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Evidence for an ATP-sensitive K^+ channel in mitoplasts isolated from *Trypanosoma cruzi* and *Crithidia fasciculata*

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ABSTRACT

Mammalian mitochondria, as well as rat, plant and *Caenorhabditis elegans* mitochondria, possess an ATP-sensitive K^+ channel (mitoK$_{ATP}$) that has been pharmacologically characterised. Opening of mitoK$_{ATP}$ and the subsequent K^+ entry into the matrix was shown to have three effects on mitochondria physiology: (i) an increase in matrix volume (swelling), (ii) an acceleration of respiration, and (iii) an increase in reactive oxygen species (ROS) production. These effects on mitochondria bioenergetics have been shown to be part of distinct intracellular signalling pathways, to protect against cell death and to modulate gene transcription. To date, such a channel or its activity has not been described in trypanosomatids. In the present study, we show pharmacological evidence for the presence of a mitoK$_{ATP}$ in trypanosomatids. Cells were incubated in a hypotonic medium followed by mild detergent exposure to isolate mitoplasts from *Trypanosoma cruzi* and *Crithidia fasciculata*. Mitoplasts swelled when incubated in KCl medium due to respiration-driven K^+ entry into the matrix. Swelling was sensitive to the presence of ATP when the mitoplast suspension was incubated in K^+-containing, but not in K^+-free, medium. The ATP inhibition of swelling was reversed by the mitoK$_{ATP}$ agonist diazoxide and the diazoxide-induced swelling was inhibited by the mitoK$_{ATP}$ blockers 5-hydroxydecanoate (5HD) or glibenclamide. Similar to mammalian and rat mitochondria, trypanosomatid mitoK$_{ATP}$ activity was modulated by the general protein kinase C (PKC) agonist phorbol 12-myristate 13-acetate (PMA) and antagonist chelerythrine. As expected, the potassium ionophore valinomycin could also reverse the ATP-inhibited state but this reversal was not sensitive to 5HD or glibenclamide. Dose response curves for ATP, diazoxide and 5HD are presented. These results provide strong evidence for the presence of an ATP-sensitive K^+ in trypanosomatid mitochondria.

1. Introduction

The parasite *T. cruzi* is the etiological agent of Chagas disease, an illness that affects approximately 13 million people in the Americas (WHO, 2005). Trypanosome-infected patients exhibit inflammatory symptoms during the acute phase of the disease and develop heart hypertrophy in its chronic stages, often leading to heart failure and death among many other symptoms (Tanowitz et al., 1992; Rassi et al., 2000; Garg, 2005).

Trypanosoma cruzi epimastigotes possess a unique and long mitochondrion localised in the anterior portion of the cell. Its mitochondrion exhibits a high rate of basal respiration, being able to generate an electrochemical gradient across the inner membrane comparable to mammalian mitochondria (Docampo and Vercesi, 1989b; Vercesi et al., 1991). This gradient creates a membrane potential that allows efficient phosphorylation of ADP, producing enough ATP to support the energy requirements of this highly motile parasite (Vercesi et al., 1991). The parasite's mitochondrial membrane potential is also important for the metabolic homeostasis of Ca^{2+} (Docampo and Vercesi, 1989a; Vercesi et al., 1990; Moreno and Docampo, 2003).

ATP-sensitive K^+ channels were first described in rat liver mitochondria (Inoue et al., 1991; Paucek et al., 1992) and were termed mitoK$_{ATP}$. More recently, mitoK$_{ATP}$ activity has been shown to be present in mitochondria from some plants (Pastore et al., 1999) and the worm *C. elegans* (Wojtovich et al., 2008). Opening of mitoK$_{ATP}$ and the subsequent K^+ entry into the matrix was shown to have three effects on mitochondrial physiology: (i) an increase in matrix volume (swelling), (ii) a mild acceleration of respiration (mild uncoupling), and (iii) a slight but significant increase in reactive oxygen species (ROS) production (Garlid and Paucek, 2003; Andrukhiv et al., 2006; Costa et al., 2006b, 2008). MitoK$_{ATP}$ activity has been implicated in several cellular pathophysiological processes, such as maintenance of mitochondrial matrix volume for efficient energy transfer to the cytosol (Kowaltowski et al., 2001; Dos Santos et al., 2002; Costa et al., 2006b), protection against myocardial necrosis induced by ischaemia-reperfusion injury (Costa et al., 2006a, 2008), protection against apoptosis (Garlid et al., 2003; O'Rourke, 2004), and production

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of ROS for gene transcription and other signalling functions (Liu et al., 2000; Garlid and Paucek, 2003; Andrukhiv et al., 2006; Costa et al., 2008).

In this study, we present evidence for the presence of mitoK$_{ATP}$ in *T. cruzi* mitochondria. Our results show that mitoplasts (mitochondria devoid of the outer membrane) isolated from *T. cruzi* swell in KCl medium. We also studied another trypanosomatid, *C. fasciculata*, and observed similar results. Mitoplast swelling in KCl medium was inhibited by ATP and by carbonyl cyanide m-chloro phenyl hydrazone (CCCP), the latter a protonophore that disrupts the electrochemical gradient, thereby abolishing the driving force for K^+ uptake. ATP inhibition was reversed by the mitoK$_{ATP}$ agonist diazoxide, and diazoxide-induced swelling was inhibited by the presence of the mitoK$_{ATP}$ blockers 5-hydroxydecanoate (5HD) or glibenclamide. Valinomycin, a potassium ionophore, also reversed the ATP inhibition but this effect was not sensitive to 5HD or glibenclamide. Importantly, the effects of ATP, diazoxide, 5HD, glibenclamide or valinomycin upon mitoplasts swelling were observed in K^+-containing, but not in a K^+-free, medium (where tetraethylammonium (TEA^+) was substituted for K^+). Similar to mammalian mitochondria, mitoK$_{ATP}$ activity in mitoplasts was also modulated by the general protein kinase C (PKC) agonist phorbol 12-myristate 13-acetate (PMA) and antagonist chelerythrine. Comparable results have been demonstrated for rat liver, heart and brain mitochondria (Jaburek et al., 1998; Kowaltowski et al., 2001; Costa et al., 2006b, 2008).

2. Materials and methods

2.1. Isolation of mitoplasts

Trypanosoma cruzi (strain Dm28c) epimastigotes or *C. fasciculata* were grown for 5 or 3 days (early stationary phase), respectively, in liver infusion broth with tryptose (LIT) medium supplemented with 100 U/ml penicillin and 10% FBS (Contreras et al., 1985, 1988). Approximately 10^{10} cells were harvested by centrifugation at 3,000*g* for 5 min and washed in cold PBS. The pellets were resuspended in hypotonic medium (30 mM NaCl, 10 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$) and incubated for 2 min to allow complete swelling. Next, 0.2% nonidet P-40 (NP-40) diluted in hypotonic medium was added to cell suspension, immediately followed by addition of 260 mM sucrose diluted in hypotonic medium. The suspension was vigorously mixed after each addition. The suspension was then centrifuged at 1,000*g* for 3 min; the supernatant was collected and centrifuged at 12,000*g* for 10 min. The pellet was resuspended in buffered sucrose medium (250 mM sucrose, 10 mM Tris–HCl pH 7.5, 1 mM EDTA), centrifuged at 12,000*g* for 2 min and finally resuspended in isotonic buffered sucrose medium at approximately 10 mg/ml. In some experiments, the final pellet was resuspended in buffered sucrose medium supplemented with 0.5 mg/ml BSA, centrifuged again at 12,000*g* for 2 min, and then BSA was removed by centrifugation in isotonic sucrose medium.

2.2. Measurement of matrix volume

Mitoplasts lose K^+ during the isolation procedure, thereby reducing their volume due to the concomitant loss of water. In the presence of respiratory substrates, K^+ re-enters the matrix driven by the electrochemical gradient generated by the respiratory chain. K^+ entry in the mitoplasts is accompanied by osmotically obligated water, which then increases matrix volume and changes the light scattered by the suspension (Beavis et al., 1985; Garlid and Beavis, 1985; Dos Santos et al., 2002; Garlid and Paucek, 2003). Therefore, changes in the light scattered by mitoplasts suspensions reflect changes in their volume. We followed the light scattering of the mitoplast suspensions at 520 nm using a Hitachi U-2000 spectrophotometer in a temperature controlled room (23 ± 1 °C). The K^+ reaction medium contained (in mM): 120 KCl, 10 Tris–HCl pH 7.2 adjusted with KOH, 1 Na_2HPO_4, 0.1 EDTA, 5 succinate, supplemented with 5 μg/ml oligomycin. Oligomycin is required to inhibit ADP phosphorylation by the ATP synthase, which would result in matrix contraction and would mask swelling induced by mitoK$_{ATP}$. Control experiments were performed in K^+-free medium, where all K^+ salts were substituted for tetraethylammonium (TEA^+) or Li^+. The results shown as "volume change (%)" were calculated by taking mitoplasts volume in KCl medium and applying to the equation $V = 100 \times [V_{(x)} - V_{(ATP)}/V_{(no\ ATP)} - V_{(ATP)}]$, where $V_{(x)}$ is the observed steady state volume at 90 s under a given experimental condition, $V_{(ATP)}$ and $V_{(no\ ATP)}$ are mitoplast volume in the presence and absence of ATP, respectively, taken as 0% and 100%. Results were considered statistically significant if $P < 0.05$ using an unpaired Student's *t*-test. Experiments were performed using three to 10 independent mitoplast preparations for each organism.

2.3. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Reduction of MTT is known to be performed by reductases present in the mitochondrial matrix (Slater et al., 1963) and is widely used as indication of mitochondria integrity and thus cell viability. The pellets from the 1,000*g* centrifugation, containing broken cells and nuclei, and from the 12,000*g* centrifugation, containing mitoplasts, were analysed for the presence of mitochondrial reductases using the MTT assay. Briefly, samples were incubated in the same buffer used in the swelling assay in the presence of 0.25 mg/ml MTT for 60 min at 28 °C. The reaction was stopped by the addition of 100 ul DMSO and the mixture was read in a spectrophotometer at 570 nm and 630 nm. The formation of the blue dye formazan was calculated by the difference of absorbance at 570 nm and 630 nm, according to Liu et al. (1997). CCCP (5 μM) was added to disrupt mitochondrial membrane potential and was used as a negative control.

2.4. Chemicals

Diazoxide, glibenclamide, 5-hydroxydecanoate, Na–ATP, PMA, chelerythrine, CCCP and oligomycin were from Sigma–Aldrich (St. Louis, MO, USA). PKCε agonist and antagonist peptides were synthesised according to published amino acid sequences (Johnson et al., 1996; Dorn et al., 1999). FBS was from Gibco BRL (USA). MTT and penicillin were from USB (Cleveland, OH, USA). NP40 was from Amresco (Solon, OH, USA). All other reagents were of analytical grade.

3. Results

3.1. Enrichment of mitochondrial function

Results in Table 1 show that mitoplasts (pellet from the 12,000*g* centrifugation) exhibited four times more reductase activity per mg of protein than the sample containing broken cells and nuclei (pellet from the 1,000*g* centrifugation). Additionally, disruption of the membrane potential by CCCP resulted in inhibition of MTT reduction to the same levels as the sample containing broken cells and nuclei, which was not affected by CCCP (Table 1). These results are in perfect agreement with the data published by Liu and co-workers (Liu et al., 1997). Although MTT reduction can be performed by other cellular compartments, none of these compartments is able to swell. Therefore, the MTT assay demonstrates an enrichment of mitochondrial function in our sample.