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In *Saccharomyces cerevisiae*, Cations Control the Fate of the Energy Derived From Oxidative Metabolism Through the Opening and Closing of the Yeast Mitochondrial Unselective Channel

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The yeast mitochondrial unspecific channel (YMUC) sensitivity to inorganic (Ca^{2+} or Mg^{2+}) or organic (hexyl or octyl-guanidine) cations was measured. The rate of oxygen consumption in State 3 and State 4, the transmembrane potential ($\Delta\psi$), mitochondrial swelling, and the polyethylene-glycol mediated recontraction were used to follow opening of the YMUC. Addition of 0.4 mM PO_4 did not close the YMUC, although it did enhance the sensitivity to Ca^{2+} (I_{50} decreased from 50 to 0.3 mM) and Mg^{2+} (I_{50} decreased from 5 to 0.83 mM Mg^{2+}). The Ca^{2+} concentration needed to close the YMUC was higher than the concentrations usually observed in the cell. Nonetheless, Mg^{2+}, Ca^{2+}, and PO_4 exhibited additive effects. These cations did not inhibit contraction of preswollen mitochondria, suggesting that the YMUC/cation interaction was labile. Octyl-guanidine (OG-I_{50} 7.5 μM) was the only cation which inhibited mitochondrial recontraction, probably as a result of membrane binding stabilization through its hydrophobic tail. The PO_4-dependent, Ca^{2+}/Mg^{2+}-mediated closure of the YMUC may be a means to control the proportion of oxidative energy producing ATP or being lost as heat.

KEY WORDS: Ca^{2+}; Mg^{2+}; octyl-guanidine; permeability transition; phosphate; energetic charge; yeast mitochondria; YMUC.

INTRODUCTION

The physiological significance of mitochondrial unspecific conductance is not clear. A large number of publications link unspecific pore opening to cell death in mammalian systems (Crompton, 1999; Lemasters *et al.*, 1998). However, other authors have proposed that unspecific channels may participate in activities such as ion detoxification or energy dissipation (Bernardi and Petronilli, 1996; Lohret and Kinnally, 1995; Zoratti and Szabo, 1995). In mammalian cells, Ca^{2+} is the candidate to be expelled from mitochondria upon opening of the permeability transition pore (PTP) and depolarization. It has been proposed that yeast would profit from this channel if it could switch mitochondrial metabolism from ATP to heat production and back (Beauvoit *et al.*, 1993; Dejean *et al.*, 2000). Yeast mass yield varies widely in a cAMP controlled fashion, probably involving mitochondria (Dejean *et al.*, 2002). The yeast mitochondrial unspecific channel (YMUC) is alternatively termed the yeast permeability transition pore (yPTP) (Jung *et al.*, 1997).

The YMUC and the PTP exhibit similar conductivity properties, but different or even opposite effector sensitivity (For reviews see, Jung *et al.*, 1997; Manon *et al.*, 1998; Zoratti and Szabo, 1995). Whether these channels are equivalent structures or serve the same functions is still

Key to abbreviations: $\Delta\psi$, transmembrane potential; HG, hexyl-guanidine; OG, octyl-guanidine; PEG, polyethylene glycol; PTP, permeability transition pore; RC, respiratory control; YMUC, yeast mitochondrial unspecific channel.

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undefined, however, the use of yeast as a model to study cell death is growing (Matsuyama *et al.*, 1999). Furthermore, the existence of a death program in yeast has been recently reported (Madeo *et al.*, 1999), opening the need to study the mechanism(s) controlling this program.

In its inner mitochondrial membrane, *S. cerevisiae,* possesses two high conductance channels exhibiting currents of 400 and 40 pS, respectively (Ballarin and Sorgato, 1995). At least one of these mildly anionic channels seems to constitute a source of unspecific permeation to ions and small solutes and it has been identified as the YMUC (Manon *et al.*, 1998). When open, the YMUC allows charge equilibration (Castrejón *et al.*,1997) and the passage of solutes as large as 1.1 kDa across the inner membrane (Jung *et al.*, 1997). In the presence of high [K^+] and in the absence of PO_4, addition of ATP, ethanol or NADH leads to mitochondrial swelling and transmembrane potential ($\Delta\psi$) depletion (Prieto *et al.*, 1992).

Different molecules control the opening of unspecific channels from different sources: the YMUC is open by ATP and respiratory substrates, while it is closed by PO_4 (Roucou *et al.*, 1997; Velours *et al.*, 1977), SO_4, AsO_4 (Cortés *et al.*, 2000), and decaVdO_4 (Manon *et al.*, 1998). In addition, it has been reported that neither Ca^{2+} nor cyclosporin-A have effects on the YMUC (Manon and Guérin, 1992; Szabo *et al.*, 1995). In contrast, the PTP is closed by ATP and cyclosporin-A, while Ca^{2+} plus PO_4 induce its opening (Halestrap and Davidson, 1990). Recently octyl-guanidine, an amphiphilic cation, has been reported to inhibit PTP opening with high affinity (Chávez *et al.*, 2000). The authors have proposed that the association between the PTP and octyl-guanidine is stabilized by insertion of the hydrophobic alkyl chain in the membrane core (Chávez *et al.*, 2000).

In regard to Ca^{2+} and yeast mitochondria, this cation does seem to regulate the activity of some enzymes such as the mitochondrial pyrophosphatase (Uribe *et al.*, 1993), while in contrast pyruvate dehydrogenase, isocitrate dehydrogenase, and oxoglutarate dehydrogenase, which are regulated by Ca^{2+} in mammalian mitochondria (Denton and McCormack, 1980), do not seem to be affected by Ca^{2+} in yeast (Nichols *et al.*, 1994). Recently, a fatty acid-activated Ca^{2+}/H^+ antiporter catalyzing the active efflux of Ca^{2+} was detected in mitochondria from *S. cerevisiae* (Bradshaw *et al.*, 2001). This efflux activity is higher than the uptake rates reported before (Uribe *et al.*, 1992). Thus, the role of Ca^{2+} in yeast mitochondria is still open to exploration. Furthermore, it has been reported that Ca^{2+} does not open the YMUC, although no efforts were made to determine whether closure was promoted (Jung *et al.*, 1997).

Another physiologically important divalent cation, Mg^{2+} reaches millimolar concentrations in both the cytoplasm and the mitochondrial matrix (Jung and Brierley, 1986; Masiacos *et al.*, 1991). In diverse biological systems, both Ca^{2+} and Mg^{2+} have been reported to have additive, synergistic or antagonistic effects (Pérez-Vázquez *et al.*, 2002; Uribe *et al.*, 1993; Zhu *et al.*, 2002). For a long time the concentration of Mg^{2+} was assumed to remain constant in the cell (Corkey *et al.*, 1986). However, recently it was demonstrated that mammalian cells expel Mg^{2+} in response to hormonal stimuli and thus, at least in some instances Mg^{2+} may regulate metabolism acting instead or in concert with Ca^{2+} (Romani *et al.*, 1993; Romani and Scarpa, 1992).

To determine whether the YMUC has a cation-sensitive site, the divalent cations Ca^{2+} and Mg^{2+} and the organic cations hexyl (HG) and octyl-guanidine (OG) were tested in isolated mitochondria. Oxygen consumption, $\Delta\psi$, and swelling were measured to determine the degree of membrane permeation. It was observed that depending on whether PO_4 was present in the medium, all four cations tested promoted coupling. In contrast, recontraction of swollen mitochondria was not inhibited by Ca^{2+}, Mg^{2+}, or HG, although it was sensitive to OG. It is suggested that, cation/membrane dissociation occurred after depletion of the $\Delta\psi$, unless there was an additional strong hydrophobic cation/membrane interaction, as seems to be the case for OG (Pressman, 1963). The possible physiological significance of the cation-mediated, PO_4-dependent regulation of the YMUC is discussed.

MATERIALS AND METHODS

Materials

All chemicals were reagent grade. Mannitol, MES, polyethylene glycol (PEG), succinate, safranine-O, $CaCl_2$, $MgCl_2 \cdot 6H_20$ and bovine serum albumin type V were from Sigma Chem Co. (St. Louis, MO). All other reagents were of the highest purity commercially available. A commercial strain of baker's yeast *Saccharomyces cerevisiae* (La Azteca, S.A.) was purchased from a local bakery and grown overnight in sterile rich medium.

Isolation of Yeast Mitochondria

Yeast (50 g) were suspended and incubated in a rich liquid medium (De-Kloet *et al.*, 1961) under aeration (3 L/min) for 8 h, washed, suspended in distilled water and starved overnight under aeration. The cells were washed by centrifugation three times and suspended in 0.6 M mannitol, 5 mM MES, 0.1% bovine serum albumin, pH 6.8 (triethanolamine). Cells were disrupted using

a Braun cell homogenizer and 0.45 mm diameter glass beads (Uribe *et al.*, 1985). From the homogenate, mitochondria were isolated by differential centrifugation in a SS34 rotor (Sorvall) (Peña *et al.*, 1977). Protein concentration was determined by the Biuret method (Gornal *et al.*, 1949)

Oxygen Consumption

The rate of oxygen consumption was measured in the resting state (State 4) and in the phosphorylating state (State 3) using an YSI model 5300 0xygraph equipped with a Clark-Type electrode at room temperature in a 3-mL chamber containing mitochondria at a final concentration of 0.5 mg prot/mL (Estabrook, 1967). The reaction mixture was 0.6 M mannitol, 5 mM Mes pH 6.8 (TEA) plus 1 μL/mL ethanol as a substrate. The concentrations of PO_4, Ca^{2+}, Mg^{2+}, and K^+ used are indicated under each figure. Stock solutions were 1.0 M $MgCl_2$, 2.0 M KCl, and either 1.0 or 0.1 M PO_4 buffer, pH 6.8 (Tris) as needed.

Transmembrane Potential ($\Delta\Psi$)

The $\Delta\Psi$ was determined using safranine-O, following the absorbance changes at 511–533 nm (Åkerman and Wikstrom, 1976) in a DW2000 Aminco spectrophotometer in dual mode.

Mitochondrial Swelling

The K^+ mediated mitochondrial swelling was determined at room temperature, following the change in absorbance at 540 nm in a DW 2000 Aminco spectrophotometer in split mode. The spectrophotometer was equipped with a magnetic stirrer (Halestrap and Davidson, 1990).

PEG-Induced Contraction of Swollen Mitochondria

To evaluate recontraction, mitochondria were subjected to K^+ mediated swelling for approximately 4 min. Once swelling stopped, as determined by stabilization of absorbance, recontraction was promoted by the addition of polyethylene glycols (PEG) of different molecular weights and followed spectrophotometrically (Jung *et al.*, 1997). The PEG concentration needed to reach 300 mOsm was determined considering their nonideal osmotic behavior (Pfeiffer *et al.*, 1995). For the 300 mOsm stock solutions, the following concentrations were used: for the 0.4 kDa PEG, 199 mM; for the 0.6 kDa PEG, 172 mM; for the 1.0 kDa PEG, 136 mM; and for the 1.45 kDa PEG, 111 mM (Pfeiffer *et al.*, 1995).

RESULTS

Opening of the YMUC results in uncoupling of mitochondria (Castrejón *et al.*, 1997; Manon *et al.*, 1998). Thus, the effects of increasing concentrations of Ca^{2+}, Mg^{2+}, HG, or OG on the rate of O_2 consumption in State 4 and in State 3, and on the respiratory control (RC) were tested (Fig. 1) in the presence of 0.4 mM PO_4. This PO_4 concentration does not close the YMUC, but does

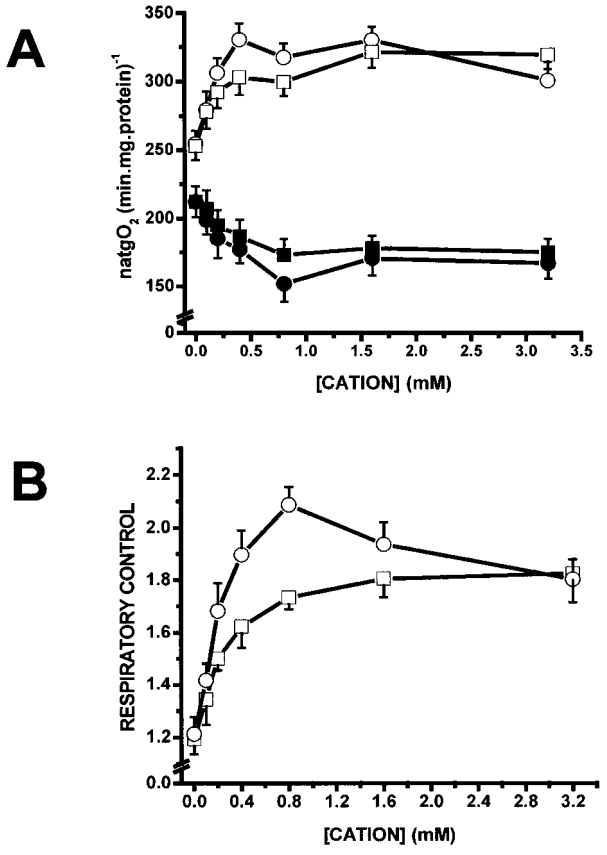

Fig. 1. Effect of Ca^{2+} or Mg^{2+} on the rate of O_2 consumption and on the respiratory control in isolated yeast mitochondria. Reaction mixture: 0.6 M mannitol, 5 mM MES, pH 6.8, 1 μL/mL ethanol, 20 mM KCl, 0.4 mM PO_4, pH 6.8 (Tris) and the indicated $CaCl_2$ and $MgCl_2$ concentrations. State 3 was induced adding 150 μM ADP. (A) Rates of oxygen consumption in State 3 (Open symbols) and State 4 (Closed symbols). ○ and ● Ca^{2+}; □ and ■ Mg^{2+}. (B) Respiratory control in the presence of ○ Ca^{2+} or □ Mg^{2+}. Each point represents the mean of five experiments ± SD.

participate in the phosphorylation reaction needed to establish State 3. In the absence of cations, the rates of O_2 consumption were as follows (Fig. 1(A)): in State 4 the rate was 212 natgO_2 (min·mg prot)$^{-1}$ and in State 3 it was 252 natgO_2 (min·mg prot)$^{-1}$ (Fig. 1(A)) yielding a RC of 1.2 (Fig. 1(B)), indicative of almost complete uncoupling. Addition of increasing Ca^{2+} or Mg^{2+} resulted in an increase in RC, which resulted from both an increase in the rate of O_2 consumption in State 3 and a decrease in State 4. For Ca^{2+}, the increase in State 3 was higher and occurred at slightly lower Ca^{2+} than Mg^{2+} (Fig. 1(A)): in the presence of 0.6 mM Ca^{2+}, the RC was 2.1 while at 1.6 mM Mg^{2+}, RC = 1.8 (Fig. 1(B)). At the highest [Ca^{2+}] or [Mg^{2+}] tested (3.2 mM), State 3 rate was 308 natgO_2 (min. mg prot)$^{-1}$ and State 4 was 170 natgO_2 (min. mg prot)$^{-1}$ (Fig. 1(A)) with an RC = 1.8 (Fig. 1(B)) for either cation.

Ca^{2+} and Mg^{2+} coexist in the cell modulating the effects of each other. In our system, both Ca^{2+} and Mg^{2+} increased RC, so it was decided to test whether these cations acted synergistically. To do so, the effect of increasing Ca^{2+} (0–0.6 mM) on the rates of O_2 consumption in State 3 and State 4 was measured in the presence of two fixed concentrations of Mg^{2+} (0.5 or 1 mM) (Table I). In the absence of Ca^{2+}, at 0.5 mM Mg^{2+}, RC = 1.65 and at 1 mM Mg^{2+}, RC = 1.78. Then, in the presence of increasing Ca^{2+} (0.1–0.6 mM), the rate of O_2 consumption in State 3 increased and State 4 decreased, with the consequent increase in RC: at 0.5 mM Mg^{2+} plus 0.5 μM Ca^{2+} or at 1 mM Mg^{2+} plus 0.3 μM Ca^{2+} the RC was 2.4. At higher Ca^{2+} the RC did not increase further (Table I). Thus, the effects of Ca^{2+} and Mg^{2+} were additive, resulting in higher RC than either cation when added alone (Fig. 1 and Table I).

In addition to Ca^{2+} and Mg^{2+}, the effects of alkyl-guanidines were analyzed (Table II). Ethyl- and butyl-guanidine did not affect mitochondrial coupling (results not shown). In contrast, hexyl-guanidine and octyl-guanidine promoted a concentration dependent increase in the RC. Their effects were similar to those of Ca^{2+} or Mg^{2+}, except the concentrations needed to couple mitochondria were lower: 25 μM hexyl-guanidine or 12.5 μM octyl-guanidine promoted the maximal increase in RC (Table II). As expected from the literature (Papa *et al.*, 1975; Pressman, 1963), at higher alkyl-guanidine concentrations inhibition of respiration was observed while no further increase in RC was observed (results not shown). Therefore, the highest alkyl-guanidine concentrations tested in all the following experiments were 25 μM hexyl-guanidine or 12.5 μM octyl-guanidine.

The coupling effects of Ca^{2+}, Mg^{2+}, and alkyl-guanidines were also evaluated by determining whether the transmembrane potential ($\Delta\psi$) was stabilized by each of these cations. In the absence of cations, only a transient $\Delta\psi$ was detected and mitochondria were depolarized within 1 min. When increasing Ca^{2+} (Fig. 2(A)) or Mg^{2+} (Fig. 2(B)) were added, a stable $\Delta\psi$ was generated, reaching the highest stability at 0.5 mM Ca^{2+} (Fig. 2(A), trace f) or higher (results not shown) and at 1.0 mM Mg^{2+} (Fig. 2(B), trace f) or higher (results not shown). Although the $\Delta\psi$ was stabilized by either cation, it did not reach the stability observed in the presence of 4 mM PO_4 (Fig. 2(A) or (B), trace g).

It was decided to test whether Ca^{2+} and Mg^{2+} exhibited additive effects on the $\Delta\psi$ (Fig. 3). Experiments were performed at 0.5 (Fig. 3(A)) or 1.0 mM Mg^{2+} (Fig. 3(B)) and increasing concentrations of Ca^{2+}. At both Mg^{2+} concentrations, Ca^{2+} addition increased the $\Delta\psi$, such that at 0.4 mM Ca^{2+} (Fig. 3(A) and (B), traces d) the $\Delta\psi$ was as stable as the control performed in the presence of 4 mM PO_4 (Fig. 3(A) and (B), traces f). These results indicated that when added together, Ca^{2+} and Mg^{2+} stabilized the

Table I. Rates of O_2 Consumption and Respiratory Control of Isolated Yeast Mitochondria in the Presence of Two Fixed Concentrations of Mg^{2+} and Increasing Concentrations of Ca^{2+}

	0.5 mM Mg^{2+}			1.0 mM Mg^{2+}		
Ca^{2+} (mM)	State 3 (natgO_2)	State 4 (min·mg protein)$^{-1}$	RC (E3/E4)	State 3 (natgO_2)	State 4 (min·mg protein)$^{-1}$	RC (E3/E4)
0	298 ± 10	179 ± 13	1.7 ± 0.1	294 ± 15	167 ± 13	1.8 ± 0.1
0.1	329 ± 12	165 ± 11	1.8 ± 0.1	293 ± 14	161 ± 11	2.0 ± 0
0.2	340 ± 13	152 ± 12	1.9 ± 0.2	293 ± 12	152 ± 12	2.2 ± 0.1
0.3	349 ± 14	148 ± 14	2.1 ± 0.1	310 ± 11	146 ± 14	2.4 ± 0.1
0.4	333 ± 14	144 ± 11	2.2 ± 0.1	322 ± 14	136 ± 11	2.4 ± 0.1
0.5	327 ± 17	140 ± 12	2.4 ± 0.1	328 ± 12	133 ± 12	2.5 ± 0.1
0.6	327 ± 11	132 ± 13	2.4 ± 0.2	326 ± 17	134 ± 14	2.4 ± 0.1

Note. Reaction mixture as in Fig. 1, except Ca^{2+} and Mg^{2+} concentrations, which are indicated. State 3 was induced by adding 150 μM ADP. Each number is the mean of five determinations ± SD.

Table II. Effect of Increasing Hexyl-guanidine or Octyl-Guanidine Concentrations on the Rate of O_2 Consumption and on the Respiratory Control of Isolated Yeast Mitochondria

HG (μM)	State 3 (natgO_2)	State 4 (min·mg protein)$^{-1}$	RC (E3/E4)	OG (μM)	State 3 (natgO_2)	State 4 (min·mg protein)$^{-1}$	RC (E3/E4)
0	258 ± 10	213 ± 9	1.2 ± 0.1	0	258 ± 8	213 ± 9	1.2 ± 0.1
5	273 ± 9	195 ± 12	1.4 ± 0.1	2.5	277 ± 10	198 ± 12	1.4 ± 0.1
10	290 ± 12	180 ± 10	1.6 ± 0.1	5	296 ± 11	185 ± 9	1.6 ± 0.1
15	306 ± 10	173 ± 11	1.8 ± 0.1	7.5	313 ± 12	175 ± 13	1.8 ± 0.2
20	317 ± 13	167 ± 10	1.9 ± 0.1	10	322 ± 11	168 ± 10	1.9 ± 0.1
25	321 ± 12	163 ± 13	2.0 ± 0.2	12.5	327 ± 10	165 ± 11	2.0 ± 0.1

Note. Reaction mixture as in Fig. 1, except for the indicated hexyl-guanidine and octyl-guanidine concentrations. State 3 was induced by adding 150 μM ADP. Each number is the mean of five determinations ± SD.

$\Delta\psi$ more efficiently than each cation by itself, supporting the idea that these two cations act synergistically to close the YMUC.

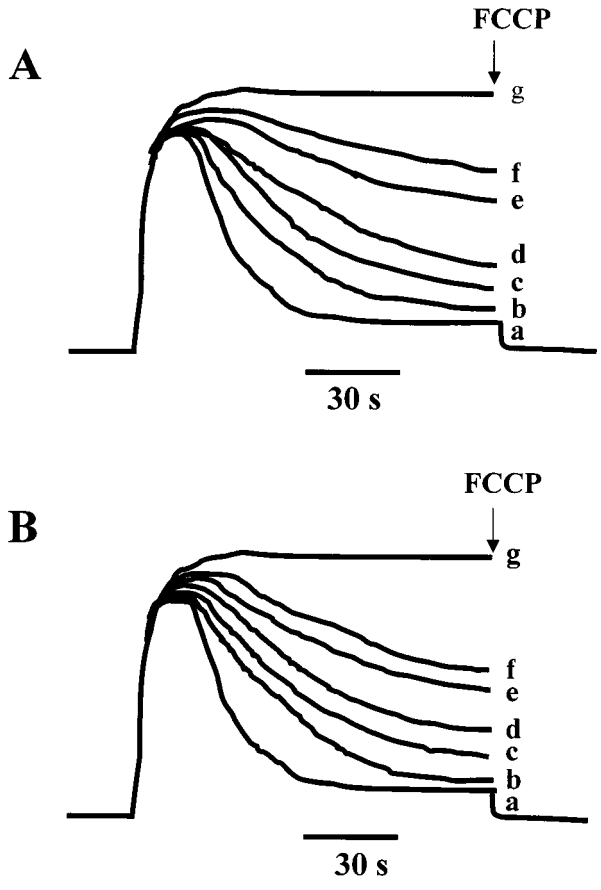

Fig. 2. Effect of Ca^{2+} or Mg^{2+} on the transmembrane potential ($\Delta\psi$) of isolated yeast mitochondria. Experimental conditions as in Fig. 1, except 10 μM safranine was added. (A) Ca^{2+} additions were a, control; b, 0.1 mM; c, 0.2 mM; d, 0.3 mM; e, 0.4 mM; f, 0.5 mM, and g, none, plus 4 mM PO_4. (B) Mg^{2+}additions were a, control; b, 0.2 mM; c, 0.4 mM; d, 0.6 mM; e, 0.8 mM, f, 1 mM, and g, none, plus 4 mM PO_4.

In regard to the alkyl-guanidines, it was observed that increasing concentrations of either HG (Fig. 4(A)) or OG (Fig. 4(B)) stabilized the $\Delta\psi$ and complete stabilization was observed at 25 μM HG (Fig. 4(A), trace e) or 12.5 μM OG (Fig. 4(B), trace e).

Initially, during the $\Delta\psi$ measurements, PO_4 was omitted. However, it was observed that the YMUC lost most of its sensitivity to each of the cations tested here, i.e., when PO_4 was absent from the reaction mixture, the concentrations of Ca^{2+}, Mg^{2+}, HG, or OG needed to observe closure of the YMUC were exceedingly high: a partially stable $\Delta\psi$ could be observed only at 50 mM Ca^{2+} or 5 mM Mg^{2+} (results not shown) and it was not possible to determine the concentration at which HG or OG closed the YMUC because the respiratory chain was inhibited at high alkyl-guanidine concentrations (results not shown) (Papa *et al.*, 1975; Pressman, 1963). Further experiments measuring O_2 consumption in State 4 and the uncoupled state confirmed that there is a large decrease in sensitivity of the YMUC to Ca^{2+}, Mg^{2+}, HG, or OG when in the absence of PO_4 (results not shown). These results indicated that the cation sensitivity of the YMUC is regulated by PO_4.

Under open YMUC conditions, the uptake of K^+ promotes energy-dependent mitochondrial swelling (Castrejón *et al.*, 1997). Thus, to test whether Ca^{2+}, Mg^{2+}, HG, or OG close the YMUC, it was decided to evaluate mitochondrial swelling in the presence of 0.4 mM PO_4. As expected from the O_2 consumption and $\Delta\psi$ experiments, increasing concentrations of each cation tested resulted in gradual inhibition of mitochondrial swelling, such that swelling was negligible at 0.5 mM Ca^{2+} (Fig. 5(A), trace f), 1.0 mM Mg^{2+} (Fig. 5(B), trace f), 25 μM hexyl-guanidine (Fig. 5(C), trace f) or 12.5 μM octyl-guanidine (Fig. 5(D), trace f). Higher concentrations of each cation did not promote higher inhibition of swelling (results not shown). When PO_4 was omitted from the reaction mixture the concentrations of each of these cations needed to inhibit swelling increased manifold, to the same extent as

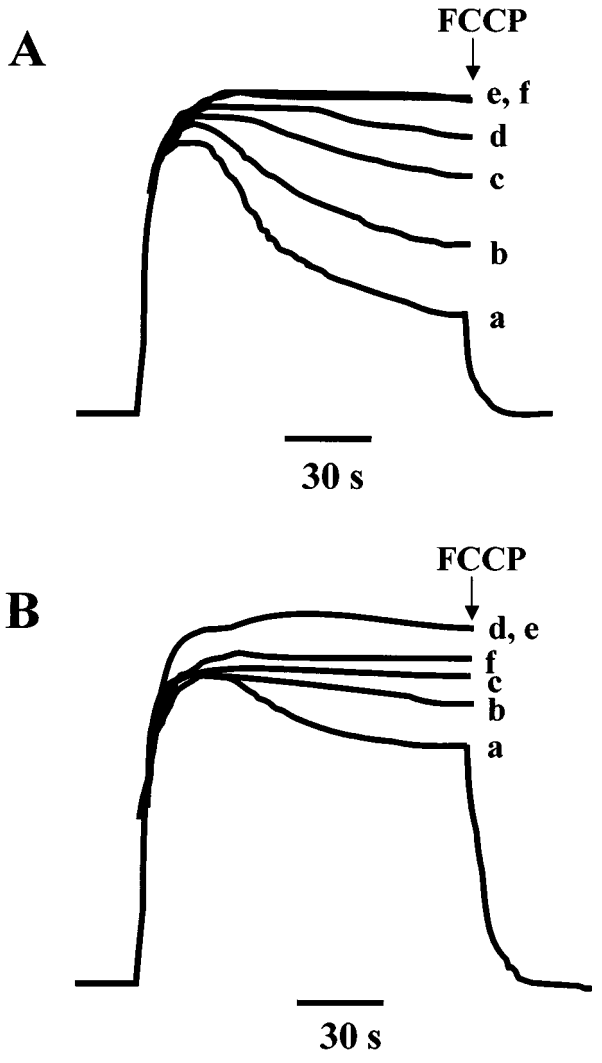

Fig. 3. Effect of increasing Ca^{2+} on the $\Delta\psi$ of isolated yeast mitochondria assayed in the presence of two fixed [Mg^{2+}]. Experimental conditions as in Fig. 2. (A) 0.5 mM Mg^{2+}, (B) 1.0 mM Mg^{2+} . Ca^{2+} additions were a, none; b, 0.1 mM; c, 0.2 mM; d, 0.3 mM; e, 0.4 mM, and f, none, plus 4 mM PO_4.

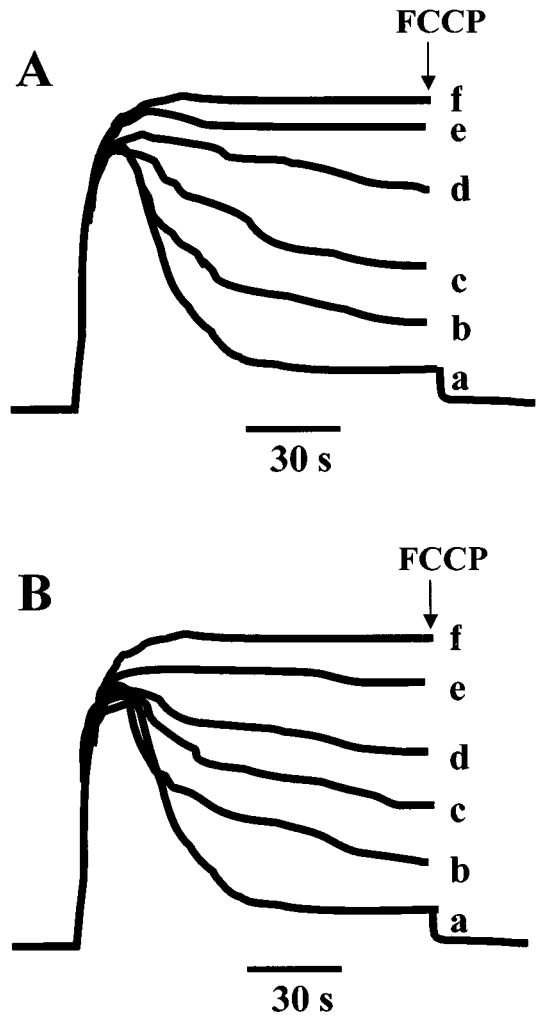

Fig. 4. Effect of hexyl-guanidine or octyl-guanidine on the $\Delta\psi$ of isolated yeast mitochondria. Experimental conditions as in Fig. 2. (A) Hexyl-guanidine concentrations were a, 0; b, 5 μM; c, 10 μM; d, 15 μM; e, 20 μM; f, 25 μM. (B) Octyl-guanidine additions were a, 0; b, 2.5 μM; c, 5 μM; d, 7.5 μM; e, 10 μM, and f, 12.5 μM.

in the O_2 consumption or in the $\Delta\psi$ experiments (results not shown).

It was decided to test whether Ca^{2+} and Mg^{2+} exhibited synergistic inhibitory effects on swelling. It was observed that in the presence of 0.5 or 1 mM Mg^{2+} and increasing concentrations of Ca^{2+}, swelling was inhibited, and that at 0.3 mM Ca^{2+} swelling was inhibited to a higher degree than when each cation was added alone, even at higher concentrations (results not shown).

To explore the mechanism of the cation/YMUC interaction, it was decided to test whether the binding of the positively charged Ca^{2+}, Mg^{2+}, or alkyl-guanidines to the YMUC was dependent on the $\Delta\psi$, which is negative inside and thus should attract cations. To do this, polyethylene-glycol (PEG)-mediated mitochondrial recontraction was evaluated in the presence of each effector. PEGs of different molecular weights have been used to estimate the size of the PTP (Pfeiffer *et al.*, 1995) or to induce recontraction of previously swollen mammalian mitochondria (Haworth and Hunter, 1979). Furthermore, in yeast mitochondria PEGs have been used to examine the size-exclusion properties of the YMUC (Jung *et al.*, 1997). Thus, to determine whether the YMUC was closed or not by Ca^{2+}, Mg^{2+}, HG, or OG, it was decided to examine the effects of four different PEGs on swollen, $\Delta\psi$-depleted mitochondria. The logic was that once the YMUC was closed, swollen, K^+ loaded mitochondria would recover

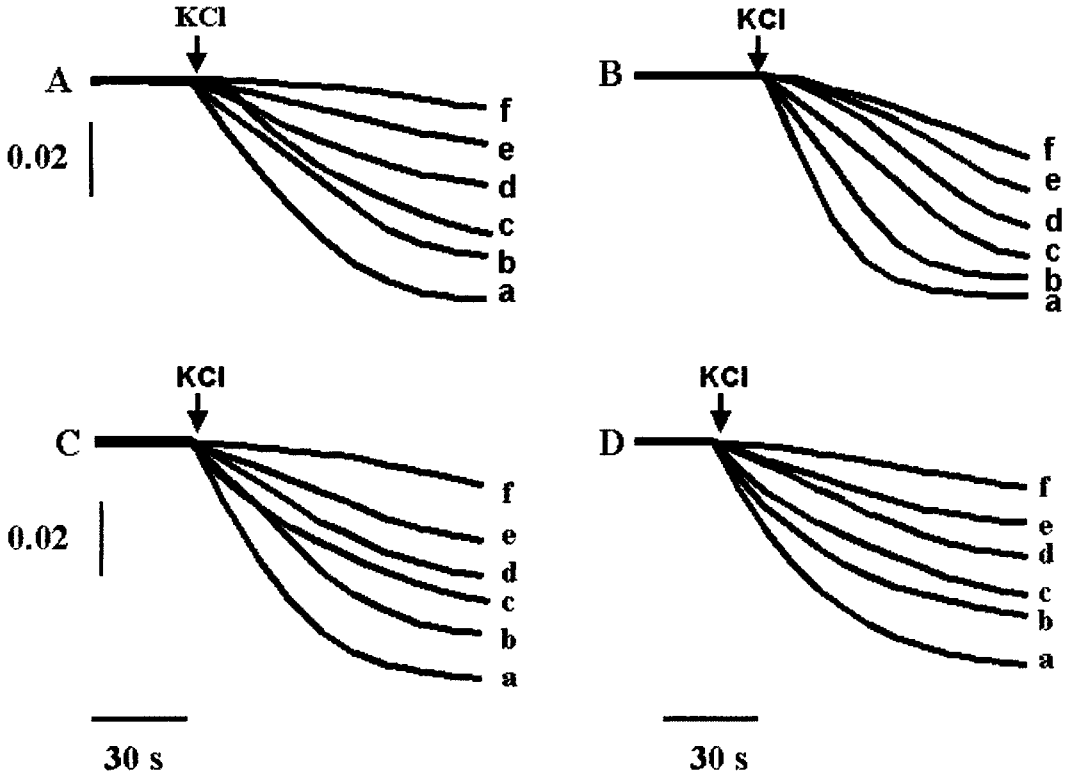

Fig. 5. Effect of Ca^{2+}, Mg^{2+}, hexyl-guanidine or octyl-guanidine on the swelling rate of isolated yeast mitochondria. Reaction mixture as in Fig. 1, except KCl (20 mM) was added at the arrow. (A) Ca^{2+} concentrations were a, 0; b, 0.1 mM; c, 0.2 mM; d, 0.3 mM; e, 0.4 mM; f, 0.5 mM. (B) Mg^{2+} concentrations were a, 0; b, 0.2 mM; c, 0.4 mM; d, 0.6 mM; e, 0.8 mM, and f, 1 mM. (C) Hexyl-guanidine concentrations were a, 0; b, 5 μM; c, 10 μM; d, 15 μM; e, 20 μM, and f, 25 μM. (D) Octyl-guanidine concentrations were a, 0; b, 2.5 μM; c, 5 μM; d, 7.5 μM; e, 10 μM, and f, 12.5 μM.

their impermeability and thus, even in the presence of a PEG-mediated increase in external osmolarity mitochondria would be unable to neither expel the K^+ from the matrix nor recontract.

Mitochondria were incubated for 2 or 3 min in the presence of 0.4 mM PO_4, 20 mM KCl and 1 μL ethanol/mL which resulted in $\Delta\psi$ depletion and swelling. Once mitochondria were swollen, each putative YMUC effector was added at the following concentrations: Control without additions (Fig. 6(A)), 4 mM PO_4 (Fig. 6(B)), 0.6 mM Ca^{2+} (Fig. 6(C)), 2 mM Mg^{2+} (results not shown), 25 μM HG (results not shown) or 15 μM OG (Fig. 6(D)). After further incubation (30 sec) recontraction of mitochondria was induced by adding PEGs of different molecular weights (Fig. 6). In the control traces it was observed that the smaller PEGs, of 0.4 (Fig. 6(A), trace b) or 0.6 kDa (Fig. 6(A), trace c), which have been reported to permeate through the open YMUC (Jung *et al.*, 1997), promoted only a mild recontraction of mitochondria. In contrast, addition of the larger PEGs, weighing 1.0 kDa (Fig. 6(A), trace d) or 1.45 kDa (Fig. 6(A), trace e) respectively, did promote recontraction. It has been suggested that recontraction occurs when addition of large PEGs which cannot cross the membrane, results in an increase in external osmolarity, forcing mitochondria to expel K^+. In contrast, when these same PEGs were tested after the addition of 4 mM PO_4 (Fig. 6(B)), which is known to close the YMUC, none of the PEGs were able to promote recontraction, indicating that the PO_4 promoted closure of the YMUC was tight enough to inhibit K^+ efflux (Fig. 6(B)). When each cation was tested to determine whether it inhibited recontraction, it was observed that Ca^{2+} (Fig. 6(C)), Mg^{2+} (results not shown) or HG (results not shown) exhibited only a mild inhibition of the mitochondrial recontraction mediated by each PEG. In contrast, OG was as efficient as PO_4 to inhibit PEG mediated recontraction, regardless of the MW of the PEG, suggesting that this cation was able to close the YMUC in uncoupled mitochondria (Fig. 6(D)). Thus, it is suggested that the cation/YMUC interaction was favored by the $\Delta\psi$ and in reciprocity, the $\Delta\psi$ was maintained by the same cation/YMUC interaction. In the case of OG, the hydrophobic interaction

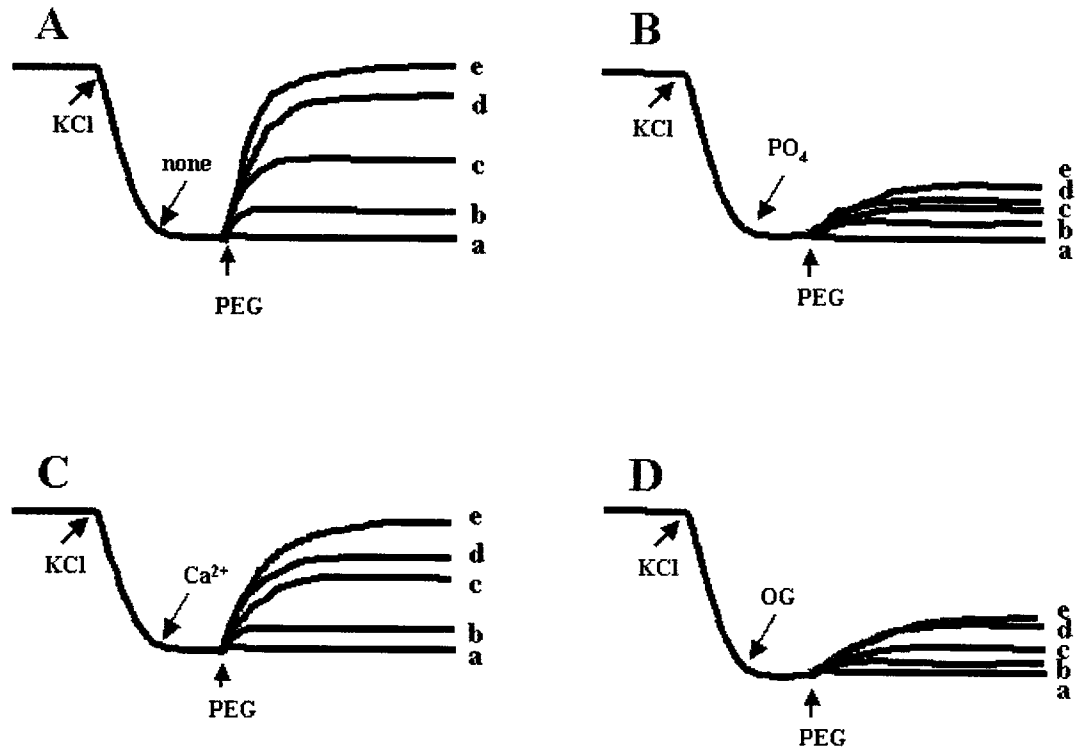

Fig. 6. Polyethylen-glycol-mediated recontraction of swollen yeast mitochondria. Effect of PO_4, Ca^{2+} or octyl-guanidine. Reaction mixture: 0.3 M mannitol, 5 mM MES, pH 6.8, 1 μL/mL ethanol, 0.4 mM PO_4, pH 6.8. KCl (20 mM) was added at the arrow. After allowing for swelling to occur, an effector was added as follows (A) None, (B) 4 mM PO4, (C) 0.6 mM Ca^{2+}, (D) 15 μM OG. After 30 s of further incubation, PEGs of different molecular weights were added to increase osmolarity by 0.3 OsM as follows: a, 0; b, 0.4 kDa and b, 0.6 kDa; c, 1 kDa, and d, 1.45 kDa. Specific PEG concentrations are listed in methods.

between the core of the membrane and the hydrophobic eight carbon tail in OG probably resulted in insertion of the molecule at its action site, even in the absence of a $\Delta\psi$.

DISCUSSION

From a physiological point of view, the regulation of the YMUC by Mg^{2+}, Ca^{2+}, and PO_4 is probably important, as wide concentration variations in all three effectors occur in concert with the energetic requirements of the cell. If this regulation occurs in vivo, the YMUC would then function as an energy dissipating valve, supporting the idea that unspecific mitochondrial permeability should serve other functions besides programmed, or accidental, cell death (Ichaz, 1997; Szabó *et al.*, 1992). The Mg^{2+} and PO_4 concentrations used were in the range observed in the cell, while the Ca^{2+} concentration needed to close the YMUC was higher (Anraku *et al.*, 1991). Nonetheless, the additive effects of all three effectors may indicate that even Ca^{2+} may be participating in vivo in the regulation of the YMUC.

In the cell, the increase in PO_4 concentration indicates a decrease in energy charge. Thus, the PO_4 mediated closure of the YMUC seems a logical adaptation as it results in an increase in the rate of ATP synthesis by mitochondria when it is needed. Once, ATP is accumulated, opening of the YMUC is promoted constituting a typical negative feed-back mechanism. In this regard, ATP would open the YMUC perpetuating the acceleration in oxygen consumption as a result of the decrease in the transmembrane potential. The opening effects of ATP on the YMUC probably occur through various mechanisms: first, through direct ATP/YMUC interaction (Prieto *et al.*, 1992; Roucou *et al.*, 1997); second, sequestering Mg^{2+} to become the ATP salt; and third, depleting the PO_4 which is used for ATP synthesis. In short, a high energy charge would result in opening of the YMUC and dissipation of energy as heat, while a low energy charge would promote YMUC closure and increased rate of ATP synthesis.

In yeast, Ca^{2+} regulates the cell cycle. Ca^{2+} is required during mitosis, a period of high energetic demand that occurs together with a rise in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) for periods lasting up to 20 min (Anraku *et al.*, 1991). Besides activating a large number of calmodulin dependent and independent pathways, the increase in $[Ca^{2+}]_{cyt}$ probably stimulates the synthesis of ATP, even before it is needed. The mechanism for increased energy conversion into ATP and then bio-mass would possibly include the closure of the YMUC observed here (Figs. 2–5). In this regard, during another high energy requiring process, namely sexual interaction, $[Ca^{2+}]_{cyt}$ rises as soon as a pheromone is detected and the cell prepares to merge into a diploid (Nakajima-Shimada *et al.*, 1991). Thus, it is logical that Ca^{2+} should work in combination with PO_4 to close the YMUC, shifting the utilization of the energy derived from oxidative metabolism toward production of ATP and other molecules. In contrast, the depletion of PO_4 , the accumulation of unused ATP and the decrease in $[Ca^{2+}]_{cyt}$ observed when the cell enters a period of rest, leads to opening of the YMUC which in turn results in dissipation of energy as heat.

In most cells, $[Mg^{2+}]$ fluctuates between 0.5 and 1.0 mM and the variation within these limits seems to regulate the activity of more than 350 enzymes (Romani and Scarpa, 2000). Considering the concentrations of Mg^{2+} which close unspecific channels, it is likely that both, the PTP (Bernardi *et al.*, 1993) and the YMUC (this paper) could be physiologically regulated by $[Mg^{2+}]$. In addition, the $[Ca^{2+}]_{cyt}$ required to inhibit the opening of the mammalian PTP by 50% (IC_{50}) has been calculated at 0.2 mM Ca^{2+} (Bernardi *et al.*, 1993) while in the YMUC the IC_{50} was estimated at 0.3 mM (this work). For Mg^{2+}, the PTP IC_{50} was 0.3 mM Mg^{2+} (Bernardi *et al.*, 1993), while for the YMUC the IC_{50} was 0.8 mM Mg^{2+} (this work). Thus, in comparison with the PTP, the YMUC seems to be less sensitive to divalent cations. There is at least another example where higher cation concentrations are needed in yeast mitochondria than in mammalian cells: the K^+/H^+ antiporter from yeast is inhibited by higher $[Ca^{2+}]$ (IC_{50} 0.82 mM) or $[Mg^{2+}]$ ($IC_{50} = 2.08$ mM) (Welihinda *et al.*, 1993) than its mammalian counterpart, which is inhibited by Ca^{2+} at $IC_{50} = 12$–18 μM or by Mg^{2+} at $IC_{50} = 50$–65 μM (Garlid, 1988).

The Ca^{2+} and/or Mg^{2+} mediated closing of the YMUC could be due to the generation of ADP and/or ATP salts as these nucleotides associated to the added divalent cation. This possibility was suggested during the oxygen consumption experiments, specially considering that cation concentrations were above the reported Kd for the $Ca{\cdot}ATP^{2-}$, $Mg{\cdot}ATP^{2-}$, and $Mg{\cdot}ADP^{-}$ salts which are 2.5×10^{-4} M, 1×10^{-4} M, and 1×10^{-3} M, respectively (Shikama, 1971). However, we believe that the cation mediated closing effects on the YMUC were due to a direct interaction with this channel because the experiments measuring $\Delta\Psi$ (Figs. 2 and 3) and swelling (Fig. 5) were performed in the absence of nucleotides and closure of the YMUC was still observed.

In regard to putative cation interaction site(s) in mitochondrial unspecific channels, the PTP open/closed probability is modulated by two divalent cation sites: (i) an internal site, where Ca^{2+} binding increases open pore probability, while the binding of other divalent cations (Sr^{2+}, Mn^{2+}) has the opposite effect (Bernardi *et al.*, 1993); and (ii) an external site where all divalent cations including Ca^{2+}, increase the closed probability (Bernardi *et al.*, 1993). So extramitochondrial Ca^{2+} would be an inhibitor of the PTP while Ca^{2+} in the matrix would trigger the opening of the pore. In the light of our results, in yeast mitochondria only the equivalent of the external site for Ca^{2+} would exist and thus in yeast, Ca^{2+} (or Mg^{2+}) would be capable of closing the pore but would be unable to promote opening.

The cation interaction site in the YMUC would be highly sensitive to PO_4 concentrations as evidenced by the fact that closure of the YMUC occurred only at exceedingly high Ca^{2+} or Mg^{2+} concentrations, unless PO_4 was added. The Ca^{2+} needed to close the YMUC decreased by at least two orders of magnitude when in the presence of a submillimolar concentration of PO_4. In addition, Ca^{2+} and Mg^{2+} exhibited synergistic effects, exhibiting a stronger coupling effect than when each cation was added alone. This additive effect was observed in all parameters tested, i.e., oxygen consumption (Table I), $\Delta\psi$ (Fig. 3), and mitochondrial swelling (results not shown).

Among the factors involved in the regulation of the cation/YMUC interactions, the $\Delta\psi$ was observed to play an important regulatory role. Once the $\Delta\psi$ was depleted and mitochondria were swollen, neither Ca^{2+} nor Mg^{2+} were able to close the YMUC and inhibit recontraction. Thus, to explore cation/YMUC interactions, we decided to test the alkyl-guanidines, which have been reported to interact with negative charges in the membrane (Gómez-Puyou and Tuena, 1977) and among which OG has recently been reported to inhibit the mammalian PTP (Chávez *et al.*, 2000). Neither ethyl- nor butyl-guanidine had any effects on the YMUC (results not shown) and thus, they were not further studied. In contrast, low concentrations of HG and OG did mimic the Ca^{2+} and Mg^{2+} effects, coupling mitochondria and maintaining a high $\Delta\psi$. Furthermore, in $\Delta\psi$ depleted, swollen mitochondria, OG was the only cation tested which was capable of inhibiting mitochondrial recontraction. The apparent stability of the OG/YMUC interaction was probably due to the anchoring

of the octyl chain in OG with the hydrophobic core of the membrane (Chávez *et al.*, 2000; Pressman, 1963). This mechanism has been previously proposed to explain the OG mediated inhibition of electron transport through the respiratory chain at the level of complex I (Papa *et al.*, 1975) and the OG mediated inhibition of mitochondrial K^+ influx in mammalians (Beaty *et al.*, 1986). Probably the interaction of the membrane with the hexyl group in HG was not sufficiently strong to anchor the molecule in the absence of a $\Delta\psi$ and this is why HG did not affect recontraction and a higher HG concentration was needed to couple mitochondria. In this regard, PTP inhibition by octyl-amine, but not by hexyl-butyl or ethyl-amine has been reported (Chávez *et al.*, 2000)

The physiological role of mitochondrial permeability transitions is not clear. Besides programmed cell death, other functions such as ion detoxification or energy dissipation have been proposed as alternatives (Bernardi, 1996; Gunter and Pfeiffer, 1990; Zoratti and Szabo, 1995). Yeast contain a mitochondrial nonspecific pore, although these cells do not contain the typical caspase cascade proteins that participate in apoptosis (Goffeau *et al.*, 1996) In addition, the control of the YMUC by Ca^{2+}, Mg^{2+}, and PO_4 seems ideally engineered to increase the efficiency of oxidative phosphorylation at times when ATP is in high demand, and switch into an energy-scape valve during the resting state.

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