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Adenine Nucleotide Translocase Mediates the K_{ATP}-Channel-Openers-Induced Proton and Potassium Flux to the Mitochondrial Matrix

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K_{ATP} channel openers have been shown to protect ischemic-reperfused myocardium by mimicking ischemic preconditioning, although their mechanisms of action have not been fully clarified. In this study we investigated the influence of the adenine nucleotide translocase (ANT) inhibitors—carboxyatractyloside (CAT) and bongkrekic acid (BA)—on the diazoxide- and pinacidil-induced uncoupling of isolated rat heart mitochondria respiring on pyruvate and malate (6 + 6 mM). We found that both CAT (1.3 μM) and BA (20 μM) markedly reduced the uncoupling of mitochondrial oxidative phosphorylation induced by the K_{ATP} channel openers. Thus, the uncoupling effect of diazoxide and pinacidil is evident only when ANT is not fixed by inhibitors in neither the C- nor the M-conformation. Moreover, the uncoupling effect of diazoxide and pinacidil was diminished in the presence of ADP or ATP, indicating a competition of K_{ATP} channel openers with adenine nucleotides. CAT also abolished K^+-dependent mitochondrial respiratory changes. Thus ANT could also be involved in the regulation of K_{ATP}-channel-openers-induced K^+ flux through the inner mitochondrial membrane.

KEY WORDS: Adenine nucleotide translocase; diazoxide; K_{ATP} channel; membrane potential; mitochondria; pinacidil; uncoupling.

INTRODUCTION

The investigations of K_{ATP} channel openers have shown their cardioprotective action in various models of ischemia/reperfusion, (see Grover and Garlid, 2000; Oldenburg *et al.*, 2002; Szewczyk and Marban, 1999; Terzic *et al.*, 2000). K_{ATP} channel openers decreased the infarct size, increased time to ischemic contracture, improved postischemic recovery of contractile function, reduced reperfusion contracture, and improved reperfusion flow in an isolated rat heart model of ischemia and reperfusion (Grover, 1994; Grover *et al.*, 2001). Moreover, these compounds suppressed the release of creatine kinase and lactate dehydrogenase during reperfusion (Grover *et al.*, 2001; Iwai *et al.*, 2000; Tanonaka *et al.*, 1999). K_{ATP} channel openers increased cell viability and reduced accumulation of Ca^{2+} in mitochondrial matrix in ischemic/reperfused (Murata *et al.*, 2001; Sato *et al.*, 2000) or hypoxic/reoxygenated (Light *et al.*, 2001) rabbit ventricular myocytes. In the investigations of skinned cardiac fibers, K_{ATP} channel openers preserved the mitochondrial oxygen consumption rate during hypoxia (Iwai *et al.*, 2000; Tanonaka *et al.*, 1999). K_{ATP} channel openers were also effective protectors of isolated mitochondria, subjected to anoxia/reoxygenation (Ozcan *et al.*, 2001, 2002). They protected mitochondrial structure from anoxia-induced damage, preserved ADP-stimulated mitochondrial respiration and ATP production, and reduced mitochondrial reactive oxygen species production at reoxygenation (Ozcan *et al.*, 2001, 2002). Although the selectivity of K_{ATP} channel openers and blockers is highly

Key to abbreviations: $\Delta\Psi$, transmembrane difference in electric potential; ANT, adenine nucleotide tranlocase; CAT, carboxyatractyloside; BA, bongkrekic acid; FCCP, carbonyl cyanide *p*(trifluoromethoxy)phenyl-hydrazone; TES, (*N*-tris[Hydroxymethyl] methyl-2-aminoethane-sulfonic acid.

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dependent on the experimental conditions (O'Rourke, 2000), the cardioprotection is supposed to be mediated by mitochondrial K_{ATP} channels (Garlid *et al.*, 1997; Gross and Fryer, 1999; Liu *et al.*, 1998; O'Rourke, 2000).

Several mechanisms of cardioprotection by K_{ATP} channel openers are currently proposed. Opening of mitochondrial K_{ATP} channel modulates production of reactive oxygen species: enhances it during the early phase of ischemia (Forbes *et al.*, 2001; Pain *et al.*, 2000; Patel and Gross, 2001), activating signal transduction pathways; but attenuates it during reperfusion (Narayan *et al.*, 2001; Vanden Hoek *et al.*, 2000), reducing injuries of oxidative stress. Mitochondrial K_{ATP} channel openers increase mitochondrial matrix volume (Garlid, 2000; Kowaltowski *et al.*, 2001), preserving functional coupling between the adenine nucleotide translocase and creatine kinase (Laclau *et al.*, 2001) and maintaining low outer mitochondrial membrane permeability for ADP and ATP (Dos Santos *et al.*, 2002). Under ischemic conditions these compounds inhibit ATP hydrolysis, and reducing Ca^{2+} uptake into the mitochondrial matrix (Belisle and Kowaltowski, 2002). Another opinion is that opening of mitochondrial K_{ATP} channels depolarizes the inner membrane of mitochondria, and therefore stimulates respiration (Holmuhamedov *et al.*, 1998), accelerates electron transfer by the respiratory chain and leads to oxidation of mitochondrial matrix flavoproteins (Liu *et al.*, 1998), and reduces Ca^{2+} uptake into and releases Ca^{2+} from the mitochondrial matrix (Holmuhamedov *et al.*, 1998, 1999).

The K_{ATP}-channel-independent targets of mitochondrial K_{ATP} channel openers (Grimmsmann and Rustenbeck, 1998; Kopustinskiene *et al.*, 2002; Kowaltowski *et al.*, 2001; Ovide-Bordeaux *et al.*, 2000; Schafer *et al.*, 1969) and blockers (Hanley *et al.*, 2002) have been revealed also in mitochondria, raising a question whether the processes activated by diazoxide and inhibited by 5-hydroxydecanoate are necessarily related to mitochondrial K_{ATP} channels (Hanley *et al.*, 2002). Thus, the cardioprotective effects exerted by K_{ATP} channel openers may be related to the direct modulation of mitochondrial functions by these compounds.

The aim of our work was to investigate the role of adenine nucleotide translocase in uncoupling of mitochondrial oxidative phosphorylation and K^+-dependent increase in mitochondrial respiration rate, induced by K_{ATP} channel openers.

MATERIALS AND METHODS

Isolation of Mitochondria

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 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). The homogenate was centrifuged at 750 *g* for 5 min, then the supernantant was recentrifuged at 6740 *g* for 10 min, and the pellet was washed once in the isolation medium without BSA, suspended in it, and kept on ice. The mitochondrial respiratory control index was used to evaluate the quality of isolated mitochondria. It was calculated from the ratio of the maximal respiration rate (in the presence of substrates and ADP (State 3) and the rate in the presence of substrates alone (State 2, 6 mM of both pyruvate and malate). The respiratory control index of our mitochondrial preparations was 7.0 ± 1.0.

Assays

The mitochondrial protein concentration was determined by the biuret method (Gornall *et al.*, 1949). The final mitochondrial protein concentration in all experiments was 0.5 mg/mL. Mitochondrial membrane potential ($\Delta\Psi$) was 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). Both pyruvate (6 mM) and malate (6 mM) were used as substrates. Mitochondrial oxygen consumption was recorded by means of the Clark-type electrode system at 37 or 25°C in KCl medium. In some experiments 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)) was used. The solubility of oxygen was taken to be 422 nmol O/mL at 37°C or 452 nmol O/mL at 25°C (Holtzman, 1976). Respiration rates were expressed as nmol $O \times min^{-1} \times$ mg mitochondrial $protein^{-1}$. $\Delta\Psi$ was measured with rhodamine 123 as a fluorescent probe (final concentration 0.1 μM) 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 added 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.

Statistical Analysis of Experimental Data

The results are presented as mean $\pm$ SE of three to five independent experiments. An analysis of variance (ANOVA) followed by Dunnet test or Tukey's multiple comparison test was used for comparisons between experimental groups. A value of $P < 0.05$ was considered statistically significant.

Reagents

All chemicals were from Sigma-Aldrich (St. Louis, MO), except carboxyatractyloside, which was from Calbiochem (La Jolla, CA).

RESULTS

We have previously shown (Kopustinskiene *et al.*, 2002) that the inhibitor of mitochondrial adenine nucleotide translocase (ANT) carboxyatractyloside (CAT), that binds ANT at sites, accessible from the mitochondrial intermembrane space (C-conformation of ANT) abolishes the potassium-independent increase in mitochondrial State 2 respiration rate, induced by the K_{ATP} channel openers diazoxide and pinacidil. To determine the importance of the conformational state of ANT for uncoupling of oxidative phosphorylation by diazoxide and pinacidil in heart mitochondria, we tested the capability of the ANT inhibitor bongkrekic acid (BA), that binds to ANT at sites, accessible from the matrix compartment of mitochondria (M-conformation of ANT), to block the decrease in $\Delta\Psi$ induced by K_{ATP} channel openers. The results in Fig. 1 show that CAT (1.3 μM) and BA (20 μM) similarly reduced diazoxide- (A) and pinacidil- (B) induced decrease in $\Delta\Psi$ in State 2 (substrate $-$ pyruvate $+$ malate). The uncoupling by diazoxide and pinacidil is thus mediated by ANT only when ANT is not fixed by inhibitors in neither the C- nor the M-conformation.

Since our results have revealed the importance of ANT in uncoupling effect of diazoxide and pinacidil, we checked whether K_{ATP} channel openers are able to interfere with mitochondrial ADP uptake. The addition of diazoxide or pinacidil caused a slight, transient increase in $\Delta\Psi$ in State 3 (pyruvate $+$ malate and 1 mM ADP; data not shown), suggesting that K_{ATP} channel openers may compete with adenine nucleotides. Indeed, in the presence of oligomycin and ADP (200 μM) or ATP (200 μM) the increase in mitochondrial respiration rate by diazoxide (Fig. 2(A)) or pinacidil (Fig. 2(B)) was diminished compared to that in the presence of oligomycin alone, indicating a competition of K_{ATP} channel openers with both ADP and ATP.

As observed previously, the action of K_{ATP} channel openers depends on their concentration: at low concentrations (up to 50 μM) they increase the K^+ flux through the inner mitochondrial membrane (Garlid, 2000; Kowaltowski *et al.*, 2001), whereas at concentrations >60 μM they directly affect mitochondrial functions (Grimmsmann and Rustenbeck, 1998; Hanley *et al.*, 2002; Kopustinskiene *et al.*, 2002; Kowaltowski *et al.*, 2001; Ovide-Bordeaux *et al.*, 2000). It was proposed that ATP-, diazoxide-, and 5-hydroxydecanoate-induced alterations in the mitochondrial respiration rate in the presence of oligomycin are related to changes in K^+ flux through the inner mitochondrial membrane (Kowaltowski *et al.*, 2001). We applied this model to test the possible participation of ANT in this process. The mitochondrial respiration rate was diminished by ATP and then restored by 30 μM of diazoxide in the presence of oligomycin in State 2 (Fig. 3). However, the capability of the mitochondrial K_{ATP} channel blocker 5-hydroxydecanoate (300 μM) to alter the mitochondrial respiration rate under these conditions depended on the temperature of incubation medium. At 37°C (Fig. 3(A)) 300 μM 5-hydroxydecanoate was not effective, whereas it abolished the diazoxide-induced increase in mitochondrial respiration at 25°C (Fig. 3(B)). Preliminary results (data not shown) have led us to assume that at 37°C higher concentrations of 5-hydroxydecanoate are needed to induce effects on mitochondrial respiration. ATP or diazoxide did not alter the mitochondrial respiration rate in the choline chloride medium devoid of K^+ (Fig. 3), confirming that the observed changes in mitochondrial respiration rate in the KCl medium are potassium-specific. Interestingly, ATP and diazoxide did not have any effect on the mitochondrial respiration rate in the presence of the ANT inhibitor CAT in the KCl medium (Fig. 3(A)). These data indicate that ANT not only participates in diazoxide- and pinacidil-induced uncoupling, but it may also mediate K^+ flux to the mitochondrial matrix induced by K_{ATP} channel openers.

DISCUSSION

Direct measurement of K^+ flux through the inner mitochondrial membrane is very complicated (Inoue *et al.*, 1991); therefore, in many cases the opening and closing of mitochondrial K_{ATP} channels is monitored by registration of the K_{ATP}- channel-openers- and blockers-induced alterations in mitochondrial swelling, $\Delta\Psi$, respiration rate, flavoprotein oxidation, and Ca^{2+} transport. However, K_{ATP} channel openers and blockers can also directly affect mitochondrial functions. The K_{ATP} channel opener diazoxide, which is thought to be selective for mitochondrial K_{ATP} channels (Garlid *et al.*, 1997; Hu *et al.*, 1999; Sato *et al.*, 1998), inhibits oxidation of succinate (Das *et al.*, 2002; Grimmsmann and Rustenbeck, 1998; Hanley *et al.*, 2002; Ovide-Bordeaux *et al.*, 2000; Schafer *et al.*, 1971) and 2-oxoglutarate (Das *et al.*, 2002). Pinacidil, which opens not only mitochondrial, but also cell membrane K_{ATP} channels (Hu *et al.*, 1999), inhibits oxidation of external NADH in submitochondrial particles (Hanley *et al.*, 2002). Both diazoxide and pinacidil at concentrations >60 μM uncouple mitochondrial oxidative phosphorylation (Kopustinskiene *et al.*, 2002;

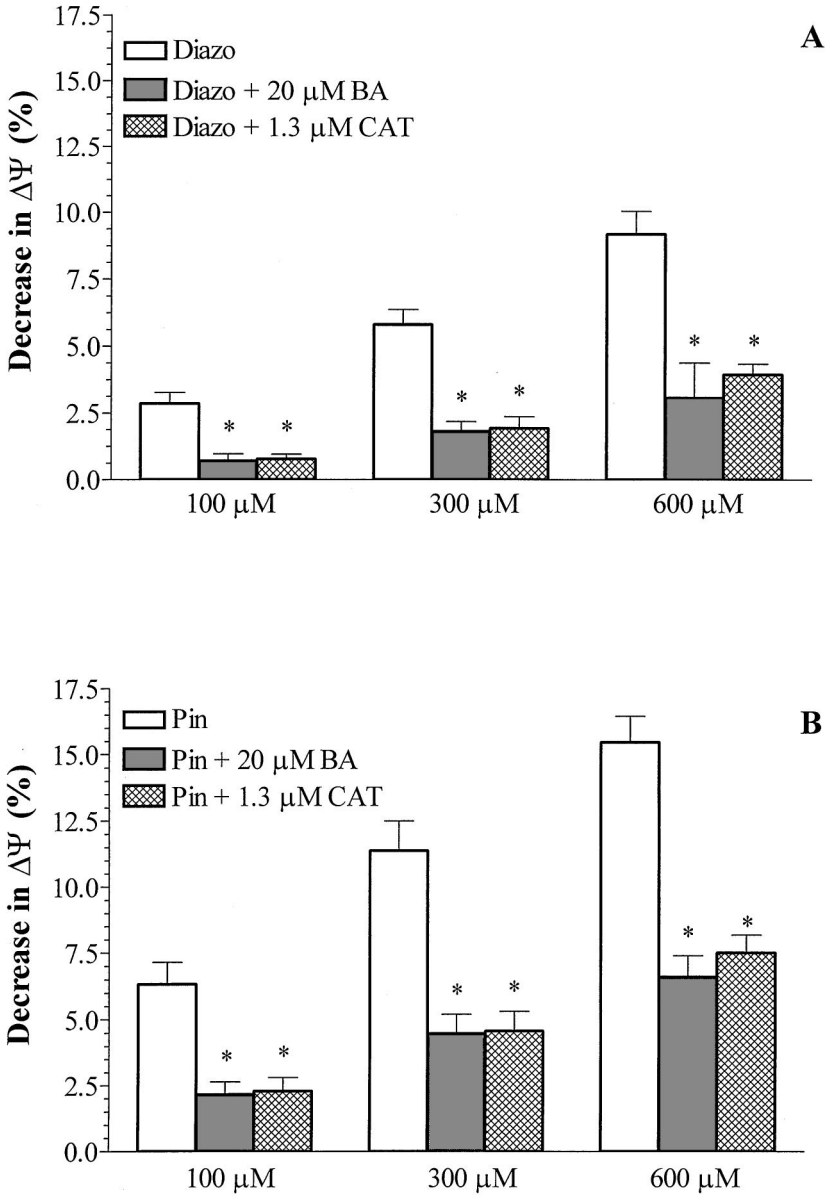

Fig. 1. Effect of bongkrekic acid (BA) and carboxyatractyloside (CAT) on diazoxide-(A) and pinacidil- (B) induced decrease in mitochondrial membrane potential ($\Delta\Psi$) in the State 2. Diazo – diazoxide, Pin – pinacidil. The decrease in $\Delta\Psi$ induced by the compounds was expressed in % of that induced by FCCP. Experiments were performed in KCl medium at 37°C, substrate – pyruvate and malate (6 + 6 mM), $n = 3$. For further experimental details, see MATERIALS AND METHODS. $^*P < 0.05$ – statistically significant effect of BA or CAT compared to control – diazoxide or pinacidil alone. The results were analyzed with one-way analysis of variance (ANOVA) followed by Dunnet posttest.

Kowaltowski *et al.*, 2001). 5-Hydroxydecanoate, a selective inhibitor of mitochondrial K_{ATP} channels (Garlid *et al.*, 1997; Hu *et al.*, 1999; Sato *et al.*, 1998), is converted to 5-hydroxydecanoyl-CoA by acyl-CoA synthase(Das *et al.*, 2002; Hanley *et al.*, 2002), and therefore may target the ANT or serve as a substrate for β-oxidation in mitochondria (Hanley *et al.*, 2002). Thus, the cardioprotective action of K_{ATP} channel openers may be related to the direct modulation of mitochondrial functions. This hypothesis is supported by recent data (Minners *et al.*, 2000, 2001)

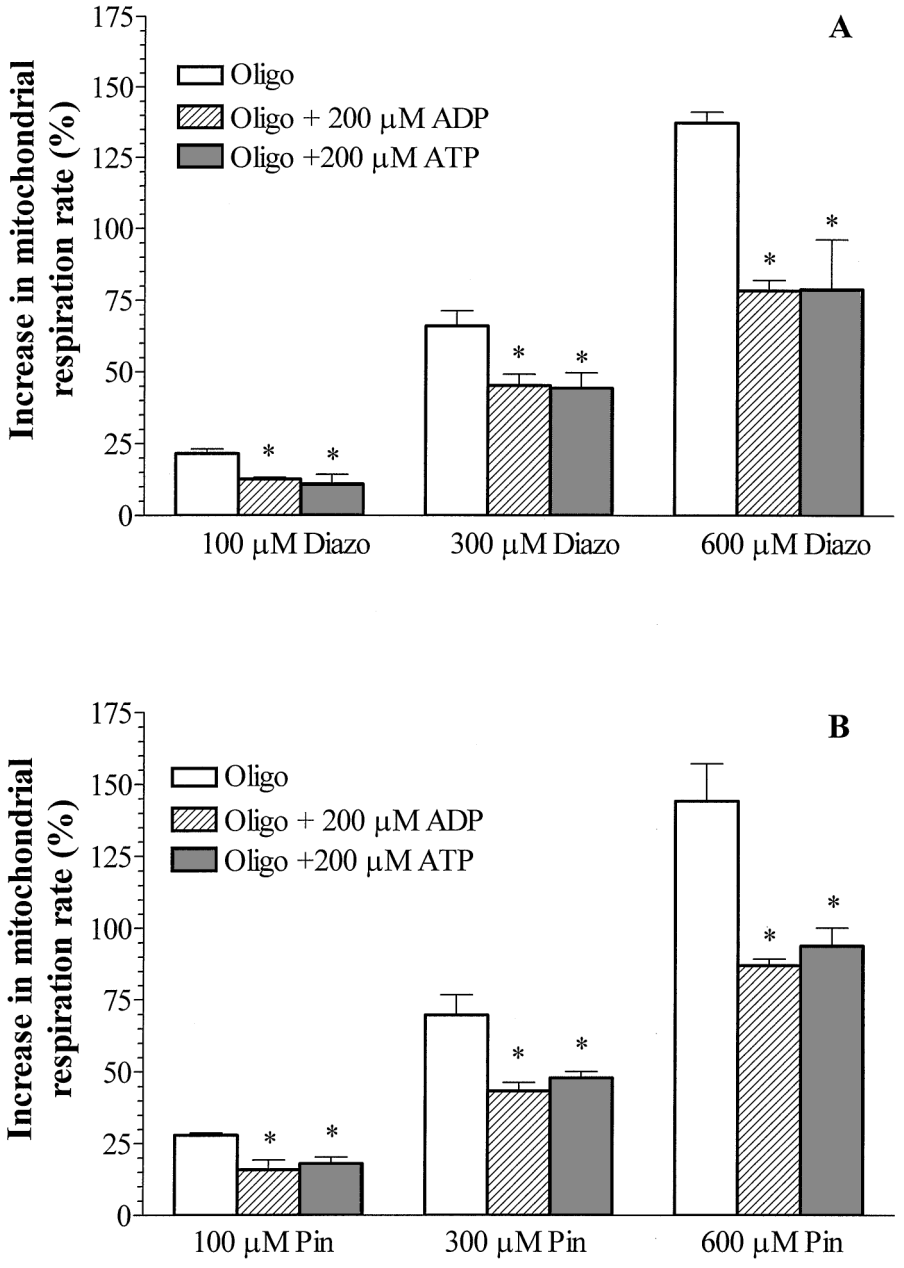

Fig. 2. Influence of ADP and ATP on diazoxide- (A) and pinacidil- (B) induced increase in State 2 respiration rate in the presence of oligomycin. Oligo – oligomycin (1 μg oligomycin/mg mitochondrial protein). Effect of diazoxide and pinacidil was expressed in % of initial respiration rate, which was 55.1 ± 1.3 nmol O min^{-1} mg $protein^{-1}$ in the presence of oligomycin, 49.5 ± 1.4 nmol O min^{-1} mg $protein^{-1}$ in the presence of oligomycin and 200 μM ADP and 48.5 ± 0.9 nmol O min^{-1} mg $protein^{-1}$ in the presence of oligomycin and 200 μM ATP. In other respects the experimental conditions were as in Fig. 1. $^*P < 0.05$ – statistically significant effect of ADP or ATP compared to control without adenine nucleotides.

that pharmacological uncoupling of mitochondrial oxidation from phosphorylation promotes preconditioning-like cardioprotection in the isolated rat heart. Also, it was reported (Ozcan *et al.*, 2002) that in isolated mitochondria subjected to anoxia/reoxygenation, K_{ATP} channel openers decreased production of mitochondrial reactive oxygen species at reoxygenation. However, that decrease was sustained in the absence of K^+ and mimicked by

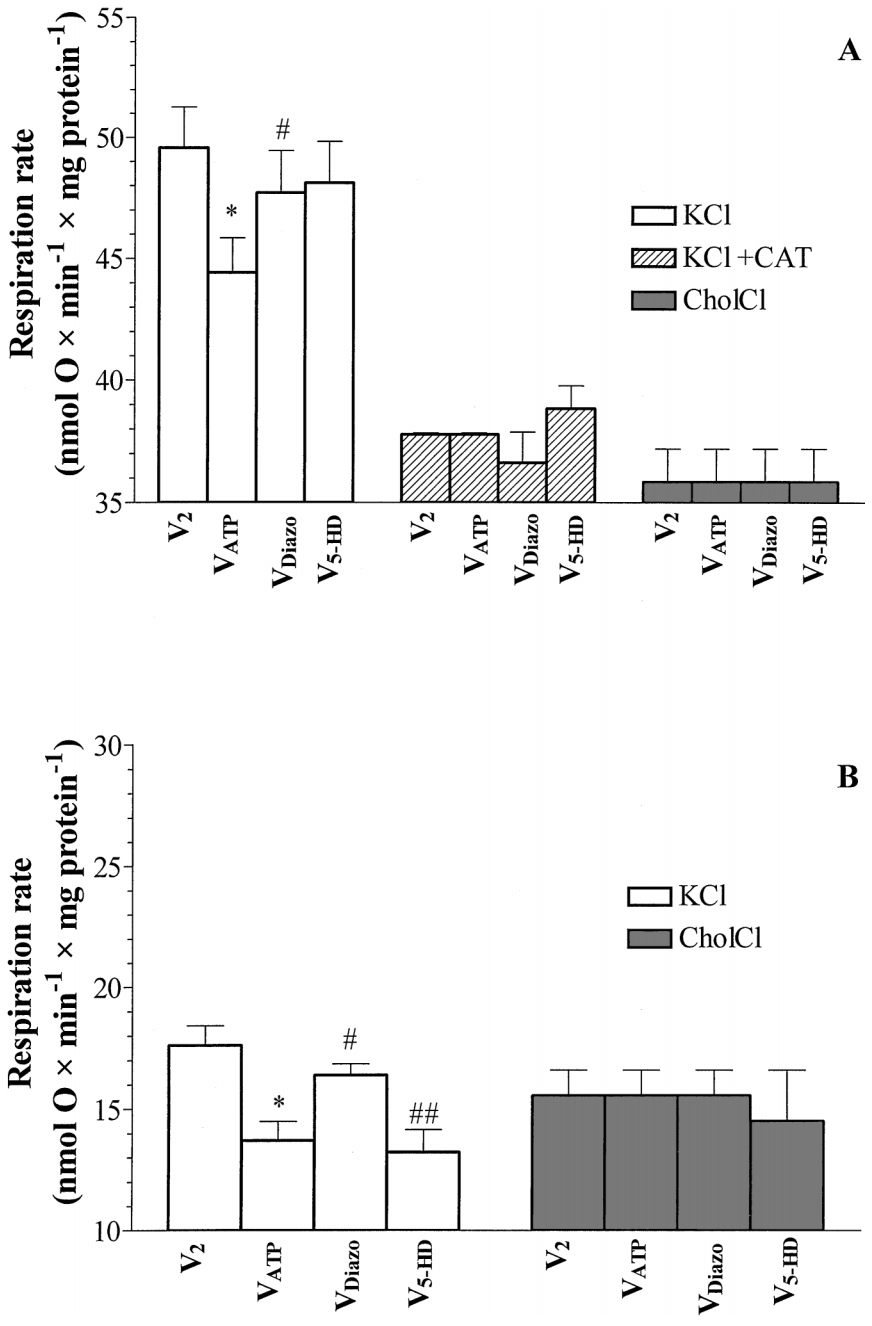

Fig. 3. Effect of ATP, diazoxide, and 5-hydroxydecanoate on the respiration rate of rat heart mitochondria. (A) Experiments were performed at 37°C; (B) at 25°C, substrate – pyruvate and malate (6 + 6 mM), $n = 5$. KCl – KCl medium, KCl+CAT – KCl medium, supplemented with 1 μM carboxyatractyloside, CholCl – choline chloride medium. V_2 – initial respiration rate, V_{ATP} – respiration rate in the presence of 200 μM ATP, V_{Diazo} – respiration rate in the presence of 200 μM ATP and 30 μM diazoxide, V_{5-HD} – respiration rate in the presence of 200 μM ATP, 30 μM diazoxide, and 300 μM 5-hydroxydecanoate. All media were supplemented with 1 μg oligomycin/mg mitochondrial protein to prevent hydrolysis of added ATP. $^{*}P < 0.05$ – statistically significant effect of ATP compared to control; $^{\#}P < 0.05$ – statistically significant effect of diazoxide compared to V_{ATP}; $^{\#\#}P < 0.05$ – statistically significant effect of 5-hydroxydecanoate compared to V_{Diazo}. The results were analyzed with repeated measures ANOVA followed by Tukey's multiple comparison test.

regulators of the mitochondrial redox state, suggesting the existence of a mitochondrial K_{ATP}-channel-independent protection pathway (Ozcan *et al.*, 2002).

In this study we have shown that K_{ATP} channel openers increase proton and potassium flux to the matrix of rat heart mitochondria via ANT. We found that CAT and BA at concentrations, which correspond to those needed for arresting State 3 respiration, similarly restored diazoxide- and pinacidil-lowered $\Delta\Psi$ of rat heart mitochondria in the State 2 in the presence of oligomycin (Fig. 1). CAT traps ANT in the C-conformation, promoting the open state of the mitochondrial permeability transition pore, whereas BA fixes ANT in the M-conformation, inhibiting pore opening (see for reviews Bernardi, 1999; Halestrap *et al.*, 2002). Our results show that the uncoupling effect of K_{ATP} channel openers is significantly reduced when ANT is fixed in either the C- or the M-conformation, indicating the importance of ANT in this process. Moreover, the analogous action of CAT and BA excludes the possibility that diazoxide- or pinacidil-induced uncoupling is a side effect due to the opening of mitochondrial permeability transition pore.

Further experiments have revealed that the diazoxide- and pinacidil-induced increase in the respiration rate in State 2 in the presence of oligomycin and substrates of ANT—ADP or ATP—was significantly diminished to approximately 2/3 of the increase in the presence of oligomycin alone (Fig. 2). The sensitivity of the uncoupling induced by K_{ATP} channel openers to both CAT and BA (Fig. 1), and to adenine nucleotides (Fig. 2), shows a similarity of this process to the uncoupling by fatty acids (Andreyev *et al.*, 1989). Our data suggest that diazoxide and pinacidil compete with both ADP and ATP. Thus, this finding should be taken into account, explaining the increase in apparent K_M for ADP after the treatment of mitochondria with diazoxide (Dos Santos *et al.*, 2002), which may be due to the competition of K_{ATP} channel openers with adenine nucleotides.

The K_{ATP} channel openers at concentrations up to 50 μM increase the K^+ flux to the mitochondrial matrix and have no additional effects on mitochondrial functions (Garlid, 2000; Kowaltowski *et al.*, 2001). It was reported that the K^+ flux through the newly opened mitochondrial K_{ATP} channels should increase the respiration rate by no more than 5% and decrease the $\Delta\Psi$ by 2–4 mV (Kowaltowski *et al.*, 2001). We have detected ATP-induced decrease in the respiration rate of isolated heart mitochondria, oxidizing pyruvate, and malate, when phosphorylation was blocked by oligomycin (Fig. 3). This decrease was reversed by 30 μM of diazoxide, but was not sensitive to 300 μM of 5-hydroxydecanoate at 37°C. Since we were not able to detect any ATP- or diazoxide-induced change of mitochondrial respiration in choline chloride medium without K^+ (Fig. 3), we have made an assumption that these alterations in mitochondrial respiration rate are related to K^+ flux. Similar effects of diazoxide were observed by other authors (Kowaltowski *et al.*, 2001) on TMPD- and ascorbate-supported mitochondrial respiration. The ATP-induced decrease in respiration rate was attributed to mitochondrial K_{ATP} channel closure, the diazoxide-induced increase—to the opening of mitochondrial K_{ATP} channel (Kowaltowski *et al.*, 2001). CAT effectively blocked the effects of ATP and diazoxide on the respiration rate (Fig. 3), suggesting that ANT also mediates the K^+ flux to mitochondrial matrix, induced by K_{ATP} channel openers.

ANT is a key energetic link between the mitochondria and cytosol, since it catalyzes the transmembrane exchange between ATP generated inside mitochondria by oxidative phosphorylation and cytosolic ADP (see for reviews Fiore *et al.*, 1998). In the early 1980s, several publications (Dragunova *et al.*, 1981; Panov *et al.*, 1980), demonstrated that ANT also controls the permeability of the mitochondrial inner membrane to K^+ and H^+. Beavis' group (Beavis *et al.*, 1993) also found that K^+ uptake and H^+ flux was reduced by the ANT inhibitor CAT. It was suggested (Panov *et al.*, 1980) that ANT acts as a specific, nucleotide-regulated channel for K^+ and H^+, allowing the passive diffusion of these ions through the inner membrane of mitochondria down the electrochemical gradient. These findings, taken together with our results, suggest that ANT by itself could be the crucial target of K_{ATP} channel openers. Since ANT is a major component of the mitochondrial inner membrane, the reduction of the K^+ and H^+ fluxes by ANT inhibitors might be due to prevention of conformation changes of ANT and thereby of mitochondrial membrane perturbation and associated cation leaks.

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