Journal of Bioenergetics and Biomembranes, Vol. 28, No. 2, 1996

The High-Conductance Channels of Yeast Mitochondrial Outer Membranes: A Planar Bilayer Study

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Received March 27, 1995; revised July 1, 1995

The high-conductance channels present in the outer membranes of wild-type and porin-less yeast mitochondria have been characterized electrophysiologically after incorporation in planar bilayer membranes. The most prominent activity was ascribed to a voltage-dependent, substate-rich, cationic channel which generally inactivated at potentials positive in the *cis* compartment, in agreement with the observations from patch-clamp experiments on porin-less mitoplasts. This channel has been identified as the so-called "peptide-sensitive channel" (PSC). We also observed similar channels displaying either no inactivation, or inactivation at both positive and negative potentials. These latter properties match those already described for mammalian and yeast PSC, respectively. These different behaviors are tentatively explained as arising from the presence, or lack of, peptides bound to the PSC. Very high conductances, apparently due to cooperative gating, were frequently displayed. In wild-type membranes, activity ascribable to the porin was also observed.

KEY WORDS: Electrophysiology; peptide-sensitive channel; mitochondrial megachannel; porin; planar bilayer; yeast mitochondria.

INTRODUCTION

We are conducting an electrophysiological investigation of the mitochondrial membranes of yeast wild-type and VDAC-less[5] strains in hopes of gathering clues on the molecular nature of the mitochondrial megachannel (for review see Zoratti and Szabò, 1994), which has been identified as the Ca^{2+}-activated "permeability transition pore" (for reviews see Gunter and Pfeiffer, 1990; Bernardi *et al.*, 1994; Halestrap, 1994; Gunter *et al.*, 1994; Zoratti and Szabò, 1995). One of the hypotheses on its still unknown components envisions its formation by a doublet of porins or of other similar channels, plus other proteins in a regulatory role (Zoratti *et al.*, 1992; Szabò and Zoratti, 1993; Szabò *et al.*, 1993; Kinnally *et al.*, 1993). The most prominent activity we observed in a patch-clamp investigation of VDAC-less mitoplasts (Szabò *et al.*, 1995) was due to a voltage-dependent, cationic pore exhibiting conductance states ranging from 100 to 650 pS (100 mM KCl) whose occupancy was influenced by the applied voltage. In the whole-cell configuration, potentials positive in the patch pipette drove the channel to long-lived closed state(s) (a process hereafter referred to as "inactivation"), and furthermore induced the channel, when open, to adopt, on average, lower conductance states than at negative pipette voltages. Importantly, the channel appeared to undergo a fast, voltage-dependent block by the pCOxIV leader peptide (Szabò *et al.*, 1995). Taken together, the pore properties strongly suggested its

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[5] Abbreviations used: PSC: peptide-sensitive channel: MMC: mitochondrial megachannel; VDAC: voltage-dependent anion channel (mitochondrial porin); Hepes: 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; pCOxIV: pre-cytochrome oxidase subunit IV.

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identification with the "peptide sensitive channel" (PSC) studied by the group of Thieffry and Henry in both mammalian (Thieffry *et al.*, 1988, 1992a,b; Henry *et al.*, 1989; Fèvre *et al.*, 1993, 1994; Chich *et al.*, 1991) and yeast (Fèvre *et al.*, 1990, 1994; Thieffry *et al.*, 1992a,b; Vallette *et al.*, 1994) mitochondria, mainly in "tip-dip" and planar bilayer experiments. This channel is probably involved in peptide translocation across the outer mitochondrial membrane (Henry *et al.*, 1989; Fèvre *et al.*, 1994; Vallette *et al.*, 1994), where it appears to be located on the basis of proteolysis experiments (Chich *et al.*, 1991). The French authors have observed the PSC to exhibit a complex voltage dependence, different from that observed by Szabò *et al.* (1995). The yeast channel was reported to occupy mostly the fully open state at low voltages, with long-lasting closures induced with slow kinetics (several seconds) by the application of potentials of either sign (Thieffry *et al.*, 1992a,b; Fèvre *et al.*, 1994). The fully open state was rapidly re-established upon returning to low voltages. These properties are very similar to those described for the isolated/reconstituted mitochondrial porin, VDAC (for reviews see, e.g., Benz, 1994; Colombini, 1994). On a faster time scale, bursts of millisecond-range fluctuations to substates, whose frequency increased with potential, occurred at positive (but not at negative) pipette ("tip-dip") or *cis*-side (bilayer) voltages (Fèvre *et al.*, 1990; Thieffry *et al.*, 1992a). The behavior of the mammalian PSC was reportedly different: it remained open at voltages negative on the cytoplasmic side of the outer membrane, and closed, with fast (1–10 ms) fluctuations, for voltages of reverse polarity (Thieffry *et al.*, 1988; Fèvre *et al.*, 1994). This gating could be eliminated by trypsin treatment (Thieffry *et al.*, 1988). No slow inactivation, analogous to that observed for the yeast PSC, was observed. Fèvre *et al.* (1994) have recently shown that addition of certain peptides restored the fast gating behavior to trypsinized mammalian PSC, and conferred similar properties to yeast PSC. This type of gating was attributed to the presence of added peptides bound in the vicinity of (or to) the channels, which would exert a fast, voltage-dependent block on the PSC (a "ball-and-chain"-type model). Bound peptides would be present in isolated mammalian mitochondria, but not in the yeast ones, hence the different gating properties.

We report here the main features of an investigation, complementary to the one described by Szabò *et al.* (1995), carried out using the planar bilayer method on fractions enriched in outer membrane of wild-type or VDAC-less yeast mitochondria.

MATERIALS AND METHODS

The preparation of mitochondria and membrane fractions has already been described (Szabò *et al.*, 1995). The resulting vesicles are mainly right-side-out (Hwang *et al.*, 1989). The various membrane fractions were analyzed by Western blot with antibodies (courtesy of G. Schatz) against the adenine nucleotide translocator and the β subunit of the F_0F_1 ATPase as markers of the inner membrane, and MAS70, an outer membrane component of the protein import machinery (Hines *et al.*, 1990) and VDAC as markers of the outer membrane, to evaluate the degree of cross-contamination, which was relatively slight, on the order of 5–10% (not shown). The presence of even this limited cross-contamination suggests caution in drawing conclusions as to the localization of the channels. VDAC was completely absent in the VDAC-less strain (HR-125-2A).

Planar bilayer experiments were conducted as described by Szabò *et al.* (1995). Bilayer membranes were composed of azolectin (Sigma) purified by acetone recrystallization. All the voltages reported in this paper are those of the *cis* chamber of the bilayer apparatus (i.e., the chamber into which the membrane vesicles were added), zero being assigned by convention to the *trans* side. Given the orientation of the vesicles, this means that the reported voltage corresponds to that of the pipette for patch-clamp experiments carried out in the cell-attached configuration. When comparing the results presented here to those in Szabò *et al.* (1995), it should be kept in mind that in that paper the voltage at the inner (matrix) side of the membrane was reported in the case of patch-clamp experiments, and that of the *trans* compartment for bilayer experiments.

Currents (cations) flowing in the *cis→trans* direction were considered to be positive and were plotted upward. Unless otherwise specified, the experiments presented were conducted in symmetrical 100 mM KCl, 100 mM sorbitol, 0.1 mM $CaCl_2$, 20 mM Hepes/K^+, pH 7.2. This medium contained about 117 mM K^+ and 100 mM Cl^-; it is referred to as "100 mM KCl." The amplified signals were recorded on tape and analyzed off-line using Axon's pClamp5.5 program set. The term "conductance" is used instead of

"chord conductance," defined as the ratio of current amplitude to applied voltage at any given voltage.

RESULTS

The major activity we observed was ascribable to a channel whose properties were very reminescent of those of the PSC channel studied in patch-clamp experiments (Szabò *et al.*, 1995). Most of the experiments presented here to illustrate its properties were performed on VDAC-less membranes to avoid the possibility of misidentification. However, this channel was also frequently observed in the wild-type outer membrane fraction as well as in the inner membrane and contact site fractions of both wild-type and VDAC-less mitochondria (not shown).

Figure 1A shows an incorporation event resulting in the introduction into the membrane of five similar channels, with the characteristics most often encountered in our experiments. The events of Fig. 1A have a conductance of about 720 pS in 300 mM KCl, corresponding to approximately 240 pS in 100 mM KCl. Conductances of 200–250 pS were very often encountered in these experiments (in 100 mM KCl), together with their approximate double, 400–500 pS. In multi-channel experiments often the current levels were close to multiples of 200–240 pS, as exemplified in Fig. 1B. However, a characteristic of the activity was that it displayed current steps of a variety of sizes. This was partly due to the presence of substates, which can be observed, for example, in Fig. 1B and in Fig. 2A. In the latter, a 230-pS level appears as a substate of a 345-pS (another often-observed size) channel. Figure 2B shows that, in analogy to the observations of patch-clamp experiments, voltage could influence the conductance displayed by the channel. In the example shown, an underlying 53-pS conductance was surmounted by a larger and "faster" one. The two conductances probably correspond to different substates of the same channel, because "coordinated" closures could be observed sometimes (Fig. 2B). The larger conductance measured 168 pS in the negative range, and 112–118 pS in the positive range. The full sizes, including the underlying 53 pS, would come to some 220 and 170 pS respectively. It should be pointed out, however, that this type of rectification was not invariably observed. In many experiments the conductance of the channel was not noticeably dependent on the voltage applied, i.e., an ohmic, nonrectifying behavior was displayed.

Observations suggesting a binary structure of the channels were plentiful. As mentioned, we often observed gating by entities displaying a conductance equal to twice (very often) or to higher multiples of 200–250 pS (see Fig. 1B). In Figs. 3A,B examples are presented showing channels capable of gating as single-step conductances of some 670 and 1020 pS respectively, and of displaying "substates" having a conductance approx. 1/2 of maximal. It is unlikely that such recordings derive from the independent gating of

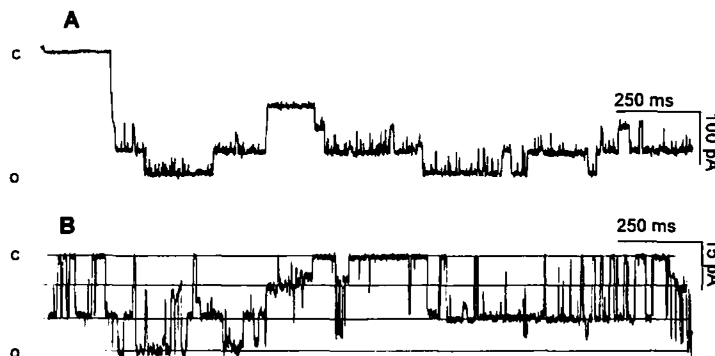

Fig. 1. The predominant conductances of VDAC-less membranes. (A) The incorporation of a vesicle containing approx. 720 pS (300 mM KCl) channels. VDAC-less outer membrane fraction. V: −60 mV. Filter: 1 KHz. Digital sampling rate: 2 kHz. The medium contained 300 mM KCl. c: closed; o: open. (B) The conductance levels measured in many experiments are multiples of 200 pS (in 100 mM KCl). A grid of lines spaced at 10 pA (200 pS) intervals is superimposed on a representative current record segment. V: −50 mV. Filter: 3 kHz. Digital sampling rate: 5 kHz.

Fig. 2. Substates. VDAC-less outer membrane fraction. Filter 1 kHz, Digital sampling rate: 2 kHz. (A) A 345-pS conductance exhibiting a 230-pS substate. V: −40 mV. (B) The conductance of the single-channel events may be influenced by voltage. See text for details. V: +60 (top), and −60 (bottom).

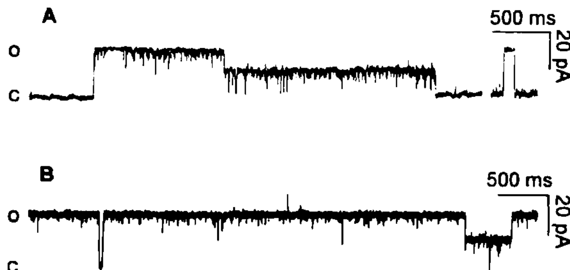

Fig. 3. Examples of putative cooperative behavior. VDAC-less outer membrane fraction. (A) A 670-pS conductance showing a substate of approximately 1/2 the maximal size. The event at right occurred about 5 sec after the one at left. V: +40 mV. Filter: 1 kHz. Digital sampling rate: 2 kHz. (B) A 1-nS channel exhibiting a 1/2 conductance substate. V: +30 mV. Filter: 1 kHz. Digital sampling rate: 2 kHz.

two or more channels, since this would require that these channels would often gate simultaneously by chance, which can be excluded on statistical grounds. In these examples, as well as in many others not shown, the size of the "full conductance state" falls in the range expected for the MMC in 100 mM KCl. The liver MMC displays a similar binary structure (Szabò *et al.*, 1992; Szabò and Zoratti, 1993). This behavior may reflect conformational substates, but it might also indicate cooperative gating by "connected" channels. Supporting this interpretation, very large conductances were also observed (see Fig. 7 below).

These channels displayed, in the majority of experiments, a strong voltage dependence. The most common behavior is illustrated in Fig. 4. Potentials negative on the *cis* side favored the open state(s), while positive voltages drove the channels to low-conductance state(s), although generally not to complete closure. The effect was stronger at higher voltages. This behavior corresponds to closure being induced by potentials positive on the cytoplasmic, and negative on the periplasmic, side. Vice versa, the open state was favored by potentials negative on the cytoplasmic side. The channels promptly returned to the open state when the voltage was switched to zero, in a manner reminescent of VDAC's behavior.

In several cases (still only a minority of experiments) we observed activity by similar channels, displaying a different type of voltage dependence. This behavior is illustrated in Fig. 5, which presents an experiment with the wild-type outer membrane fraction. The recording shows two approx. 240-pS conductances which frequently closed or opened together. The polarity of the applied voltage influenced the gating kinetics: at negative voltages closures were more frequent, as reflected by the sizes of the peaks in the conductance histograms. However, the closures were brief, i.e., there was no inactivation at either polarity. In a small minority of cases, the channels displayed a tendency to inactivate at both positive and negative voltages, while the open state was favored at low potentials. This behavior, which resembles that of isolated VDAC, is illustrated in Fig. 6.

The kinetic behavior of these channels was rather variable, so that a generally valid description would be difficult to make. Both a fast and a slow component were however often present, as can be observed, for example, in Fig. 2. As illustrated by Szabò *et al.* (1995), the channels were cationic, with P_K/P_{Cl} ratios of 4–6.

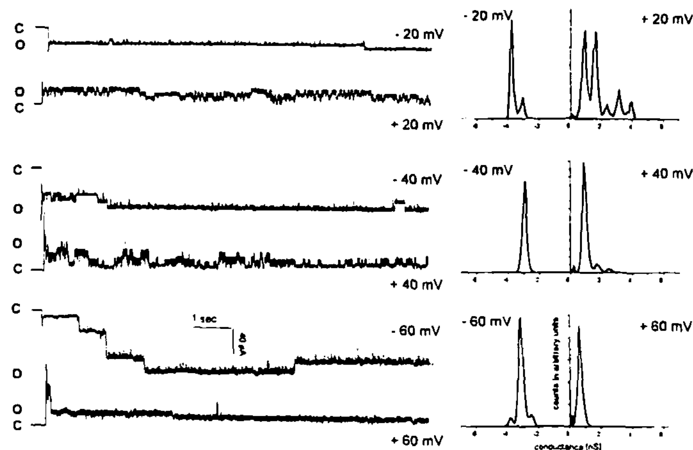

Fig. 4. The most commonly displayed voltage dependence of the PSC channel. Experiment in 300 mM KCl. VDAC-less outer membrane fraction. Left side: representative current traces illustrating the behavior upon application of the specified voltages. The capacitance-charging current spikes at the beginning of the traces signal the application of voltage. Filter: 3 kHz. Digital sampling rate: 5 kHz. Right side: the corresponding conductance histograms, derived from the current amplitude histograms by dividing the current amplitude abscissa values by the applied voltages. The area under each peak is proportional to the fraction of time during which the bilayer had the corresponding conductance. Histogram data were collected over approx. 100-sec intervals. Compare to Figs. 5 and 6.

With wild-type outer membranes, we also observed a channel with a maximum conductance of about 450 pS (100 mM KCl) and very low anionic selectivity, which was driven to long-lasting closures by voltages of either polarity in approximately symmetrical fashion (not shown). These properties are very similar to those of VDAC, with which the channel can be tentatively identified. Remarkably, this VDAC activity appeared in no more than five experiments out of 40.

Huge conductances, ranging in size up to 5 nS, were observed with both wild-type and VDAC-less membranes (Fig. 7). They were not observed in patch-clamp experiments, so that their presence in planar bilayer experiments might derive from aggregations induced by the experimental conditions. We point out here that cooperative behavior resulting in huge conductances as well as in conductances having twice the size of one porin was also observed by us in planar bilayer experiments involving reconstitution of purified VDAC molecules (Zoratti *et al.*, 1992; Szabò *et al.*, 1993). These megaconductances displayed either voltage-induced inactivation (Fig. 7A,B) or an asymmetrical voltage dependence (Fig. 7C). In this latter case they were driven to closed state(s) by positive *cis* potentials, while negative voltages favored the open state(s).

Relatively small (<100 pS) channels were also present in both membrane fractions. Their characterization was made difficult by the concomitant presence of larger conductances.

DISCUSSION

One first consideration concerning the body of data collected on VDAC-less membranes is that the activity displayed was more varied than that observed in patch-clamp experiments on the corresponding mitoplasts (Szabò *et al.*, 1995). In patch-clamp experiments on the native VDAC-less membranes, only one large channel, namely the putative PSC, with the properties summarized in the introduction, was observed. A channel with the same properties was also clearly recognizable in bilayer experiments, in which it constituted the most prominent activity. Its most characteristic feature may be considered to be the rapid inactivation induced by positive *cis* voltages (Fig. 4). However, in a minority of the experiments we also observed channels of similar size displaying an effect

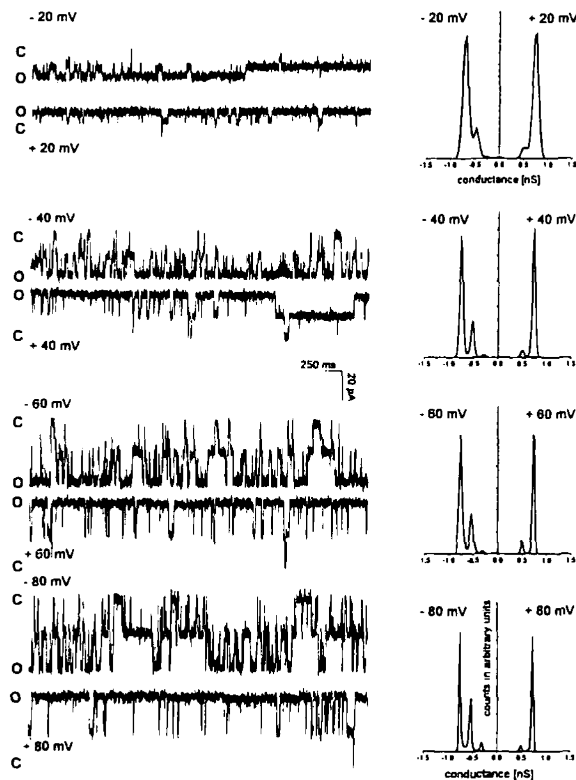

Fig. 5. PSC channel(s) displaying no voltage-dependent inactivation. Wild-type outer membrane fraction. Left side: exemplificative current traces recorded at the indicated voltages. Filter: 3 kHz. Digital sampling rate: 2 kHz. Right side: corresponding bilayer conductance histograms based on data collected over approx. 60 sec of recording at each voltage. Compare to Figs. 4 and 6.

of voltage polarity on the gating characteristics, but no inactivation (Fig. 5). This behavior is strikingly reminiscent of that reported for mammalian and yeast PSC exposed to nonpermeant peptides and to subsequent washing [compare the traces in Fig. 5 and those, e.g., in Figs. 4–8 of Fèvre *et al.* (1994)]. In a few cases, the channel displayed a tendency to inactivate with slow kinetics at both positive and negative voltages (Fig. 6), analogous to the behavior of purified/reconstituted VDAC. This latter behavior resembles that described by the French colleagues for yeast PSC (Fèvre *et al.*, 1990; Thieffry *et al.*, 1992a).

Can this varied behavior be reconciled with the "ball-and-chain"-type model (see the Introduction)? The inactivation at positive *cis*-side potentials (Fig. 4) fits very neatly: it is precisely the behavior expected if a basic (positively charged) peptide were bound in the vicinity of the PSC on the *cis* (outer) side, and were driven by *cis*-positive potentials to block the pore. This is the behavior generally observed in patch-clamp experiments (Szabò *et al.*, 1995). The relatively fast closures observed in a minority of the bilayer experiments, more frequent at negative *cis* potentials (Fig. 5), might be explained by envisioning a similar mechanism involving, however, a peptide bound on the opposite side of the channel (*trans*-facing, inner). The obvious differences (compare Figs. 4 and 5) suggest that the discrepancy is not due to the occasional incorporation of an inside-out-vesicle. They might instead derive from an asymmetry in the channel structure, which might accommodate the peptide less snugly at its inner mouth. Alternatively, a different folding of the peptide itself (which presumably had been translocated through the pore) might be invoked. The inactivation with slow kinetics at both positive and negative potentials (Fig. 6; Fèvre *et al.*, 1990; Thieffry *et al.*, 1992a), a behavior observed by us only in a handful of cases, is more difficult to explain. It might represent the intrinsic voltage dependence of the channel without a bound peptide. The presence of a polycation bound to the channel might indeed be expected to influence the voltage dependence of its intrinsic gating, in addition to acting as a blocker. In this hypothetical framework, the data of the French colleagues and ours become reconcilable if it is assumed that for some unknown reason their membrane preparations do not conserve "outer" peptides bound to the PSC, while ours do.

Partial closures to conductance substates (e.g., Fig. 2A) were much more prominent in bilayer recordings than in patch-clamp ones. This might tentatively be attributed to the difference in the phospholipid compositions of the planar bilayer and of the native membrane. The bilayer experiments also offered evidence of cooperative behavior. Huge conductances, not observed in our patch-clamp experiments, at times characterized the recordings (Fig. 7). Aggregation phenomena induced by the experimental conditions might account for this. In many cases the channels characteristically displayed a "1/2 conductance substate" (examples in Fig. 3A,B), suggesting the existence of doublets of pores. Fèvre *et al.* (1994) have fitted some of their data on the basis of such a model (see Fig. 8 in that paper). This observation, which has no parallel in our patch-clamp experiments (Szabò *et al.*, 1995), is of particular relevance, since a binary structure is a characteristic of the MMC studied in RLM (Szabò *et al.*, 1992; Szabò and Zoratti, 1993). It suggests that an MMC-like channel might be present, or form, in yeast membranes, including VDAC-less ones. On the other

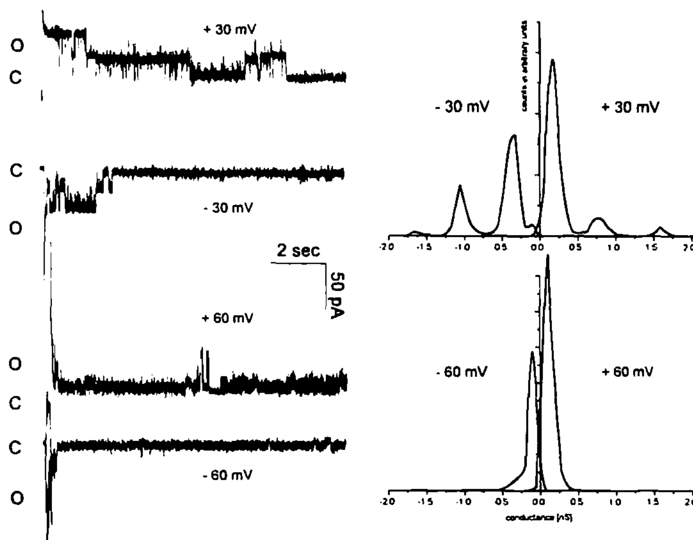

Fig. 6. Inactivation of the PSC channel by voltages of either sign. This figure illustrates a behavior displayed only in a small minority of cases. VDAC-less outer membrane fraction. Left side: current traces recorded at the specified voltages. The capacitance-charging current spikes signal the onset of voltage. Filter: 1 kHz. Digital sampling rate: 5 kHz. Right side: the corresponding capacitance histograms, data collected over approx. 50-sec intervals. Compare to Figs. 4 and 5.

hand, many membrane proteins are known or thought to function as dimers.

The general statement can be made that the recordings from wild-type and VDAC-less preparations were remarkably similar. Activity resembling that expected of VDAC was observed, but it was not as prominent as might have been predicted on the basis of the high VDAC content of the wild-type outer membrane. As already pointed out by Thieffry *et al.* (1992b), reliably distinguishing the VDAC and the PSC is not easy, also because the response to voltage jumps exhibited by purified/reconstituted VDAC is not completely symmetric (Zoratti *et al.*, 1992; Szabò *et al.*, 1993). Cooperation by VDAC molecules might give rise to some of the megaconductances observed with wild-type membranes.

The possibility that the PSC might intrinsically have a voltage dependence similar to VDAC's, and the similarities in the range of conductances displayed by the two channels point to analogies between these pores. A parallel might be drawn with the situation prevailing in Gram-negative bacteria, which possess more than one porin, and express preferentially one or the other depending on conditions. The PSC and VDAC might be two representatives of a multi-component mitochondrial porin family. It is quite clear that any PSC isolation project ought to utilize VDAC-less mitochondria as starting material. The presence of VDAC may be expected to complicate any electrophysiological assay designed to detect the channel in membrane fractions.

Finally, the question of whether or not yeast mitochondria undergo the permeability transition (Szabò and Zoratti, 1994, 1995) gains further significance in the light of our results. As already mentioned, while patch-clamp experiments on VDAC-less organelles did not offer evidence of MMC-like activity, in bilayer experiments we often observed channels having the appropriate conductance and a binary structure (Fig. 3). It remains to be verified whether these channels (in planar bilayers) would be affected by pharmacological agents known to act on the MMC. At present it seems possible that a couple of PSC channels, rather than a couple of porins, might sometimes (or always) constitute the MMC.

ACKNOWLEDGMENTS

The preparation of the yeast mitochondria and membrane fractions was carried out in the laboratory of Prof. G. Schatz (Biozentrum, Basel), whom we

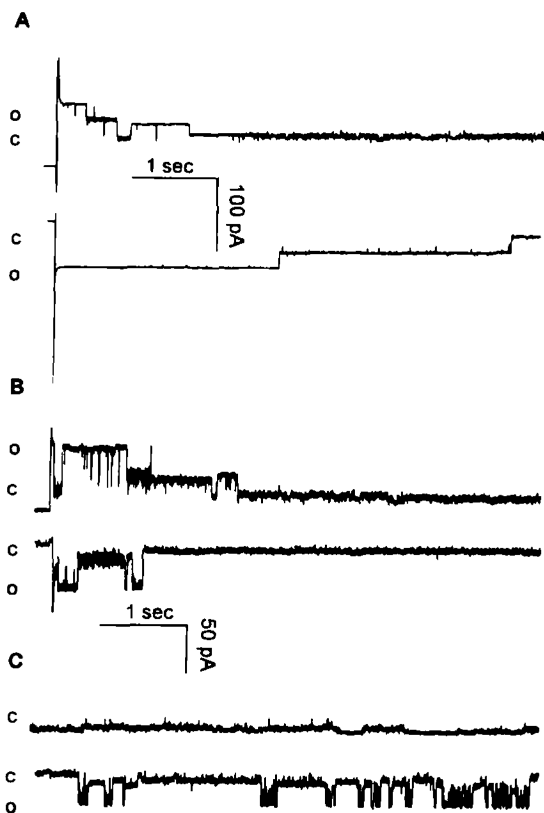

Fig. 7. Megaconductances and their voltage dependence. Filter: 1 kHz. Sampling rate: 5 kHz. (A) nS-level conductances with symmetrical (VDAC-like) voltage dependence in the wild-type outer membrane fraction. V: +45 (top) and −45 (bottom) mV. Capacitive current spikes signal the onset of voltage. The transitions correspond to 1.0 and 1.3 nS (100 mM KCl). (B) The same type of behavior observed with the VDAC-less outer membrane fraction. V: +20 (top) and −20 (bottom) mV. The highest transitions correspond to approx. 1.1, 1.7, and 2.3 nS. (C) Megaconductances appearing only at one voltage polarity (negative). Wild-type outer membrane fraction. V: + 10 (top) and − 10 (bottom) mV. The highest transitions correspond to approx. 2.7 nS (100 mM KCl).

thank for hospitality and useful discussions. We also thank K. Suda for instruction and help with the preparations, as well as Drs. C. Cola and T. Starc for performing some experiments. I. Szabò's stay in Basel was financed by an EMBO short-term fellowship, G. Bathori's stays in Padova by an EEC grant (ERB-CIPA-CT-92-2259/3577) and by an EMBO short-term fellowship, and D. Wolff's stays in Padova by grant FONDECYT 1930909 and by a CNR grant under the CNR/CONICYT program of international cooperation. This work was financed in part by the European Economic Community under the program "Science."

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