Comparison of mitochondrial cationic channels in wild-type and porin-deficient mutant yeast

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Bilayers were formed at the tip of microelectrodes from a suspension of proteoliposomes derived from wild-type and porin-deficient mutant yeast mitochondria. In both preparations, identical cationic channels of large conductance were recorded. This result rules out any relationship between this channel and the outer membrane voltage-dependent anion channel, the activity of which is carried by porin. The ionic selectivity and the voltage-dependence of the yeast cationic channel suggest that it is related to that recently described in mammalian mitochondria. This hypothesis is further supported by the fact that both channels are blocked by a mitochondrial addressing peptide.

Ionic channels; Mitochondria; Porin-deficient mutant; (Yeast)

1. INTRODUCTION

It is now well established that mitochondrial membranes contain several types of ionic channels [1–5]. The best characterized is the VDAC (voltage-dependent anion channel) [6]. Its activity is carried by a protein of about 30 kDa, the mitochondrial porin, a major protein of the outer membrane which forms two-dimensional crystalline arrays [7]. The VDAC has been observed in planar bilayers where it is inserted from the aqueous phase in the presence of detergent [1]. Using a different approach, we have recently shown that bilayers formed at the tip of microelectrodes from a suspension of proteoliposomes enriched in mitochondrial membranes contain a cationic channel of large conductance [5]. In those experiments, we never detected channels having the properties reported for VDAC. Therefore, it was difficult to rule out the possibility that the activity was carried by porin and that differences in electrical properties originated in methodological differences. In yeast, viable porin-deficient mutants can be obtained [8–10]. The aim of the present work was to look for the existence of yeast mitochondrial cationic channels both in wild-type and in mutants, in order to clarify the relationship between the VDAC and the newly characterized cationic channel. In addition, the mammalian cationic channel is blocked by a peptide with a mitochondrial addressing sequence [11], which makes the channel a possible candidate as a protein translocator. Since the peptide used was derived from a yeast addressing sequence, it was important to test whether such an effect was also observed in an homogeneous yeast system.

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Abbreviations: VDAC, voltage-dependent anion channel; TEA, tetraethylammonium

2. MATERIALS AND METHODS

2.1. *Yeast strains and mutants*

Wild-type yeast strain DBY 747 and its porin-deficient derivative B_5 [10] were grown at 28°C on 2% Bacto-peptone, 1% yeast extract (Difco) medium supplemented with 3% glycerol as a carbon source.

2.2. *Preparation of mitochondria*

Cells were collected at the late exponential phase of growth and mitochondria were prepared after treatment of the cells with zymolyase (Miles) [12].

2.3. *Preparation of proteoliposomes*

Liposomes were prepared by sonicating to clarity a mixture (7:3) of bovine brain phosphatidylethanolamine and phosphatidylserine (Avanti Polar, Birmingham, USA) in 20 mM Hepes buffer (pH 7.5) at a final lipid concentration of 10 mg/ml. Biological membranes were centrifuged at 35000 × *g* for 20 min and resuspended at 0.08–2 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.

2.4. *Electrical recording*

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 in-

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to 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 [13–15]. Current measurements were made using a patch clamp amplifier (List EPC 7). The bath potential was taken as the reference voltage. Data were filtered at 10 kHz and stored on video tape.

3. RESULTS

An important proportion of bilayers enriched in wild-type yeast mitochondria exhibited a complex conductance pattern in which several discrete levels could be clearly distinguished (fig.1A). Around −30 mV, the current fluctuated mainly between 3 discrete levels separated by jumps of about 330 pS. Decreasing the voltage below −30 mV increased the probability of the lowest conductance levels, the channel remaining at its lowest conductance level if the voltage was applied for a sufficient time (~30 s). Applying a brief pulse of opposite polarity immediately restored the full conductance state. Around 0 mV, only very brief closures were detectable. At positive potentials, much faster fluctuations between several discrete levels were observed, the probability of the lowest conductance levels increasing with the pipette voltage. In many patches, periods of several seconds during which only brief (~1 ms) closures occurred at a relatively low frequency alternated with periods of intense activity. Due to the very fast kinetics at these potentials, conductance jumps were difficult to evaluate. However, jumps of 100, 140, 240 and 330 pS were reproducibly found in all records at potentials above 50 mV. These jumps were always present together in the different patches. Decreasing the protein/lipid ratio of the proteoliposomes down to 0.003 (w/w) increased the probability of finding empty patches, but did not permit the observation of channels having only one, or a subset, of the conductance jumps listed above. The activity illustrated in fig.1A is thus likely that of a complex channel rather than that of different channels present simultaneously in the bilayer. This is in good agreement with the observation in the richest fractions of noisy records having 5 conductance levels separated by jumps of 330 pS at negative potentials, which presumably resulted from the presence of two channels in the patch.

In none of the above experiments was an electrical activity resembling that of the VDAC found. In particular, the voltage-dependence of the channels was always asymmetrical in contrast to that of the VDAC and the kinetics were much faster than those reported for the VDAC [16]. In order to test whether the porin molecule could carry the electrical activity that we observed (our experimental conditions possibly altering the porin channel properties), experiments were carried out with the same protocol using mitochondria from a porin-deficient mutant in place of wild-type mitochondria. This mutant has been constructed by integration of the URA_3 gene (1.1 kb *Hin*dIII DNA fragment) within the *Eco*RV–*Nco*I gap of the porin gene. Transcriptional activity of the modified porin gene was completely abolished [10]. The electrical activity recorded from the mutant preparation was the same as that described above for the wild type. None of the current jumps were eliminated and the potential-dependence was identical for both preparations (fig.1B). Moreover, long closures at negative potentials and alternating periods of low and high activity above

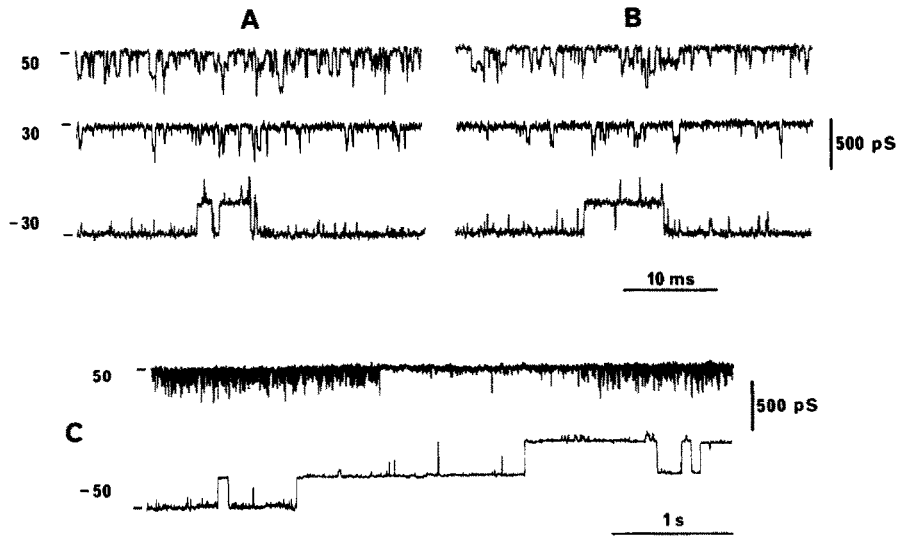

Fig.1. Samples of current fluctuations recorded from bilayers formed by apposition of monolayers at the tip of microelectrodes. Monolayers derived from proteoliposomes containing membranes from wild-type (A) or porin-deficient mutant (B) yeast mitochondria. Sampling: 20 kHz. Filter: 5 kHz. The pipette potential (indicated at the left of each trace) was switched every 5 s from $+V$ to $-V$. (C) Current fluctuations recorded in steady-state conditions at +50 mV and −50 mV using a slower time scale from bilayers enriched in porin-deficient mutant mitochondrial membranes. At −50 mV, 60 pS jumps not found in all preparations are superimposed on the characteristic 330 pS jumps. Sampling: 20 kHz. Filter: 5 kHz at 50 mV and 500 Hz at −50 mV. The bar indicates the highest conductance level.

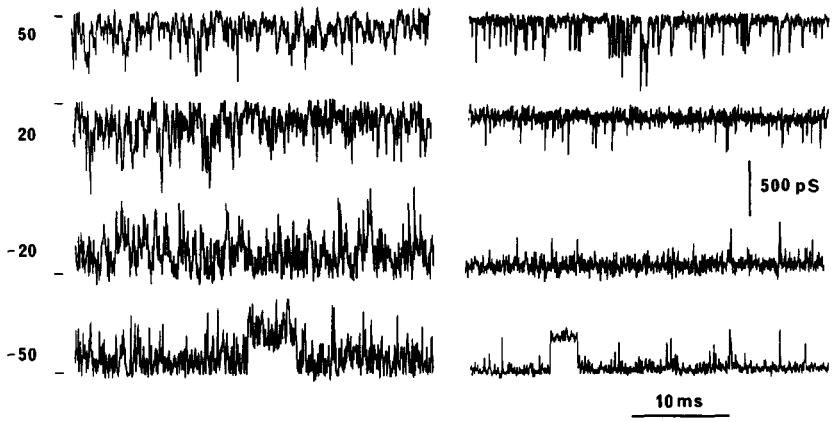

Fig.2. Reversible blockade of the yeast cationic mitochondrial channel by the mitochondrial addressing peptide MLSLRQSIRFFKY. A bilayer containing the channel from a porin-deficient strain was transferred to a bath containing the peptide at a concentration of 100 μM. Immediately after transfer, a blockade characterized by numerous brief transitions to the lowest conductance levels occurred (left). After transfer of the tip back to a peptide-free bath, the blockade was rapidly relieved (right). Sampling: 20 kHz. Filter: 5 kHz. The pipette potential is indicated at the left of each trace. The bar indicates the highest conductance level.

40 mV, which characterized the activity of wild-type channels, were similarly observed in mutant preparations (fig.1C).

The channel selectivity was investigated by computing the shift of the reversal potential of the 330 pS jumps, observed mainly at negative potential, following changes of the bath ionic composition as previously described [5]. The following sequence was found for the permeabilities:

$$p_K > p_{Na} > p_{TEA} \approx p_{Cl}$$

with $p_{Na}/p_{Cl} \approx 3$. This channel is thus slightly cationic and is permeant to large molecules like TEA.

The mammalian mitochondrial cationic channel is blocked by a mitochondrial addressing peptide of 13 residues (MLSLRQSIRFFKY) [11], the first 12 of which constitute the N-terminal end of cytochrome *c* oxidase subunit IV precursor from yeast *Saccharomyces cerevisiae* [17]. The blockade properties are consistent with a translocation of the peptide through the channel [11]. The effect of this peptide was investigated in bilayers containing the mitochondrial channel of wild-type or porin-deficient mutants. For both preparations, transfer of the bilayer to a bath containing the peptide at a concentration of 100 μM induced immediately a blockade of the channel which was reversible by washing out of the peptide (fig.2). The blockade was characterized by a tremendous increase in the number of brief closures superimposed on the spontaneous activity. It increased with decreasing potentials down to about −20 mV, then slightly decreased.

4. DISCUSSION

We have found that yeast mitochondrial membranes contain a complex channel with multiple conductance levels. This channel is slightly cationic and is permeant to large ions like TEA. Its potential-dependence is asymmetrical with respect to 0 mV. This channel differs from the VDAC in many respects: conductance, selectivity, kinetics and potential-dependence. The two channels are thus likely to be different from one another. But, since we never observed the VDAC under our experimental conditions, it could be suspected that both the VDAC and the cationic channel were associated with the same protein, differences in properties resulting from differences in experimental conditions. Alternatively, the cationic channel and the VDAC could be associated with different proteins, the VDAC being inactivated at some stage of our protocol. The fact that the same activity was found in mitochondrial membranes from wild-type and porin-deficient mutants definitely rules out the possibility that the mitochondrial cationic channel is associated to the porin molecule. Since channels having the properties of VDAC were never identified in bilayers deriving from wild-type yeast proteoliposomes, it has to be concluded that VDAC activity is indeed irreversibly lost during the experimental protocol. Both freezing and thawing and the long stay of the porin molecule in the monolayer at the air/water interface might be deleterious for its activity. This is likely to explain why we never detected the VDAC activity in bilayers prepared from mammalian mitochondria. Such an inactivation might, however, be useful in cases where channels of large conductance or present at high density might mask the activity of other less fragile channels.

Using another experimental approach, Dihanich and co-workers recently reported the existence of a slightly cationic pore in the outer membrane of porin-deficient yeast mutants [4]. The current fluctuations controlled by this pore are similar to those shown in fig.1B (slow time scale). In addition, the conductance and the selec-

tivity of this pore are close to those found in the present work. It is thus likely that the same channel was detected in both studies. The cationic channel that we recorded from yeast mitochondria is not strictly identical to that of mitochondria from mammalian tissues [5]. In the latter, conductance jumps are smaller by about 30% and the potential-dependence is steeper. However, it is noteworthy that a voltage-dependence closely related to that of the yeast cationic channel has been found for the cationic channel of human spermatozoa (unpublished results). Both yeast and mammalian channels have the same selectivity and close upon application of a sufficient transbilayer potential. They might thus be supposed to have similar physiological roles. This idea is further supported by the fact that a mitochondrial addressing sequence was found to block the yeast and mammalian channels in the same way. For both preparations, the blockade characteristics were identical. As discussed previously for mammalian channels [11], those characteristics are consistent with a translocation of the peptide through the channel.

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