Properties of the mitochondrial peptide-sensitive cationic channel studied in planar bilayers and patches of giant liposomes

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ABSTRACT A voltage-dependent cationic channel of large conductance is observed in phospholipid bilayers formed by the tip-dip method from proteoliposomes derived from mitochondrial membranes. It is blocked by peptide M, a 13 residue peptide having the properties of a mitochondrial signal sequence. To verify the reliability of the experimental approach, mitochondrial membranes from bovine adrenal cortex or porin-deficient mutant yeast were either fused to planar bilayers or incorporated in giant liposomes which were studied by patch clamp. Cationic channels were found with both techniques. They had the same conductance levels and voltage-dependence as those which have been described using the tip-dip method. Moreover, they were similarly blocked by peptide M. The voltage-dependence of block duration was analyzed in planar bilayer and tip-dip records. Results strengthen the idea that peptide M might cross the channel. Other mitochondrial channels were observed in planar bilayers and patch clamp of giant liposomes. Because they were never detected in tip-dip records, they are likely to be inactivated at the surface monolayer used to form the bilayer in this type of experiment.

INTRODUCTION

In recent years, the study of mitochondrial channels has gained considerable attention. For a long time, investigations were restricted to the VDAC[1] (voltage dependent anion channel) (1), a large poorly selective pore of the outer membrane. Biochemical and morphological studies revealed that VDAC was a major component of the outer membrane where it forms a bidimensional lattice (2). The outer membrane, thus, has a very high and poorly selective conductance which, a priori, makes it difficult to search for possible channels of other types. No similar problem exists for the study of the inner membrane where an anionic voltage-dependent channel was unambiguously characterized by patch clamp of mitoplasts derived from giant mitochondria (3). The channel was further identified both in liposomes and in planar bilayers enriched in inner membrane fractions (4). More recently, a channel of small conductance, highly selective for K^+ and sensitive to ATP, was found in patches from fused giant mitoplasts (5). Other channels have been recorded from mitoplasts, but they have not yet been as well characterized (3, 6).

The problem of outer membrane channels is more confusing. Tedeschi and co-workers (7) could show by patch clamp of giant mitochondria that the macroscopic currents recorded from outer membranes could not be accounted for by VDAC only. Therefore, they proposed that another class of channels still not described was present in those membranes. Using the tip-dip method (8–10), we found a voltage-dependent cationic channel of large conductance in bilayers derived from mammalian mitochondrial membranes (11). Further biochemical studies indicated that it was localized in the outer membrane, possibly at the level of contact sites between inner and outer membranes (12). This channel is blocked by a mitochondrial addressing peptide (13) and was thus, referred to as PSC (peptide sensitive channel). Surprisingly, no channel with similar properties was identified by other authors who used patch-clamp methods to study the electrical activity of giant liposomes enriched in rat brain mitochondrial membranes (14). The origin of such a discrepancy, thus, needed to be analyzed. Because we had exclusively used records from bilayers formed at the tip of microelectrodes by the tip-dip method, possible distortions induced by our protocol had to be ruled out.

Moreover, using the tip-dip technique, we found that yeast mitochondria have a cationic channel whose properties (15) are close, but not strictly similar, to those of channels from mitochondrial membranes of porin-deficient mutants incorporated into planar bilayers by detergent addition (16, 17). It was also interesting to evaluate in yeast the reliability of our technical approach.

In the present paper, we show that mammalian and yeast cationic mitochondrial channels can be observed in bilayers formed by three different methods (planar bilayer, patch clamp of giant liposomes and tip dipping) and that most of their properties do not depend on the technique used.

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[1] *Abbreviations used in this paper:* peptide M-MLSLRQSIRFFKY; POPC-1-palmitoyl-2-oleoyl phosphatidylcholine; POPE-1-palmitoyl-2-oleoyl phosphatidylethanolamine; POPS-1-palmitoyl-2-oleoyl phosphatidylserine; PSC-peptide sensitive channel; VDAC-voltage dependent anion channel.

MATERIAL AND METHODS

Preparation of biological membranes

Bovine adrenal cortex mitochondria

Bovine adrenal cortex mitochondria were prepared by differential centrifugation as described earlier (13).

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Yeast mitochondria

Mitochondria from the porin-deficient derivative B_5 from yeast strain DBY 747 were kindly provided by Dr G. Lauquin. They were prepared as described earlier (15).

Preparation of proteoliposomes

Tip-dip and planar bilayer experiments

Liposomes were prepared by sonicating to clarity a mixture (7:3) of bovine brain phosphatidylethanolamine and phosphatidylserine (Avanti Polar Lipids, Inc., Birmingham, AL) in 20 mM Hepes buffer (pH 7.5) at a final lipid concentration of 10 mg/ml. Biological membranes were centrifuged at 35,000 *g* for 20 min and resuspended at 0.08–1 mg protein/ml in 0.15 M NaCl/20 mM Hepes buffer pH 7.5. A 75-μl aliquot of the phospholipid solution was added to 25 μl of the membrane solution. The mixture was frozen in liquid nitrogen and kept at −80°C. Before use, the mixture was thawed at room temperature and submitted to two additional cycles of freezing and thawing.

Patch-clamp experiments

Giant liposomes were prepared by a modification of the procedure of Criado and Keller (18). Asolectin (lecithin type II-S, Sigma Chemical Co., St. Louis, MO), purified according to Kagawa and Racker (19), was sonicated to clarity in 100 mM KCl, 10 mM Hepes buffer (pH 7) at a final lipid concentration of 10 mg/ml. The liposome suspension was kept frozen at −70°C. 1–2 μl of the biological membrane solution (10 μg protein) were added to 100 μl of the liposome suspension. The mixture was centrifuged at 100,000 *g* for 40 min in an Airfuge (Beckman Instruments, Palo Alto, CA). The pellet was resuspended in 20 μl of 10 mM Hepes buffer pH 7 and 6-μl aliquots were deposited in wells of a microtitration plate and dehydrated for 1 h in a vacuum dessicator in the presence of silicagel. Rehydration was performed overnight at 4°C after adding 10 μl of a buffer containing 150 or 300 mM KCl, 10 mM Hepes (pH 7).

Electrical recording

Microelectrodes

Microelectrodes were pulled from Vitrex borosilicate hematocrit tubes (Modulohm I/S, Herlev, Denmark) using either a PC 84 or a P 87 puller (Sutter Instrument Co., San Rafael, CA). In 150 mM NaCl, their resistance was 3–10 MΩ. For tip-dip experiments, their tips were fire polished.

Planar bilayers

Synthetic phospholipids (1-palmitoyl-2-oleoyl phosphatidylcholine (POPC), 1-palmitoyl-2-oleoyl phosphatidylethanolamine (POPE), 1-palmitoyl-2-oleoyl phosphatidylserine (POPS), Avanti Polar Lipids, Inc.) in chloroform solutions were mixed as required, dried under nitrogen and dissolved in *n*-decane at a final concentration of 20 mg/ml. Planar lipid bilayers were formed by the Mueller-Rudin method (20). Solutions were 10 mM Hepes-NaOH pH 7.4 (*trans*) and 150 mM NaCl, 10 mM Hepes-NaOH pH 7.4 (*cis*). 100 μl of a proteoliposome suspension, prepared as described above, were added to 100 μl of a 300-mM sucrose solution and the mixture was sonicated for 30 s. 5–15 μl of this suspension were added to the *cis* compartment right against the lipid membrane and the transbilayer current was monitored under voltage clamp using a low noise I/V converter designed by Hanke and Miller (21). When a channel insertion was detected, solutions were made symmetrical by adding NaCl from a 3-M solution to the *trans* compartment. Data, which were filtered at 5 kHz by the electronics, were stored on video tape. Unless otherwise specified, voltages are given by reference to that of the *trans* compartment defined as zero voltage.

Patch-clamp of giant liposomes

1 μl of the giant liposome suspension was added to 1.5 ml of a solution containing 150 or 300 mM NaCl (or KCl), 5 mM $MgCl_2$, 20 mM Hepes-NaOH (or Hepes-KOH) pH 7. Microelectrodes were filled with the same solution as the chamber. After formation of a tight seal, the patches were gently withdrawn. Current was monitored through a RK 300 amplifier (Biologic, Claix, France), filtered at 30 kHz and stored on digital tape. The bath potential was taken as the reference voltage.

Tip dip

Surface monolayers were formed by adding 20 μl of the proteoliposome suspension to 200 μl of a solution (150 mM NaCl, 1 mM $MgCl_2$, 20 mM Hepes pH 7.3). Microelectrodes were introduced into the bath under positive pressure. Bilayers were formed by the tip-dip method. The tip was briefly exposed to the air and reimmersed in the bath (8–10). Current measurements were made using a patch clamp amplifier (List EPC 7). Data were filtered at 10 kHz and stored on video tape. The bath potential was taken as the reference voltage.

Peptide M

Peptide M, a 13-residue peptide (MLSLRQSIRFFKY), was synthesized by Neosystems (Strasbourg, France). The first twelve residues, which have the property of a mitochondrial signal sequence, constitute the NH_2-terminal end of cytochrome *c* oxidase subunit IV precursor from yeast *Saccharomyces cerevisiae* (22).

Analysis of the voltage-dependence of block durations

The duration of the closures induced by peptide M was analyzed at potentials at which the channels had no fast spontaneous activity. Because most of the channels had three main conductance levels separated by jumps of equal amplitude, the channel was considered as "dimeric." Only closures from the fully open to the intermediate levels were considered. At each potential, the mean duration was computed from the slope of linear regression fitting duration histograms in semilogarithmic plot.

RESULTS

Most of the results reported below have to be compared to those which we previously reported using tip-dip records (11, 13, 15). For brevity, they will not be summarized, the number of the figures from these references to which the present data can be directly compared will be indicated in the legends.

Adrenal cortex mitochondria

Planar bilayers

Liposomes identical to those used for the formation of the surface monolayer in tip-dip experiments (except an additional sonication step, see Methods) were fused to a POPE/POPS (7:3) preformed bilayer. With this technique, fusion of a liposome containing channels is detected by a sudden increase of the membrane conductance. In about half of the experiments, fusion events induced a current which, in the presence of the *cis/trans* ionic gradient and at zero mV, was flowing from the *cis* to the *trans* compartment, thus, indicating cation selectivity. Once *cis* and *trans* solutions were made symmetrical, current fluctuations were observed (Fig. 1) which were very similar to those of PSC studied in tip-dip records. First, there exist three main conductance levels separated by jumps of ~200 pS in 150 mM NaCl solutions

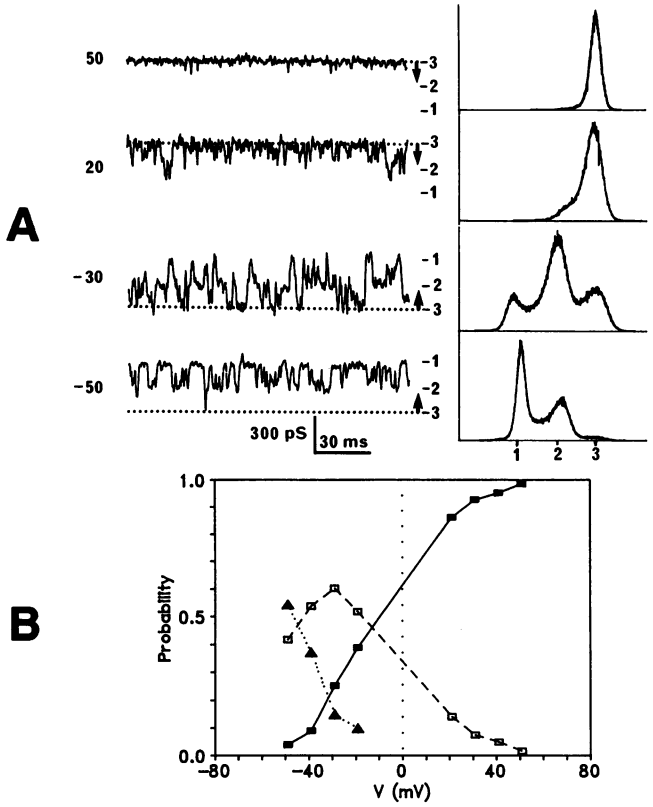

FIGURE 1 Mitochondrial cationic channel from bovine adrenal cortex observed in a POPE/POPS (7:3) planar bilayer. (*A*) samples of current traces (*left*) and conductance histograms (*right*) at different potentials indicated at the left of each trace. The numbers 1, 2, and 3 in the histograms refer to the three main levels of increasing conductance indicated at the right of the current traces. The vertical line indicates the zero conductance level. Histograms were computed for continuous periods of at least 19 s at each potential. To be compared to Fig. 1 of reference 11. (*B*) probability of the conductance levels 1 (▲), 2 (□), and 3 (■) as a function of the potential. Data filtered at 2.5 kHz and sampled at 5 kHz. To be compared to Fig. 2 of reference 11. For this figure and the following ones, the channel maximum conductance is indicated in each current trace by a dotted line. At the end of the trace, an arrow starting from this line points to the direction of channel closures.

(Fig. 1, −30 mV). Second, the current fluctuates between these levels with fast kinetics. Third, a similar voltage-dependence is found using either technique. Fully open at positive potentials, the channel turns to the lowest conductance levels when potential decreases below 0 mV. No long lasting closure takes place when fixed potentials are maintained for periods of several min.

In the experiment shown in Fig. 1, the maximum conductance was ~750 pS and the lowest of the three main conductance levels had a "residual conductance" of ~300 pS. Brief closures of ~100 pS below this level occurred rarely at negative potentials (Fig. 1, −50 mV), but no complete closure to the zero current level could be observed. That this 300 pS level is part of the PSC channel or that it results from the presence of another conductance in the liposome which contained the PSC channel is still unclear. However, in five trials where PSC channels were recognized, the "residual conductance" was never smaller than 300 pS and could be as large as 1 nS.

Trypsin eliminated in two steps the voltage-dependence, leaving channels fully open at all potentials except for rare and brief (≈1 ms) closures (Fig. 2 *A*).

Both native and trypsinized channels were reversibly blocked by peptide M. Blockade of a trypsinized channel is shown in Fig. 2 *B*. The effect is voltage dependent and is observed irrespective of the side of the chamber to which peptide is added provided the right potential polarity is applied. It is characterized by brief transitions to the lowest conductance levels. Their number increases when the driving force for the peptide (which carries four positive charges) is increased (data not shown).

Neither the channel properties nor the characteristics of the blockade by peptide M were changed when a POPE/POPC mixture was used in place of the POPE/POPS mixture to form the bilayer.

Conductance changes due to channels which clearly were not PSC (showing, unlike the PSC, anionic selectivity or/and very slow kinetics) were often observed with the adrenal cortex preparation. They were not analyzed in this study.

Patch clamp of giant liposomes

Channels with similar characteristics were found in patches excised from giant proteoliposomes (Fig. 3).

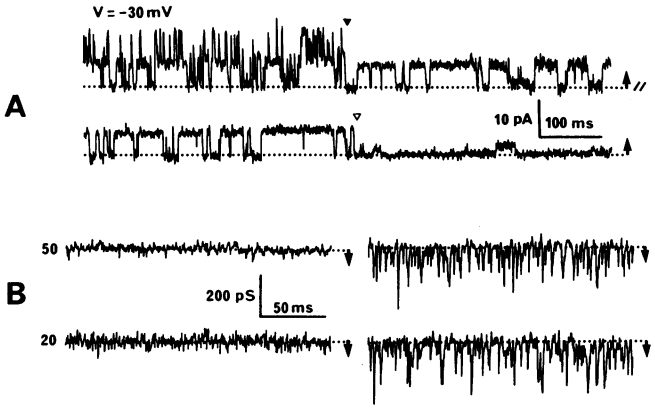

FIGURE 2 Sensitivity to trypsin (*A*) and blockade by peptide M (*B*) of a mitochondrial cationic channel from bovine adrenal cortex observed in a POPE/POPS (7:3) planar bilayer. The channel shown in Fig. 1 was exposed to trypsin, added at a concentration of 10 μg/ml to the *trans* compartment, and the potential was clamped at −30 mV. (*A*) After a delay of 65 s, flickering between levels 1 and 2 was abolished (▼) whereas jumps between levels 2 and 3 still occurred at a lower rate. 100 s latter (*second line*), those jumps in turn irreversibly disappeared (▽), the channel remaining at its higher conductance level at all potentials (*B, left*). To be compared to Fig. 5 of reference 11. Addition of peptide M to the *cis* compartment, at a final concentration of 10 μM, induced an immediate blockade characterized by brief closures to the lowest conductance levels (*B, right*). Filtering and sampling as in Fig. 1. To be compared to Fig. 5 of reference 13.

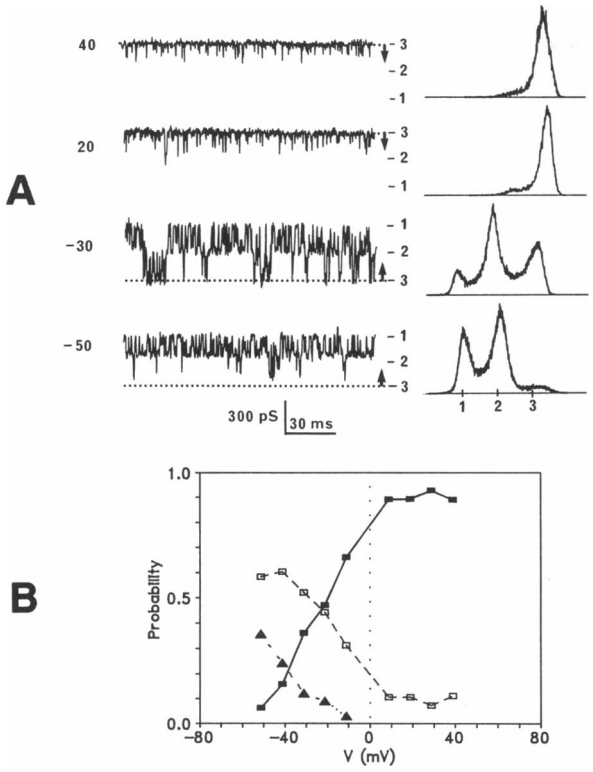

FIGURE 3 Mitochondrial cationic channel from bovine adrenal cortex observed in a patch excised from a giant asolectin proteoliposome. Refer to Fig. 1 for explanations. To be compared to Fig. 1 of reference 11.

Conductance jumps between levels 1 and 2 and levels 2 and 3 were consistently ~200 pS in 150 mM NaCl and 450 pS in 300 mM KCl. There was a slight variability in the voltage dependence of the flicker observed for potentials at which the channel closes, extreme types corresponding to type I and type II channels previously described in tip-dip records (11).

Trypsin and peptide M, respectively, abolished the voltage dependence and blocked the channels exactly as they did for channels incorporated in tip-dip or planar bilayers (data not shown).

In addition to PSCs, other unrelated conductance jumps were also present in those patches, but were not analyzed in the present work.

Porin-deficient yeast mitochondria

Planar bilayers

Proteoliposomes from porin-deficient mutant yeast were fused to planar bilayers. In all cases, the conductance increase due to channel insertion appeared as a current flowing from the *cis* to the *trans* compartment, indicating cation selectivity. The typical activity, found after solutions were made symmetrical by NaCl addition, is shown in Fig. 4. At potentials between 30 and −30 mV, the channel is mostly open. At potentials above 30 mV, the signal is generally noisy, with fast fluctuations between three main conductance levels. In fact, it appears to be composed of jumps of 150–240 pS difficult to measure precisely. Probability of the lowest conductance levels increases with potential. Below −30 mV, transient closures (in the range of tens of milliseconds) to levels respectively about 330 and 660 pS below the maximum conductance occur after voltage application. In fact, they may vary from 260 to 340 pS within the same record. For both positive and negative voltages, long lasting closures occur when the potential is maintained for long durations (Fig. 4, *right*). One observes then, two closures of ~330 pS. They occur faster at potentials of larger magnitude. Times of several tens of seconds are required around −30 mV, whereas a few seconds only are generally necessary around −80 mV. The fully open state can be restored by a brief pulse to 0 mV or to a voltage of reverse polarity.

In 150 mM NaCl, the maximum conductance was ~900 pS. The minimum conductance, observed during fast flickering persisting during long lasting closures, was ~100 pS (Fig. 4, *right,* trace at 85 mV).

The selectivity of the channel was studied under bionic conditions or NaCl gradient and, from the reversal potentials corrected for junction potentials, the following sequence was found for permeabilities:

$$p_K > p_{Na} > p_{TEA} > p_{Cl},$$

with $p_{Na}/p_{Cl} \approx 5$. The conductance was higher in 150 mM KCl, the two main jump values being 500 pS as compared to 330 pS in 150 mM NaCl.

Peptide M induced a reversible voltage-dependent block on both sides of the membrane (Fig. 5). The pep-

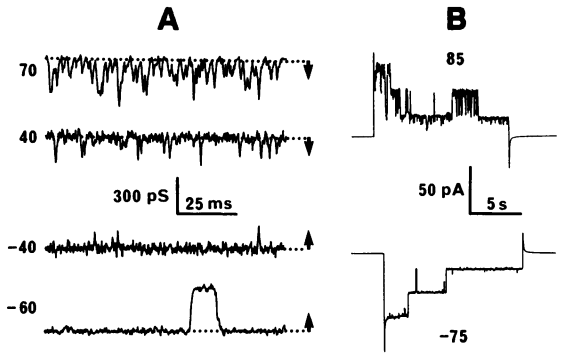

FIGURE 4 Typical activity of a porin-deficient yeast mitochondrial cationic channel recorded from a POPE/POPS (7:3) planar bilayer. (*A*) Samples of current recorded at different potentials indicated at the left of each trace. Data filtered at 2.5 kHz and sampled at 5 kHz. (*B*) Slow kinetics closures of the channel during prolonged voltage pulses. The potential was maintained for several s at 0 mV before switching to 85 mV (*top*) or −75 mV (*bottom*). Data filtered at 100 Hz and sampled at 200 Hz. For *A* and *B*, voltages are given by reference to that of the *cis* compartment defined as zero voltage. To be compared to Fig. 1 of reference 15.

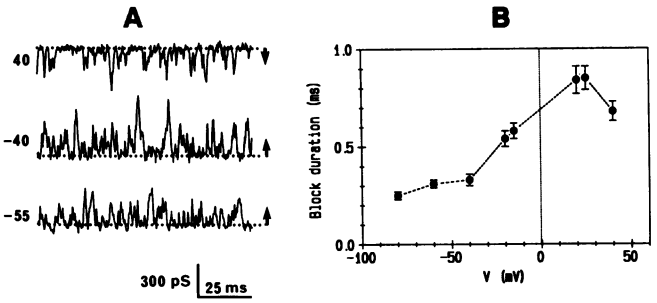

FIGURE 5 Blockade by peptide M of a porin-deficient yeast mitochondrial cationic channel incorporated in a planar bilayer. (*A*) the channel shown in Fig. 4 was exposed to peptide M added to the *cis* compartment at a final concentration of 20 μM. Samples of current recorded at different potentials indicated at the left of each trace. Data filtered at 2.5 kHz and sampled at 5 kHz. To be compared to Fig. 2 of reference 15. (*B*) Voltage dependence of the duration of peptide induced closures for another channel recorded by the same method. The analysis was carried out using a channel in which current fluctuated between only two levels. Standard errors are indicated by bars. Data filtered at 5 kHz and sampled at 20 kHz. Voltages are given by reference to that of the *cis* compartment defined as zero voltage.

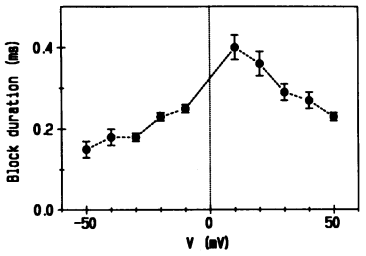

FIGURE 7 Voltage-dependence of the duration of peptide M induced closures in a tip-dip record. The bilayer was formed from a monolayer derived from proteoliposomes enriched in porin-deficient mutant yeast mitochondrial membranes. Standard errors are indicated by bars. Data filtered at 5 kHz and sampled at 20 kHz.

tide induced closures increased in number with the magnitude of the driving force for the peptide (Fig. 5, *left*) whereas their duration first increased, then decreased (Fig. 5, *right*) while increasing the voltage of the compartment containing the peptide.

Patch-clamp of giant liposomes

Similar channels were found in all the patches where current jumps were present (Fig. 6, *left*). However, they generally did not exhibit slow closing kinetics during long voltage pulses. The conductance jumps were 330 pS in 150 mM NaCl and 480 pS in 150 mM KCl. Selectivity

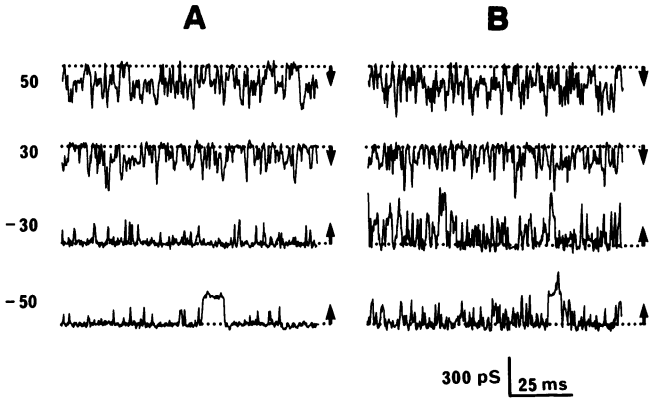

FIGURE 6 Typical activity and blockade by peptide M of a porin-deficient yeast mitochondrial cationic channel recorded from a patch excised from a giant asolectin proteoliposome. (*A*) Samples of current recorded at different potentials indicated at the left of each trace before peptide addition. (*B*) currents recorded at the same potentials in the presence of 10 μM peptide M in the bath. Current was filtered at 2.5 kHz and sampled at 5 kHz. To be compared to Fig. 2 of reference 15.

measurements using a ten-fold NaCl gradient (15 mM in the pipette, 150 mM in the bath) indicated a ratio p_{Na}/p_{Cl} of the order of 4.5.

Peptide M induced a voltage-dependent reversible block (Fig. 6, *right*) similar to that described above.

Tip-dip method

The properties of the cationic channel found in tip-dip bilayers enriched in mitochondrial membranes from either wild-type or porin-deficient mutants have been previously reported (15). Additional analysis of the voltage-dependence of the blockade induced by peptide M was performed to compare more closely the effect seen using tip-dip records to that observed in planar bilayers. The duration of peptide induced closures was determined at different potentials (Fig. 7). As described above for planar bilayers, this duration first increased with the depolarization of the compartment where the peptide was applied, was maximum around 20 mV, then decreased. However, for the same transmembrane voltages, the block times were shorter in tip-dip than in planar bilayers.

DISCUSSION

Peptide M sensitive channels having all the characteristics of the mammalian or yeast PSCs described using tip-dip records (cationic selectivity, voltage-dependence, presence of two conductance jumps of equal amplitude, sensitivity to peptide M) have been identified in both patches of giant liposomes and planar bilayers. This rules out the possibility that the tip-dip method could have led to an erroneous characterization. Only small differences were noted. The kinetics of flicker was slower in planar bilayer experiments than in microelectrode records (compare Fig. 1 to Fig. 3). The selectivity of yeast channels for cations, estimated from giant liposome patches or planar bilayers ($p_{Na}/p_{Cl} \approx 5$), was slightly higher than that we reported from tip-dip experiments ($p_{Na}/p_{Cl} \approx 3$). Slow kinetics closures of yeast channels in patch-clamped liposomes was less frequent than in planar bi-

layers. The channel properties may, thus, be affected by the physico-chemical properties of the membrane. For other parameters, such as the value of the conductance jumps or the voltage-dependence of the fast kinetics events, the variability within records obtained by different methods was not higher than that observed among different tip-dip patches derived from the same mitochondrial proteoliposome preparation.

Because the seal resistance of patches formed at the tip of microelectrodes cannot be known, no conclusion about the PSC lowest conductance level was presently available. Data obtained using planar bilayers show that, both in mammal and in yeast preparations, a residual current flows in the lowest conductance state. Because this current was observed in all records, it is unlikely to be due to another channel and one may conclude that PSC does not completely close. A similar situation stands for the VDAC (23).

A feature of the PSC of potential functional importance is that it is blocked by peptide M, a mitochondrial addressing sequence. The complex voltage dependence of the blockade led us to suggest that the peptide could cross the channel at high enough potentials (13). In the present work, the peptide effect was confirmed in records from giant liposomes and planar bilayers. In the latter, using POPE/POPC mixture in place of POPE/POPS did not change the peptide effect, showing that an electrostatic interaction between the peptide and the lipids making the membrane was not the mechanism underlying the observed blockade. The voltage dependence of the duration of closures induced by the peptide were studied in tip-dip and in planar bilayers. Longer blocks were found using the latter, indicating again that the channel properties could be affected by the physico-chemical properties of the membrane. However, the profiles of voltage-dependence of block duration measured with both techniques were strikingly similar (Figs. 5 and 7). The fact that block duration decreases when the compartment containing the peptide is depolarized above 0 mV (potentials decreasing below 0 mV in the graphs of Figs. 5 and 7) is in good agreement with the previous observation (13) that, in the same conditions, the blockade no longer increases, which is in complete contradiction with a classical Woodhull type of block (24). In fact, this result strongly strengthens the idea that peptide M might go through the channel.

Our data concerning the mitochondrial cationic channel of porin-deficient mutant yeast are in good agreement with those which were reported for a similar material by Benz and co-workers (16, 17), except for voltage-dependence. The pore studied by these authors was voltage-independent up to voltages of 100 mV (16). By contrast, we found with the three techniques we used that both fast and slow kinetics conductance jumps were clearly voltage dependent. A possible origin for such a discrepancy might be the use of detergent. In a recent attempt to solubilize and reconstitute functional adrenal cortex PSC in tip-dip bilayers, we observed that the proteins forming the channel, which was successfully reconstituted after solubilization by a moderate concentration of detergent, retained their ability to form pores if higher detergent concentrations were used. However, their voltage-dependence was then altered (F. Fèvre et al., unpublished results). A similar situation might then occur for the yeast PSC.

Up to now, no channels having the properties of mammalian PSC have been reported by other authors. In fact, the number of studies in which identification of this channel could have been reasonably expected is very small. Because PSC is localized in outer membrane (12), its observation requires the elimination of the huge conductance due to the high density of VDAC channels. This precludes using direct patch-clamp of the native outer membrane, because no blocker of VDAC is presently available. One possibility is to use bilayers in which the channel density is low enough to permit single-channel recording. As far as we know, only two papers report experiments in which either outer or whole membrane mitochondrial channels were studied in these conditions (14, 25). In both cases, experiments were carried out by patch-clamp of giant liposomes using a protocol very similar to that used in the present study. In the work Moran and co-workers carried out using rat brain mitochondria (14), a voltage-dependent 250-pS channel found in outer membrane fractions might be related to the PSC. However, because its selectivity was not established, no conclusion can be presently drawn. Experiments reported in the second paper (25) were carried out using *Neurospora* mitochondria. Because only slow activities were looked at, the authors do not exclude the possibility that some channels were not resolved. Moreover, because the selectivity of the channels they described was not studied, it cannot be ruled out that at least some of those channels had a cationic selectivity. In fact, as long as fast kinetics are not considered, the voltage-dependence of the cationic channel is characterized, like that of the VDAC (23), by slow closing kinetics (Fig. 4) and fast opening.

For tip-dip and planar bilayer records used in the present work, the starting material was identical (liposomes in which mitochondrial proteins were incorporated by three cycles of freezing and thawing). Channels were further incorporated by directly fusing the liposomes with planar bilayers but they had to stay in a surface monolayer when the membrane was formed by tip dipping, a step which might cause channel protein denaturation. In agreement with this hypothesis, only PSC were observed with the tip-dip method whereas, with planar bilayers, conductances induced by non-PSC channels were often seen when membrane preparation from adrenal cortex mitochondria was used. Thus, some mitochondrial channels different from PSC are likely to be inactivated during air exposure at the monolayer interface.

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