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Diazoxide and Pinacidil Uncouple Pyruvate–Malate-Induced Mitochondrial Respiration

Dalia M. Kopustinskiene,[1,3] Jovita Jovaisiene,[2] Julius Liobikas,[1] and Adolfas Toleikis[1]

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We investigated the effects of K_{ATP} channel openers diazoxide and pinacidil on the respiration rate and membrane potential ($\Delta\Psi$) of rat heart mitochondria, oxidizing pyruvate and malate. Diazoxide and pinacidil (58.8–1348.3 μM) increased the V_2 (-ADP) respiration rate accordingly by 13–208% and 30–273% and decreased the $\Delta\Psi$ by 2–17% and 6–55%. These effects were also similar in the respiration medium without K^+. Moreover, carboxyatractyloside completely abolished diazoxide- and pinacidil-induced uncoupling, indicating a role for the mitochondrial adenine nucleotide translocase in this process.

KEY WORDS: Diazoxide; pinacidil; uncoupling; mitochondria; adenine nucleotide translocase.

INTRODUCTION

Since 1989, a number of pharmacological studies have shown that K_{ATP} channel openers protect ischemic-reperfused myocardium by mimicking ischemic preconditioning, and these protective effects are abolished by K_{ATP} channel blockers (for review, see Grover and Garlid, 2000; Szewczyk and Marban, 1999; Terzic *et al.*, 2000). Until recently, the effects of openers have been believed to be due to the opening of K_{ATP} channels in cell plasma membranes. However, several recent studies indicate that the cardioprotection is mediated by mitochondrial K_{ATP} channels (Garlid *et al.*, 1997; Gross and Fryer, 1999; Liu *et al.*, 1998).

There are several different opinions about the results of the mitochondrial K_{ATP} channel opening. Mitochondrial K_{ATP} channels together with the K^+/H^+ antiporter are believed to maintain K^+ homeostasis within the mitochondrion and thereby to control mitochondrial volume (Garlid, 2000; Kowaltowski *et al.*, 2001). The second putative functional role of mitochondrial K_{ATP} channels is enabling the formation of ΔpH along with membrane potential ($\Delta\Psi$) (Czyz *et al.*, 1995). Opening of mitochondrial K_{ATP} channels depolarizes the inner membrane of mitochondria, stimulates respiration, reduces Ca^{2+} uptake into and releases Ca^{2+} from the mitochondrial matrix (Holmuhamedov *et al.*, 1998, 1999). Also, mitochondrial K_{ATP} channel opening accelerates electron transfer by the respiratory chain and leads to net oxidation of the mitochondria (Liu *et al.*, 1998).

In many investigations, K_{ATP} channel openers diazoxide, which was shown to be selective to mitochondrial K_{ATP} channel, and pinacidil are used in concentrations $\geq$100 μM (Holmuhamedov *et al.*, 1998; Hu *et al.*, 1999; Ozcan *et al.*, 2001), and the obtained effects are explained as being due to the opening of K_{ATP} channels. However, several recent studies show that K_{ATP} channel openers at $\geq$100 μM also have other effects which are not related to the channel opening and depend on the used respiratory substrates (Grimmsmann and Rustenbeck, 1998; Kowaltowski *et al.*, 2001; Ovide-Bordeaux *et al.*, 2000).

The aim of our work was to investigate the effects of K_{ATP} channel openers diazoxide and pinacidil (58.8–1348.3 μM) on the respiration rate and membrane

Key to abbreviations: $\Delta\Psi$, transmembrane difference in electric potential; ANT, adenine nucleotide translocase; BSA, bovine serum albumin; CAT, carboxyatractyloside; FCCP, carbonyl cyanide *p*(trifluoromethoxy)phenylhydrazone; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid; V_2 mitochondrial respiration rate with 6 mM of both pyruvate and malate; V_3 maximal mitochondrial respiration rate in the presence of 1 mM ADP; V_{cytc} maximal respiration rate in the presence of 1 mM ADP and 17 μM cytochrome *c*.

[1]Institute for Biomedical Research, Kaunas University of Medicine, Eiveniu str. 4, LT-3007 Kaunas, Lithuania.

[2]Department of Biology, Vytautas Magnus University, Vileikos str. 8, LT-3035 Kaunas, Lithuania.

[3]To whom correspondence should be addressed; e-mail: bioch@kmu.lt.

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potential of rat heart mitochondria, oxidizing physiological substrates pyruvate + malate. Our results show that diazoxide- and pinacidil-induced uncoupling is not due to K^+ flux but is mediated by adenine nucleotide translocase (ANT).

MATERIALS AND METHODS

Preparation

The experiments were carried out on mitochondria isolated from male Wistar rat hearts by differential centrifugation procedure. After decapitation hearts were excised and rinsed in ice-cold isolation medium, containing 220 mM manitol, 70 mM sucrose, 5 mM *N*-tris[hydroxymethyl]methyl-2-aminoethane-sulphonic acid (TES), and 0.5 mM EGTA (pH 7.4, adjusted with Trizma base; 2°C). Mitochondria were isolated in the same medium supplemented with 2 mg/mL bovine serum albumin (BSA; fraction V, A4503, Sigma). Homogenate was centrifugated for 5 min at $750 \times g$, then supernatant was centrifugated for 10 min at $6740 \times g$ and the pellet was washed once in the isolation medium without bovine serum albumin (BSA), suspended in it and kept on ice.

Assays

The mitochondrial protein concentration was determined by biuret method (Gornall *et al.*, 1949). The final mitochondrial protein concentration in all experiments was 0.5 mg/mL. Mitochondrial oxygen consumption and $\Delta\Psi$ were determined at 37°C in KCl medium (120 mM KCl, 5 mM KH_2PO_4, 5 mM TES, and 1 mM $MgCl_2$; pH 7.4, adjusted with Trizma base, 37°C) or choline chloride medium (120 mM choline chloride, 5 mM NaH_2PO_4, 5 mM TES, and 1 mM $MgCl_2$; pH 7.4, adjusted with Trizma base, 37°C). Both pyruvate and malate (6 mM) were used as substrates. Mitochondrial oxygen consumption was recorded by means of the Clark-type electrode system. The solubility of oxygen was taken to be 422 ng atoms/mL (Holtzman, 1976). Respiration rates were expressed as ng atoms O/(min mg) mitochondrial protein. $\Delta\Psi$ of mitochondria was measured with rhodamine 123 (final concentration 0.1 μM), as a fluorescent probe using the excitation at 503 nm and emission at 527 nm (Emaus *et al.*, 1986) with the Hitachi F4000 fluorometer. The difference in fluorescence between mitochondria after adding carbonyl cyanide *p*(trifluoromethoxy)phenylhydrazone (FCCP) (0.4 μM) and without it was taken as 100%, and decrease in the $\Delta\Psi$ by tested compounds was expressed in percentage of FCCP effect. Swelling of nonrespiring mitochondria was recorded as the decrease of light scattering at 540 nm with the Hitachi 557 spectrophotometer in potassium acetate medium (120 mM potassium acetate, 10 mM Tris-HCl, 0.5 mM EGTA; pH 7.4 with Trizma base, 25°C) supplemented with 2 μM rotenone and 0.5 μM valinomycin (Kowaltowski *et al.*, 2001; Nicholls and Ferguson, 1992; Schonfeld *et al.*, 2000). The results are presented as means ±SE of three independent experiments in Fig. 3, where missing error bars were smaller than symbol size.

Reagents

Carboxyatractyloside was from Calbiochem (La Jolla, CA, USA); all other chemicals were from Sigma-Aldrich (St Louis, MO, USA).

RESULTS

Before all experiments we checked the quality of our mitochondrial preparations. The respiratory control index (V_3/V_2) was 6.8 ± 0.7 and 6.1 ± 0.4, accordingly in KCl medium and in choline chloride medium. Cytochrome *c* effect (V_{cytc}/V_3) was 1.14 ± 0.01 in both cases.

Figure 1 shows the effect of diazoxide (A) and pinacidil (B) on V_2 and $\Delta\Psi$ of isolated rat heart mitochondria, respiring on pyruvate and malate (6 + 6 mM) in KCl and choline chloride media. Both compounds in a concentration-dependent manner increased V_2 and decreased $\Delta\Psi$. This effect was similar in the presence and in the absence of K^+ in the media, indicating the direct uncoupling of respiration.

For detailed investigation of the uncoupling effect of diazoxide and pinacidil, we measured swelling of nonrespiring mitochondria in potassium acetate medium in the presence of valinomycin. In these conditions swelling starts only in the presence of protonophore (Kowaltowski *et al.*, 2001; Nicholls and Ferguson, 1992; Schonfeld *et al.*, 2000). We obtained that diazoxide and pinacidil induced mitochondrial swelling (Fig. 2 (A) and (B)). Thus, our results show that both diazoxide and pinacidil act as protonophores.

To elucidate the uncoupling mechanism of diazoxide and pinacidil, we tested whether carboxyatractyloside (CAT), which partially reverses fatty acid-mediated uncoupling (Skulachev, 1999; Wojtczak and Wieckowski, 1999), can also abolish diazoxide- and pinacidil-mediated uncoupling. At first, we checked what concentration of CAT can completely block V_3 in our mitochondrial preparations in KCl medium, and obtained the maximal inhibition at 0.86 μM CAT. This concentration of CAT was

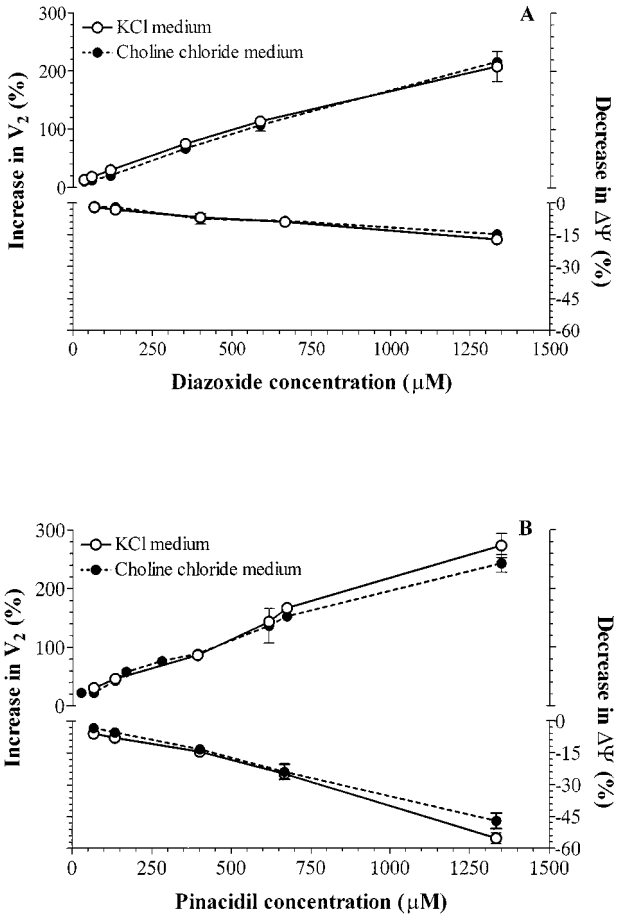

Fig. 1. Effect of diazoxide (A) and pinacidil (B) on the respiration rate (V_2) and $\Delta\Psi$ of rat heart mitochondria, $n = 3$. Effect of both compounds on V_2 was expressed as percentage of initial respiratory rate, which was 63.8 ± 2.9 ng atoms O/(min mg) protein in KCl medium and 60.2 ± 6.2 ng atoms O/(min mg) protein in choline chloride medium. Decrease in the $\Delta\Psi$ by tested compounds was expressed in percentage of FCCP effect. For details, see Materials and Methods.

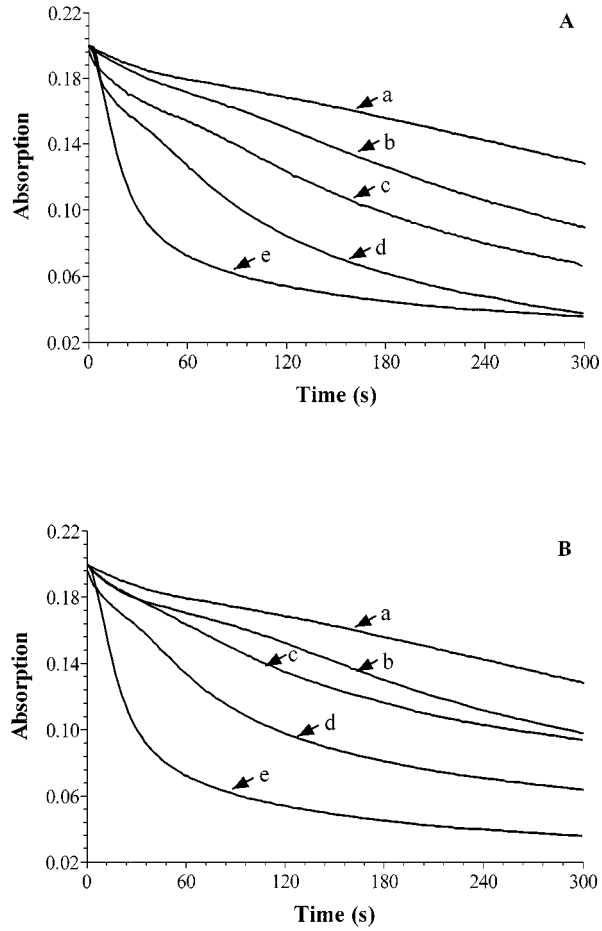

Fig. 2. Diazoxide (A) and pinacidil (B) supported swelling of rat heart mitochondria in potassium acetate medium with 2 μM rotenone and 0.5 μM valinomycin. (a) control, (b) 100 μM, (c) 360 μM, (d) 1000 μM diazoxide (A) or pinacidil (B), (e) 2 μM FCCP. Similar curves were obtained in 3 independent experiments.

chosen for further experiments. We observed that neither diazoxide nor pinacidil (60–1000 μM) stimulated V_2 when mitochondria were incubated with 0.86 μM CAT (Fig. 3 (A) and (B)). These results indicate a role for ANT in diazoxide- or pinacidil-induced uncoupling of mitochondria.

DISCUSSION

Although in numerous studies the effects of diazoxide or pinacidil ($\geq$100 μM) on the mitochondrial function (Holmuhamedov *et al.*, 1998, 1999; Liu *et al.*, 1998; Ozcan *et al.*, 2001; Wang *et al.*, 2001) are associated with the mitochondrial K_{ATP} channel opening (Hu *et al.*, 1999), several studies have shown that these compounds also have the effects, unrelated to K^+ flux (Grimmsmann and Rustenbeck, 1998; Kowaltowski *et al.*, 2001; Ovide-Bordeaux *et al.*, 2000; Schafer *et al.*, 1969). We found that diazoxide and pinacidil already at $\approx$60 μM had uncoupling properties, e.g., they increased V_2 and decreased $\Delta\Psi$ of isolated rat heart mitochondria, respiring on physiological substrates pyruvate and malate ($6 + 6$ mM) in KCl medium (Fig. 1A and B, open symbols). We also checked whether the uncoupling effect of diazoxide and pinacidil remains in the medium without K^+. Both compounds depending on their concentration increased V_2 and decreased $\Delta\Psi$ similarly in the absence and in the presence of K^+ (Fig. 1 A and B).

Recent studies show that the additional effects of K_{ATP} channel openers on the mitochondrial function depend on the used respiratory substrates (Grimmsmann

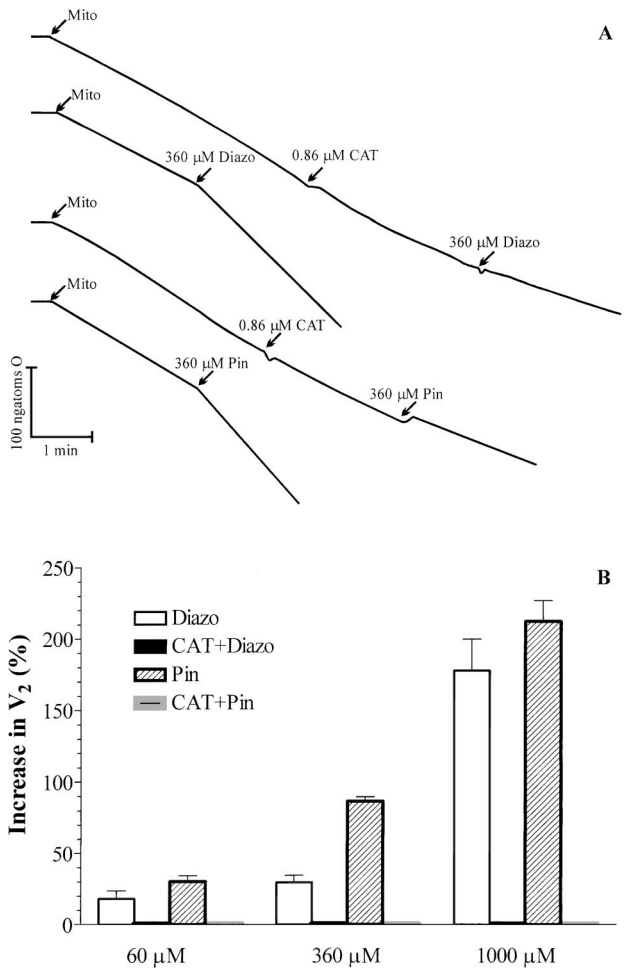

Fig. 3. Influence of 0.86 μM CAT on the effect of diazoxide and pinacidil on the mitochondrial V_2 respiration rate. (A) typical respiratory curves; (B) calculated data, $n = 3$. Mito (mitochondria), Diazo (diazoxide), Pin (pinacidil). Experiments were performed in KCl medium.

and Rustenbeck, 1998; Kowaltowski *et al.*, 2001; Ovide-Bordeaux *et al.*, 2000). Therefore, we have chosen the swelling model of nonrespiring mitochondria in potassium acetate medium to confirm that diazoxide and pinacidil directly uncouple mitochondria. Nonrespiring mitochondria do not swell in potassium acetate medium in the presence of valinomycin, because only undissociated acetic acid can cross the mitochondrial membrane. Therefore, swelling is initiated only in the presence of protonophore (Kowaltowski *et al.*, 2001; Nicholls and Ferguson, 1992; Schonfeld *et al.*, 2000). Both diazoxide and pinacidil increased mitochondrial swelling under these conditions (Fig. 2 A and B), indicating that they have protonophoric properties.

Our previous results showed that a common supplement, BSA, of media for mitochondrial investigations binds diazoxide (Kopustinskiene *et al.*, 2000). It is also known that diazoxide is bound by human plasma albumin (Sellers and Koch-Weser, 1974). These data and our finding that diazoxide and pinacidil directly uncouple mitochondria led us to the assumption that uncoupling mechanism of these compounds may be at some extent similar to the uncoupling by fatty acids. There are many studies showing that ANT participates in fatty acid-mediated uncoupling, which can be partially reversed by CAT (for reviews, see Wojtczak and Wieckowski, 1999; Skulachev, 1999). Therefore, we tested whether CAT can abolish uncoupling by diazoxide and pinacidil. When mitochondria were preincubated with 0.86 μM CAT, neither diazoxide nor pinacidil (60–1000 μM) were able to stimulate V_2 (Fig. 3 (A) and (B)). CAT was also effective when it was added after diazoxide or pinacidil. Thus, our results show that ANT participates in diazoxide- or pinacidil-induced uncoupling of mitochondria.

In conclusion, our results demonstrate that diazoxide and pinacidil starting at 60 μM and depending on their concentration increase V_2 and decrease $\Delta\Psi$ of isolated rat heart mitochondria. The uncoupling effect of both compounds is mediated by ANT and not connected to K^+ flux.

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