Published in final edited form as:

Int J Parasitol. 2009 July 15; 39(9): 955–961. doi:10.1016/j.ijpara.2009.01.002.

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 characterized. 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 signaling 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.

Keywords

Mitochondrial ATP-sensitive K⁺ channel; Mitoplasts; Trypanosome; *Crithidia*

1. Introduction

The parasite *Trypanosoma 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

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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 localized 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 $\text{mitoK}_{\text{ATP}}$. More recently, $\text{mitoK}_{\text{ATP}}$ activity has been shown to be present in mitochondria from some plants (Pastore et al., 1999) and the worm *Caenorhabditis elegans* (Wojtovich et al., 2008). Opening of $\text{mitoK}_{\text{ATP}}$ 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). $\text{mitoK}_{\text{ATP}}$ 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 ischemia-reperfusion injury (Costa et al., 2006a, 2008), protection against apoptosis (Garlid et al., 2003; O'Rourke, 2004), and production of ROS for gene transcription and other signaling 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 $\text{mitoK}_{\text{ATP}}$ 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, *Crithidia 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 $\text{mitoK}_{\text{ATP}}$ agonist diazoxide, and diazoxide-induced swelling was inhibited by the presence of the $\text{mitoK}_{\text{ATP}}$ 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, $\text{mitoK}_{\text{ATP}}$ 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 *Crithidia 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 MgCl₂) 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 ($23 \pm 1^\circ\text{C}$). The K⁺ reaction medium contained (in mM): 120 KCl, 10 Tris-HCl pH 7.2 adjusted with KOH, 1 Na₂HPO₄, 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 analyzed 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 µl 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). PKC ϵ agonist and antagonist peptides were synthesized 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 coworkers (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.

3.2. Evidence for the presence of mitoK_{ATP} in mitoplasts isolated from *T. cruzi*

Fig. 1 shows that mitoplasts isolated from *T. cruzi* epimastigotes swelled when incubated in isotonic K⁺ medium supplemented with the respiratory substrate succinate (K⁺, line a). Swelling was also observed in the absence of succinate, presumably due to the presence of endogenous substrates. However, addition of succinate significantly increased the reproducibility of the results; thus it was present in all experiments performed in this study. Swelling was inhibited in the presence of ATP (K⁺, line b), and the ATP-inhibited state was reversed by the addition of the K_{ATP} channel agonist diazoxide, resulting in swelling comparable to that observed in the absence of ATP (K⁺, line c). Diazoxide-induced swelling was blocked in the presence of the mitoK_{ATP}-specific antagonist 5HD (K⁺, line d) or by the unspecific K_{ATP} antagonist glibenclamide (not shown). Addition of the potassium ionophore valinomycin to ATP-inhibited mitoplasts resulted in swelling similar to that observed with no ATP or in the presence of diazoxide (K⁺, line e). Because valinomycin effects are independent of mitoK_{ATP}, valinomycin-induced swelling was not sensitive to 5HD (Table 2). Experiments performed with BSA-washed mitoplasts or addition of BSA (0.05 mg/ml) to the assay had no effect on valinomycin-induced swelling (data not shown), so we can exclude valinomycin-induced side effects such as uncoupling due to valinomycin ion-pairing with free fatty acids.

To investigate the nature of the observed swelling in mitoplasts (Fig. 1, K⁺), we performed experiments to determine the ion specificity of the swelling. To test the hypothesis that we were indeed measuring a K⁺-specific channel, we repeated the experiments in Fig. 1 using a medium where K⁺ was substituted for the artificial cation TEA⁺ (Fig. 1, TEA⁺). As predicted, if all conditions were kept the same, no swelling was observed when K⁺ was not present.

Fig. 2 summarizes and extends some of the data shown in Fig. 1. The two columns on the left show the diazoxide-induced reversal of ATP-inhibited swelling and its inhibition by 5HD. The next three columns show the effect of PKC modulators upon mitoplast swelling. In the presence of ATP, the general PKC agonist PMA induces matrix swelling to the same extent as diazoxide. This swelling was inhibited by the presence of the general PKC inhibitor chelerythrine or by 5HD. PMA-induced swelling was not inhibited by the PKC ϵ -specific antagonist peptide

ϵV_{1-2} (data not shown). Accordingly, the PKC ϵ -specific agonist peptide, $\psi\epsilon$ RACK, was ineffective in reversing the ATP inhibition of matrix swelling (data not shown).

Table 2 summarizes a series of controls performed to validate the results shown in Figs. 1 and 2. The table shows that mitoplast matrix volume: (i) did not change if Li⁺ substituted for K⁺, showing that the swelling observed in Fig. 1 (K⁺) and Fig. 2 is K⁺-dependent; (ii) was not influenced by the presence or absence of ATP in Li⁺ medium (i.e., in the absence of K⁺); (iii) increased upon incubation with valinomycin and ATP in K⁺ but not Li⁺ medium, demonstrating the specificity of the effects of valinomycin for K⁺; (iv) did not change in K⁺ medium in the presence of the protonophore CCCP, demonstrating that the observed swelling is driven by the membrane potential built up by the respiratory chain.

3.3. Biochemical characterization of mitoK_{ATP} from *T. cruzi*

As a preliminary biochemical characterization of the mitoK_{ATP} activity present in mitoplasts from *T. cruzi*, we performed a detailed study of the effect of ATP, diazoxide or 5HD on mitoplast swelling in K⁺-medium. Fig. 3A shows that increasing doses of ATP progressively decreased matrix swelling, with an apparent K_i of 5.5 mM (Hill coefficient = 2.5). ATP-induced changes were not observed when mitoplasts were incubated in TEA⁺-medium (Fig. 1, TEA⁺).

Next, mitoplasts incubated with inhibitory concentrations of ATP were exposed to the mitoK_{ATP} opener diazoxide. Fig. 3B shows that increasing concentrations of diazoxide induced increased rates of swelling. The apparent K_{1/2} observed in an average of three independent experiments was 90 μ M (Hill coefficient = 1). The two data points that are outside the fitted curve (black diamonds) show that higher concentrations of diazoxide inhibit mitoplast swelling, possibly by inhibition of the respiratory chain enzyme succinate dehydrogenase (Kowaltowski et al., 2001; Garlid and Paucek, 2003; Costa et al., 2006b). Fig. 3C shows that the pharmacological open state (in the presence of 8 mM ATP and 400 μ M diazoxide) was progressively inhibited by increasing concentrations of mitoK_{ATP}-specific blocker 5HD (apparent K_i of 371 μ M and Hill coefficient = 1). Also, preliminary data shows that this pharmacological open state is inhibited by 32 μ M glibenclamide, a non-selective potassium channel blocker. These results suggest that mitoK_{ATP} from *T. cruzi* has similar pharmacology to mitoK_{ATP} from rat heart, brain or liver mitochondria.

3.4. Evidence for the presence of mitoK_{ATP} in mitoplasts isolated from *C. fasciculata*

Mitoplasts isolated from another trypanosomatid, *C. fasciculata*, responded similarly to mitoK_{ATP} modulators (Fig. 4). Thus, mitoplasts isolated using the same protocol used for *T. cruzi* showed ATP-sensitive swelling when incubated in K⁺ medium but not in TEA⁺ medium (lines a and b); diazoxide reversed the ATP inhibition (line c), which was blocked by the presence of 5-HD (line d) or glibenclamide (not shown). Valinomycin also reversed the ATP-inhibition (line e). These results suggest a widespread presence of mitoK_{ATP} in all life forms of mitochondrion-containing trypanosomatids.

4. Discussion

MitoK_{ATP} was initially described in rat liver mitochondria (Inoue et al., 1991; Paucek et al., 1992) and, in subsequent years, mitoK_{ATP} activity was described in other tissues such as heart, brain and kidney (Paucek et al., 1992; Garlid et al., 1996; Bajgar et al., 2001; Cancherini et al., 2003; Sato et al., 2006). MitoK_{ATP} activity was also described in mitochondria of some plants such as durum wheat, spinach, rye, barley and potato (Pastore et al., 1999), but ATP-insensitive K⁺ channel activity was described in potato, tomato and maize mitochondria (Ruy et al., 2004). More recently, mitoK_{ATP} activity was also shown in mitochondria isolated from the

worm *C. elegans* (Wojtovich et al., 2008). The demonstration that mitoK_{ATP} is also present in nematodes led these authors to suggest that the ATP-sensitive K⁺ channel is an evolutionary conserved entity (Wojtovich et al., 2008).

The evidence presented here suggests the existence of mitoK_{ATP} in mitochondria from the trypanosomatids *T. cruzi* and *C. fasciculata*. Mitoplasts isolated from these parasites swell in K⁺-containing but not K⁺-free medium (Figs. 1 and 4). That this swelling was driven by respiratory chain-built membrane potential was confirmed by the effect of the protonophore CCCP, which abolishes the electrochemical gradient generated by respiring mitochondria, thus eliminating the driving force for potassium uptake (Table 2). Swelling in K⁺ medium was inhibited by ATP (Fig. 3A), and this inhibition could be reversed by the presence of the K⁺ channel opener diazoxide (Fig. 3B) or the K⁺ ionophore valinomycin (Figs. 1 and 4). Diazoxide-induced opening of mitoK_{ATP} was reversed by the mitoK_{ATP} blocker 5HD (Fig. 3C) or by glibenclamide (not shown). As expected, valinomycin-induced changes in mitoplast volume was insensitive to mitoK_{ATP} blockers (Table 2). These data demonstrate unequivocally the existence of mitoK_{ATP} in the mitochondria of these two trypanosomatids. Preliminary experiments suggest that mitoK_{ATP} activity is also present in *T. cruzi* amastigotes (data not shown).

Trypanosoma cruzi mitoK_{ATP} and mitoK_{ATP} from other organisms appear to be sensitive to similar compounds although with different affinities. Whereas ATP inhibition for rat and *C. elegans* mitoK_{ATP}-mediated K⁺ flux is in the micromolar range (Paucek et al., 1992; Wojtovich et al., 2008), we found that the K_{1/2} for *T. cruzi* mitoK_{ATP} is higher than 5 mM. Moreover, depending on the species, plant mitoK_{ATP} was shown to be sensitive (Pastore et al., 1999) or even insensitive to ATP (Ruy et al., 2004). Although ATP inhibition of plant mitoK_{ATP} is Mg²⁺-independent (Pastore et al., 1999), in heart, liver and brain mitochondria ATP inhibition of mitoK_{ATP} is Mg²⁺-dependent (Paucek et al., 1996; Yarov-Yarovoy et al., 1997; Jaburek et al., 1998). Further studies will have to be performed to characterize the effect of Mg²⁺ on the ATP inhibition of protozoan mitoK_{ATP}. Trypanosome mitoK_{ATP} activation by diazoxide exhibited a higher K_{1/2}, suggesting a lower affinity of the channel for this drug compared with mammalian, plant and *C. elegans* mitoK_{ATP} (Garlid et al., 1997; Pastore et al., 1999; Wojtovich et al., 2008). We observed that protozoan mitoK_{ATP} is sensitive to glibenclamide, similar to rat, mammalian and worm mitoK_{ATP} (Jaburek et al., 1998; Wojtovich et al., 2008) but different than plant mitoK_{ATP} (Pastore et al., 1999). Higher concentrations of 5HD were also required to fully inhibit the protozoan mitoK_{ATP}. Interestingly, it is commonly found that trypanosomatids require higher concentration of drugs to observe the same effects when compared with higher eukaryotes. Such is the case, for example, for staurosporine (Ha et al., 2004; Braga and de Souza, 2006) and PMA (Sato et al., 1998; Santander et al., 2002). Although we do not envision a direct therapeutic use against *T. cruzi* for the drugs used in our study, the observed differences suggest that the trypanosome ATP-sensitive K⁺ channel could be structurally different from that found in its mammalian hosts, and therefore could be a potential target for a therapeutic strategy. Also, the differences in K_{1/2} observed for diazoxide activation and 5HD inhibition of mitoK_{ATP} might be used in the therapeutic setting to distinguish the effects of K_{ATP} activation in the host versus the trypanosomatid.

Similar to mammalian and rat mitoK_{ATP}, trypanosomatid mitoK_{ATP} is activated by the general PKC agonist PMA to the same extent as the mitoK_{ATP} opener diazoxide (Fig. 2), but not by the PKCε-specific agonist peptide ψεRACK (data not shown). Accordingly, the mitoK_{ATP} blocker 5HD or the general PKC inhibitor chelerythrine blocked the swelling induced by PMA (Fig. 2), but the PKCε-specific antagonist peptide εV₁₋₂ was ineffective (data not shown). These results differ than those found for rat and mammalian mitoK_{ATP} (Costa et al., 2005; Costa and Garlid, 2008), suggesting that the PKC that modulates mitoK_{ATP} in trypanosomatids is a distinct isoform or is not sensitive to the peptides designed specifically

to modulate mammalian PKC ϵ (Johnson et al., 1996; Souroujon and Mochly-Rosen, 1998; Dorn et al., 1999; Murriel and Mochly-Rosen, 2003; Budas and Mochly-Rosen, 2007; Churchill and Mochly-Rosen, 2007). Several lines of evidence suggest that PKC isoforms are present in higher eukaryote mitochondria. PKC ϵ was shown to phosphorylate and co-immunoprecipitate with cytochrome oxidase subunit IV in rat neonatal cardiomyocytes (Ogbi and Johnson, 2006; Guo et al., 2007). Jaburek and co-workers showed that PKC ϵ and mitoK_{ATP} co-purify in a diethylaminoethyl (DEAE)-cellulose column and co-reconstitute in lipid vesicles in a functional complex (Jaburek et al., 2006). Accordingly, Costa and co-workers showed that PKC ϵ is retained in an active form in rat heart mitoplasts (Costa et al., 2006a; Costa and Garlid, 2008). Furthermore, proteomic approaches have provided evidence for the presence of several kinase signaling cascades in mitochondria (Agnetti et al., 2007). However, although PKC has not been explicitly found in the trypanosome genome (Parsons et al., 2005), PKC-like activity is a very important signaling function and has already been observed in *T. cruzi* (de Carvalho and de Souza, 1987; Santander et al., 2002). Thus, it is not surprising to find PKC-like activity within trypanosomatid mitochondria regulating mitoK_{ATP} opening. All of these regulatory differences observed between mammalian and *T. cruzi* mitoK_{ATP} might be due to the loss of some regulatory cofactor during the isolation procedure or differences in the channel subunit composition. Both explanations are equally possible and the elucidation of these differences will have to wait for molecular identification of mitoK_{ATP} and of PKC isoforms in trypanosomatids.

Due to its protective properties against cardiac ischemia/reperfusion injuries (Garlid et al., 2003; Teshima et al., 2003; Costa et al., 2005; Raval et al., 2007; Costa and Garlid, 2008; Fornazari et al., 2008), mitoK_{ATP} has been commonly viewed only as part of the protective signaling pathways. Several arguments exist against this common view. MitoK_{ATP} is present in rats, organisms that do not suffer from cardiac diseases. Additionally, it would be naïve to think that there is selective pressure to express a protein throughout the lifetime of a given organism, mammals for example, if it would only be necessary in the senescent stages (Garlid et al., 2006). The results shown in this paper extend our knowledge of mitoK_{ATP} presence to the Protozoa group, and provide support to the hypothesis put forth by Garlid et al. (2006). They hypothesized that mitoK_{ATP} has some basic functions in the bioenergetics of the cell, such as maintenance of matrix volume for adequate energy transfer from the inner membrane space to the cytosol (Dos Santos et al., 2002; Garlid et al., 2006) and maintenance of matrix enzyme activity for proper ATP production (Garlid et al., 2003; Garlid and Paucek, 2003). It has long been known that proper rates of ATP production are achieved by maintenance of mitochondria volume. If mitochondria shrink past a critical point, several enzymes do not function adequately and respiration slows down, impairing ATP production. Low respiration rates are also known to induce deleterious ROS production (Chandel and Budinger, 2007; Clanton, 2007). Thus, matrix volume regulation is of the utmost importance for the maintenance of appropriate energy flow from mitochondria to the cytosol and for control of ROS generation. Indeed, it has already been hypothesized that mitoK_{ATP} could prevent excessive and deleterious ROS production (Garlid et al., 2003; O'Rourke, 2004; Costa et al., 2006a; Costa and Garlid, 2008) or participate in intracellular calcium homeostasis (Kersten et al., 1997; Holmuhamedov et al., 1999; Liu et al., 2005) due to its ability to decrease mitochondrial membrane potential, albeit this decrease is only a few millivolts (Kowaltowski et al., 2001).

Mitochondria are intrinsically involved in several intracellular signaling events beyond ATP production, such as production of ROS for activation of signaling kinases (Costa et al., 2008) or gene transcription (Chandel and Budinger, 2007), homeostasis of cytosolic calcium (Kowaltowski, 2000), prevention of necrosis (Crompton, 1999), and supplying the cytosol with Krebs cycle intermediates (Scholte, 1988). Additionally, mitochondria are key players in apoptotic signaling, since they house several anti-apoptotic as well as pro-apoptotic factors

and enzymes (Fleury et al., 2002; Jeong and Seol, 2008). Due to its protective role against apoptosis and necrosis, mitochondria play a central role in protection against ischemia/reperfusion injury (Di Lisa et al., 2007). Thus, it is tempting to speculate regarding possible functions for mitoK_{ATP} in trypanosomatids. *Trypanosoma cruzi* and *C. fasciculata* could use mitoK_{ATP} and the K⁺ cycle in order to achieve maximum efficiency for specific processes that require mitochondria participation. As was hypothesized for mammalian mitoK_{ATP}, opening of protozoan mitoK_{ATP} by a physiological agent as PMA suggests that the channel is part of the regular cellular bioenergetic processes, such as the response to environmental stress occurring when *T. cruzi* moves from the insect gut to the vertebrate host. Protozoan mitoK_{ATP} might also participate in the regulation of cytosolic Ca²⁺ or in the process of programmed cell death (Debrabant et al., 2003; Debrabant and Nakhasi, 2003). Future studies are necessary to test these hypotheses and uncover physiological functions of mitoK_{ATP} in trypanosomatids.

Acknowledgments

Crithidia fasciculata cultures were kindly donated by Dr. Maria Cristina Machado Motta (Instituto de Biofísica Carlos Chagas Filho - UFRJ, Rio de Janeiro, Brazil). The authors would like to express their gratitude for the excellent technical support of Nilson J. Fidêncio and Vanessa M. dos Santos. Help with the genome search by Dr. Fabricio K. Marchini and critical reading of the manuscript by Dr. Christian M. Probst are greatly appreciated. This investigation received financial support from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), FIOCRUZ, and NIH (grant 5R01AI050196.02). A.D.T.C. and M.A.K hold CNPq research fellowships.

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Fig. 1.

Trypanosoma cruzi mitoplasts swell in K⁺ but not in tetraethylammonium (TEA⁺) medium. Shown are changes in absorbance of mitoplast suspensions incubated in KCl (K⁺) or TEA-Cl (TEA⁺). Mitoplasts (0.1 mg) were suspended in each medium (0.1 ml) in the presence of oligomycin and the respiratory substrate succinate. Conditions for both K⁺ and TEA⁺ were as follows: (a) no additions; (b) 8 mM ATP; (c) 8 mM ATP + 400 μM Dzx; (d) 8 mM ATP + 400 μM diazoxide (Dzx) + 1.5 mM 5-hydroxydecanoate (5HD); (e) 8 mM ATP + 1.6 μM valinomycin. Traces are representative of three to 10 independent preparations.

Fig. 2.

Effect of protein kinase C (PKC) agonists and antagonists on mitochondrial ATP-sensitive K^+ channel (mitoK_{ATP}) activity. Shown is a comparison between the effects of diazoxide (Dzx), 5-hydroxydecanoate (5HD), and the general PKC agonist phorbol 12-myristate 13-acetate (PMA) and antagonist chelerythrine on mitoplast volume. *Trypanosoma cruzi* mitoplast suspensions (1 mg/ml) were incubated in KCl medium in the presence of 8 mM ATP and each of the following: diazoxide (400 μ M), diazoxide plus 5HD (1.5 mM), PMA (4 μ M), PMA plus chelerythrine (6.5 μ M), PMA plus 5HD (1.5 mM). Steady state volumes measured at 90 s in the absence and presence of ATP were taken as 0 and 100%, respectively (see Materials and methods). Data are shown as mean $\pm$ S.D. of at least three independent experiments. * $P < 0.05$ versus diazoxide alone; ** $P < 0.05$ versus PMA alone.

Fig. 3. Pharmacological modulation of mitochondrial ATP-sensitive K⁺ channel (mitoK_{ATP}) activity. Shown are dose response curves for ATP (A), diazoxide (B), and 5-hydroxydecanoate (5HD) (C). Mitoplasts (1 mg/ml) were incubated in the presence of oligomycin and: (A) increasing concentrations of ATP; (B) 8 mM ATP plus increasing concentrations of diazoxide (Dzx); (C) 8 mM ATP plus 400 μM diazoxide plus increasing concentrations of 5HD. Data for each point on the dose response curves, measured as the steady state volume at 90 s (see Materials and methods), are an average of the results obtained from two to five independent preparations.

Fig. 4. Mitoplasts from *Crithidia fasciculata* are sensitive to mitochondrial ATP-sensitive K^+ channel (mitoK_{ATP}) agonists and antagonists. Shown are changes in absorbance of mitoplast suspensions incubated in KCl (K^+) or tetraethylammonium chloride (TEA-Cl) (TEA^+). Mitoplasts (0.1 mg) were suspended in each medium (0.1 ml) in the presence of oligomycin and the respiratory substrate succinate. Conditions for both K^+ and TEA^+ were as follows: (a) no additions; (b) 8 mM ATP; (c) 8 mM ATP + 400 μ M diazoxide (Dzx); (d) 8 mM ATP + 400 μ M Dzx + 1.5 mM 5-hydroxydecanoate (5HD); (e) 8 mM ATP + 1.6 μ M valinomycin. Traces are representative of three to five independent preparations.

Table 1

Succinate-dependent reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).

Experimental condition	Specific activity ($A_{570\text{nm}} - A_{630\text{nm}}$ /mg protein/h)
Mitoplasts	1.99 ± 0.05
Mitoplasts + CCCP	0.38 ± 0.02
Broken cells/nuclei	0.37 ± 0.02
Broken cells/nuclei + CCCP	0.36 ± 0.02

MTT reduction by isolated mitoplasts (1 mg/ml) or broken cells (1 mg/ml) was measured in KCl medium containing succinate (see Materials and methods for exact medium composition). The reaction was allowed to proceed for 60 min at 28 °C in the presence of 0.25 mg/ml MTT before solubilization with DMSO. After solubilization, the mixture was analyzed in a spectrophotometer at 570 nm, using the reading at 630 nm as the reference wavelength. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) (5 μ M) was added to inhibit MTT reduction. Data are the mean $\pm$ S.D. from two independent experiments

Table 2

Swelling rates of control experiments.

Experimental condition	Volume change at 90 s (%)
CCCP (5 μ M)	3 $\pm$ 2
no ATP in LiCl	13 $\pm$ 6
+ ATP in LiCl	20 $\pm$ 3
+ ATP + valinomycin (1 μ M) in LiCl	17 $\pm$ 5
+ ATP + valinomycin (1 μ M) in KCl	87 $\pm$ 8
+ ATP + valinomycin (1 μ M) + 5HD in KCl	92 $\pm$ 6

Mitoplasts matrix volume was measured as described in Materials and methods.

Experimental conditions and drug concentrations, except where noted, were the same as in Fig. 1. Steady state volumes measured at 90 s in the absence and presence of ATP were taken as 0 and 100%, respectively.

CCCP, carbonyl cyanide m-chlorophenylhydrazone; 5HD, 5-hydroxydecanoate.