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Induction of a non-specific permeability transition in mitochondria from *Yarrowia lipolytica* and *Dipodascus* (*Endomyces*) *magnusii* yeasts

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Abstract In this study we used tightly-coupled mitochondria from *Yarrowia lipolytica* and *Dipodascus* (*Endomyces*) *magnusii* yeasts, possessing a respiratory chain with the usual three points of energy conservation. High-amplitude swelling and collapse of the membrane potential were used as parameters for demonstrating induction of the mitochondrial permeability transition due to opening of a pore (mPTP). Mitochondria from *Y. lipolytica*, lacking a natural mitochondrial Ca^{2+} uptake pathway, and from *D. magnusii*, harboring a high-capacitive, regulated mitochondrial Ca^{2+} transport system (Bazhenova et al. J Biol Chem 273:4372–4377, 1998a; Bazhenova et al. Biochim Biophys Acta 1371:96–100, 1998b; Deryabina and Zvyagilskaya Biochemistry (Moscow) 65:1352–1356, 2000; Deryabina et al. J Biol Chem 276:47801–47806, 2001) were very resistant to Ca^{2+} overload. However, exposure of yeast mitochondria to 50–100 μM Ca^{2+} in the presence of the Ca^{2+} ionophore ETH129 induced collapse of the membrane potential, possibly due to activation of the fatty acid-dependent Ca^{2+}/nH^{+}-antiporter, with no classical mPTP induction. The absence of response in yeast mitochondria was not simply due to structural limitations, since large-amplitude swelling occurred in the presence of alamethicin, a hydrophobic, helical peptide, forming voltage-sensitive ion channels in lipid membranes. Ca^{2+}- ETH129-induced activation of the Ca^{2+}/H^{+}-antiport system was inhibited and prevented by bovine serum albumin, and partially by inorganic phosphate and ATP. We subjected yeast mitochondria to other conditions known to induce the permeability transition in animal mitochondria, i.e., Ca^{2+} overload (in the presence of ETH129) combined with palmitic acid (Mironova et al. J Bioenerg Biomembr 33:319–331, 2001; Sultan and Sokolove Arch Biochem Biophys 386:37–51, 2001), SH-reagents, carboxyatractyloside (an inhibitor of the ADP/ATP translocator), depletion of intramitochondrial adenine nucleotide pools, deenergization of mitochondria, and shifting to acidic pH values in the presence of high phosphate concentrations. None of the above-mentioned substances or conditions induced a mPTP-like pore. It is thus evident that the permeability transition in yeast mitochondria is not coupled with Ca^{2+} uptake and is differently regulated compared to the mPTP of animal mitochondria.

Keywords Ca^{2+} · Yeast mitochondria · *Dipodascus* (*Endomyces*) *magnusii* · *Yarrowia lipolytica* · Membrane potential · Mitochondrial permeability transition · Swelling · Yeast

Abbreviations

ΔΨ	mitochondrial transmembrane potential
Atr	atractyloside
ANT	adenine nucleotide translocase
BSA	bovine serum albumin
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
CsA	cyclosporin A

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DTT	dithiothreitol
EGTA	ethylene glycol-bis(aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid
PhAsO	phenylarsine oxide
mPTP	permeability transition pore
Pal	palmitic acid
Pi	inorganic phosphate
PP	pyrophosphate
RC	respiratory control ratio
Tris	tris(hydroxymethyl)aminomethane

Introduction

Studies over the past decade have led to the recognition that mitochondria in animal cells are implicated not only in energy conversion, integrated cellular metabolism, and signal transduction, but also in initiating cell death (apoptosis and necrosis), primarily due to two complementary mitochondrial functions, i.e., generation of reactive oxygen species, potent triggers of apoptosis, and mitochondrial location of some apoptotic factors. Two major mechanisms for release of mitochondrial apoptotic factors into the cytosol were suggested**.** One involves recruitment, activation, conformational rearrangement, and insertion in the mitochondrial outer membrane of pro-apoptotic Bax, a member of the Bcl-2 family proteins. Bax insertion in the mitochondrial outer membrane is modulated by the mitochondrial complex of the translocase of the outer membrane (called TOM) (Colin et al. 2009) and requires cardiolipin (Lucken-Ardjomande et al. 2008).

The other mechanism relays on increased mitochondrial conductance due to opening of some pores in the inner mitochondrial membranes: 1) A non-specific, cyclosporin A (CsA) -sensitive, Ca^{2+}/P_i-dependent, mildly anionic pore known as the mitochondrial permeability transition pore (mPTP) (see review by Bernardi et al. 2006). The molecular nature of the mPTP remains controversial but current evidence implicates a matrix protein, cyclophilin-D and two inner membrane proteins, the adenine nucleotide translocase (ANT) and the phosphate carrier. Inhibition of mPTP opening can be achieved with inhibitors of each component, but targeting cyclophilin-D with CsA and its non-immunosuppressive analogues is the best described (see, Leung and Halestrap 2008); 2) A nonproteinous pore, formed by Ca^{2+} in the presence of saturated fatty acids and distinguished from the classical one by its insensitivity to CsA, a nonselective permeability to divalent cations, and an ability to be spontaneously closed (Mironova et al. 1997, 2001; Sultan and Sokolove 2001); 3) A CsA-sensitive pore induced by high extramitochondrial phosphate concentrations at acidic pH values (Kristian et al. 2001; Knorre et al. 2003).

Pore opening allows free passage of protons across the inner membrane, leading to dissipation of the proton-motive force, i.e. the mitochondrial transmembrane potential ($\Delta\Psi$) and the ΔpH gradient, causing mitochondrial dysfunction, disruption of ionic homeostasis, massive swelling of mitochondria, rupture of the outer membrane, and release of proapoptotic factors.

Apoptosis, a form of programmed cell death playing a central role in development and homeostasis of metazoan organisms (Kerr 2002), recently was also discovered in yeasts as a response to many intra- or extracellular factors (see, Almeida et al. 2007, 2008; Eisenberg et al. 2007; Low et al. 2008; Madeo et al. 2004; Madeo and Fröhlich 2008; Perrone et al. 2008; Reiter et al. 2005; Schmitt and Reiter 2008; Severin et al. 2008) or during the aging process (Laun et al. 2006, 2008; Herker et al. 2004). Moreover, many similarities occur between apoptotic programs in yeast and mammalian cells (Ludovico et al. 2002; Madeo et al. 2002; Fahrenkrog et al. 2004; Fannjiang et al. 2004; Silva et al. 2005; Wissing et al. 2004). In yeast mitochondria, some of the apoptotic factors are also located in the intermembrane space. However, mechanisms underlying their release from mitochondria into the cytosol remain uncertain. Yeast cells lack Bcl-2 family proteins and information about an mPTP-like pore in yeast mitochondria is scarce, fragmentary, and contradictory.

Mitochondria from the *D. magnusii* yeast have a competent respiratory chain with all three points of energy conservation and in contrast to mitochondria from other well characterized yeast species, harboring a high-capacity, regulated Ca^{2+} transport system (Bazhenova et al. 1998a and b; Deryabina and Zvyagilskaya 2000; Deryabina et al. 2001), but did not exhibit the typical mPTP (Deryabina et al. 2004). Also, *S. cerevisiae* mitochondria accumulated large amounts of Ca^{2+} in the presence of the Ca^{2+}-ionophore ETH129 without pore opening (Jung et al. 1997). On the other hand, a channel described in *S. cerevisiae* mitoplasts with high peak conductances (1–1.5 nS) could be equivalent to an mPTP (Lohret and Kinnally 1995). Wild-type *S. cerevisiae* cells were very resistant to high concentrations of Ca^{2+} and P_i or *t*-BOOH, but $\Delta\Psi$ collapsed in the presence of Ca^{2+} and the dithiol-binding reagent phenylarsine oxide (PhAsO) (Kowaltowski et al. 2000). In *S. cerevisiae* yeast, the existence of an unspecific mitochondrial pore (also termed yeast mitochondrial unspecific channel) has been reported (Prieto et al. 1992, 1995, 1996; Manon and Guerin 1998; Manon et al. 1998; Roucou et al. 1997; Gutiérrez-Aguilar et al. 2007). This yeast pore exhibited a similar cutoff size to that of the mammalian mPTP, was active *in situ*, and was inhibited upon ATP depletion **(**Manon and Guerin 1998). So, at present, it is difficult to construct a comprehensive picture of mPTP induction in yeast mitochondria using as a background the above-mentioned conflicting data.

In this study we used yeasts *Y. lipolytica* and *D. magnusii*, good alternatives to *S. cerevisiae*, as having strictly aerobic metabolism, combined with the functioning of energy coupling in Complex I (Andreishcheva et al. 1997), that may be involved in mPTP with an increased open probability when flux increases (see, Bernardi et al. 2006), and well-organized cristae, ensuring less structural limitations to observe appreciable swelling of mitochondria. Genetic tractability of *Y. lipolytica* (Kerscher et al. 2002) allows us to reveal components analogous to those involved in the mPTP of animal mitochondria and the same orthologs of apoptotic factors found in *S. cerevisiae* (unpublished data). We examined (to our knowledge, for the first time) responses of *Y. lipolytica* and *D. magnusii* mitochondria to almost all conditions known to induce permeability transition in animal mitochondria, i.e., elevated [Ca^{2+}] in different combinations with varying concentrations of P_i, palmitic acid, SH-reagents, carboxyatractyloside (an inhibitor of ADP/ATP translocator), as well as depletion of intramitochondrial adenine nucleotide pools, deenergization of mitochondria, and shifting to acidic pH values in the presence of high [P_i]. Here we present unequivocal evidence that in yeast mitochondria a mPTP-like pore, if it ever occurs, is differently regulated than in mammalian mitochondria, and give some plausible explanations of this phenomenon. Some of these data have been reported at meetings (Zvyagilskaya et al. 2006, 2008).

Materials and methods

D. magnusii yeast, strain VKM Y261, was cultivated in agitated Ehrlenmeyer flasks in batches of 100 ml in glycerol-containing medium (Zvyagilskaya et al. 1981) at 28°C. *Y. lipolytica* strain was obtained by RZ as a pure isolate from epiphytic microflora of salt-excreting leaves of arid plants (Negev Desert, Israel) and identified as anamorpha of *Y. lipolytica* (Wick.) van der Walt and Arx. (Zvyagilskaya et al. 2001a, b). *Y. lipolytica* cells were routinely grown in succinate-containing semi-synthetic medium (Andreishcheva et al. 1997). Yeasts were harvested

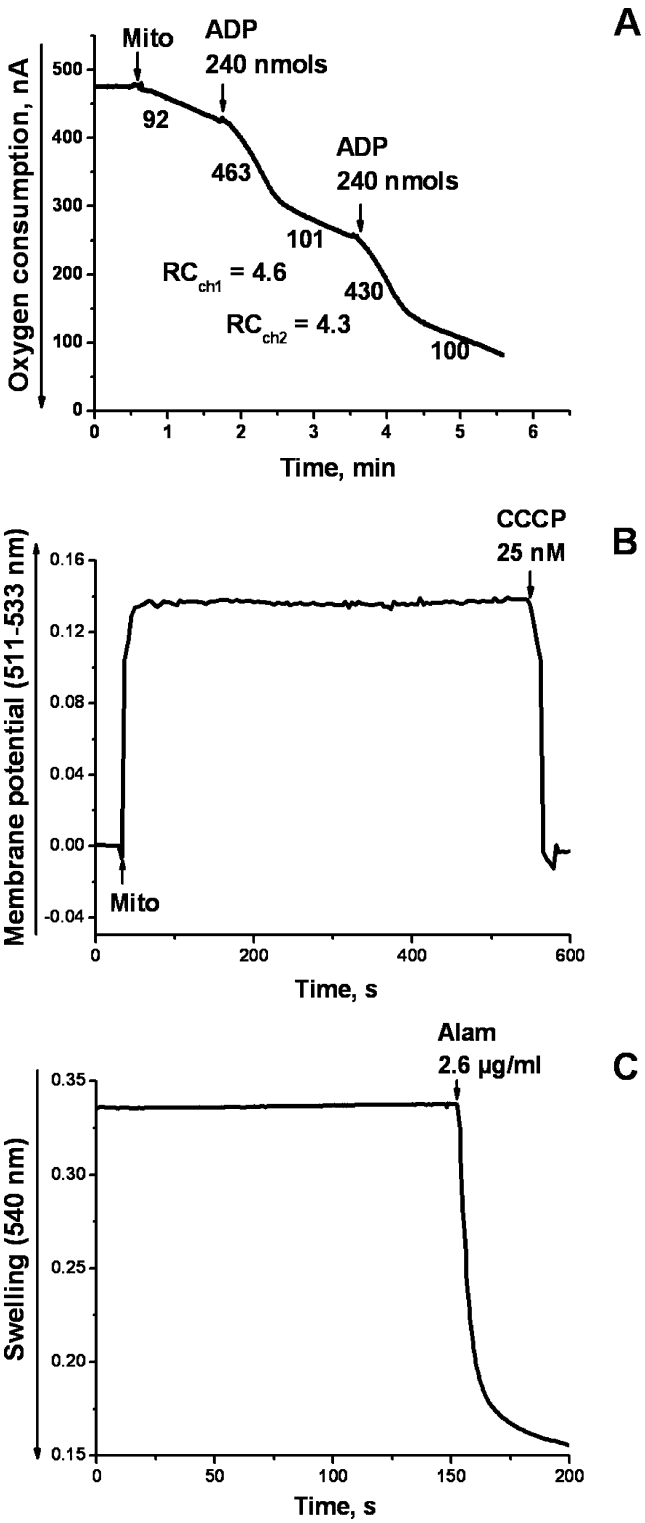

Fig. 1 Energy parameters of *Y. lipolytica* mitochondria. **a** Amperometric recording of oxygen consumption by mitochondria *Y. lipolytica* respiring on pyruvate + malate. Numbers adjacent to traces are respiration rates in ng-atoms of O/min/mg of mitochondrial protein. Respiratory control ratios (determined as a ratio of state 3 respiration to state 4 respiration) upon successive additions of ADP were 4.6 and 4.3. ADP/O ratios upon successive additions of ADP were 2.9 and 2.8. The incubation medium contained 0.6 M mannitol, 0.2 mM Tris-phosphate, pH 7.2, 20 mM Tris-pyruvate and 5 mM malate as respiratory substrates, and mitochondria corresponding to 0.5 mg mitochondrial protein, added at Mito. **b** Recording of ΔΨ of *Y. lipolytica* mitochondria, respiring on 20 mM succinate. ΔΨ was maintained for at least 10 min. **c** *Y. lipolytica* mitochondria, respiring on 20 mM succinate, did not swell in the isotonic incubation medium. Where indicated, alamethicin (Alam) and CCCP were added. Amounts added are seen in the figure

at the late exponential growth phase. Cell growth was monitored at 590 nm (OD_{590}).

Mitochondria from *D. magnusii* cells were prepared according to the protocol described in (Bazhenova et al. 1998a, b). Mitochondria from *Y. lipolytica* cells were prepared as described in (Zvyagilskaya et al. 2001a, b) with minor modifications. Briefly, cells were harvested at the late exponential growth phase (OD_{590}=7.0–7.2), washed twice with ice-cold water, resuspended (0.1 g wet cells/ml) in pre-spheroplast buffer (50 mM Tris-HCl buffer, pH 8.6, 4 mM dithiothreitol), incubated at room temperature for 10–15 min, then diluted with ice-cold water, pelleted at 3,000 g for 10 min, washed twice to remove excess dithiothreitol, and incubated at 28°C under gentle stirring for 15–20 min in spheroplast buffer (10 mM HEPES-buffer, pH 7.2, 1.1 M sorbitol) with Zymolyase 20 T from *Arthrobacter luteus* (ICN Biomedicals) added to a final concentration of 2.5 mg/g cells. Spheroplast formation was monitored by measuring the osmotic fragility of a 100-µl sample of cells after dilution 1:10 in distilled water. The spheroplasts were rapidly cooled, pelleted by centrifugation at 3,000 g for 10 min, washed gently twice in post-spheroplast buffer (1.1 M sorbitol, 5 mM $MgSO_4$, pH adjusted to 7.2), resuspended (0.1 g wet cells/ml) in grinding buffer (10 mM Tris-HCl, pH 7.2, containing 0.4 M mannitol, 1 mM EDTA, 0.05 mM EGTA, and 4 mg/ml BSA), and disrupted in an all-glass Dounce homogenizer (Kontes, Vineland, NJ, USA) with a low clearance pestle. The suspension was diluted with isolation buffer (10 mM Tris-HCl, pH 7.2, 0.6 M mannitol, 0.05 mM EDTA, 0.05 mM EGTA, and 4 mg/ml BSA) and centrifuged at 2000 g for 10 min. The supernatant was centrifuged once more at 7000 g for 20 min. The resulting pellet was washed in 10 mM Tris-HCl, pH 7.2, containing 0.6 M mannitol and 4 mg/ml BSA, resuspended in a smaller volume of the same buffer, and used immediately. Mitochondrial preparations thus obtained were fully active for at least 3 h.

Mitochondrial adenine nucleotide pools were depleted by supplementing grinding medium and washes with 10 mM pyrophosphate (Asimakis and Sordahl 1981). Deenergization of mitochondria was monitored after 1 min incubation in 0.6 M mannitol containing antimycin A (8 µg/ml) and oligomycin (20 µg/ml).

Oxygen consumption by mitochondria was monitored amperometrically with a Clark-type oxygen electrode. Unless otherwise specified in figure legends, the incubation medium contained 0.6 M mannitol, 2 mM Tris-phosphate, pH 7.2–7.4, 20 mM Tris-pyruvate, 5 mM malate or 20 mM

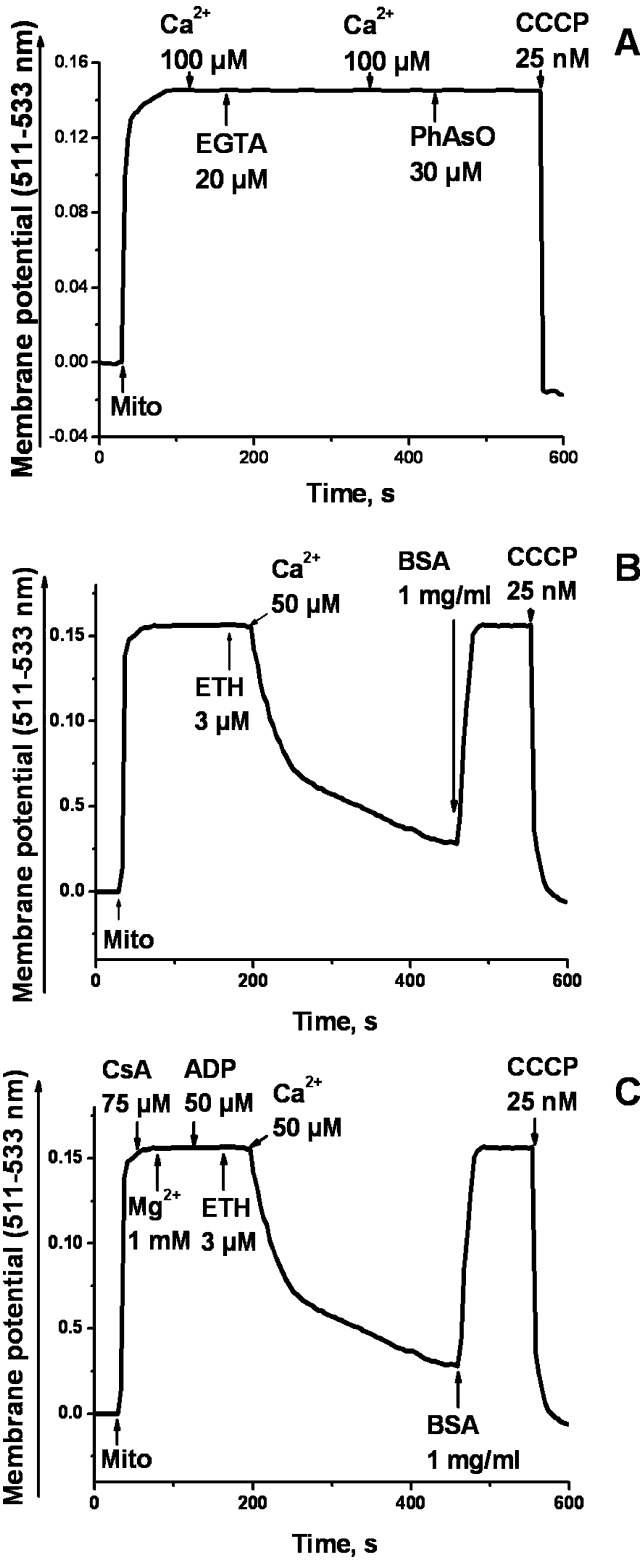

Fig. 2 Effects of Ca^{2+}, EGTA, PhAO, ETH129, BSA, CsA, ADP, and Mg^{2+} on the ΔΨ generated by *Y. lipolytica* mitochondria, respiring on succinate. Experimental conditions were as in Fig. 1b. Additions and amounts are given in the figure. **a** Moderate Ca^{2+} concentrations did not disturb the ΔΨ-related absorbance even in the presence of small concentrations of EGTA and PhAO. **b** A combination of Ca^{2+} and ETH 129 (ETH) produced a substantial fatty acid-dependent decrease in Δψ, abolished by BSA. **c** The Ca^{2+}-ETH-induced decrease in Δψ was insensitive to CsA, ADP, and Mg^{2+}, all known as closers of the canonical CsA-sensitive pore

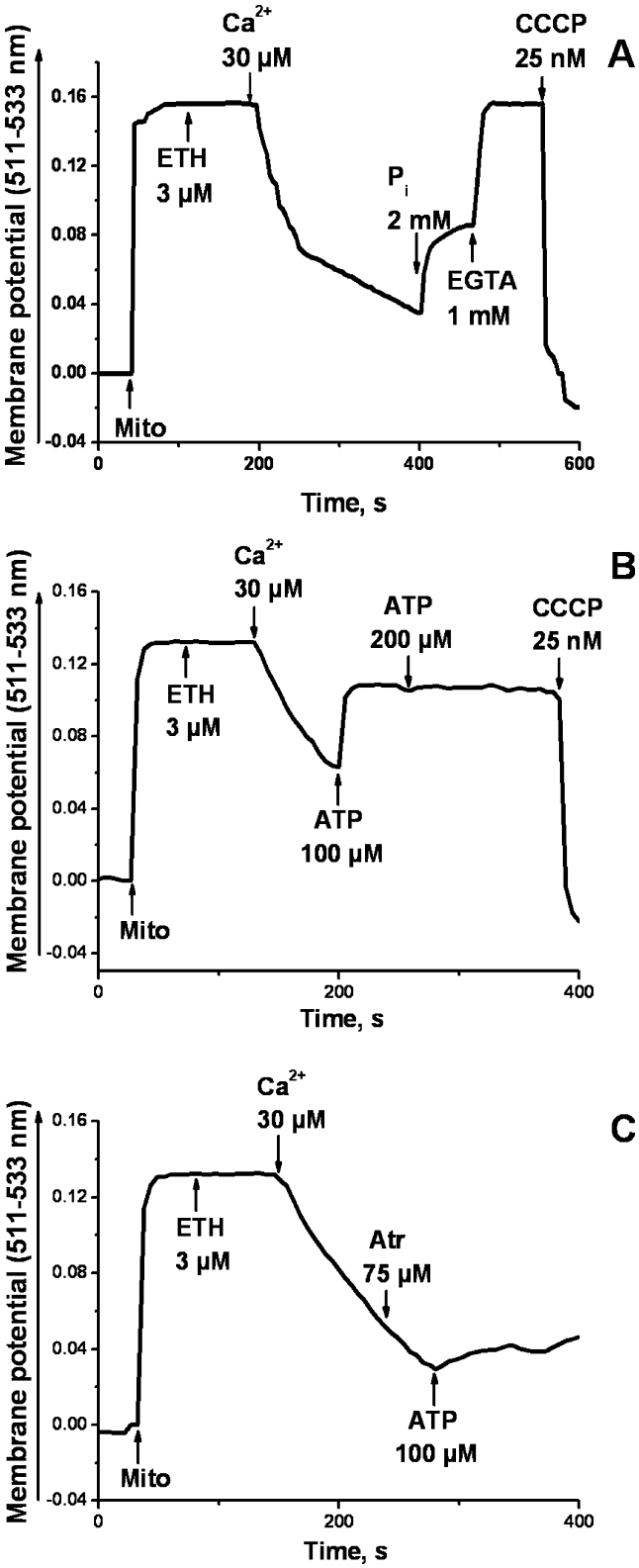

Fig. 3 The Ca^{2+}-ETH-promoted decline in $\Delta\psi$ was partially restored by P_i (A) and ATP (B). The experimental conditions were as in Fig. 1b. Additions and amounts are seen in the figures. **a** Control experiments showing changes in $\Delta\psi$ induced by Ca^{2+}, Pi, EGTA and CCCP. **b** Effects of additions of various amounts of ATP and CCCP are shown. **c** The "recoupling" effect of ATP was specific as inferred from its substantial decrease by atractyloside (Atr)

Tris-succinate, and mitochondria corresponding to 0.5 mg protein/ml. Respiratory rates were expressed as ng-atoms O/(min per mg mitochondrial protein). Respiratory control and ADP/O ratios were calculated according to Chance and Williams (1955). Membrane potential was measured with 10–20 μM safranine O as a probe with a Beckman dual beam spectrophotometer using 511 nm and 533 nm as the measuring and reference wavelength, respectively. Mitochondrial volume changes were monitored as changes in A_{540} with a Hitachi-557 spectrophotometer (Japan). Mitochondrial protein was determined using the Bradford method (Bradford 1976) with BSA as standard.

Zymolyase 20 T (from *Arthrobacter luteus*) was purchased from ICN Biomedicals (Tokyo) and yeast extract from Difco (USA). Mannitol, sorbitol, pyruvic acid, malic acid, succinic acid, ADP, ATP, EDTA, ethylene glycol-bis(aminoethyl ether), *N,N,N′,N′*-tetraacetic acid (EGTA), fatty-acid-free BSA, spermine, oligomycin, antimycin A, carboxyatractyloside, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), Tris, dithiothreitol, CsA, PhAsO, and $CaCl_2$ were purchased from Sigma-Aldrich, Coomassie G-250 and safranine O from Serva (DE), ETH129 from Fluka (Switzerland). Other reagents of the highest quality were from domestic suppliers.

Results and discussion

All yeast mitochondrial preparations used were fairly intact, as inferred from high respiratory rates in state 3 respiration (in the presence of ADP), metabolic state regulation upon successive additions of ADP, high respiratory control ratios (RC) ranging from 4 to 5 upon oxidation of NAD-dependent substrates, ADP/O ratios close to the theoretically expected maxima (Fig. 1a), and from some additional tests on structural integrity of the outer and inner mitochondrial membranes (Data not shown). Respiring mitochondria maintained their $\Delta\Psi$ for a long time (Fig. 1b) without any disturbance in light scattering even in hypotonic incubation medium (Fig. 1c).

According to the purpose of this study, we examined practically all conditions known to induce mPTP in animal mitochondria. Collapse of $\Delta\Psi$ and high-amplitude swelling of mitochondria were used as parameters demonstrating mPTP-like pore induction.

First we found that tightly-coupled *Y. lipolytica* mitochondria lack a natural Ca^{2+}-transporting system as they were unable to transport Ca^{2+} even in the presence of spermine, ADP, and NADH, known to improve the kinetic

properties of the Ca^{2+} uniporter in mammalian and yeast mitochondria (Bazhenova et al. 1998a, b). Therefore, it was not surprising that *Y. lipolytica* mitochondria were very resistant to Ca^{2+}, not responding to elevated [Ca^{2+}] even in the presence of micromolar concentrations of EGTA (usually added to block possible reversed activity of the Ca^{2+} uniporter, thus sustaining high [Ca^{2+}] in the matrix space, needed for induction of mPTP opening) and bifunctional hydrophobic SH-reagent phenylarsine oxide (PhAsO) (Fig. 2a), all known to promote mPTP opening in mammalian mitochondria (see, Bernardi et al. 2006). Addition of the uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) induced a sudden collapse of $\Delta\Psi$ due to dissipation of the electrochemical proton gradient. In the presence of the Ca^{2+} ionophore ETH129 yeast mitochondria accumulated Ca^{2+} with pronounced membrane depolarization (uncoupling) (Fig. 2b), suggesting futile Ca^{2+} cycling under these conditions, presumably as a result of activation of a Ca^{2+}/nH^{+} antiporter. Since addition of fatty acid-free BSA totally restored the initial $\Delta\Psi$ value (Fig. 2b), we concluded that activation of the Ca^{2+}/H^{+} antiporter in *Y. lipolytica* mitochondria is dependent on endogenous fatty acids as was previously found in *S. cerevisiae* mitochondria (Bradshaw et al. 2001). The ETH129+Ca^{2+}-induced decline in the $\Delta\Psi$ was insensitive to CsA, Mg^{2+}, and ADP (Fig. 2c), all known to close mPTP in mammalian mitochondria (see, Bernardi et al. 2006), and partially rescued by Pi (Fig. 3a) and ATP (Fig. 3b); the "recoupling" ATP effect was specific, being almost totally abolished by atractyloside, an inhibitor of ANT (Fig. 3c).

In contrast to animal mitochondria, *Y. lipolytica* mitochondria did not swell in mannitol-based medium when exposed to high [Ca^{2+}], even in the presence of ETH129, thus showing no induction of pore opening. (Fig. 4a). The absence of response in yeast mitochondria to ETH129+Ca^{2+} was not simply due to structural limitations, since large-amplitude swelling occurred in the presence of alamethicin, which being incorporated into mitochondrial membranes forms channels with a diameter of 1 nm that are permeable to divalent cations and low-molecular weight compounds, resembling in this respect the Ca^{2+}-dependent mPTP. Closely similar results were obtained with *D. magnusii* mitochondria, possessing a high-capacitive Ca^{2+} transport system (Bazhenova et al. 1998a, b). Thus, energized mitochondria did not display increased membrane permeability in response to elevated [Ca^{2+}] and a number of mPTP promoters.

It has previously been demonstrated that deenergization of mitochondria or depletion of the intramitochondrial pools

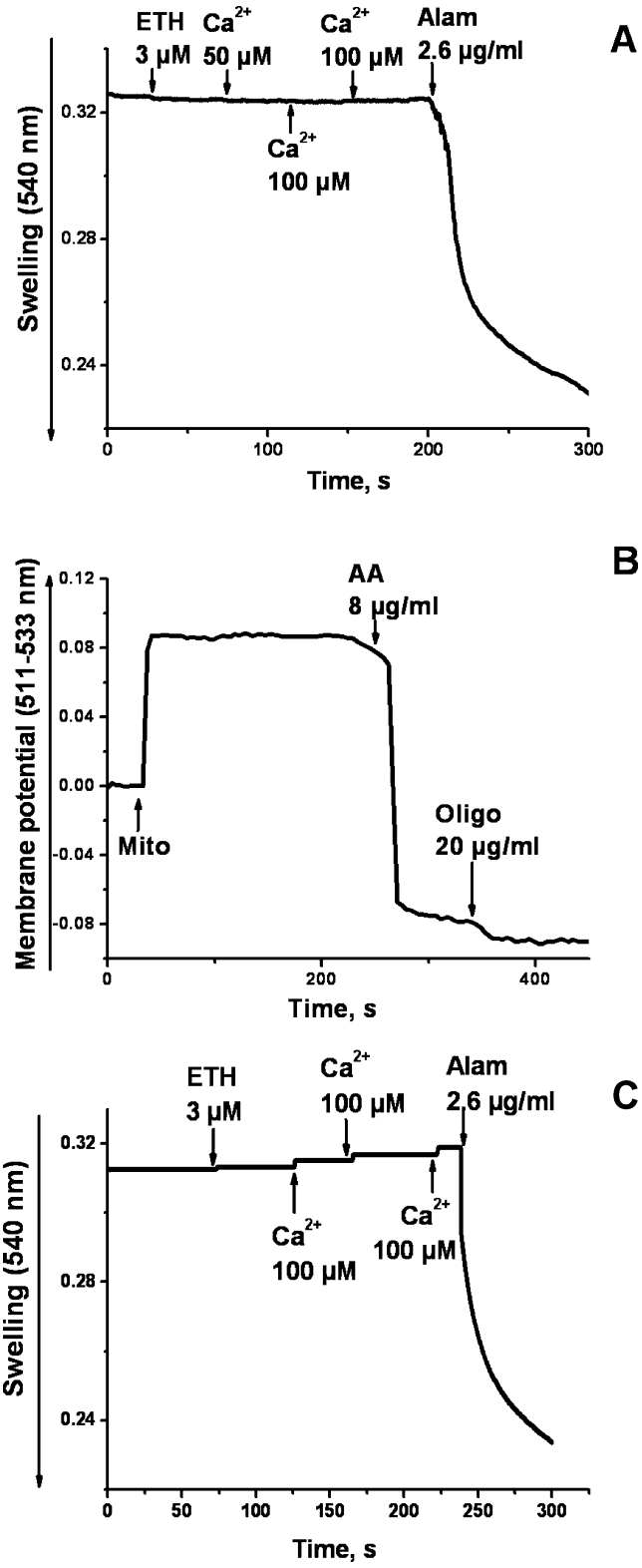

Fig. 4 Recording of swelling (A and C) and $\Delta\psi$ generation (B) of *Y. lipolytica* mitochondria under various conditions. **a** No Ca^{2+}-induced swelling (decrease in A_{540}) was observed in the presence of ETH. Experimental conditions as in Fig. 1c. Where indicated, ETH, Ca^{2+}, and alamethicin were added. **b** Decreased $\Delta\psi$ generated by *Y. lipolytica* mitochondria respiring on endogenous substrates collapsed in the presence of antimycin A (8 μg/ml) and oligomycin (20 μg/ml). **c** Deenergized *Y. lipolytica* mitochondria did not swell in Ca^{2+}-containing mannitol-based medium. The basal incubation medium contained also antimycin A (8 μg/ml) and oligomycin (20 μg/ml)

of adenine nucleotides (see, Bernardi et al. 2006) make animal mitochondria more sensitive to massive Ca^{2+} loads and thus promote the opening of the mPTP. Therefore, the effects of potential mPTP opening inducers on deenergized *Y. lipolytica* mitochondria were studied. For this purpose, the mitochondrial suspension was incubated with antimycin A, an inhibitor of the respiratory chain (to prevent oxidation of endogenous substrates) and oligomycin (an inhibitor of energy transfer). Figure 4b clearly shows that antimycin A caused a total collapse of $\Delta\Psi$, while addition of oligomycin hardly had any further effect. In contrast to animal mitochondria, deenergized yeast mitochondria were resistant to Ca^{2+} load (in the presence of ETH129), showing no swelling (Fig. 4c) under these conditions and, therefore, no pore induction.

For depletion of adenine nucleotide pools, yeast mitochondria were incubated with 10 mM pyrophosphate (PP). As is well known (Asimakis and Sordahl 1981), PP is transported into mitochondria via the ANT, being exchanged for mitochondrial ATP and thus lowering the intramitochondrial ATP pool, especially in the presence of oligomycin, preventing ATP formation from ADP. Control amperometric experiments showed that PP itself had no impact on the respiratory rate, however preventing ADP-induced stimulation of respiration in state 4 (Fig. 5a), which would agree with the notion that in yeast mitochondria PP competes with ADP for the binding site on the ANT. PP itself had no impact on $\Delta\Psi$ (Fig. 5b). PP-treated *Y. lipolytica* and *D. magnusii* mitochondria thus responded to Ca^{2+} load without pore opening (Fig. 5c). Almost the same results were obtained with *D. magnusii* mitochondria (Data not shown).

Our next step was to examine the effects of combined action of Ca^{2+} and saturated fatty acids leading in mammalian mitochondria to formation of a non-proteinaceous pore, distinguishable from the classical one by its insensitivity to CsA, and its ability to be spontaneously closed (Mironova et al. 2001; Sultan and Sokolove 2001). To our surprise, we did not find any information concerning responses of yeast mitochondria to saturated fatty acids. Therefore, we tested effects of palmitic, stearic and pentadecanoic acids on energy parameters of *Y. lipolytica* mitochondria. Palmitic acid (the same results were obtained with stearic and pentadecanoic acids) added to *Y. lipolytica* mitochondria respiring on NAD-dependent substrates increased the respiration rate in state 4 (Fig. 6a), which is indicative of uncoupling. Subsequent additions of Ca^{2+} did not trigger additional uncoupling. Complementary to these results, palmitic acid induced depolarization of the

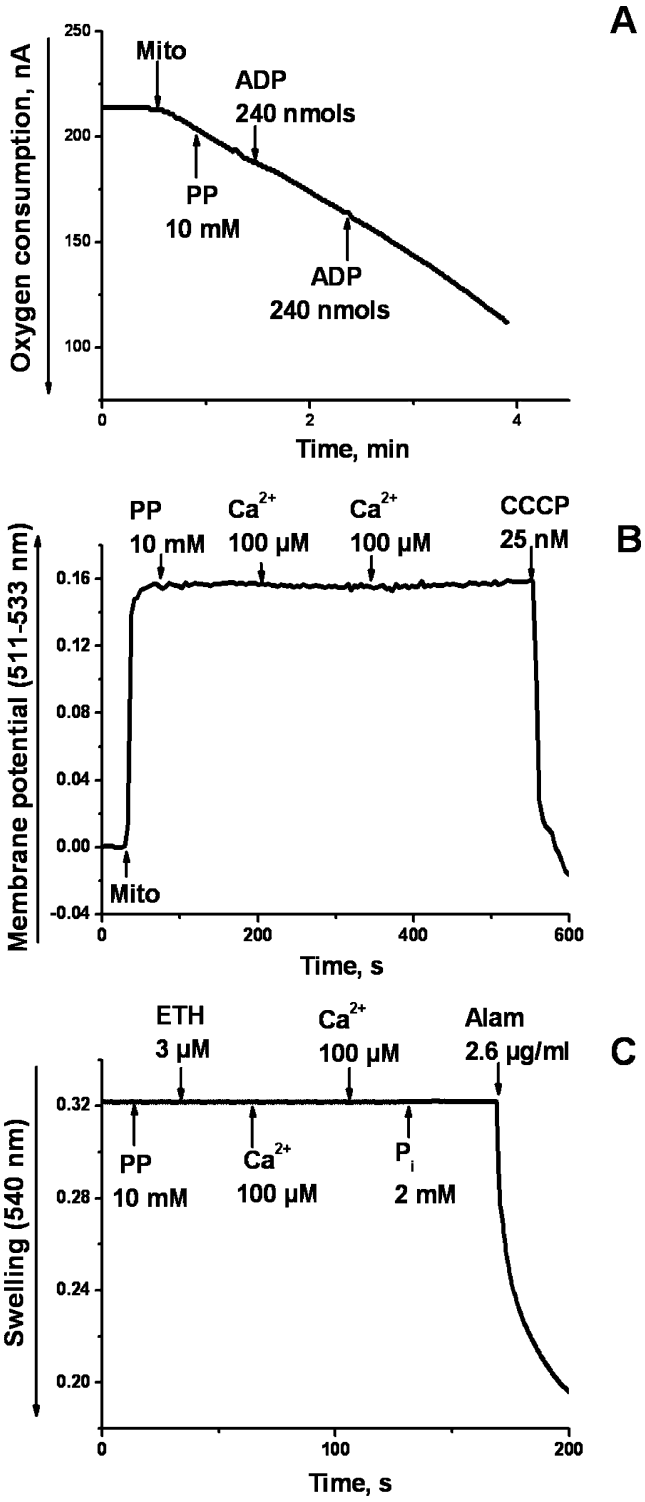

Fig. 5 Recording of oxygen consumption (A), $\Delta\psi$ generation (B) and swelling (C) of *Y. lipolytica* mitochondria treated with pyrophosphate (PP) to deplete the adenine nucleotide pool. Additions are indicated in the figure. **a** PP prevents transition of state 4 respiration to state 3 respiration upon additions of ADP. The basal incubation medium was as in Fig. 1a. **b** PP had no effect on response of *Y. lipolytica* mitochondria to Ca^{2+}. **c** *Y. lipolytica* mitochondria with depleted adenine nucleotide pool did not swell in the Ca^{2+}-containing mannitol-based medium

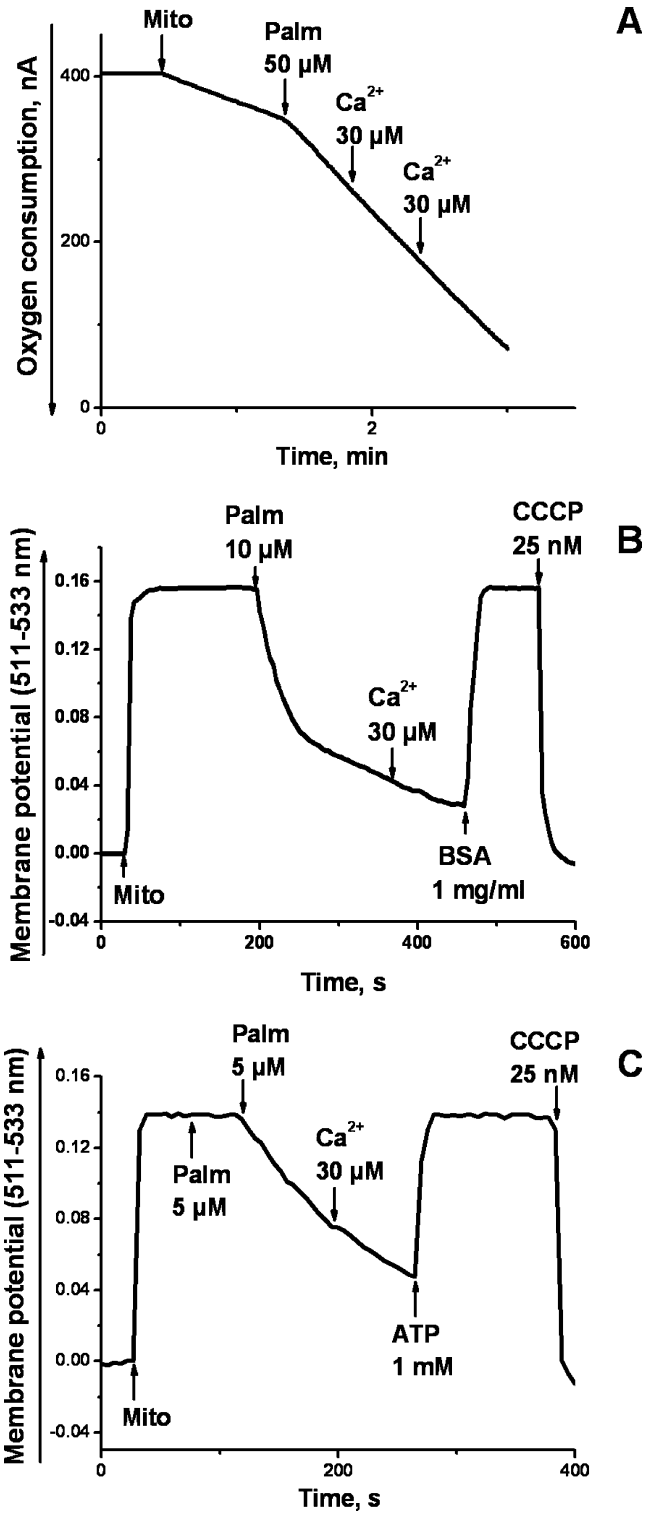

Fig. 6 Uncoupling effect of palmitic acid on *Y. lipolytica* mitochondria was reversed by BSA and ATP. Additions are indicated in the figure. **a** Palmitate increased the respiratory rate with no further stimulation by Ca^{2+}. Experimental conditions as in Fig. 1a. **b** Addition of palmitate reduced the Δψ rapidly, then at slower rate. Experimental conditions as in Fig.1b. Subsequent addition of Ca^{2+} did not promote further Δψ decline. The palmitate-induced Δψ decrease was recovered by addition of BSA or by ATP (**c**)

inner membrane, and this effect was abolished by BSA (Fig. 6b), probably due to binding saturated fatty acids with high affinity, and ATP (Fig. 6c). ATP and BSA added before the fatty acids prevented their uncoupling effect (Data not shown). ETH129 itself left the membrane potential unchanged (Fig. 7a), however subsequent addition of Ca^{2+} caused an additional decrease in ΔΨ (Fig. 7b), most likely due to the already described fatty acid-dependent activation of the Ca^{2+}/nH^{+} antiporter (see Fig. 2b). EGTA prevented this additional decline in ΔΨ induced by ETH129 and Ca^{2+} (Fig. 7b). Moderate concentrations of Ca^{2+} and palmitic acid did not perturb changes in the organelle volume (Fig. 7c), thus definitely indicating lack of pore induction in yeast mitochondria under these conditions. Almost the same results were obtained with *D. magnusii* mitochondria (Data not shown).

In contrast to mammalian mitochondria (Kristian et al. 2001), the yeast mitochondria appeared to be very resistant to Ca^{2+} load in the presence of high (10 mM) [Pi] at acidic pH values (to 6.0) even in hypotonic media (Fig. 8a and b).

Thus, none of the well-established mPTP promoters were able to induce high-amplitude swelling of *Y. lipolytica* mitochondria. In contrast to data obtained by Kowaltowski et al. (2000) on *S. cerevisiae* spheroplasts, no Ca^{2+}-dependent permeabilization of tightly-coupled and deenergized or adenine nucleotide-depleted *Y. lipolytica* mitochondria was obtained even in the presence of agents (PhAsO and Pi, for example) that are known to be potent inducers of the mPTP in animal mitochondria. A decrease in the ΔΨ observed on permeabilized *S. cerevisiae* spheroplasts in the presence of excessive (0.5 mM) Ca^{2+} concentrations and the prooxidant PhAsO (Kowaltowski et al. 2000) might be due to inhibition of oxygen consumption by PhAsO and, more likely, activation of Ca^{2+}-dependent lipases leading to accumulation of free fatty acids and, ultimately, to activation of the fatty acid-dependent Ca^{2+}/nH^{+}-antiport, as described by Bradshaw et al. (2001) and confirmed by us (Fig. 4). With this information, we believe that the lack of Ca^{2+}-dependent permeabilization is a common trait of yeast mitochondria.

This could be due to 1) lack of some component needed for the formation of a mPTP, 2) different regulation of its opening, i.e. non-stimulation by Ca^{2+}, Pi, and prooxidants, or 3) presence of potent inhibitors of mPTP opening. In regard to mechanism (1), there are several components suggested to take part in the mPTP formation, though there is some uncertainty of which indeed are necessary (see "Introduction"). One possible candidate for altered function in yeast mitochondria is cyclophilin D, a mitochondrial protein, the CsA receptor, that is engaged in the mPTP development (Nicolli et al. 1996; Leung and Halestrap 2008). A cyclophilin family with rather uncertain functions was found in yeast mitochondria. The only conclusively defined function is their involvement in the protein folding process (Matou-

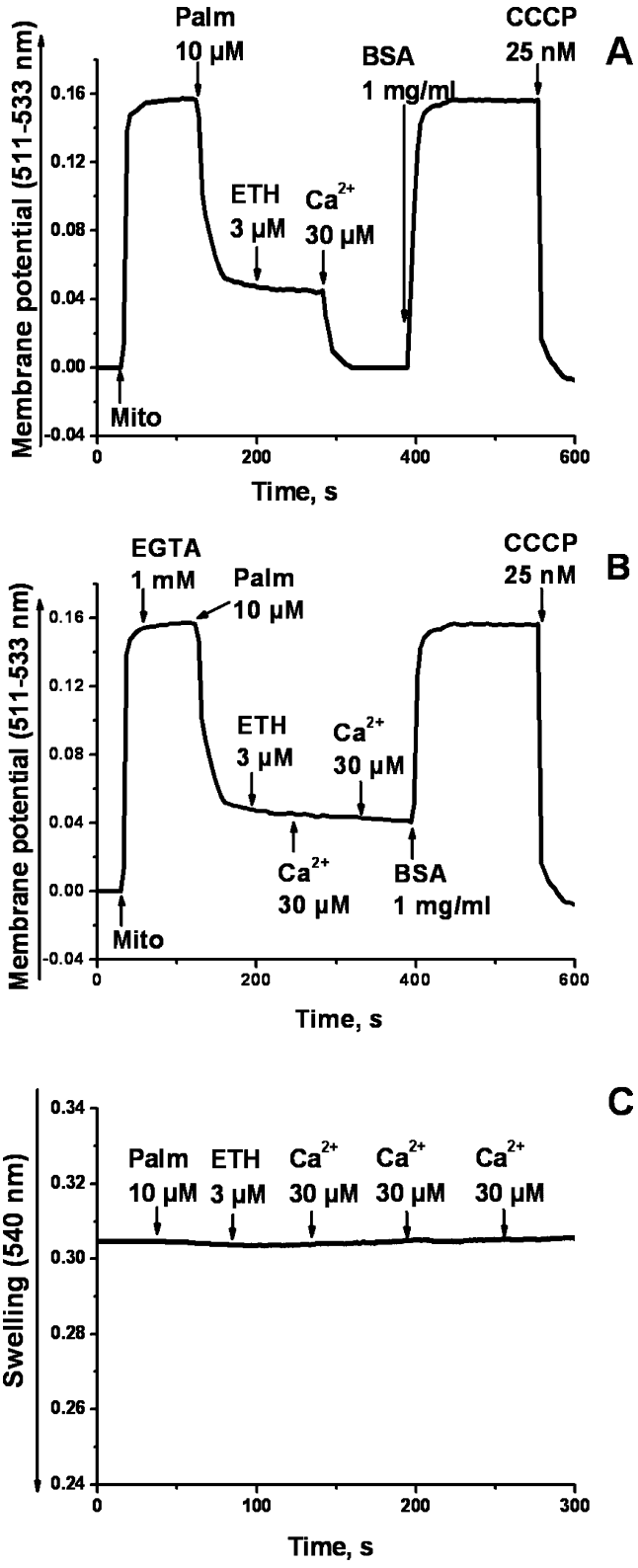

Fig. 7 Combined action of palmitate, ETH129 and Ca^{2+} on $\Delta\psi$ generation (A and B) and swelling (C) of *Y. lipolytica* mitochondria. Additions are indicated in the figure. **a** Palmitate induced depolarization of the inner membrane, subsequent addition of ETH129 and Ca^{2+} caused an additional decrease in $\Delta\Psi$. Membrane depolarization was totally recovered by BSA. **b** EGTA prevented the additional decline in $\Delta\Psi$ induced by ETH129 and Ca^{2+}. **c** *Y. lipolytica* mitochondria treated by palmitate, ETH129 and Ca^{2+} did not swell in the mannitol-based medium

schek et al. 1995). It is conceivable that yeast mitochondrial cyclophilins may fulfill functions not related to the mPTP, since yeast mitochondria are not sensitive to CsA.

As for mechanism 2), different regulation of mPTP opening, it has been shown that yeast (*S. cerevisiae*) mitochondrial permeabilization was rather insensitive to Ca^{2+} (Jung et al. 1997). This may be true of *Y. lipolytica* and *D. magnusii* as well. The calcium sensor of an mPTP may be absent or less sensitive. The matrix [Ca^{2+}] may also be kept below the threshold by relatively high concen-

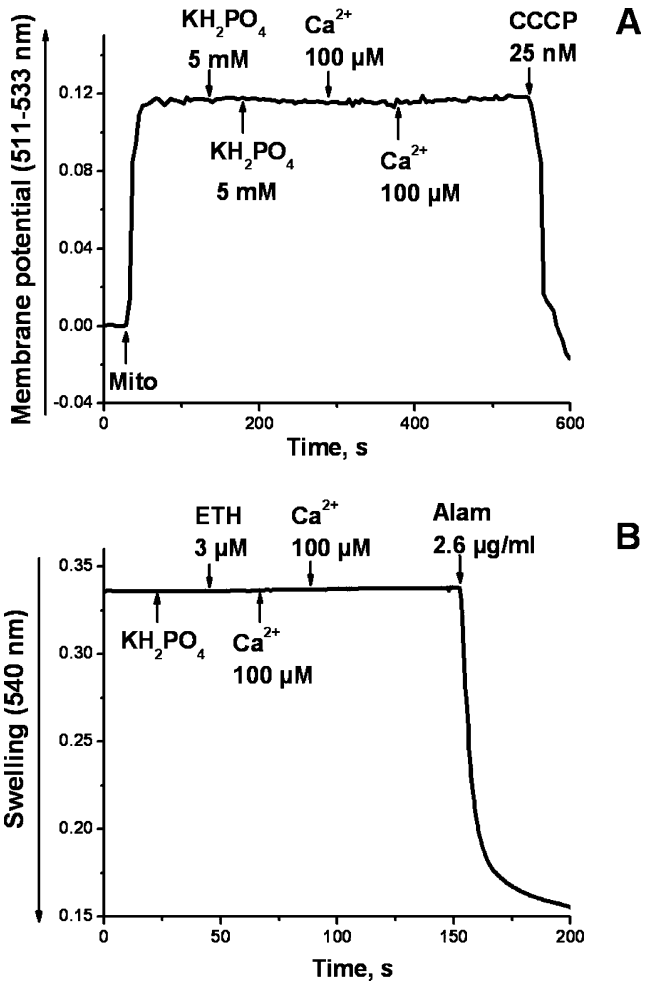

Fig. 8 *Y. lipolytica* mitochondria did not respond to KH_2PO_4 and Ca^{2+} even at acidic pH values. Additions are indicated in the figure. **a** The incubation medium was as in Fig. 1b but the pH was 6.5. **b** *Y. lipolytica* mitochondria did not swell in the mannitol-based medium. pH 6.5, supplemented with 10 mM KH_2PO_4 and Ca^{2+}. Additions included ETH129 and alamethicin where indicated

trations of Pi that precipitates the calcium. There might also be a higher sensitivity to inhibitory cations like Mg^{2+} and polyamines. These may also be responsible for mechanism 3) if their concentration is high in these yeast cells, which has not been measured. More likely, especially in oxidative stress, is potent antioxidant defense, including thioredoxin peroxidase, two catalases, two superoxide dismutases (localized in the cytosol and mitochondria, respectively), cytochrome c peroxidase (localized in the intermembrane space), glutathione, and metallothionein (see, Jamieson 1998). Indeed, *Y. lipolytica* and *D. magnusii* mitochondria proved to be much more resistant to oxidative stress than liver mitochondria.

To summarize, we have provided new insights into properties of yeast mitochondria (uncoupling effect of saturated fatty acids and activation of the Ca^{2+}/nH^{+} antiporter upon concerted action of Ca^{2+} and the Ca^{2+}-ionophore ETH129, for example), as well as into mechanisms underlying an mPTP-like pore formation in yeast mitochondria.

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