from 0.01 *M* to 4 *M*) (Colombini, 1986). For example, the conductance measured at 0.1 *M* KCl is approximately 10-fold less than at 1 *M* KCl (Roos, Benz, and Brdiczka, 1982; Freitag, Neupert, and Benz, 1982; Ludwig, Benz, and Schultz, 1989; Ludwig et al., 1986, 1988; De Pinto et al., 1991a) (Table 1) (see, however, Mirzabekov et al., 1993).

The high conductance, however, is the expression of a state that VDAC assumes only under limited conditions, that is, when the applied potential difference is of low magnitude

TABLE 1
Main Single Channel Conductances Found in the Outer Mitochondrial Membrane

Method	Conductance (pS) in 0.15 *M* salt	VDAC responsible for activity	Voltage dependence	Ref.
Planar bilayer	600–750*	Yes	Symmetrical	1, 2
	315*	No	n.d.	3
	420*	No	Symmetrical	4
Tip-dip	>440**, >660**	No	Asymmetrical	5, 6
Patch clamp of mitochondria	99, 152, 220, 307	?***	Asymmetrical	7
Patch clamp of proteoliposomes	97, 156, 244, 535	?***	Asymmetrical	7
	650	Yes	Symmetrical	8

Note: n.d.: not determined; *: reported values were calculated from those found at 0.1 M KCl; **: the first value is found in mammalian outer membranes (5), the second is found in yeast outer membranes (6); ***: VDAC was detected immunologically (7).

1. Colombini (1989), 2. Benz (1990), 3. Dihanich et al. (1989), 4. De Pinto et al. (1991a), 5. Thieffry et al. (1988, 1992), 6. Fèvre et al. (1990), 7. Moran et al. (1992), 8. Wunder and Colombini (1991). For further details see text.

(from 0 mV to approx ± 20 mV) (Benz, 1990; Colombini, 1980; Smack and Colombini, 1985; Doring and Colombini, 1985a; Benz and Brdiczka, 1992; Schein, Colombini, and Finkelstein, 1976; Ludwig et al., 1986, 1988; Colombini et al., 1987; Forte, Adelsberger-Mangan, and Colombini, 1987; De Pinto et al., 1989). In fact, the macroscopic conductance-voltage curve for rat liver VDAC of Figure 4 is taken as proof that channels switch to lower conducting levels at potential differences higher than approximately ± 20 mV. Under these conditions, VDAC is still open (because it can allow ions to permeate), albeit the conductance decreases the higher the potential difference. In contrast to the maximal conducting state, called the open state, the multiple sublevels are defined closed states. Apparently, VDAC is incapable of having a completely nonconductive conformation, even at potential differences higher than 80 mV (Ludwig et al., 1986; De Pinto et al., 1989; Benz, 1990; Benz and Brdiczka, 1992). Conversely, Mirzabekov et al. (1993) have shown that exposure to voltages higher than ± 60 mV induces an irreversible decrease of VDAC single channel conductance to only few percentages of the full open state.

The ability to close symmetrically on voltage increase is common to VDAC of different origins. What apparently varies, is the probability of closure for a given magnitude of the applied potential. This can be better appreciated from the reported values of n, which can be considered as a quantitative expression of a channel voltage sensitivity (i.e., the higher the value, the greater the steepness of the conductance-voltage curve), if the conductance-voltage data are analyzed assuming a two-state system with a Boltzmann distribution (Ehrenstein, Lecar, and Nossal, 1970; Schein, Colombini, and Finkelstein, 1976). Values around 2 (De Pinto et al., 1987, 1989, 1991a; Adelsberger-Mangan and Colombini, 1987; Ludwig et al., 1988; Ludwig, Benz, and Schultz, 1989; Benz, Kottke, and Brdiczka, 1990) or around 4 (Schein, Colombini, and Finkelstein, 1976; Colombini, 1983; Bowen, Tam, and Colombini, 1985; Doring and Colombini, 1985a; Smack and Colombini, 1985; Forte, Adelsberger-Mangan, and Colombini, 1987) but also

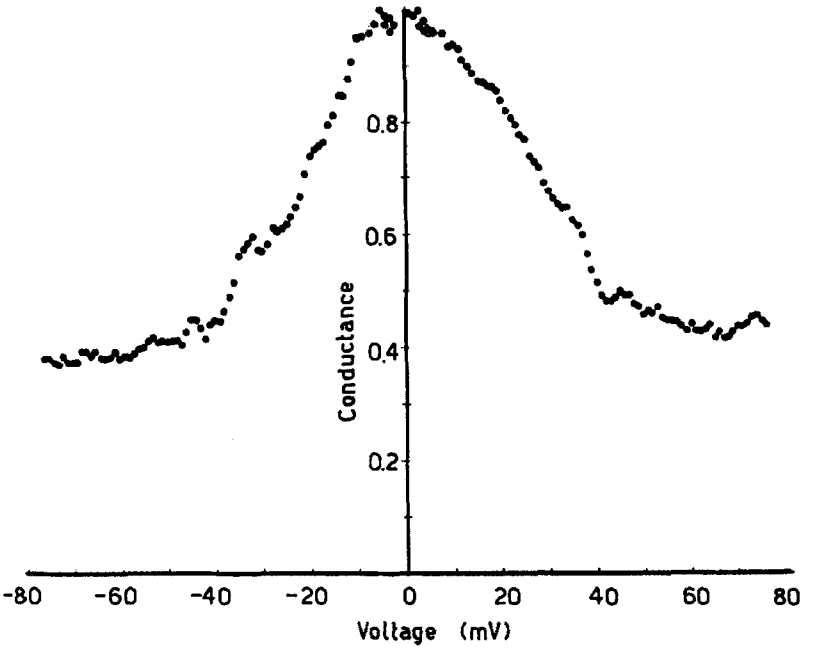

FIGURE 4. Conductance-voltage relationship found for rat liver VDAC. VDAC was isolated according to Colombini (1983) and was added to the *cis* side of the planar bilayer setup. Solutions were symmetrical 1 *M* LiCl, 5 m *M* $CaCl_2$, 50 m*M* LiMops, pH 7.2. Triangular voltage waves, applied in the direction of decreasing electric fields, were used to measure the macroscopic current of the multichannel-containing bilayer. The conductance at each voltage was normalized to the maximal conductance found. (Modified from Doring, C. and Colombini, M., *Biophys. J.*, 45, 44–46, 1984. With permission.)

higher than 6 (Mirzabekov and Ermishkin, 1989; Ermishkin and Mirzabekov, 1990) have been determined. By following the same analysis as just referenced, the value of the membrane potential (Vo) at which the VDAC population has an equal (0.5) probability to be in the open or closed state has been evaluated. In general, with either set of results, Vo increased as n decreased, which indicates an unchanged value for the energy needed to close the channel (Ehrenstein, Lecar, and Nossal, 1970; Schein, Colombini, and Finkelstein, 1976).

It is not known whether the discrepancy concerning n is methodological. There are indeed differences in the bilayer lipid composition, in the methods both to form the planar bilayer (see Section II) and to isolate the outer membrane or VDAC (with or without detergents). As for the voltage control, either triangular waves (Schein, Colombini, and Finkelstein, 1976; Colombini, 1980; Smack and Colombini, 1985; Doring and Colombini, 1985a; Forte, Adelsberger-Mangan, and Colombini, 1987) or discrete voltage steps (Ludwig et al., 1986; Benz, 1990; Benz and Brdiczka, 1992) were generally applied. Inherent to these distinctive protocols is the measurement of the current at different times after the voltage change, and there is no stringent proof against the possibility that evaluations correspond to different stages of the channel relaxation process. However, according to Ludwig et al. (1986), who described by far the lowest response to voltage of VDAC from rat brain (n being 1), the different values could be suggestive of different roles of the outer membrane in different cells. As it stands, lack of consensus on the n value even for identical VDACs (Forte, Adelsberger-Mangan, and Colombini, 1987; Ludwig et al., 1988, Colombini, 1983; Doring and Colombini, 1985a; Benz, Kottke, and Brdiczka, 1990; Schein, Colombini,

and Finkelstein, 1976; Ludwig, Benz, and Schultz, 1989) makes it difficult to derive any reliable conclusions from these data.

As shown in Figure 4, the response of VDAC to electric fields of opposite direction looks essentially symmetrical. There have been reports in favor of an asymmetrical behavior of VDAC, however, where the current decrease of single- or multi-channel-containing bilayers occurred preferentially at only one sign of the applied voltage (Roos, Benz, and Brdiczka, 1982; Freitag, Neupert, and Benz, 1982; Fiek et al., 1982; De Pinto et al., 1985; Pfaller et al., 1985; Ludwig et al., 1988; Benz, 1990). Although the validity of these results has been disputed for the use of solvent-containing membranes (Colombini, 1983, 1989), they could now assume a renewed relevance in the light of recent evaluations of VDAC stoichiometry (see Section IIIC).

Two independent approaches have been used to estimate the diameter of the channel in the open state. One relies on the conductance value (γ) for a single VDAC, assumed as a 6-nm long cylinder (1) filled with a solution whose specific conductivity (σ) equals that of the external aqueous medium. Thus, with σ of 110 mS/cm for the 1 *M* KCl solution, the diameter (2r) of the pore, readily calculated from the equation (Benz et al., 1978)

$$\gamma = \sigma\pi r^2/1$$

has been found to vary from a minimum of 1.3 nm [for *Paramecium* VDAC, whose conductance is exceptionally low (Ludwig et al., 1988)] to approximately 1.7 nm for VDAC of other cells (see Benz, 1990). Although questioned by others (Colombini et al., 1987), the applicability of this equation was considered justified by the almost constant value of the γ/σ ratio found at any KCl concentration (Roos, Benz, and Brdiczka, 1982; Ludwig, Benz, and Schultz, 1989; Ludwig et al., 1986, 1988).

The other determination, based on the permeability exclusion of molecules with known Stokes-Einstein radius from liposomes containing outer membranes (Colombini, 1980), has provided a twofold higher value of 4 nm. Given the heterogeneity in the molecular weight of the polyethylene glycol found unable to permeate proteoliposomes (Colombini, 1980), this value has been judged frequently to be an overestimate (Ludwig et al., 1988; Benz, 1990).

A kinetic description of single channels (in terms of dwell time distribution) in planar bilayers has never really been tackled, primarily because of the slow kinetics apparently relating to the transitions of VDAC between different levels of conductance at any fixed applied potential (but see Ermishkin and Mirzabekov, 1990; Mirzabekov et al., 1993). Indeed, the only reported feature is of at least 1 min for VDAC full open lifetime at low potential differences (Roos, Benz, and Brdiczka, 1982), and Figure 5 confirms that the steady state behavior under voltage clamp is rather sluggish. Additional data came from the analysis of conductance changes of a bilayer after insertion of single active channels at one given potential difference (as exemplified in Figure 3A, right side) that indicated that closing transitions of different size are only rare events at low voltages (Colombini, 1980; Freitag, Neupert, and Benz, 1982; Roos, Benz, and Brdiczka, 1982; Fiek et al., 1982). According to Roos, Benz, and Brdiczka (1982), these have a magnitude of approximately 50% of the maximal conductance, whereas Colombini (1980) found two definite substates of 1 and 3.5 nS, respectively. As the magnitude of the applied electric field increases, the number and frequency of the closing transitions increase accordingly (Roos, Benz, and Brdiczka, 1982). VDAC has then been best described as a channel with one, large, open state and several different low conducting states (Colombini, 1979, 1980; Ermishkin and Mirzabekov, 1990; Schein, Colombini, and Finkelstein, 1976; Freitag, Neupert, and Benz, 1982; Roos, Benz, and Brdiczka, 1982).

On the other hand, determination of the time constants for the transition from the open to the closed states (or vice versa) has been estimated with more confidence in multichannel-containing bilayers, by following changes in current on voltage steps of substantial magnitude. It was then unambiguously defined that the rate at which channels opened, on passing from high to low potentials, is very fast, with a value that must be lower than the resolution time (of approximately 1 ms) of the particular recording instrumentation connected to the planar bilayer setup (Colombini, 1979; Schein, Colombini, and Finkelstein, 1976; Ludwig, Benz, and Schultz, 1989; Ludwig et al.,

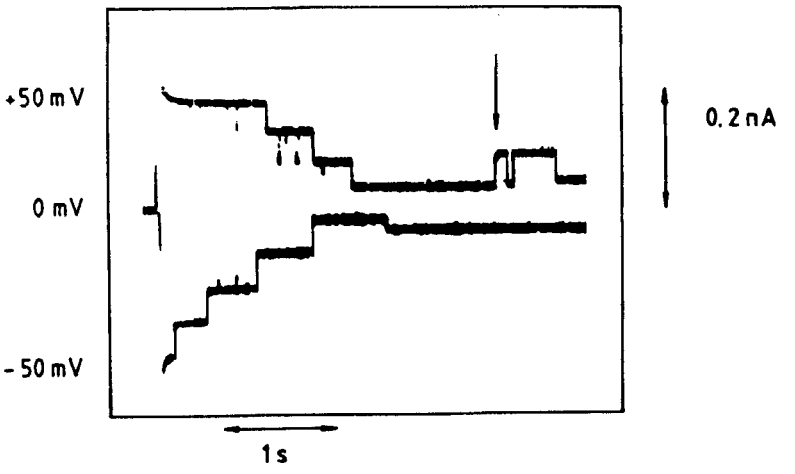

FIGURE 5. Recording of VDAC single channel events with the planar bilayer technique. VDAC from *Paramecium aurelia* was isolated according to De Pinto et al. (1987) and added to the *cis* side. Currents were recorded from planar bilayers initially containing 3 channels (upper trace) and then, after insertion of an additional one (arrow), 4 VDAC channels (lower trace). Solutions were symmetrical 0.5 *M* Tris HCl, pH 7.2. The voltage protocol consisted in a pulse from 0 mV to + 50 mV, followed by a step from 0 mV to − 50 mV. (From Ludwig, O., Benz, R., and Schultz, J. E., *Biochim. Biophys. Acta,* 978, 319–327, 1989. With permission.)

1986, 1988). Conversely, a rate on the timescale of seconds characterized the closing phenomenon occurring when the applied potential changed from low to high values (Schein, Colombini, and Finkelstein, 1976; Colombini, 1979; Freitag, Neupert, and Benz, 1982). Closure was described by a single exponential decay, whose rate constant decreased with increasing of the voltage steps (Colombini, 1989; Ludwig, Benz, and Schultz, 1989; Ludwig et al., 1986, 1988).

As expected from the large current mediated by the channel, access of ions to the water-filled pore of VDAC is restricted more by the mobility of the ions in the aqueous phase (Ludwig et al., 1988) than by a selectivity filter (see legend to Figure 1). In spite of this, VDAC is able to discriminate between ions of different valence, and, for what has been interpreted as extremely relevant to the physiology of the outer membrane (see Section III.D), VDAC changes ionic selectivity on increase of the potential difference. Indeed, in the presence of equally mobile ions, VDAC in the open state conducts anions with a slight preference; vice versa, at high potential differences, when VDAC is in the closed state, the current is mainly due to cations. By measuring reversal potential values in the presence of a KCl gradient (see Section II), the Cl^- over K^+ permeability ratio for the open state was found to be $\leqslant 2$ (Roos, Benz, and Brdiczka, 1982; Ludwig, Benz, and Schultz, 1989; De Pinto et al., 1985, 1987, 1989, 1991a; Ludwig et al., 1986, 1988; Colombini, 1979; Smack and Colombini, 1985; Doring and Colombini, 1985a; Forte, Adelsberg-Mangan and Colombini, 1987; Adelsberger-Mangan and Colombini, 1987), whereas the K^+ over Cl^- permeability ratio for the closed states varied extensively (described later in this section).

Implicit to the phenomenology of conductance decrease occurring with increasing membrane potentials (Figure 4) is the notion that VDAC changes conformation accordingly and that each closed conformation is the more energetically stable for that given voltage value. There are two mechanisms generally at work in voltage-dependent channels to sense a variation in the electric field and to respond with a change in the channel conformation, that is, a movement of charged domains or a change of the dipole moment in the direction of the electric field. In the case of VDAC, at variance from the suggestion

that both positive and negative charges participate in large numbers in the gating mechanism (Mirzabekov and Ermishkin, 1989; Ermishkin and Mirzabekov, 1990), only a set of positive charges was identified by Colombini and co-workers by several lines of evidence, which are now summarized.

One clue was provided by following changes in the voltage sensitivity consequent to the chemical reaction of rat liver VDAC with succinic anhydride, whose derivatization of positive amino residues converts them to negative carboxyl groups. Thus, modification of VDAC positive charges was expected to result in a parallel change of the voltage sensitivity. The effect of the reagent was tested on VDAC in either the open or the closed state (at low or high potential difference, respectively) and under conditions whereby the addition of the reagent occurred at positive or negative potential (to only one side of the bilayer). With VDAC in the open state, loss of the typical response to voltage of the macroscopic conductance was observed irrespective of the sign of the voltage present at the side of reagent addition (Doring and Colombini, 1984, 1985a). If, on the contrary, succinic anhydride was added to VDAC in the closed state, then voltage desensitization was detected preferentially with the reagent added to the negatively charged side, supporting the view that the conformation achieved at negative voltages is different from that at positive voltages and that only the former would facilitate the access of the anhydride to the gating charges (Doring and Colombini, 1985b). In any case, once succinylation took place, diminution or elimination of voltage dependence was possible at either sign of the potential difference (Doring and Colombini, 1985a, 1985b). Despite other effects observed after derivatization (loss of conductance or complete closure of channels), these results were nevertheless rationalized by a model where the molecular bases for the responses of VDAC to positive and negative potentials were based on the same set of fixed positive charges, asymmetrically located, to form two separate gating systems operative under opposite electric fields (Doring and Colombini, 1985b).

An important corollary to these experiments was the observation of a parallel change in selectivity of the open state from anionic to cationic (Doring and Colombini, 1985a). This result allowed the proposition that the same set of positive charges was responsible also for the preference of VDAC to conduct anions at low potentials. In view of the low selectivity, these charges would then contribute to creating within the pore the proper electric environment for flow of anions, rather than be directly involved in ion conduction.

Further evidence for a gating mechanism involving movement of positive charges was provided by other approaches. Bowen, Tam, and Colombini (1985) eliminated the voltage dependence by titration of amino groups with increasing pH. The pK_a value of 10.6 led to the implication of the positive charges of lysines in the voltage sensitivity.

Additional proofs for the presence of charges at the gate of the channel came from Mangan and Colombini (1987) who searched for compounds able to alter the voltage dependence indirectly by an electrostatic interaction with the gating mechanism. One was the highly negatively charged dextran sulfate with a too large molecular weight (8 kDa) to enter the pore. Dextran sulfate needed a negative potential to be effective, which means that, under these conditions, it accumulated at the local positive region in the vicinity of the mouth arising from anion depletion at the negative potential side of the channel (see inset of Figure 4 in Mangan and Colombini, 1987). In contrast to succinylation, the action of dextran sulfate was to increase dramatically both the rate and the extent of closure of VDAC even at those (low) potentials at which the channel is usually kept in the open state (n increasing up to 14-fold, with no substantial change in nV_o at 62 μM dextran sulfate). As predicted, the effect on the steepness of the voltage dependence increased with voltage and the amount of added reagent, resulting in an increase of the probability of finding the channel in the closed state at any negative potential difference.

Within the same strategy, another negatively charged molecule was tested (Colombini et al., 1987), the so-called König's polyanion (König et al., 1977, 1982), a copolymer of methacrylate, maleate, and styrene (in a 1:2:3 ratio) with a molecular weight of 10 kDa. The behavior of this amphipathic molecule was more complex, probably because it can bind to the lipid-protein

boundaries through its lipophilic portion. In fact, on the one hand, Colombini et al., (1987) found that it could affect VDAC voltage sensitivity similarly to dextran sulfate when added to the negative side of the membrane. On the other hand, there was an additional clear block on some channels, not necessarily voltage dependent (Colombini et al., 1987), but also a stabilization of the open state taking place when the polyanion was added to the positive side of the bilayer (De Pinto et al., 1989; Benz et al., 1988; Benz, Kottke, and Brdiczka, 1990).

At all events, if the closed sate were associated with movement of charges, as proposed, then a change in selectivity would be expected to occur even under unmodified conditions (i.e., without added effectors). This was indeed proved by Ludwig, Benz and Schultz (1989), De Pinto et al. (1989), and Zhang and Colombini (1990), who showed that on closure at high potential differences, VDAC was clearly preferring K^+ over Cl^-, even though more than one value for the cationic permeability seemed to correspond to any of the closed states (Colombini, 1989; Zhang and Colombini, 1990).

Experiments were carried out to determine whether the lower conductance of the closed conformation depended merely on a local constriction of the channel entrance and/or on a more generalized diminution of the pore with reduction of the volume available for the flow of ions. Both the diameter decrease of around 20% [estimated from fluxes of radiolabeled glucose (Colombini, 1986)] and the substantial volume change [measured under osmotic stress (Zimmerberg and Parsegian, 1986)] of VDAC at high potential differences supported the notion that the conformational change consequent to closure was inducing a steric block over the entire length of the channel.

Subsequently, Colombini et al. (1987) determined a smaller diameter value of 1.8 nm from the largest nonelectrolyte still permeable through liposomes containing VDAC in the presence of König's polyanion and attributed it to the closed state of the channel. If a reduced permeability cut-off is clearly evident, at face value there seems to be no formal proof for identity of polyanion molecular action in systems (such as the planar bilayer and liposomes) that cannot be controlled in the same way. For example, no potential difference was applied in liposomes. In addition, complications arise from the behavior of the anion copolymer in planar bilayers where, if a negative potential seems necessary for VDAC closure in the presence of polyanion (Colombini et al., 1987; De Pinto et al., 1989; Benz et al., 1988; Benz, Kottke, and Brdiczka, 1990), prevention of closure at potential values as high as 80 to 100 mV is, on the other hand, demonstrated for the reagent added at the positive side of the membrane (De Pinto et al., 1989; Benz et al., 1988; Benz, Kottke, and Brdiczka, 1990). Finally, complete block of VDAC was also observed (Colombini et al., 1987). Pertinent to these uncertainties are the results of De Pinto et al. (1990), where no change in the chromatographic properties of VDAC (in terms of shield or exposure of charges) was found in the presence of high concentrations of König's copolymer and in the absence of any membrane potential.

In essence, the picture that has emerged from functional studies in planar bilayers is that VDAC has properties highly conserved in all eukaryotic cells. These include a large conductance, a poor selectivity for small ions, and the presence of two positively charged sensors that are responsible for the steep symmetrical closure in response to potential differences of opposite sign.

2. Patch Clamp and Tip-dip Studies

This rather uniform electric picture of the outer mitochondrial membrane was upset when other methods were used to study its electrophysiology, even though, with these methods, diverging results have sometimes been obtained.

The suspicion that the outer membrane *in situ* was not behaving as previously described was first aroused when a patch pipette was applied to intact liver mitochondria (i.e., to the outer membrane) (Figure 6A) that had been obtained from mice treated with cuprizone, a copper-chelating agent that induces the formation of liver mitochondria of larger size (Suzuki, 1969; Tandler and Hoppel, 1973). This experiment showed a channel that could not be readily identified as VDAC for its lower conductance value (Sorgato, Keller, and Stühmer, 1987). Other experiments of this type also resulted in few data consistently reminiscent of the VDAC behavior in planar bilayers (Tedeschi, Mannella, and Bowman, 1987).

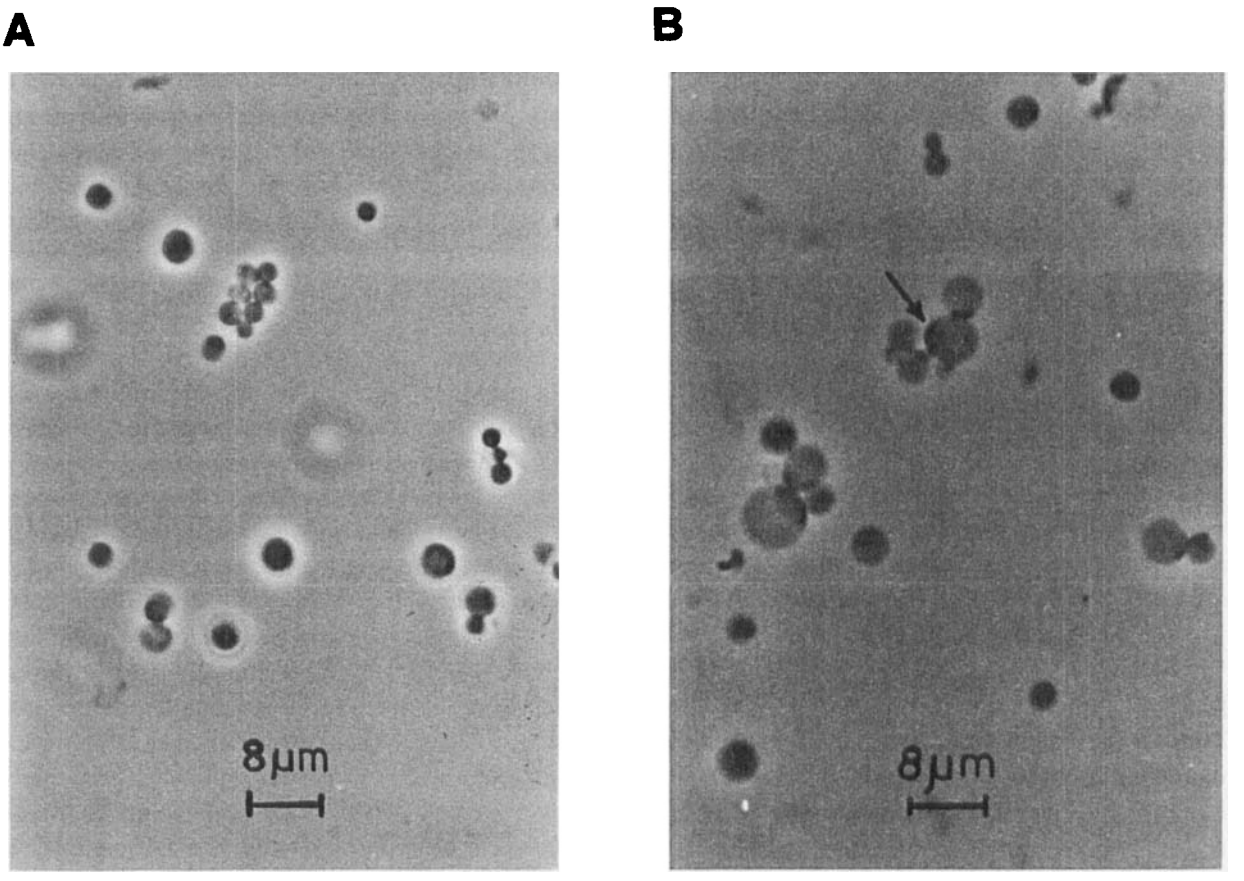

FIGURE 6. Liver mitochondria (A) and mitoplasts (B) visualized with phase contrast microscopy. Mitochondria, obtained from mice either treated (here) or not (see text) with cuprizone, are subjected to osmotic stress for removal of the outer membrane. Pieces of the outer membrane can remain attached to the inner one through tight connections, giving rise to the so-called cap regions (indicated by the arrow). Thus, in patch clamp experiments with mitoplasts, care is taken to place the pipette outside the clearly visible cap regions.

In fact, only in rare instances did the macroscopic conductance-voltage relation show the typical bell-shaped curve (as in Figure 4). At best, a conductance decrease on augmenting the potential (as expected for VDAC-containing membranes) was detected when the electric field was of one particular direction (positive relative to the pipette potential). Under the same conditions but with negative potentials, an increase in current was otherwise detected. Thus, a possible second channel-forming protein was hypothesized in the outer mitochondrial membrane. In subsequent studies, the mode of action of this non-VDAC channel was further explored (Kinnally, Tedeschi, and Mannella, 1987), and a model was developed to explain the voltage-dependent increase of the outer membrane conductance (Tedeschi, Kinnally, and Mannella, 1989; Kinnally et al., 1989). More precisely, the experimentally observed distinct kinetics for the exponential increase of the conductance at negative potentials were taken as suggestive of a first phase, with activation of single channels, and of a later phase, where channels would assemble together to give rise to a higher conductance oligomeric entity.

If this model fits well with the experiments on the temperature sensitivity of the aggregated state (Kinnally, Tedeschi, and Mannella, 1987), no evidence of such behavior was found in the only other study of integral mouse liver mitochondria carried out with the patch clamp (Moran et al., 1992). Discrepancy becomes more significant in the light of the presumed density of the hypothetical second channel (and of VDAC) in the outer membrane that, parenthetically, would

have made the use of low salt medium (10 m*M* KCl) almost compulsory for successful experiments (Tedeschi, Mannella, and Bowman, 1987; Kinnally, Tedeschi, and Mannella, 1987). On the contrary, Moran et al. (1992) showed not only recordings of single channels with fast kinetics of the open-close transitions, but also that these were measurable at physiological 150 m*M* KCl (Figure 7). Undoubtedly, the *in situ* analysis revealed new features of the outer membrane. In more detail, at least five sets of conductances could be distinguished on the basis of single-channel conductance and voltage sensitivity: one, apparently voltage independent, of less than 70 to 80 pS, the other of 99, 152, and 220 pS, which showed a higher open probability at positive potential values of the pipette (Table 1). A conductance of 307 pS, similar in magnitude to that previously described for the outer membrane (Sorgato, Keller, and Stühmer, 1987), was also present. Finally, reversal potential determinations in outer membranes containing liposomes demonstrated a general cationic selectivity.

Integral liver mitochondria from mice not treated with cuprizone, such as those used by Moran et al. (1992), have such small dimensions as to render problematic the application of the patch clamp. Contrary to mitoplasts (see Figure 6B), in whole mitochondria, the outer and inner membranes cannot be distinguished with a phase contrast microscope (see Figure 6A) and, therefore, the possibility that the pipette is testing the inner, and not the outer, membrane cannot be ruled out *a priori*. However, two pieces of evidence militate against this hypothesis. One is that channels with the same combined characteristics (conductance values associated with the voltage-dependence and cationic selectivity) are not found in the inner membrane (Section IV.A). The second argument regards the almost identical results obtained by patch clamping the outer membrane that had been isolated from rat brain and reconstituted in liposomes without detergents (Moran et al., 1992). These two sets of observations thus make one confident of the location of the found channels.

Consistent with the possibility of recording discrete single-channel currents in the outer membrane, is the report where the tip-dip method (whereby membranes form bilayers at the tip of a pipette) was used to study mitochondrial electrophysiology. With both mitochondrial membranes, mainly from bovine adrenal medulla or cortex (Thieffry et al., 1988, 1992; Henry et al., 1989), or with the outer membrane isolated from rat or ox liver (Chich et al., 1991), slightly ca-

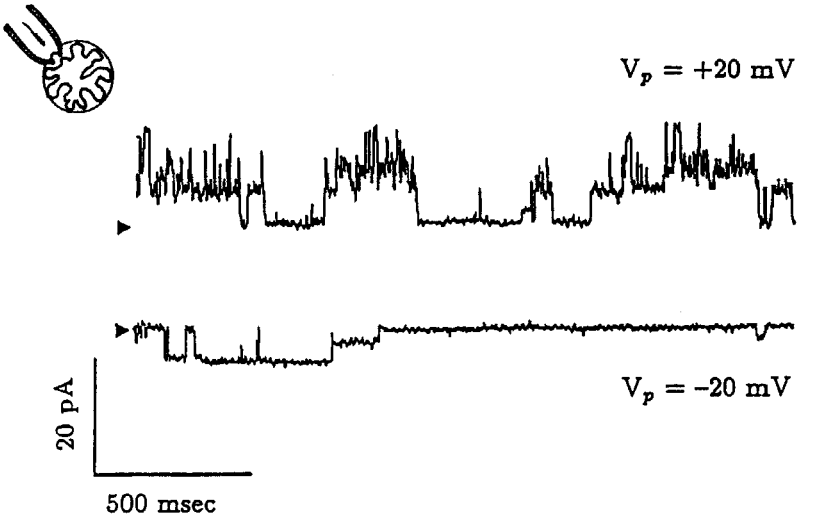

FIGURE 7. Recording of currents from an integral mouse liver mitochondria with the patch clamp technique. The configuration used was a mitochondria-attached patch, and solutions were symmetrical 150 m*M* KCl, 0.1 m*M* $CaCl_2$, 20 m*M* Hepes-KOH, pH 7.2. Arrows indicate 0 current levels. The sign of the voltage (Vp) refers to that of the pipette. (From Moran, O. et al., *Eur. Biophys. J.*, 20, 311–319, 1992. With permission.)

tionic conductances were found ($pNa^+/pCl^- \approx 4$) rapidly fluctuating in a voltage-asymmetrical fashion between at least three levels separated by 220 pS transitions (in 150 m*M* NaCl). This channel is usually referred to as PSC (peptide-sensitive channel) (see Section IIID.2). Confirmation of these results has been obtained recently with membranes incorporated in planar bilayers or in patches of giant liposomes (Thieffry et al., 1992). The different electric features, the sensitivity to proteases of the voltage-dependent fluctuations (Thieffry et al., 1988; Chich et al., 1991), and a similar behavior detected both in wild-type and VDAC-deficient yeast mitochondria (Fèvre et al., 1990; Thieffry et al., 1992) strongly indicated that the channel-forming protein was not VDAC (Table 1).

Despite the different approaches and the inhomogeneous results, the experimental evidence gathered with techniques other than the planar bilayers is indeed suggestive of an outer membrane electric behavior not under the control of VDAC. It is thus important to discuss the findings encountered by Wunder and Colombini (1991) in their patch clamp investigation in physiological salt of proteoliposomes containing *Neurospora crassa* mitochondrial membranes. Here, the major conductance value found, the slow and fast kinetics displayed by the membrane patch in the closing and opening events on voltage changes, together with the expected effect of König's polyanion were interpreted as strongly supporting a primary role for VDAC (see Table 1). At variance with the results from planar bilayers, however, a current decrease in proteoliposomes was frequently prevented at high negative command voltages. The proposed explanation envisaged a curvature present in the patch membrane (and absent in the planar bilayer), which would be the indirect cause of a structural hindrance for the movement of the VDAC-positive voltage sensor at negative membrane potentials. Significantly, a similar asymmetrical response to voltage was also found by Moran et al. (1992) in the native membrane. Thus, at this point, the crucial issue is to understand which of the two situations, the planar bilayer or the membrane under a patch, is the closest to the physiological membrane.

If we can take refuge in methodological causes to explain different data obtained with reconstituted systems (alternative procedures used to isolate the biological samples and/or to prepare the proteoliposomes), the same cannot be advocated when comparison is made with *in situ* studies. Thus, in the first instance, very few points of convergency are obvious. To our minds, the simplest possible explanation is that one or more channels different from VDAC are present and active in the outer membrane of mitochondria other than *Neurospora crassa*. Indeed, it is in this fungus where the highest concentration of VDAC was estimated [higher than 50% of the total protein content (Mannella, 1982) and around $4 \cdot 10^4$ VDAC/μm^2 in phospholipase A_2-treated membranes (Mannella, 1984)]. Hence, the results of the patch clamping by Wunder and Colombini (1991) would fit the expectations of this hypothesis, with VDAC being the most detectable channel in fungal membranes, and at the same time, would not necessarily disagree with the diverging features encountered by others in the patch clamping of mammalian outer membranes where VDAC is present, but to a lower extent (De Pinto et al., 1987). Nevertheless, it is important to recall here that mammalian VDAC was detected immunologically in both native and reconstituted outer membranes analyzed with the patch clamp (Moran et al., 1992).

As discussed later (Section III.D.1), a protein, isolated from the mitochondrial intermembrane space, was proved to interact potently with VDAC in planar membranes (Holden and Colombini, 1988). In principle, its presence in the patched integral mitochondria should be ensured during the isolation of the organelle and could therefore provide the sought explanation of the unorthodox, although presumably more physiological, behavior of the native membrane. The question is why the same electric pattern is detected in liposomes with the isolated membrane, where the modulator is likely to be lost. Along a similar line of thought, the possibility should be considered that VDAC interacts with other membrane components, whose attachment may remain unaffected by proteoliposomes formation in the absence of detergents. In fact, recent isolation protocols have demonstrated that VDAC

is associated with other membrane proteins (Section III.D.4).

3. Other Channels in the Outer Membrane

As mentioned before, application of the patch clamp technique to whole mitochondria indicated that the outer membrane may harbor a channel different from VDAC. This view was proven for the cationic channel also detected in yeast mutants lacking VDAC (Thieffry et al., 1988, 1992; Henry et al., 1989; Fèvre et al., 1990; Chich et al., 1991). Interestingly, Dihanich et al. (1989), by examining detergent-solubilized outer membranes of a similar yeast mutant in planar bilayers, concluded that the observed slightly cationic channel of 210 pS conductance (in 0.1 *M* KCl), which had more rapid kinetics than standard VDAC (Table 1), could nevertheless be present along with VDAC in the outer membrane of wild-type yeast mitochondria. Similarly, De Pinto et al. (1991a) were able to identify a second pore-forming protein, with molecular mass and electric features close to, yet distinguishable from, VDAC, in the liver outer membrane of the fish *Anguilla anguilla* (Table 1).

Finally, in reconstituted and in *in situ* experiments with the outer mitochondrial membrane of rat brain and mouse liver, respectively, much smaller conductances than those described hitherto (in the range of approximately 20 to 50 pS in 150 m*M* KCl) and apparently voltage-independent were recorded (Moran et al., 1992). The interesting aspect of this finding is that entities of the same kind have been detected in the inner membrane and contact sites fraction (see Sections IV.A.2, IV.A.4).

B. Structural Features of VDAC from Electron Microscopy, Molecular Biology, and Biochemical Studies

1. Electron Microscopy

A considerable effort has been devoted to the understanding of the molecular structure of VDAC, and recognition must undoubtedly be paid to the remarkable body of work provided by Mannella and co-workers, who tackled this problem first with electron microscopic techniques (Mannella and Bonner, 1975a; Mannella, 1981, 1982) well before there was knowledge of the protein primary sequence (Mihara and Sato, 1985). The outer membrane used in these studies is that from the fungus *Neurospora crassa*. In negatively stained specimens, VDAC crystalline arrays were observed to form sporadically (Mannella, 1982; Mannella and Frank, 1982) or in larger amounts after membrane treatment with soluble phospholipase A_2 (Mannella, 1984). This finding greatly helped the study of two-dimensional crystals by transmission electron microscopy not only on specimens negatively stained by different procedures (Mannella, 1984; Mannella, Radermacher, and Frank, 1984; Mannella and Guo, 1990; Guo and Mannella, 1992; Guo et al., 1992), but also on unstained membranes, either frozen dried and metal shadowed (Thomas et al., 1991) or simply frozen hydrated (Guo, 1990; Mannella, Guo, and Cognon, 1989). Recently, the outer mitochondrial membrane has been examined with a scanning transmission electron microscope (Thomas et al., 1991). All this effort has resulted in important information with respect to the surface topology and three-dimensional reconstruction of the VDAC channel, despite the still low resolution obtained (1 to 2 nm).

With any specimen, the electron microscopic images have constantly shown the presence of cell units composed of six circular elements, identified with certainty as VDAC proteins by immunogold electron microscopy (Mannella and Colombini, 1984). These are mainly disposed in parallelogram arrays with a twofold rotational symmetry (Figure 8A) (Mannella, 1986; Mannella, Colombini, and Frank, 1983; Mannella, Ribeiro, and Frank, 1986). Figure 8B shows a three-dimensional computer-reconstructed image of both sides of frozen-dried and metal-shadowed outer membranes where channels appear structurally symmetrical (Thomas et al., 1991).

Considering each element of the unit cell, this could be best represented by a hollow cylinder normal to and entirely traversing the membrane (Mannella, 1989; Mannella, Radermacher, and Frank, 1984; Mannella, Guo, and Cognon, 1989) with a length of 3 to 3.5 nm (Guo et al.,

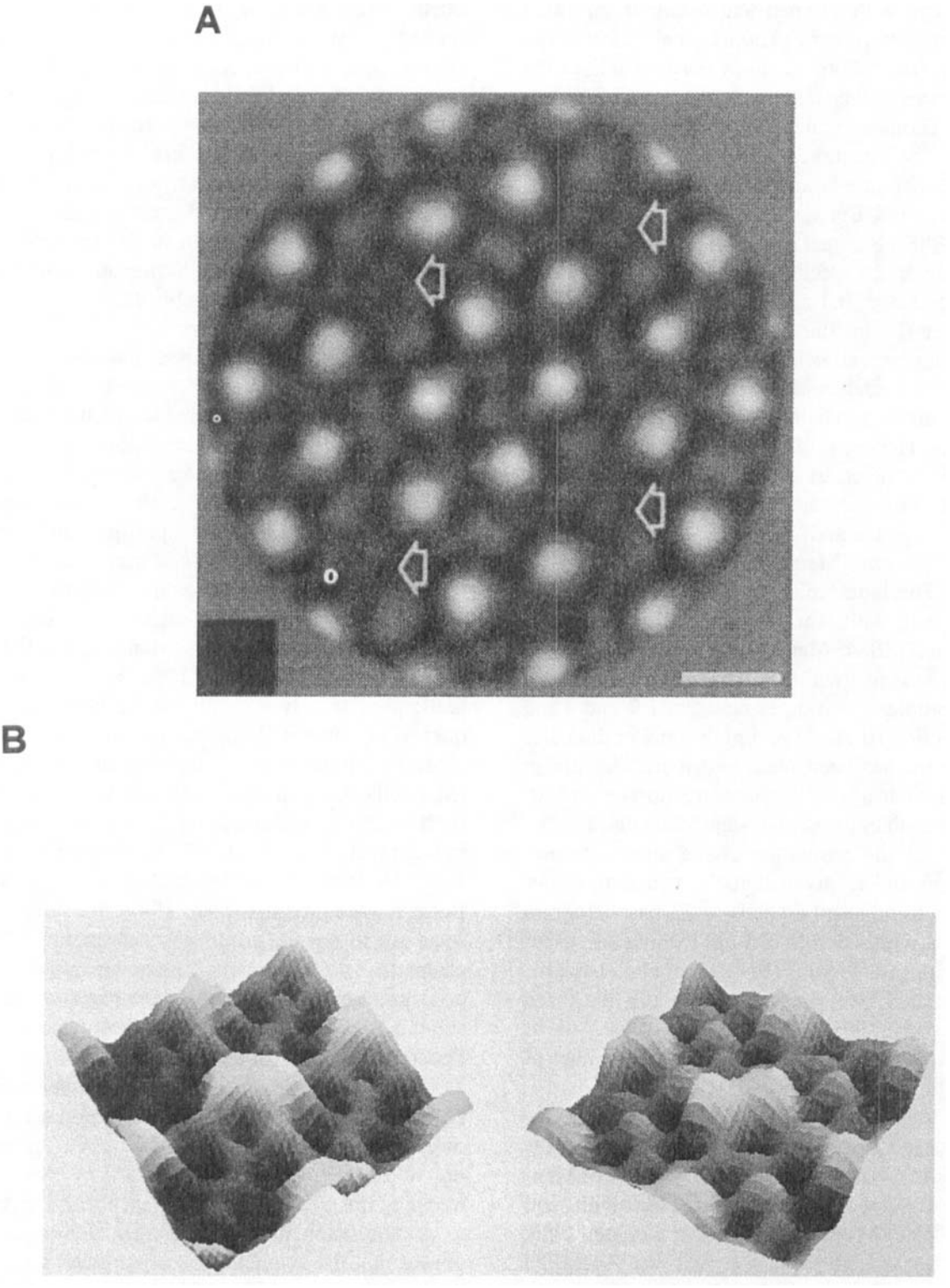

FIGURE 8. (A) Correlation average of electron microscopic images of oblique arrays of VDAC channels from unstained, frozen-hydrated *Neurospora crassa* outer mitochondrial membranes treated with soluble phospholipase A_2. Within the space limited by arrows, a unit cell composed of 6 elements is visible. The low contrast regions correspond to water-filled pores, the darker regions to the protein. In particular, arrows indicate the protein arms extending laterally at the corners of the unit cell. Bar: 5 nm. For details see text. (From Mannella, C. A., Forte, M., and Colombini, M., *J. Bioenerg. Biomembr.*, 24, 7–19, 1992. With permission). (B) Three-dimensional image reconstruction of the top and bottom surfaces of the two-dimensional arrays of *Neurospora crassa* outer mitochondrial membranes. Membranes were treated with soluble phospholipase A_2, frozen-dried, and Pt-shadowed. White plateaus correspond to VDAC protein domains more exposed to the membrane surface. (From Thomas, L. et al., *J. Struct. Biol.*, 106, 161–171, 1991. With permission.)

1992) and with symmetrical openings on either side of the membrane (Thomas et al., 1991). The majority of the protein does not protrude to the membrane surface (Mannella, Guo, and Cognon, 1989; Thomas et al., 1991), which could well explain the general resistance of VDAC to proteolysis (Mannella and Bonner, 1975a; Freitag, Neupert, and Benz, 1982; Holden and Colombini, 1988). Refined crystal-modeling studies on frozen-hydrated specimens have pointed to a mean value of 3.8 ± 0.1 nm for the projected cylinder diameter (Mannella, Guo, and Cognon, 1989). The cylinder wall is most probably made of antiparallel β strands with residues alternatively facing the inside and outside of the pore (see Section III.B.2). Hence, given a value of approximately 0.5 nm for the protrusion of the residues on either side of the wall, the outer diameter would be around 4.8 nm and the inner (lumen) diameter about 2.8 nm (Mannella, Guo, and Cognon, 1989). The latter value for the water-filled pore agrees well with other structural data (Mannella and Frank, 1984; Mannella, Ribeiro, and Frank, 1986) but is just within that estimated from functional studies that ranges between 1.7 and 4 nm (see Section III.A.1). A slightly smaller diameter of 2.2 nm has been measured more recently in crystalline arrays of the outer membrane embedded in aurothioglucose (Guo and Mannella, 1992).

As for the estimation of the outer diameter of the cylinder, given that the center-to-center distance of adjacent crystals in the parallelogram lattice is within 4.5 to 5.5 nm (Mannella, 1986; Mannella and Frank, 1984; Mannella, Ribeiro, and Frank, 1986), the value of 4.8 nm has posed severe constraints to the thickness of the wall of the cylinder and, consequently, to the number of β sheets forming the wall.

As mentioned, VDAC channels crystallize into ordered arrays that, however, may have different lattice angles (Mannella 1984, 1986; Mannella and Guo, 1990; Mannella, Colombini, and Frank, 1983; Mannella, Ribeiro, and Frank, 1986; Thomas et al., 1991). In phospholipase A_2-treated membranes, the most frequently observed of such polymorphs is the so-called "oblique" lattice, bearing an angle of 109° between the lattice vectors, a and b, of 13.3 nm and 11.5 nm, respectively. In this array, lateral protein arms are seen to extend into the phospholipid domain from each corner of the unit cell (Figure 8A). A less frequent arrangement is that defined as "contracted", that is, adjacent rows in the arrays appear to slide with respect to each other, leading to a diminished unit cell area and an angle of 99° (with a = 13 nm, and b = 10 nm). Another major difference between these two polymorphs is the absence of the lateral arms in the contracted lattice. Hence, the oblique state appears to be stabilized by the interaction of the protein arm with the bilayer, and any detrimental effect on this interaction would possibly facilitate transitions to the contracted form.

Two experimental conditions have been found to increase the percentage of the contracted lattice concomitant to the disappearance of the oblique array. One was a prolonged incubation with phospholipase A_2, which provoked shrinkage of the lipid patches located outside the channel hexamer, indicating that loss of phospholipids was a necessary factor for the phase transition to take place (Manella, 1986). The other was the addition of organic molecules such as the König's synthetic anionic copolymer (Mannella and Guo, 1990; Guo and Mannella, 1992) and of a synthetic peptide corresponding to the targeting sequence of subunit IV of the mitochondrial respiratory chain enzyme cytochrome oxidase (Mannella, Guo, and Dias, 1992). The choice of such molecules was intentional: the polyanion for inducing closure of VDAC (Colombini et al., 1987; De Pinto et al., 1989; Benz et al., 1988; Benz, Kottke, and Brdiczka, 1990); the targeting sequence to test the possible involvement of the channel in the import machinery for mitochondrial precursors (including cytochrome oxidase subunit IV) *en route* to their final destination (see Section III.D.2).

Analysis of the outer membrane in the presence of either molecule showed increased disorder and contraction of the crystal lattice, pointing to an interaction with VDAC. In fact, by mapping the areas of negative stain exclusion due to accumulation of the molecules, it was concluded that the synthetic precursor binds specifically to the arm regions of VDAC arrays (Mannella, Guo, and Dias, 1992), whereas polyanion, given its amphipathic nature, would probably bind to protein-lipid interface areas (Guo and Mannella, 1992). Regardless of the mode, direct or indirect, either molecule would nevertheless interact with the arms and cause their freeing from

the bilayer and array contraction (Guo, 1990; Mannella, Guo, and Dias, 1992). Therefore, the occurrence of two events seems necessary for phase transition, displacement of arms, and loss of phospholipids.

In light of these results, one might expect a direct link between crystal polymorphism and VDAC functional states (open and closed). However, this does not appear to be the case because of the recent finding of lack of correlation between type of array and size of the pore diameter, that is, the large and restricted diameter are present in both oblique and contracted arrays (Guo and Mannella, 1992). On the other hand, Kön-ing's polyanion was shown to favor the reduction of the projected lumen diameter of a single VDAC from 2.8–2.2 nm to 1.4–1.7 nm (Mannella and Guo, 1990; Guo and Mannella, 1992) in line with the diminished permeability of polyanion-treated VDAC (Section III.A.1). These results have thus been interpreted as indicative of a relation between structural changes of VDAC crystals (although not linked necessarily to contraction) and functional states of the channel and are the basis for a proposed mechanism of closure (Section III.B.2).

No doubt, the possibility of having crystals in the membrane has provided the necessary starting point for the three-dimensional study of an integral membrane protein in its natural milieu. As it stands, however, suggestion of a functional significance of crystallization, albeit fascinating, sounds premature. Possibly, in such aggregated state, VDAC behavior could be influenced by the vicinity of other VDAC molecules. However, one still has to understand whether VDAC particular arrangement in hexameric clusters, seen abundantly at the electron microscope after phospholipase A_2 treatment, occurs as such *in vivo* and in mitochondrial outer membranes other than those from *Neurospora crassa*. Apparently, in mammalian mitochondria, phosphotungstate and silicotungstate, not phospholipase A_2, can induce the formation of paracrystals whose arrays are more similar to those of sarcoplasmic reticulum Ca^{2+}-ATPase than to VDAC crystals in *Neurospora crassa* (Mannella et al., 1992; see also Parson, Williams, and Chance, 1966). Mannella (1987, 1988, 1989, 1990) put forward the hypothesis that VDAC could organize itself naturally in periodic arrays on action of endogenous phospholipase A_2. This enzyme is activated by calcium and, thus, different calcium levels (i.e., different cell-activated states) would in turn regulate the formation of the arrays and also, presumably, the physiology of the outer membrane. In this respect, it is certainly unfortunate that the electric characteristics of the outer membrane have never been analyzed under controlled phospholipase activity.

2. Molecular Biology and Biochemical Studies

Of all mitochondrial channels, VDAC is the only protein whose primary structure is known from cDNA sequencing or amino acid determination: 282 to 283 amino acids compose the fungal *Neurospora crassa*, the yeast *Saccharomyces cerevisiae*, and the human B lymphocytes and skeletal muscle VDAC (Mihara and Sato, 1985; Forte, Guy, and Mannella, 1987; Kleene et al., 1987; Kayser et al., 1989; Jürgens et al., 1991). In all cases, the protein has a basic and relatively hydrophilic character [45.5% of polar and charged amino acids in yeast VDAC (Forte, Guy, and Mannella, 1987) and 56% in the human VDAC (Kayser et al., 1989)]. Sequence homology among the polypeptides of different origins is not high: 43% between fungal and yeast protein (Kleene et al., 1987) and 24% and 29% between the human and the yeast and fungal VDAC, respectively (Kayser et al., 1989).

In spite of the low similarity of the amino acid sequence, evolutionary pressure has not evolved in changed structural-functional correlations in the light of the highly conserved electric behavior of VDAC in planar membranes (see Section III.A.1). As for the secondary structure predictions, the many strands with alternating polar and apolar residues, found repeatedly in VDAC of different origins, have been judged suitable for constructing the wall of the pore with antiparallel β sheets (Kleene et al., 1987; Forte, Guy, and Mannella, 1987; Blachly-Dyson et al., 1989; De Pinto et al., 1991b) in analogy with bacterial porins (Weiss et al., 1991), which nevertheless bear no sequence homology with VDAC and display different electric patterns (Benz, 1985; Forte, Guy, and Mannella, 1987). The alternating residues would ideally satisfy the necessary hydro-

phobic (with the bilayer) and hydrophilic (with the water-filled pore) interactions of the pore (Kleene et al., 1987; Forte, Guy, and Mannella, 1987; Blachly-Dyson et al., 1989; De Pinto et al., 1991b). However, a different number of β strands has been predicted, connected by proline or adjacent charged amino acids: 15 to 19 (Forte, Guy, and Mannella, 1987) and 12 (Blachly-Dyson et al., 1989, 1990; Peng et al., 1992a) for yeast VDAC; 12 to 16 for yeast and fungal VDAC (Kleene et al., 1987); 16 for human VDAC (De Pinto et al., 1991b).

In no case were hydrophobic stretches detected that are compatible with α helixes sufficiently long to span the bilayer (Forte, Guy, and Mannella, 1987; Kleene et al., 1987). Yet, the overall amphipathic nature of the NH_2-terminal in all polypeptides consistently argues for an α helix conformation in this region. The actual location of this strand (composed of 18 to 24 amino acids, depending on the origin), whether embedded in the membrane (Blachly-Dyson et al., 1989, 1990; and Peng et al., 1992a) or exposed to the surface (De Pinto and Palmieri, 1992; Forte, Guy, and Mannella, 1987; De Pinto et al., 1991b), is still a matter of controversy.

In general, the choice of the number and of the type of intramembrane strands is based primarily on the use of secondary structure prediction algorithms (Fasman, 1990). However with VDAC, other pieces of information have contributed to select one or the other model. For instance, Blachly-Dyson et al. (1989, 1990) and Peng et al. (1992a) have elegantly combined molecular genetics and biophysics to locate a certain number of amino acid residues inside or outside the membrane. They have done so by monitoring the ion selectivity of the open state (by reversal potential value determination) of VDAC isolated from yeast strains bearing one or more mutations at selected sites, in particular, at charged amino acids. The rationale underlying this work was that an altered selectivity would have been consistent with the location of the replaced amino acid in an intramembrane strand and an unchanged selectivity with the replaced amino acid being outside. Following this reasoning, data available from 29 of such mutants have led to the proposal of the channel wall made of 12 β strands plus the NH_2-terminal α helix (Blachly-Dyson et al., 1990) (Figure 9A). The rest of the protein (approximately 50%) would instead protrude from either side of the pore.

It may be too simplistic to assign such a definite result to the substitution of an amino acid and the more so in the case of a charged residue. Of course, the interpretation of an unequivocal relation between selectivity and position of a mutated amino acid, inside or outside the channel's lumen, may be valid. On the other hand, it is not easy to discard the possibility that local perturbations after single amino acid substitutions may propagate over a short or long range, sufficient to provoke a disturbance both of the pore's secondary structure and of selectivity. The almost unchanged conductance found in VDAC's mutants (Blachly-Dyson et al., 1989, 1990; Peng et al., 1992a) may be at least one reason to question this hypothesis, but not to exclude it at this time.

De Pinto et al. (1991b) followed a completely different approach for establishing the transmembrane topology of mammalian VDAC. Using biochemical (proteases) or immunological (with antibodies raised against the whole protein or a synthetic peptide mimicking the N-terminal piece) assays on integral and broken ox heart mitochondria, they concluded that whereas the majority of the VDAC polypeptide is embedded in the bilayer (for a total of 16 β strands), the NH_2-terminal α helix and two major interconnecting loops are exposed to the cytoplasmic side of the membrane (Figure 9B). This leads to an asymmetric disposition of VDAC in terms of structure and charge distribution. That several of the positively charged residues were likely to be located on the hydrophilic surface of the channel had already been suspected by testing the interaction capacity with ion exchange columns by VDAC dissolved with detergents of different chemicophysical properties (Palmieri and De Pinto, 1989; De Pinto et al., 1990). A fairly good homology found in the sequence of the α helix of phylogenetically distant VDACs and its particular location have led De Pinto et al. (1991b) to support the involvement of the strand as a voltage sensor. Interestingly, the same number of β strands and a similar charge disposition was found for each subunit of the porin channel of

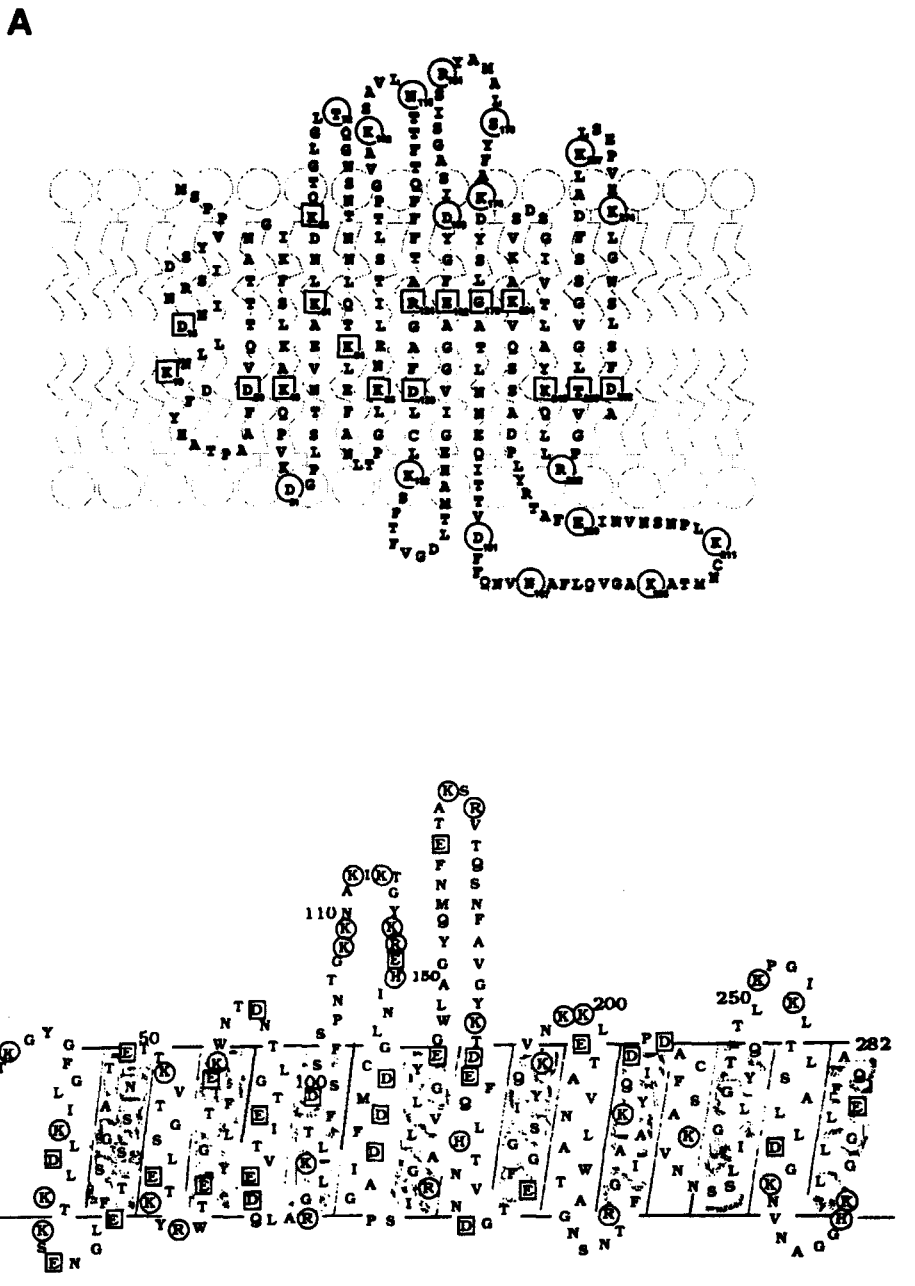

FIGURE 9. Model of transmembrane arrangement for yeast (A) and human (B) VDAC. (A) A barrel consisting of 1 α helix and 12 β strands is proposed for yeast VDAC after site-directed mutations. Substitution of boxed residues altered the channel's selectivity, whereas no change was found for mutations at the circled residues (Blachly-Dyson et al., 1990). (From Mannella, C. A., Forte, M., and Colombini, M., *J. Bioenerg. Biomembr.*, 24, 7–19, 1992. With permission.) (B) A barrel consisting of 16 β strands is proposed for human VDAC after biochemical and immunological assays (De Pinto et al., 1991b). Note that the N-terminal α helix and the major loops face the cytoplasmic side of the membrane. Boxed residues are positively charged and circled residues negatively charged. (From De Pinto, V. and Palmieri, F., *J. Bioenerg. Biomembr.*, 24, 21–26, 1992. With permission.) For details see text.

Rhodobacter capsulatus, whose crystal structure has been determined down to 1.8 Å (Weiss et al., 1991).

What is the relation of a particular number of strands to the overall geometry of the channel? As discussed recently by Mannella, Forte, and Colombini (1992), the hypothetical number of strands (mainly in the β sheet conformation) that would form a cylinder with a fixed C_α-backbone diameter (for instance, of 3.8 nm, as in Mannella, Guo, and Cognon, 1989) can vary somewhat, provided that the inclination of the strands with respect to the long axis of the channel, assumed uniform for simplicity, is also varied. Hence, no tilting is required with 23 strands fitting the channel wall. Otherwise, a tilt of 35° is necessary for about 18 strands and one of 60° if the wall is lined by only 13 strands. Of course, the length of the cylinder would shorten with the increased tilting. According to Peng et al. (1992b), who favor the smallest number of total strands (13), such a hypothetical decrease in length would respond nicely to three-dimensional reconstruction images of electron micrographs, showing that VDAC is, in fact, shorter than the membrane (Thomas et al., 1991). Nonetheless, as pointed out by Hille (1984), a channel must have a minimum length to ease entrance of ions restricted otherwise by the phospholipid barrier. In the case of VDAC, the resulting length of 2.2 nm for the cylinder (Mannella, Forte, and Colombini, 1992) seems perhaps too short to justify its large conductance.

Two models have been proposed to depict tentatively the structural alterations encountered by VDAC when passing from the open to the closed state, essentially from the following experimental results. The first result is that the voltage sensor is made of positive charges that need to move along the entire electric field to account for the voltage-dependent closure (Bowen, Tam, and Colombini, 1985; Doring and Colombini, 1985a; Adelsberger-Mangan and Colombini, 1987; Forte, Adelsberger-Mangan, and Colombini, 1987). The second result is that on closure VDAC undergoes a large change in conductance, ion selectivity, and volume concomitant to a drastic reduction of the pore diameter (Colombini, 1986, 1989; Benz, 1990; Zimmerberg and Parsegian, 1986; Zhang and Colombini, 1990; Ludwig, Benz, and Schultz, 1989; Benz, Kottke, and Brdiczka, 1990; Colombini et al., 1987; De Pinto et al., 1989). Finally, energy difference between the open and closed states is minimal (Bowen, Tam, and Colombini, 1985). Consensus over these data is widespread except for the location of the voltage sensor, which is supposedly intralumenal in one case or in someway linked to an external domain in the other.

The first model was proposed by Colombini (Colombini, 1989; Zhang and Colombini, 1990) and supported further by site-directed mutagenesis experiments showing that lysines, crucial for voltage gating and selectivity mechanisms (see Section III.A.1), were part of the wall of VDAC pore in the open state (Blachly-Dyson et al., 1989, 1990) but not after closure (Peng et al., 1991, 1992a). The transition from open to closed was then postulated to occur by a physical dislocation of β strands from the interior to the exterior of the channel associated with an overall decrease of the circumference of the pore wall.

The second model (Mannella, 1990; Guo and Mannella, 1992) stemmed from observations of electron microscopic pictures of VDAC whose large or reduced diameter could be best correlated, respectively, with the presence, or the absence, of the extended protein arm (see Section III.B.1). Hence, closure was hypothesized to be consequent to the insertion of the flexible arm into the lumen of the channel, causing partial blockage but not necessarily a decrease per se of the outer wall circumference. As a working hypothesis, in this model the protein arm (or a domain interacting with it) is best identified with the voltage sensor, and other data pointing to the involvement in the gating process of two superficially exposed lysines per VDAC monomer (De Pinto et al., 1990) corroborate the proposal.

C. Stoichiometry

At this point, it should be rather clear that the picture of VDAC, emerging from both structural and sequence analyses, is that of a barrel with a thin wall able to accommodate only a single layer of β strands. But, what is the molecular stoichiometry of the channel? Interpretations of almost all biochemical and electrophysiological data have long suggested a contribution of two subunits to the formation of

a functional channel (Colombini, 1986, 1989; Lindén and Gellerfors, 1983; Mangan and Colombini, 1987; Holden and Colombini, 1988; Zhang and Colombini, 1990; Schein, Colombini, and Finkelstein, 1976; Pfaller et al., 1985; Blachly-Dyson et al., 1989, 1990). A strong piece of evidence for this hypothesis is the quasi-symmetrical response of VDAC in planar bilayers to potential differences of opposite sign (Colombini, 1986; Benz, 1990) or to the addition to both sides of the membrane of natural or synthetic effectors (Doring and Colombini, 1985a; Mangan and Colombini, 1987; Holden and Colombini, 1988; Zhang and Colombini, 1989; Dill, Holden, and Colombini, 1987; Benz, Kottke, and Brdiczka, 1990; Colombini et al., 1987). This was interpreted as an availability of the voltage sensor, or of the binding sites for the added molecules, to either exposed faces of the channel. Thus a dimer, composed of two identical subunits but with opposed orientation, seemed the most plausible hypothesis (Colombini, 1989; Doring and Colombini, 1985b; Blachly-Dyson et al., 1989, 1990).

The question of stoichiometry has been approached also by calculating the relative surface area occupied by VDAC in projected density maps, which led Mannella (1986, 1987) to suspect that the aperture of the pore was too small to accommodate two polypeptide chains. Based on other arguments, Forte, Guy, and Mannella (1987) and De Pinto et al. (1991b) also favored a stoichiometry of 1. Apparently, two additional pieces of evidence have recently reinforced such a conclusion: the mass per unit area estimated by scanning transmission electron microscopy of VDAC crystals (Thomas et al., 1991) and genetic manipulations combined with electric measurements (Peng et al., 1992b). In the latter case, experiments were carried out with VDAC extracted from yeast strains containing both the wild-type gene and a mutant gene altered specifically so as to yield functional channels with unaltered conductance but with different ion selectivity (Blachly-Dyson et al., 1990). With a dimer, the expectation was the recording of intermediate selectivity displayed by the chimeric protein. Failure to observe such values has thus been recognized as strong proof for a monomeric form of the channel (Peng et al., 1992). Yet, a slight uncertainty surrounds the validity of this conclusion, as the possibility of a nonfunctional association between the wild-type and mutant monomers was not considered.

Unequivocal evidence that the molecular stoichiometry of VDAC is in fact 1 would undoubtedly represent an important contribution to the field of mitochondrial channels. Thus, the implication of such information would allow a much more confident construction of the secondary structure of VDAC. At the same time, however, it could question the validity of the interpretation of many electrophysiological data as the basis of the proposition of a dimer and, in particular, of the symmetric response of VDAC to voltages of opposite sign (Figure 4). Indeed, it should be considered that symmetric behavior is typical of VDAC in planar bilayers but not of "patched" mitochondria (Moran et al., 1992).

In recent work intended to clarify the diverging results obtained with the two methods, Mirzabekov et al. (1993) found criteria whereby the behavior of the outer membrane in bilayers was consistent with that displayed in a pipette patch, except for the voltage sensitivity, which remained symmetrical. In light of all this, there are two possibilities. One is that the VDAC monomer harbors two gating sensors [as already suggested for the dimeric form (Colombini, 1989)], each of which responds to the electric field of one particular direction only when the protein is in planar bilayers. The second is that VDAC inserts in bilayers in both directions. As naïve as this latter possibility may appear, it seems, to our knowledge, that its veracity has never really been probed thoroughly, despite some incongruous data of VDAC in planar bilayers. For example, Benz (1990); Colombini (1986); Roos, Benz, and Brdiczka (1982); Freitag, Neupert, and Benz (1982); Fiek et al. (1982); De Pinto et al. (1985); and Ludwig et al. (1988) did describe an asymmetrical dependence in terms of extent and kinetics of closure to potential differences of opposite sign, but then these data were either not supported sufficiently (Benz, 1990) or interpreted as being the result of some imperfection in the planar membrane or in the protein itself (Colombini, 1983, 1986, 1989).

D. Physiology of the Outer Mitochondrial Membrane Channels

Our previous concentration on the structural and electric characterization of the outer membrane should not obscure other observations pointing to an involvement of VDAC and of other channels in the physiology of mitochondria. The most important clues to this issue are now discussed.

1. VDAC as a Controlled Pathway for Mitochondrial Metabolites

The study of the permeability of the outer mitochondrial membrane goes back to the 1960s (O'Brien and Brierly, 1965; Pfaff et al., 1968), if not earlier (Werkheiser and Bartley, 1957), when a generalized permeability to small metabolites, except for macromolecules such as cytochrome *c* (Wojtczak and Zalustra, 1969), was found. At the same time, Parson, Bonner, and Verboon (1965), with electron microscopic investigations, found the presence of cavities in negatively stained outer membranes of mung bean mitochondria [but not of rat liver (Parson, Williams, and Chance, 1966)], subsequently identified by X-ray diffraction and biochemical characterization with a 30-kDa polypeptide (Mannella and Bonner, 1975a, 1975b).

Yet, it was only after the discovery of large pores with electrophysiological techniques (Schein, Colombini and Finkelstein, 1976; Colombini, 1979) that a closer search for proteins at the basis of the outer membrane permeability eventually started. Measurements of permeability in proteoliposomes showed that polypeptides of approximately 30 kDa allowed passage of molecules with molecular weight of 6 to 8 kDa (Zalman, Nikaido, and Kagawa, 1980; Lindén, Gellerfors, and Nelson, 1982a) and these corresponded to the channel-forming proteins in planar bilayers (see Section III.A.1). VDAC is one of the (if not the) most abundant proteins of the outer mitochondrial membrane of primitive and advanced eukaryotic cells (Mannella and Bonner, 1975a; Mannella 1982; Freitag, Neupert, and Benz, 1982; Forte, Adelsberger-Mangan, and Colombini, 1987; Fiek et al., 1982; Reizman et al., 1983; De Pinto et al., 1987), and the overall weight of evidence from different approaches points strongly to this protein as the conduit for the exchange of metabolites through the outer membrane. Frequently, this function has been related to that of porins of the outer envelope of Gram-negative bacteria (Benz, 1985).

The molecular sieving attributed to VDAC does not axiomatically imply a relevance to the physiology of mitochondria. This would be the case if the access to VDAC were regulated so to allow, or hinder, the passage of metabolites and metal ions essential for mitochondrial processes. Evidence accumulated from *in vitro* experiments with planar bilayers argues in favor of such a possibility, as the highly conserved voltage-gating properties of VDAC cause the induction of substantial changes in pore size, permeability, and selectivity in response to only discrete differences in the electric environment (see Section III.A.1). The proof both of these changes also in integral mitochondrial suspensions and of the existence of a potential difference across the outer membrane should be cardinal to this view.

As one approach to this question, Benz et al. (1988) and Benz, Kottke, and Brdiczka (1990) treated suspensions of rat liver mitochondria with the synthetic negatively charged copolymer (König's polyanion) (see Section III.A.1) to ascertain whether the impairment of oxidative phosphorylation and of several inner membrane carriers observed previously in mitochondria in the presence of the copolymer (König et al., 1977, 1982) could be reinterpreted on the basis of a restricted permeability of adenine nucleotides through the outer membrane itself. In fact, addition of the reagent to integral mitochondria prevented activation of the intermembrane-located adenylate kinase and creatine kinase by added ADP or ATP, respectively. Moreover, polyanion inhibited access of intramitochondrially synthesized ATP to exogenous hexokinase, which is supposed to bind to the cytoplasmic side of VDAC (Lindén, Gellerfors, and Nelson, 1982b; Fiek et al., 1982). As parallel experiments in planar bilayers proved that VDAC in the polyanion-induced closed state was definitively preferring cations over anions (Benz, Kottke, and Brdiczka, 1990), all these results were taken to support the notion of the possible regulation of mitochondrial metabolism by the outer membrane through VDAC permeability changes (Benz et al., 1988;

Benz, Kottke, and Brdiczka, 1990; Benz and Brdiczka, 1992).

However, this interpretation contains some elements of uncertainty. We have already alluded to the risk of inferring that conclusions obtained in one system under some conditions are transportable to others kept under varied experimental constraints (see Section III.A.1). In this particular case, the fact that the change in selectivity (from anionic to cationic) of VDAC by polyanion (added on either side of planar bilayers and in the presence of a high electric field) made sense with the impermeability to negatively charged nucleotides of polyanion-treated nonrespiring mitochondria does not necessarily exclude different causes as the basis of a similar phenomenology. To overcome this obstacle, Benz et al. (1988) proposed that a difference in surface potential between the two faces of the outer membrane (Benz, 1985) would provide the necessary requirements for a similar action of polyanion in intact mitochondria and planar bilayers. Unless more compelling evidence is given, however, it is not yet possible to exclude other, perhaps unspecific, effects of polyanion on the outer membrane permeability of the isolated organelle.

A second impetus for the acceptance of the regulation of mitochondrial metabolism by VDAC has come from the isolation from the intermembrane space of different eukaryotic mitochondria (*Neurospora crassa*, potato or rat liver) of a negatively charged protein that is able to affect, in the same way, VDAC derived from other sources (Holden and Colombini, 1988; Liu and Colombini, 1991). This conserved ability was interpreted as a recognition of a crucial role exerted by the protein on VDAC.

Indeed, in planar bilayers, in the presence of a negative field at the side of the addition, nanomolar quantities produced a strong increase in the extent and rate of VDAC closure at high potential differences and inhibition of the rate of the reopening process that is commonly observed when voltage is switched from high to low values. A possible binding of the protein to the channel was then proposed (Holden and Colombini, 1988; Liu and Colombini, 1992a). A permeability change of the outer membrane of intact potato mitochondria was also attributed to the added natural modulator, as oxygen consumption by the respiratory chain and activity of adenylate kinase did not respond normally to ADP supply (Liu and Colombini, 1992b). Although one is forced to envisage that the natural protein, present in the intermembrane space, is lost or becomes inactive during isolation of mitochondria, the discovery of a natural effector that is able to modulate VDAC behavior is certainly of great interest and encourages the belief of a controlled permeability of the outer membrane to metabolite fluxes. Particularly suggestive is to imagine that, *in vivo*, no membrane potential is needed to change the permeability of the outer membrane but that the protein has a different capability to bind to the channel depending on the prevailing metabolic states of the cell. Alternatively, as already proposed (Liu and Colombini, 1992a), the protein could simply greatly amplify the effects of variations of the potential difference across the outer membrane.*

If the latter hypothesis is compatible with the electrophysiological experiments, it poses the question of how and if the outer membrane can maintain a potential difference when ions can flow through VDAC, even in the more closed states. Still, however unproved, there is no stringent reason for denying the existence of a small membrane potential generated by nondissipative processes, such as a Donnan potential, for example, that could be regulated by mechanisms like protein phosphorylation, ion binding, etc. (Liu and Colombini, 1991, 1992a, 1992b). On the other hand, at this stage, other suggestions for the existence of an intrinsic membrane potential due to asymmetry of membranes or of an electric coupling between the inner and outer membrane (Benz, 1990; Benz and Brdiczka, 1992) seem more conjectural.

* A strong indication in favor of the physiological importance of VDAC comes from the recent discovery of multiple isoforms of the channel in the human genome. Two of these isoforms (HVDAC1 and HVDAC2) are found to be expressed in many human cell lines and to produce functional channels when expressed in a yeast strain lacking wild-type VDAC (Blachly-Dyson, E., Zambrowicz, E. B., Yu, W. H., Adams, V., McCabe, E. R. B., Adelman, J., Colombini, M., and Forte, M., Cloning and functional expression in yeast of two human isoforms of the outer mitochondrial membrane channel, VDAC, *J. Biol. Chem.*, 268, 1835–1841, 1993).

2. VDAC or Other Outer Membrane Channels as Pathways for Mitochondrial Precursors

The majority of mitochondrial proteins are encoded by the nuclear genome and synthesized on the cytoplasmic ribosomes. Proteins belonging to the inner membrane bear targeting information either in a region close to the N-terminal of the mature protein or in a cleavable N-terminal peptide extension (Hartl and Neupert, 1990. See also Arizmendi et al., 1992; Walker et al., 1992). One of the most intriguing steps in mitochondrial biogenesis is the binding of precursors to, and their passage across, the two membranes to reach the functional destination. A great effort has been devoted in the past few years to clarify this complex process and recent reviews present in detail the most probable mechanisms at the basis of translocation of proteins (Hartl and Neupert, 1990; Pfanner and Neupert, 1990; Baker and Schatz, 1991; Pfanner et al., 1992). Here, we are concerned merely with understanding whether, as proposed by some, VDAC may be part of the import apparatus, given its abundance in the outer membrane and the suggestion that translocation takes place through water-filled pores (Pfanner et al., 1987).

Mannella and co-workers have investigated this possibility by looking at modifications in VDAC crystalline arrays induced by apocytochrome *c* or by a 20 amino acid synthetic peptide corresponding to the presequence of cytochrome oxidase subunit IV (Mannella, Ribeiro, and Frank, 1987; Mannella, 1989; Mannella, Guo, and Dias, 1992). These electron microscopic studies have shown that structural changes occurred in either case and that the site of binding for both the peptide and apocytochrome *c* was at the level of the protein arms extending laterally from VDAC lumen (Mannella, Guo, and Dias, 1992) (see Section III.B.1). Whereas such results would indicate an interaction of the channel with preproteins, at least under certain conditions, other data do not support the notion of a direct involvement of VDAC in the translocation of precursors.

For example, it seems now rather clear that in yeast and fungal outer membranes, most precursors [including subunit IV of cytochrome oxidase (Eilers, Hwang, and Schatz, 1988)] bind to proteinaceous receptors before following a common path, represented by insertion into a general insertion protein, and subsequent translocation at sites where the two membranes are in close vicinity (contact sites). An exception is, however, apocytochrome *c*, which enters the intermembrane space without assistance of surface receptors (Stuart, Nicholson, and Neupert, 1990). In any case, none of the proteins so far found to be associated with the outer membrane import machinery (a total of seven) can be identified with VDAC (Gillespie, 1987; Vestweber et al., 1989; Baker et al., 1990; Hines et al., 1990; Steger et al., 1990; Söllner et al., 1989, 1990, 1991).

Another argument against VDAC involvement comes from electrophysiological experiments performed with ox adrenal cortex and yeast mitochondrial membranes, where a slightly cationic channel (see Section III.A.2) was shown to be blocked by a synthetic polypeptide comprising the first 12 amino acids of the presequence of cytochrome oxidase subunit IV (Henry et al., 1989; Thieffry et al., 1992). For this reason, the channel is named PSC (peptide-sensitive channel). Of particular interest here is that a similar block is detected in VDAC-deficient yeast mutants (Fèvre et al., 1990; Thieffry et al., 1992). Hence, as far as our knowledge of mitochondrial biogenesis stands, if a role of VDAC in the import process seems improbable, the candidature of PSC appears better founded.

3. VDAC as Part of a Microcompartment Essential for Exploitation of Mitochondrial Matrix ATP

Mitochondrial contact sites are those regions of apposition between the inner and outer membranes that resist a mechanical or osmotic stress. First recognized in thin sectioned mitochondria (Hackenbrock, 1968), subsequent freeze-fracture studies have shown that these interactions can lead to as much as semifusion events (Van Venetië and Verkleij, 1982; Knoll and Brdiczka, 1983) and that their frequency depends on the functional state of the organelle, maximal under active oxidative phosphorylation (Knoll and Brdiczka, 1983; Bücheler, Adams, and Brdiczka, 1991). In the light of these features and of the short matrix-cytoplasm distance, contact sites

have been advocated to play a key role in several mitochondrial functions, including regulation of peripheral kinases through the population of VDAC present in these regions (Adams et al., 1989).

More explicitly, the direct link suggested for kinases and VDAC functional state (open or closed) (see Section III.D.1) would be particularly relevant in the case of contact sites whose extensive electric, biochemical, and immunological characterization has, in fact, described these areas enriched of VDAC channels, of hexokinase [bound to the cytoplasmic side of VDAC with more affinity than elsewhere (Weiler et al., 1985; Ohlendieck et al., 1986; Dorbani et al., 1987; Kottke et al., 1988)], and of enzymes located behind the outer membrane, such as nucleoside diphosphokinase, and creatine kinase (Adams et al., 1989; Kottke et al., 1988, 1991). Significantly, the adenine nucleotide translocator of the inner membrane is also preferentially located in these regions (Bücheler, Adams, and Brdiczka, 1991). Taken together, these data have suggested the occurrence of a microcompartment made of inner and outer membrane components, the first containing the adenine nucleotide translocator and the second VDAC, functionally coupled to peripheral kinases (Adams et al., 1989; Kottke et al., 1988, 1991).

In the case of mitochondrial creatine kinase, the envisaged mechanism in this specialized environment, whereby creatine is immediately phosphorylated with ATP exported by the adenine nucleotide translocator, is certainly in line with the large body of evidence reported for the preferred access of mitochondrial creatine kinase to matrix ATP (Jacobus, 1985; Bessman and Carpenter, 1985; Jacobus, Moreadith, and Vandegaer, 1983). As for VDAC, the closed cationic state induced by the potential difference generated by capacitative coupling between the two membranes (Benz, 1990; Benz and Brdiczka, 1992), would permit passage across the outer membrane of the positively charged creatine but not of the negatively charged adenine nucleotides and phosphocreatine. Thus, steady state conditions between mitochondrial and cytosolic phosphate potentials would be achieved after phosphocreatine flux through the open anionic VDAC, placed elsewhere from contact sites, and the activity of the cytosolic creatine kinase (Adams et al., 1989; Benz, Kottke, and Brdiczka, 1990). If such a model has some support from recent data on the structure and localization of the mitochondrial creatine kinase (Kottke et al., 1991; for an updated review see Wallimann et al., 1992), the role depicted for VDAC in such a scheme would automatically exclude the involvement of the channel in the claimed preferential access of hexokinase to matrix ATP in contact sites (Benz, Kottke, and Brdiczka, 1990), where an open state is obviously necessary. This contradiction may, however, bear only a relative importance after the rigorous reinvestigation of the subcellular location of hexokinase receptors demonstrating that, contrary to previous studies (Craven, Goldblatt, and Basford, 1969; Lindén, Gellerfors, and Nelson, 1982b; Fiek et al., 1982; Dorbani et al., 1987; Kottke et al., 1988; Felgner, Messer, and Wilson, 1979), in normal tissues the hexokinase preferentially occupies nonmitochondrial membrane receptors (Parry and Pedersen, 1984, 1990; Arora, Parry, and Pedersen, 1992). Additionally, Kabir and Nelson (1991) have shown that in rat brain mitochondria hexokinase utilizes external ATP more efficiently than ATP from oxidative phosphorylation.

Yet, in rapidly growing and highly glycolytic tumor cells, the overexpressed hexokinase does associate with an outer mitochondrial membrane receptor complex, a constituent of which is VDAC (Nakashima, 1989; Nakashima et al., 1986). This interaction per se is sufficient to explain the higher resistance of the bound enzyme to product inhibition by glucose-6-phosphate. At the same time, if receptors were located at contact sites, the proximity of the enzyme active site to VDAC would be ideal for hexokinase to utilize efficiently mitochondrially synthesized ATP, as found in tumor cells, simply by the direct permeation of ATP through VDAC open channels (Arora and Pedersen, 1988).

4. Association of VDAC with Primary and Secondary Benzodiazepine Receptors and with the Plasma Membrane

Recently, several drugs have been demonstrated to bind to secondary receptors located in mitochondria (for a review, see McEnery, 1992).

By testing in mitochondria of neuronal and non-neuronal origin drugs belonging to the anxiolytic benzodiazepine family, or of their antagonists, a few functions have been shown to be affected (McEnery, 1992). Among others, steroidogenesis is perturbed, possibly by a hampered transfer across mitochondrial membranes of steroid substrates through the benzodiazepine receptor. Therefore, of particular interest to the topic of mitochondrial outer membrane channels is the report of the identification (Snyder, McEnery, and Verma, 1990) and purification (McEnery et al., 1992) from rat kidney mitochondria of a multisubunit benzodiazepine receptor, apparently composed of an 18-kDa protein, the adenine nucleotide translocator and VDAC, the latter holding the drugs recognition site.

Equally interesting is the finding in mammalian brain of an apparently new member of the VDAC protein family associated with the central benzodiazepine receptor that corresponds to the heterooligomer γ-aminobutyric acid (GABA) receptor, type A, known to mediate Cl^- currents (Verdoorn et al., 1990). In fact, one of the polypeptides systematically copurified with the receptor major subunits has been demonstrated to be a 36 kDa protein, bearing over 70% identity with human B lymphocytes VDAC sequence, and with the same electric characteristics in bilayers as all other VDACs of mitochondrial origin (Bureau et al., 1992). The functional role of the protein remains to be understood. In any case, even if a nonmitochondrial location were undisputedly proved, the behavior in neuronal plasma membranes of the 36-kDa protein has to be entirely different from that envisaged to occur in the outer mitochondrial membrane, despite the striking similarities in planar bilayers. Plausibly, a reciprocal modulation with either the $GABA_A$-receptor proteins or with some other unidentified membrane components must occur (Bureau et al., 1992).

Contrary to the findings of Lindén et al. (1984), an extramitochondrial location of VDAC has been supported also by cytotoxicity and indirect immunofluorescence studies of the plasma membrane of a variety of human cells (Thinnes et al., 1989; König et al., 1991; Jürgens et al., 1991; Thinnes, 1992). This had led to the suggestion that VDAC could diversify its function, depending on the kind of membrane harboring the channel (Thinnes, 1992).

IV. CHANNELS IN THE INNER MITOCHONDRIAL MEMBRANE

A. Electrophysiological Characteristics of the Inner Membrane

Almost all of the electrophysiological studies of the inner mitochondrial membrane have been carried out by applying the patch clamp technique directly to inner membrane vesicles (mitoplasts, figure 6B), obtained from mitochondria after removal of the outer membrane either by osmotic shock or French press. Larger vesicles after fusion of mitoplasts have also been used, and fractions of the inner membrane reconstituted in liposomes were characterized with the patch clamp and in planar bilayers. Several distinct ion channels have thus been described on the basis of conductance value, voltage dependence, and sensitivity to added molecules (see Table 2).

To commence this part of the review, some clarification is needed. As generally symmetrical 150 m*M* KCl solutions were used, unless otherwise specified, all data are implicitly referred to this ionic condition. Moreover, the sign of the reported potential difference is that found in the mitochondrial matrix.

1. Voltage-Dependent 107 pS Channel

A conductance of 107 pS (mCS, mitochondrial Centum pico-Siemens) was the first described in the inner membrane of mitochondria (Sorgato, Keller, and Stühmer, 1987) (Table 2). Originally, it was found by patch clamping liver mitoplasts of cuprizone-fed mice (see Section III.A.2), but then was found in mitoplasts of untreated mice and rat liver, of mice heart, and of rat brown adipose tissue, and in reconstitution experiments (Klitsch and Siemen, 1991; Petronilli, Szabo', and Zoratti, 1989; Sorgato et al., 1989; Moran et al., 1990; Inoue et al., 1991; Kinnally et al., 1991). The current range of values attributed to this channel vary from approx-

TABLE 2
Main Single Channel Conductances Found in the Inner Mitochondrial Membrane

Conductance (pS) in 0.15 *M* salt	Voltage dependence	Selectivity	Effectors	Ref.
9.7	No	Cationic	ATP	1*
107	Yes	Anionic	Purine nucleotides (3); Ca^{2+} (5)	1*, 2, 3, 4, 5
>1000**	Yes	Unselective	Me^{2+}, ADP, H^+(7,8); Ca^{2+}(5,7,8)	4, 6

Note: All reported values were obtained by use of the patch clamp technique; *the salt concentration was 100 m*M*; **the conductance has multiple sublevels.

1. Inoue et al. (1991), 2. Sorgato, Keller, and Stühmer (1987), 3. Klitsch and Siemen (1991), 4. Petronilli, Szabo', and Zoratti (1989), 5. Kinnally et al. (1991), 6. Kinnally, Campo, and Tedeschi (1989), 7. Szabo' and Zoratti (1992), 8. Szabo', Bernardi, and Zoratti (1992). For further details see text.

imately 90 pS to 130 pS (Sorgato et al., 1989; Petronilli, Szabo', and Zoratti, 1989; Moran et al., 1990; Antonenko et al., 1991; Inoue et al., 1991; Klitsch and Siemen, 1991; Campo, Kinnally, and Tedeschi, 1992). This variability could be due to some methodological cause or, as recently observed (Ballarin, Sorgato, and Moran, unpublished), to an asymmetry of the channel itself, resulting in current rectification at negative potentials. Possible substates of the mCS channel have also been observed, ranging between 30 and 60 pS (Sorgato et al., 1989; Klitsch and Siemen, 1991).

From reversal potential measurements, the mCS channel was found to be slightly anionic [the permeability ratio of Cl^- over K^+ being 4.5 (Sorgato, Keller, and Stühmer, 1987)], a feature confirmed by Klitsch and Siemen (1991). Yet, the strongest characteristic is the voltage dependence. When the electric behavior of the entire inner membrane is analyzed (as with a whole mitoplast configuration, Figure 10A), the quiescent current at negative potentials increases importantly with membrane depolarization (Sorgato, Keller, and Stühmer, 1987; Sorgato et al., 1989; Klitsch and Siemen, 1991; Moran and Sorgato, 1992). This voltage dependence is retained by the mCS channel (Figures 10B, C) (Sorgato, Keller, and Stühmer, 1987; Sorgato et al., 1989; Moran et al., 1990; Klitsch and Siemen, 1991; Inoue et al., 1991; Antonenko et al., 1991; Campo, Kinnally, and Tedeschi, 1992) so as to justify the conclusion that this conductance is the major factor responsible for the electric behavior of the whole inner membrane (Sorgato, Keller, and Stühmer, 1987; Sorgato et al., 1989; Klitsch and Siemen, 1991; Moran and Sorgato, 1992). A similar sigmoidal curve obtained for the open probability of the mCS channel and for the macroscopic current at different voltages is a further, important support of this conclusion (Ballarin, Sorgato, and Moran, 1993).

The kinetic analysis of single channels has allowed more than one open and closed state to be ascribed to the mCS conductance. In fact, the typical bursts of activity of variable duration per se are indicative of the existence of at least two closed states (Sorgato, Keller, and Stühmer, 1987; Klitsch and Siemen, 1991; Antonenko et al., 1991; Campo, Kinnally, and Tedeschi, 1992; Ballarin, Sorgato, and Moran, 1993). To quantify them, analysis of the distribution of the open and closed times has been carried out at opposite potential differences. At positive potentials, the open distribution has been characterized by a single exponential of 6.9 ms (at 60 mV: Sorgato, Keller, and Stühmer, 1987) and of 52 ms (at 40 mV: Ballarin, Sorgato, and Moran, 1993) or by

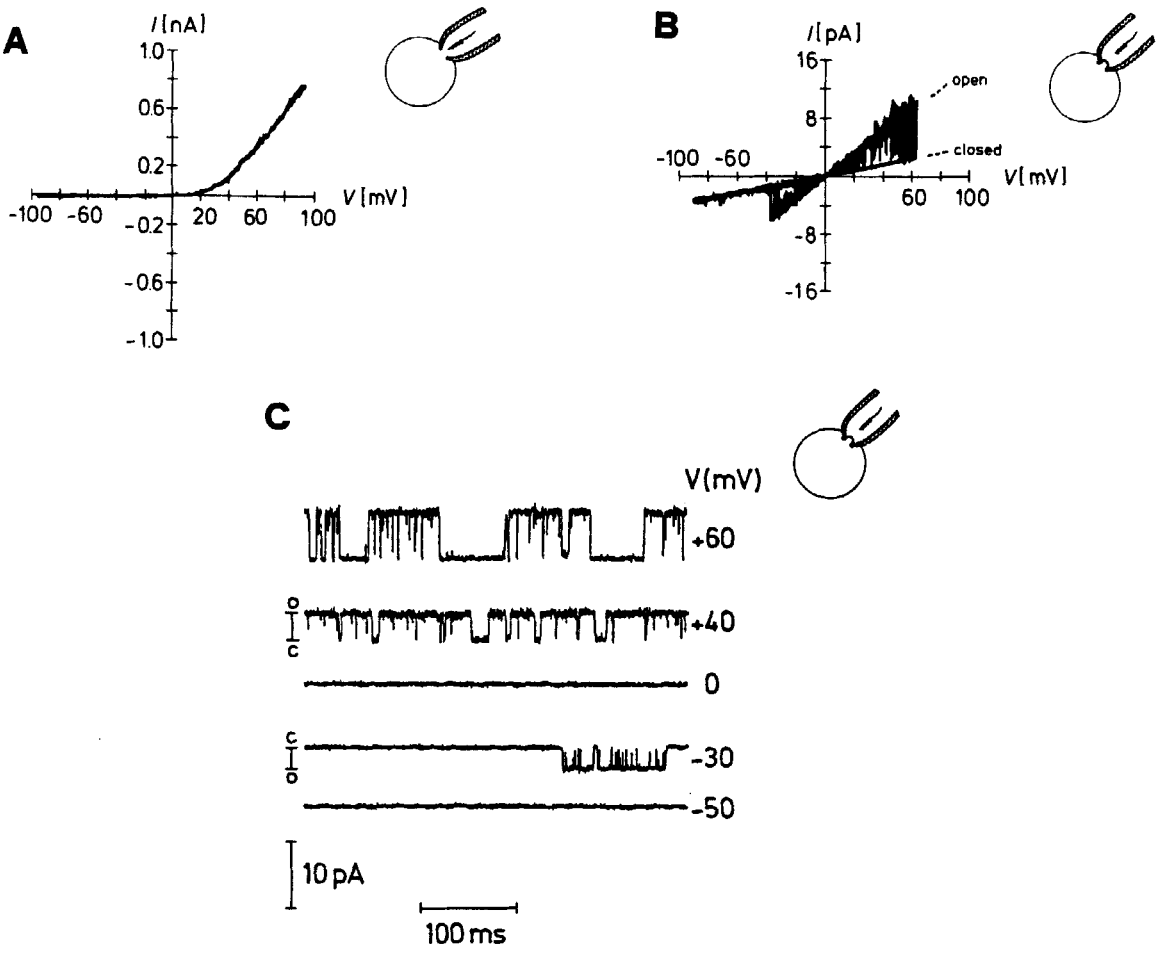

FIGURE 10. Current traces recorded from the inner membrane of mitochondria with the patch clamp technique. (A) Steady state current-voltage relation in a whole mitoplast patch. (B) Steady state current-voltage relation in a mitoplast-attached patch, showing the gating properties of a single channel. (C) Records of the 107 pS channel at different voltages, from a mitoplast-attached patch. Note the higher open probability at potential differences of positive sign. Recordings were obtained from mouse liver mitoplasts in symmetrical 150 m*M* KCl, 0.1 m*M* $CaCl_2$, 20 m*M* Hepes-KOH (pH 7.2). The sign of the voltage (V) refers always to the interior of the vesicle. (Reprinted by permission of *Nature*, 330, 498–500; Copyright © 1987 Macmillan Magazines Limited.)

two exponentials of 0.75 and 58 ms, respectively (at 50 mV: Klitsch and Siemen, 1991). Possibly, the very fast component detected in the latter report (with brown adipose tissue mitoplasts) might have escaped detection in the other works (with liver mitoplasts) because of the lower bandwidth used in data analysis. In any case, it is the slower time constant that is markedly voltage-dependent as it decreases more than sixfold when the potential is made negative (Klitsch and Siemen, 1991; Ballarin, Sorgato, and Moran, 1993). Although more variable, other observations on the mean open time are consistent with this outlined trend (Antonenko et al., 1991; Campo, Kinnally, and Tedeschi, 1992).

For the closed time distribution, two rather fast constants (of 0.17 and 1 ms) were found in brown adipose mitoplasts that, however, showed

very little voltage sensitivity (Klitsch and Siemen, 1991). Conversely, a fast component, of between 0.5 and 2 ms, independent of the sign of the potential difference, was identified in liver mitoplasts, as well as a slow component, the value of which increased with increasing polarization, from approximately 40 ms at 40 mV to around 10 s at −30 mV (Sorgato, Keller, and Stühmer, 1987; Ballarin, Sorgato, and Moran, 1993).

In addition to these determinations, the observation stands of different periods of time necessary to activate or deactivate the channel. This is evident when applying voltage steps from negative to positive values or vice versa. Hence, if the applied potential is changed from negative to positive full activation of the membrane patch takes longer, the more negative is the starting potential (from a few seconds to tens of seconds) (Sorgato, Keller, and Stühmer, 1987; Klitsch and Siemen, 1991; Ballarin, Sorgato, and Moran, 1993). In contrast, by switching from positive to negative potentials, the decrease of activity is immediate with a time constant of the order of msec (see, for example, Figure 3A of Sorgato, Keller, and Stühmer, 1987 or Figure 5 of Ballarin, Sorgato, and Moran, 1993).

In view of these observations, it is possible to hypothesize a linear scheme for the transitions of the mCS channel to the various conductance states (Ballarin, Sorgato, and Moran, 1993). One or, perhaps two, open states and several closed states are envisaged, as any of the closed conformations are influenced differently by the potential sign. When the potential from negative is switched to positive, the time needed for the channel to finally reach the open state will clearly depend on the closed starting state determined by the magnitude of the negative potential, whereas the reverse transition, from the open to the closed state, will be almost immediate.

The mCS channel has been tested with a variety of drugs, some of pharmacological interest, some because of their known interaction with the inner membrane of mitochondria. Of the latter molecules, which include quinine (Sorgato, Keller, and Stühmer, 1987), cyclosporine A (Szabo′ and Zoratti, 1991, 1992; Szabo′, Bernardi, and Zoratti, 1992), oligomycin, *N, N′*-dicyclonexylcarbodiimide, and bongkrekic acid (Sorgato et al., 1989), only antimycin A, a blocker of the activity of the respiratory chain (van Keulen and Berden, 1985), was found to affect the channel (Campo, Kinnally, and Tedeschi, 1992). At micromolar concentrations, the action of antimycin A was reversible [contrary to its effect on the respiratory chain proteins (Slater, 1973; van Keulen and Berden, 1985)], and dose dependent. Antimycin A also markedly decreased the mean open probability at positive potentials. As the single channel conductance remained unaltered, but not the mean duration of the open state, antimycin A was imputed to diminish the sensitivity to voltage of the channel voltage sensor (Campo, Kinnally, and Tedeschi, 1992).

Substances, some of which affect channels of certain plasma membranes, have been tested to establish whether there exists a relationship between the mCS conductance with the better known plasma membrane channels. 4-4′-Diisothiocyanatostilbene-2,2′-disulfonic acid (White and Miller, 1981), decamethonium (Albuquerque, Daly, and Warnick, 1991), Zn^{2+} (Stanfield, 1970), Gd^{3+} [an inhibitor of pressure activated channels (Yang and Sachs, 1989)] had no effect (Sorgato et al., 1989). Conversely, Antonenko et al. (1991) observed complex modifications on the mCS channel by cationic amphiphiles such as amiodarone (an antiarrythmic) and propranolol (a β-blocker) in that the single channel conductance increased by 40% concomitant with a drastic reduction of the mean open state probability at positive potentials. Amiodarone and propranolol are known to interact with an inner mitochondrial membrane protein called anion channel (Beavis, 1989) (see Section IVB) and also to block Ca^{2+} and Na^{+} plasma membrane channels (Kolhardt and Fichtner, 1988; Nishimura, Follmer, and Singer, 1989). However, as these effects are different from those observed on the mCS channel, either a direct action takes place (Campo, Kinnally, and Tedeschi, 1992) or an unspecific perturbation at the protein-lipid interaction may be expected for their lipophilic character (Seeman, 1972).

Whereas the mCS channel is largely unaffected by variations of 3 pH units (from 6 to 9) on the matrix or cytoplasmic side of the channel-containing membrane (Sorgato, Keller, and Stühmer, 1987), a possible modulation by Ca^{2+} is not yet clarified. According to Kinnally et al. (1991), to detect the channel, it is necessary, but

sometimes not sufficient, to use Ca^{2+}-free media during isolation of mitochondria and mitoplasts. Indeed, the best way to activate the mCS channel irreversibly was found by having Ca^{2+} present during the preparation of mitoplasts and to remove it afterwards with EGTA washings. On other observations, inactivation of the channel was attributed to Ca^{2+} acting on the cytoplasmic side of the inner membrane, probably by interaction with an unidentified regulatory protein, whereas, once activated, Ca^{2+} would inhibit the mCS channel from the matrix side.

This complex scheme of modulation is apparently contradicted by the finding of mCS activity in mitoplast irrespective of the presence (Klitsch and Siemen, 1991; Petronilli, Szabo', and Zoratti, 1989; Szabo', Bernardi, and Zoratti, 1992) or absence (Sorgato, Keller, and Stühmer, 1987; Sorgato et al., 1989) of Ca^{2+} chelators and by the invariant features retained on selective decrease or increase of the Ca^{2+} content of the medium used in the patch clamp experiments (Sorgato, Keller, and Stühmer, 1987; Sorgato et al., 1989; Klitsch and Siemen, 1991; Szabo' and Zoratti, 1992; Szabo', Bernardi, and Zoratti, 1992). Therefore a final adjudication on the cause of mCS silent patches, commonly experienced by many laboratories (Sorgato, Keller and Stühmer, 1987; Kinnally et al., 1991; Klitsch and Siemen, 1991; Szabo' and Zoratti, 1991), seems not possible in the light of these results. At all events, Campo, Kinnally, and Tedeschi (1992) and Kinnally, Antonenko, and Zorov (1992) have viewed their observations on the action of Ca^{2+}, antimycin A, and the cationic amphiphiles as supporting a nonlinear model for the mCS activity, in which a few functional states have been introduced by necessity.

According to Szabo' and Zoratti (1992), neither m*M* Mg^{2+} nor μ*M* ADP modify the mCS behavior with access available on the matrix side, and Inoue et al. (1991) showed similar lack of effect by m*M* ATP concentrations. Conversely, Klitsch and Siemen (1991) reported important decreases, partially reversible, of the current mediated by a whole mitoplast patch on micromolar additions of adenosine and guanosine tri- and diphosphate, as well as of guanosine monophosphate. However, as for preparation of mitoplast, the membrane is necessarily stretched. It is debatable whether, in this latter case, the site of action of nucleotides can be safely attributed to only the cytoplasmic side of the membrane.

Reconstitution experiments for identifying the protein responsible for the mCS conductance have been carried out with an inner membrane fraction isolated from ox heart mitochondria by its incorporation first in planar bilayers (Stühmer et al., 1988), then in liposomes sufficiently enlarged for patch clamp experiments (Sorgato et al., 1989; Moran et al., 1990). Channels similar to those observed *in situ* were detected, especially in the latter case. Such successful reconstitution, which involves careful dehydration-rehydration steps (Criado and Keller, 1987), was then extended to either the whole inner membrane or to both mitochondrial membranes, with similar possibilities of detection of the mCS conductance (Moran et al., 1992). A slightly higher conductance (130 to 140 pS), sometimes retaining the strong mCS voltage dependence, was also reported in proteoliposomes with a mitochondrial fraction containing a much lower number of bands than reported before (Costa, Kinnally, and Diwan, 1991; Paliwal, Costa, and Diwan, 1992), which certainly renders highly promising the efforts for the final isolation of the protein responsible for mCS activity.

2. Low Conductance Channels

A plethora of conductances ≤ 50 pS have been described for the inner membrane, as either substates of the mCS channel, previously mentioned, or distinctive entities in reconstituted (Mironova et al., 1981; Moran et al., 1990; Costa, Kinnally, and Diwan, 1991; Paliwal, Costa, and Diwan, 1992) or in *in situ* experiments (Antonenko, Kinnally, and Tedeschi, 1991; Inoue et al., 1991; Moran et al., 1992). Of these, an exquisitely K^+-selective channel, with no clear voltage dependence and of 9.7 pS conductance (with 100 m*M* K^+ in the pipette and 33.3 m*M* K^+ plus 66.7 m*M* Na^+ in the bath), was observed in large vesicles obtained by fusion of liver mitoplasts (Inoue et al., 1991). This small conductance has a particular relevance for its sensitivity to drugs and physiological effectors (Table 2). In fact, the specific inhibition by m*M* ATP or by 4-aminopyridine (Meves and Pichon, 1977) and glybenclamide (Sturgess et al., 1985), block-

ers of the plasma membrane K^+ channel, have allowed the suggestion of a link with the ATP-sensitive K^+ channel family described for some cell membranes (Bean and Friel, 1990; Ashcroft, Harrison, and Ashcroft, 1984).

Other low conductances (15 to 70 pS in 150 m*M* KCl) were found in liver mitoplasts associated with an increase of membrane current originating specifically on alkalinization of the matrix side (Antonenko, Kinnally, and Tedeschi, 1991). The specificity of the current and the inhibition by low pH, increasing Mg^{2+}, or by cationic amphiphiles and tributyltin can be judged sufficient criteria for distinguishing it from that mediated by the 9.7 pS ATP-sensitive channel.

A focused search for channel-forming proteins of the inner mitochondrial membrane has just begun. The first evidence for a mitochondrial channel other than VDAC, however, came in 1981, when Mironova et al. showed in planar bilayers K^+ currents induced by an ethanol-extracted mitochondrial protein of about 60 kDa. On average, voltage-dependent conductance amplitudes were multiples of 24 pS (in 100 m*M* KCl), with the most frequent being 175 pS transitions. Given the high selectivity for K^+, this activity was then tentatively attributed to the mitochondrial K^+ transporting systems. More recently, patch clamp experiments of liposomes containing a 57-kDa protein (isolated from detergent-solubilized submitochondrial particles chromatographed on immobilized quinine) showed a 40 pS channel (in 150 m*M* KCl), with no apparent voltage-gating properties (Paliwal, Costa, and Diwan, 1992). Of interest is its resemblance to low conductances detected in mitoplasts (Kinnally, Campo, and Tedeschi, 1989; Moran et al., 1992) and in proteoliposomes containing a contact sites-enriched fraction (Moran et al., 1990).

3. nS Channel Activity with Multiple Substates

Along with the mCS channel, much higher conductances have been reported to be harbored in patches of the inner membrane. Kinnally, Campo, and Tedeschi (1989) and Petronilli, Szabo', and Zoratti (1989) first reported peak conductances in mouse and rat liver mitoplasts of 1 to 1.3 nS, accompanied by multiple substates broadly covering the range < 0.1 to 1.0 nS, with a 550 pS as the predominant level. In either report, the frequent simultaneous presence in a patch of this multifarious behavior was taken to indicate that it might be the expression of a same phenomenon and was therefore referred to as multiconductance channel (Kinnally et al., 1991) or mitochondrial megachannel (Szabo' and Zoratti, 1991). After an initial divergence, it now appears that the peak state often gets stabilized by negative potential differences, whereas at positive voltages the open probability of the several substates increases (Petronilli, Szabo', and Zoratti, 1989; Campo, Kinnally, and Tedeschi, 1992; Zorov, Kinnally, and Tedeschi, 1992; Zorov et al., 1992; Kinnally, Antonenko, and Zorov, 1992).

Not all observations made by the two groups, however, adhere to a single pattern of behavior. Besides attribution of widespread unselectivity (Szabo' and Zoratti, 1992) or of a slight cationic preference proved for some sublevels (Kinnally, Campo, and Tedeschi, 1989), the major discrepancy lies with the role of Ca^{2+} present during or after isolation of mitochondria. More particularly, Kinnally et al. (1991) demonstrated the necessity for μM Ca^{2+} concentrations during the isolation of mitochondria and mitoplasts to activate irreversibly the multiconductance channel from the cytoplasmic side of the membrane (in 96% of the cases). Chelation of the ion or exposure to it of the matrix side of the inner membrane (as in excised patches) would otherwise cause blockage of activity. On the other hand, data presented by Petronilli, Szabo', and Zoratti (1989), Szabo' and Zoratti (1991, 1992), and Szabo', Bernardi, and Zoratti (1992) do not agree with the postulated requirement for free Ca^{2+} in mitochondral isolation buffers (which contain EGTA). Still, when present in the patch clamp experimental medium (by acting most probably on the matrix side), 100 μM Ca^{2+} can give rise to as much as 70% patches with megachannel activity (Szabo' and Zoratti, 1991). Further additions of similar quantities either increase the conductance of already active membranes or awake silent patches (Szabo' and Zoratti, 1992; Szabo', Bernardi, and Zoratti, 1992).

Apparently, voltage steps higher than $\pm$ 60 mV can replace Ca^{2+} in inducing a progressive increase of opening transitions to the final con-

ductance of 1 nS, if not higher, of patches of heart and liver inner membranes. Under these conditions, alternative modes of behavior have been identified (Zorov et al., 1992; Campo, Kinnally, and Tedeschi, 1992; Zorov, Kinnally, and Tedeschi, 1992). These include strong dependence of the multiconductance activity on voltage and long stability of the open or closed states, usually at negative and positive voltages, respectively; fast openings and closures at a minimum of nine conductance levels; a combination of these two modes (as already seen by Petronilli, Szabo′, and Zoratti, 1989), whereby negative voltages would induce long lasting openings and positive voltages causing frequent transitions to lower conductance levels. Two gating behaviors could then be singled out, interconverting spontaneously or by voltage changes (Zorov et al., 1992). Interestingly, the same stepwise decrease of the peak current induced by voltage is also observed with various drugs such as amiodarone, propranolol and quinine (Antonenko et al., 1991; Zorov et al., 1992), antimycin A (Campo, Kinnally, and Tedeschi, 1992), and cyclosporine A (Szabo′ and Zoratti, 1991; Zorov, Kinnally, and Tedeschi, 1992).

In front of the observation of nS activity of mitoplasts, it seems necessary to address the question of the coexistence in an inner membrane patch of the mCS channel with this large set of high conductances, provided by some (Petronilli, Szabo′, and Zoratti, 1989; Kinnally et al., 1991; Szabo′ and Zoratti, 1991, 1992; Kinnally, Antonenko, and Zorov, 1992; Szabo′, Bernardi, and Zoratti, 1992) but denied by others (Sorgato, Keller, and Stühmer, 1987; Sorgato et al., 1989; Inoue et al., 1991; Klitsch and Siemen, 1991), who reported most frequent conductances of ≤ 100 pS, of mCS, or of maximally 200 pS. Detailed inspection of the methods used to prepare mitochondria and mitoplasts, and of other technical steps required to warrant the recording of the high current transitions, apparently confirms that lack of detection should not be caused by methodological differences. Two other pieces of evidence support this difficulty. One is the behavior of the macroscopic current of the inner membrane that most probably reflects the voltage dependence of mCS single channels (see Figure 10). The other comes from reconstitution experiments where high conductances are infrequent, voltage independent, and without any clear cooperative pattern (Sorgato et al., 1989; Moran et al., 1992; see Section IV.B).

4. Electrophysiology of a Fraction Enriched in Contact Sites

Moran et al. (1990) patch clamped proteoliposomes containing a membrane fraction isolated from rat brain mitochondria (Sandri, Siagri, and Panfili, 1988) whose biochemical features could be ascribed to contact sites (Kottke et al., 1988). Electron microscopy of this preparation shows double-membrane vesicles with points of adhesion as well as mitoplasts with large remnants of the outer membrane tightly connected to the inner one (Moran and Sorgato, 1992). In contrast to the clear picture obtained by patch clamping the inner membrane *in situ* (Sorgato, Keller, and Stühmer, 1987), the electric analysis of the contact sites proteoliposomes was by far less uniform. A whole range of small conductances, from 6 to around 40 pS, was identified, along with much higher conductances, the most frequent being of 475 and 550 pS. Interestingly, a 535 pS conductance was also found in proteoliposomes containing the isolated outer membrane (Moran et al., 1992) and is considered the predominant substate of the 1 to 1.3 nS peak conductance of the inner membrane (Petronilli, Szabo′, and Zoratti, 1989; Zorov et al., 1992). Neither the 475 nor the 550 pS channel showed voltage gating, but the 475 pS frequently could be distinguished by transitions to two sublevels, of 245 and 373 pS, respectively. Rarely, higher currents were observed, with maximal conductance of approximately 1 nS, again lacking control by voltage.

B. Physiology of the Inner Mitochondrial Membrane Channels

A very tight control of the permeability of the inner membrane of mitochondria is crucial to the validity of the chemiosmotic tenets of energy transduction (Mitchell, 1966). The concept, however, that ATP production and other essential functions are based on exploitation of the electrochemical proton gradient maintained across the inner membrane *a priori* should not bias one neg-

atively against the hypothesis of the presence of ion channels in the inner membrane. Understandably, a critical attitude may stem from the difficulty in imagining a physiological role for the high ion fluxes mediated by most inner membrane channels, which in principle would rapidly provoke depolarization and disruption of energy-linked membrane functions. It should be noted, however, that in almost all cases, voltage or mitochondrial components have been shown to prevent opening of the channels under the conditions pertinent for ATP synthesis (see Sections IV.A.1 and IV.A.2 and following). On the other hand, as far as our knowledge stands, the *in vitro* determined electrophysiology may be somewhat deceiving. Matrix or intermembrane regulators, or other vital connections such as the cytoskeleton (Lindén et al., 1989; Lin, Krockmalnic, and Penman, 1990), forcedly removed during mitoplasts preparation, could modify the electric behavior of the inner membrane in the intact organelle. Moreover, if the role of these channels were not axiomatically linked to permeation of ions as for classical excitable elements, then no incompatibility with mitochondrial bioenergetics would exist.

These arguments seem tenable, particularly with respect to the mCS channel found in a few mammalian tissues (liver, heart, and brown adipose tissue), whose functional identity is well established and is distinguishable by many criteria from the other conductances of the inner membrane (see Section IV.A.1). The channel is inoperative at the high negative membrane potentials sustained by mitochondria, and thus the interesting challenge is to discover if and why it becomes active under nonphysiological voltages, or if there are interactions with modulators able to overcome the voltage-dependent closure and consequently ensure operation at more physiological membrane potentials. Clearly, there is no plausible reason why mitochondria should maintain an inoperative protein. Even if we are some way from understanding the *raison d'etre* of this channel, there are grounds for optimism. For example, translocations of macromolecules (Vestweber and Schatz, 1989; Baker and Schatz, 1991; Pfanner et al., 1992) and/or gap junction-like physical connections within cardiac mitochondria (Amchenkova et al., 1988) could plausibly exploit the powerful conduits represented by water-filled pores (Blobel, 1980; Blobel and Dobberstein, 1975; Singer, Maher and Yaffe, 1987).

If mCS channels represented the contribution of the inner membrane to the transport machinery of macromolecules and if import occurred at the limited number of contact sites apparently existing in a mitochondrion (Rassow et al., 1989), then it makes sense to find them closed at physiological voltages in swollen mitoplasts without, or possibly with damaged, intermembrane junctions. Recent models for translocation of mitochondrial precursors through these regions where the perfect alignment of the two distinct halves of the import apparatus is dynamic (Hwang, Wachter, and Schatz, 1991; Pfanner et al., 1992), would be consistent with this hypothesis, which is also corroborated by the lack of a mCS conductance as such in proteoliposomes presumably containing integral contact sites (Moran et al., 1990; but see Moran and Sorgato, 1992). Available experimental evidence is undoubtedly insufficient for substantiating this hypothesis, but the recent demonstration of an endoplasmic reticulum channel involved in protein translocation (Simon and Blobel, 1991) is a good precedent for pursuing it. Vice versa, if the mCS channels were part of low resistance junctions observed to connect mitochondria (Amchenkova et al., 1988) and visualized to transmit in the long distance $\Delta \mu_{H+}$ in organelle clusters (Skulachev, 1990), then the high open probability of the mCS channel at 0 mV finds an automatic explanation in the lack of electric difference across the connecting junctions.

Other functions for the mCS conductance, such as heat producer, has been recently denied by Klitsch and Siemen (1991). These authors have demonstrated that the channel is present in mitoplasts of brown adipose tissue, but that it is distinct from the uncoupling protein responsible for the nonshivering thermogenesis (Nicholls, Cunningham, and Rial, 1986). Similarly to the role postulated for the ATP-sensitive K^+-selective 9.7 pS conductance, which becomes operative under the stressed condition of low content of matrix ATP (Inoue et al., 1991), Klitsch and Siemen (1991) have suggested a role in volume regulation for the mCS channel. Mitochondria are known to possess efficient apparatus for maintaining volume homeostasis, which possibly include the electrophoretic activity of an anion

transporter (for reviews see Garlid and Beavis, 1986; Beavis, 1992). The physiological effectors found to regulate this protein (pH, Mg^{2+}), however, have no such action on the mCS conductance, which therefore, unless differently sustained, must be regarded as a separate protein even if with a putative similar function. Rather, the current activated by alkaline pH and Mg^{2+} depletion (Antonenko, Kinnally, and Tedeschi, 1991) has a higher probability of identity with this anion uniporter.

A different line of reasoning is applicable for the much larger conductances, observed to aggregate together also with varying stoichiometry at physiological potentials to yield preferentially the 1 to 1.3 nS conductance (Kinnally, Campo, and Tedeschi, 1989; Petronolli, Szabo′, and Zoratti, 1989). Because of the opposite results on the timing and side of action of Ca^{2+} in the activation process of this phenomenon, two interpretations have been put forward. Based primarily on the consequence of the presence or the absence of Ca^{2+} during mitoplasts preparation (Kinnally et al., 1991) and on the relevance of Ca^{2+} for the structural integrity of contact sites (Sandri, Siagri, and Panfili, 1988), Kinnally, Antonenko, and Zorov (1992) have favored an association of the high current transitions either to whole contact sites (with channels of the inner and outer membrane working in tandem) or only to the inner membrane component present outside the regions of close membrane apposition.

On the other hand, study of factors able to regulate the nS transitions in mitoplast patches has allowed Szabo′ and Zoratti (1991, 1992) and Szabo′, Bernardi, and Zoratti (1992) to propose, with circumstantial arguments, the identification of the mitochondrial megachannel activity with the permeability transition pore, long suspected to mediate uncoupling of oxidative phosphorylation (with loss of volume homeostasis capacity and matrix content) in mitochondria damaged by a variety of agents (for a recent review on the subject, see Gunter and Pfeiffer, 1990). Indeed, the dramatic acceleration of the permeability process observed in mitochondrial suspensions (first treated with the perturbing agent phenylarsine oxide) on Ca^{2+} addition, and the competitive action of divalent metal ions, of small acidic transitions, and of the specific inhibitor cyclosporin A in delaying or inhibiting this process (Bernardi et al., 1992), compare well with the varied open-close probability of the megachannel of a Ca^{2+}-treated patch in the presence of the above factors, of Ca^{2+} chelators, and ADP (Szabo′ and Zoratti, 1992; Szabo′, Bernardi, and Zoratti, 1992). Clearly, this large and unspecific permeability demands pathological events, the reoxygenation-induced tissue injury, for example (Crompton and Costi, 1988, 1990; Halestrap and Davidson, 1990), given that the physiological matrix concentration of ADP, Mg^{2+}, and protons are in the right range for ensuring closure of the pore (Szabo′ and Zoratti, 1992; Szabo′, Bernardi, and Zoratti, 1992).

Matrix Ca^{2+} overload plays a major role in inducing or stimulating the process of generalized mitochondrial permeability (Gunter and Pfeiffer, 1990). Exposure of mitoplasts to about 100 μM Ca^{2+} before electric recordings fulfills the expectations for the finding of the megachannel activity (Szabo′ and Zoratti, 1991, 1992; Szabo′, Bernardi, and Zoratti, 1992). Similar to the evidence with mitochondrial suspensions (Haworth and Hunter, 1979; Bernardi et al., 1992), the opening of the pore also in a patch has an all-or-nothing character (Szabo′, Bernardi, and Zoratti, 1992), which implies a tightly coordinated process in ensuring the opening of the pore. Plausibly, this could be due to protein association (as in Fagian et al., 1990) or to the simultaneous activation of crucial factors. All of these results thus render puzzling the incapability of some inner membrane patches to show nS transitions in the presence of high Ca^{2+} levels (see Section IV.A.3). Hence, unless other explanations are placed on these discrepant observations, one may tentatively suggest that some membranes are particularly refractory to such type of damage.

V. CONCLUSIONS AND PERSPECTIVES

Undoubtedly, much progress has been achieved as a result of the electrophysiological studies of the inner and outer mitochondrial membranes. Nevertheless, many questions remain unanswered.

Research is clearly more advanced for the first discovered outer membrane channel, VDAC, for which important structural, molecular, and biochemical information is available. Now that

the molecular stoichiometry appears known, strategies of genetic engineering will certainly expedite the understanding of how the channel operates. However, the functional picture of the outer membrane that is emerging from recent studies is certainly different and more complex than that provided by classic experiments with planar bilayers. It poses questions as to the behavior of the outer membrane *in situ* and indicates that conductances other than VDAC play some roles in the physiology of the membrane. In any case, the possible association of VDAC with receptors in mitochondria and the presence of VDAC-like proteins in the mammalian plasma membrane indicate that there may be diversified functions and modes of regulation belonging to this channel.

Inevitably, application of the patch clamp technique to mitochondria has taken its electrophysiological analysis to another level, allowing the study of membranes (especially the outer) under conditions unexpectedly close to physiological. Thanks to this technique, the inner membrane has also been shown to harbor several conductances, some of which may be linked to physiological roles, whereas some may be induced by pathological events. The exact function and the molecular identity of proteins responsible for these conductances are as yet unknown, but information is growing steadily. The likely possibility of an early identification of these proteins and the putative assignment of the 9.7 pS conductance to the plasma membrane family of ATP-sensitive K^+-channels encourage optimism for the future. In any case, it is clear that, despite its recent advent, mitochondrial electrophysiology has disclosed unexpected features of transport systems occurring in the organelle and reinforced the recognition of the widespread location of highly conductive channels in membranes not involved in rapid electric signaling.

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