

Direct Effects of K_{ATP} Channel Openers Pinacidil and Diazoxide on Oxidative Phosphorylation of Mitochondria *in situ*

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Key Words

Mitochondria *in situ* • Oxidative phosphorylation • K_{ATP} channel openers • Skinned cardiac fibers • Pinacidil • Diazoxide • Uncoupling

Abstract

K_{ATP} channel openers protect ischemic-reperfused myocardium by mimicking ischemic preconditioning, however, the protection mechanisms have not been fully clarified yet. Since the skinned fibers technique gives an opportunity to investigate an entire population of mitochondria in their native milieu, in this study we have investigated the effects of K_{ATP} channel openers pinacidil and diazoxide on the respiration rate of rat heart mitochondria *in situ*, oxidizing physiological substrates pyruvate and malate (6+6 mM). Respiration rates were recorded by the means of Clark-type oxygen electrode in the physiological salt solution (37°C). Our results showed that both pinacidil and diazoxide (60-1250 μ M) in a concentration-dependent manner increased pyruvate-malate supported State 2 respiration rate of skinned cardiac fibers (59.1 ± 5.1 nmol O/min/mg fiber dry weight, RCI 2.6 ± 0.2 , $n=4$) by 15-120%. Moreover, diazoxide did not affect, whereas pinacidil (60-1250 μ M) decreased the State 3 respiration rate of skinned cardiac fibers (116.6 ± 13.6 nmol O/min/mg fiber dry

weight, RCI 2.3 ± 0.2 , $n=4$) by 4-27%. Thus, common effect for both K_{ATP} channel openers is uncoupling of pyruvate and malate oxidizing mitochondria in skinned cardiac fibers, whereas pinacidil under same conditions also inhibits mitochondrial respiratory chain. Since mitochondria *in situ* resemble to the great extent mitochondria *in vivo*, our results suggest that uncoupling and/or respiratory chain inhibition could play a role in the cardioprotection by K_{ATP} channel openers.

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Introduction

The ability of ATP-sensitive potassium (K_{ATP}) channel openers to protect heart from ischemic damage in the manner similar to naturally occurring phenomenon-ischemic preconditioning, has led to extensive studies of these compounds (for reviews, see [1-5]). The K_{ATP} channel openers-induced cardioprotection is supposed to be mediated by their interactions with mitochondria which are the main ATP-generating sites in the cell. However, the mechanism of action of K_{ATP} channel openers has not been completely elucidated yet. It is evident that K_{ATP} channel openers not only increase K^+ flux to mitochondrial matrix [6, 7] but also directly affect oxidative

phosphorylation in mitochondria [8-10].

Main currently proposed mechanisms of cardioprotection by K_{ATP} channel openers include: (a) mitochondrial swelling [6, 7, 11], resulting in increased fatty acid oxidation, mitochondrial respiration and ATP production [12]; (b) changes in ROS level [13-16]: enhanced ROS production during early phase of ischemia which leads to activation of signal transduction pathways and decreased ROS production during reperfusion which reduces injuries of oxidative stress; (c) inhibition of ATP hydrolysis during ischemia [17, 18] resulting in decreased Ca^{2+} uptake and preserved ATP in the cells; (d) respiratory inhibition [8, 10, 19, 20] which triggers ROS signalling pathway; (e) mild uncoupling [9, 11, 21, 22], resulting in stimulation of mitochondrial respiration, oxidation of mitochondrial matrix flavoproteins, suppressed ROS production and reduced Ca^{2+} uptake to mitochondrial matrix.

Most investigations on the direct effects of K_{ATP} channel openers on mitochondrial functions are done on isolated mitochondria. However, mitochondrial isolation leads to the damage of mitochondrial connections with intracellular structures (cytoskeleton, contractile apparatus, membraneous systems). The skinned fibers technique [23] gives an opportunity to investigate an entire population of mitochondria in their native milieu. Therefore, we have investigated the effects of K_{ATP} channel openers pinacidil and diazoxide on the respiration rate of rat heart mitochondria *in situ*, oxidizing physiological NAD-dependent substrates pyruvate and malate.

Materials and Methods

Isolation of skinned cardiac fibers and heart mitochondria

The experiments were carried out on skinned cardiac fibers and mitochondria isolated from male Wistar rat hearts. The use of rats was in accordance with the European Community Council Directive 86/609/EEC and approved by the Ethics Commission on Animal Experiments of Kaunas University of Medicine.

For the isolation of skinned cardiac fibers hearts were excised and rinsed in ice-cold 0.9% KCl solution. The bundles of cardiac fibers, approximately 0.3-0.4 mm in diameter, were prepared by using sharp-ended needles from the muscle strips cut out from the left ventricular endocardium in ice-cold preparation solution containing 20 mM imidazole, 20 mM taurine, 0.5 mM dithiothreitol, 7.1 mM $MgCl_2$, 50 mM 2-(N-Morpholino)ethanesulfonic acid (MES), 5 mM ATP, 15 mM phosphocreatine, 2.62 mM CaK_2EGTA and 7.38 mM K_2EGTA (ionic strength of the solution 160 mM, free Ca^{2+} 0.1 μM , free

Mg^{2+} 3 mM; pH 7.0, adjusted with KOH), then permeabilized by saponin (50 $\mu g/ml$, 30 min), washed for 10 min in a physiological salt solution containing 20 mM imidazole, 20 mM taurine, 0.5 mM dithiothreitol, 1.61 mM $MgCl_2$, 100 mM MES, 3 mM KH_2PO_4 , 2.95 mM CaK_2EGTA and 7.05 mM K_2EGTA (ionic strength of the solution 160 mM, free Ca^{2+} 0.1 μM , free Mg^{2+} 1 mM; pH 7.1, adjusted with KOH). All procedures were carried out under intensive shaking (120 times/min). The washed bundles of fibers were rinsed once in the physiological salt solution, transferred into the tubes with the same solution and kept on ice.

Mitochondria were isolated by differential centrifugation procedure. Hearts were excised and rinsed in ice-cold isolation medium, containing 220 mM mannitol, 70 mM sucrose, 5 mM N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic 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×g, then supernatant was centrifugated for 10 min at 6740×g and the pellet was washed once in the isolation medium without BSA, suspended in it and kept on ice. The mitochondrial protein concentration was determined by biuret method [24]. The final mitochondrial protein concentration in all experiments was 0.5 mg/ml.

Oxygen consumption assays

Respiration rates of isolated mitochondria and skinned cardiac fibers oxidizing pyruvate and malate (6+6 mM) were determined in physiological salt solution (20 mM imidazole, 20 mM taurine, 0.5 mM dithiothreitol, 1.61 mM $MgCl_2$, 100 mM MES, 3 mM KH_2PO_4 , 2.95 mM CaK_2EGTA and 7.05 mM K_2EGTA (ionic strength of the solution 160 mM, free Ca^{2+} 0.1 μM , free Mg^{2+} 1 mM; pH 7.1, adjusted with KOH)) at 37°C by the means of the Clark-type oxygen electrode. Where indicated, respiration rates of isolated mitochondria 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). Respiration rates were expressed as nmol O/min/mg mitochondrial protein or fibers' dry weight. Dry weight = wet weight before respiration measurement/factor 'W'. The factor 'W' was calculated to be 4.85 for heart muscle fibers [25]. The solubility of oxygen was taken to be 422 nmol O/ml at 37°C [26].

Statistical analysis

The results are presented as means \pm S.E. of 4 independent experiments. Statistical analysis was performed using paired Student's t-test and $P < 0.05$ was taken as the level of significance. Where missing, error bars were smaller than symbol size.

Results and Discussion

In saponin-skinned cardiac fibers the intracellular structures (cytoskeleton, contractile apparatus, membraneous systems) are well preserved and the

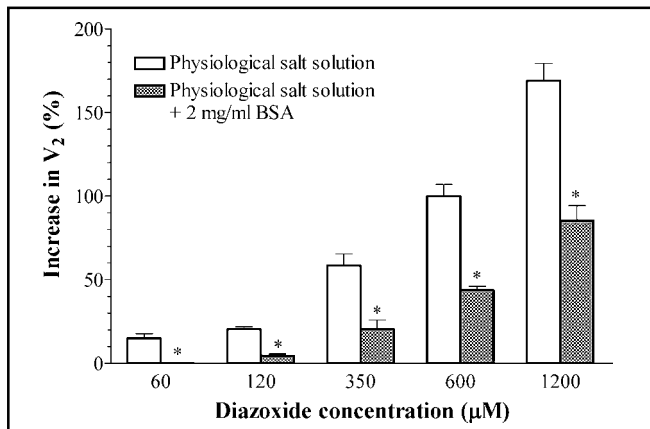


Fig. 1. Influence of BSA on the effect of diazoxide on mitochondrial respiration rate in the State 2. Effect of diazoxide on the State 2 respiration rate (V_2) was expressed in % of initial respiration rate, which was 95.7 ± 7.8 nmol O/min/mg protein (RCI 4.7 ± 0.1) in physiological salt solution and 75.1 ± 5.8 nmol O/min/mg protein (RCI 6.1 ± 0.6) in physiological salt solution, supplemented with BSA. Experiments were performed at 37°C , substrate-pyruvate and malate (6+6 mM), $n=4$. * $p<0.05$ vs control.

morphological appearance of mitochondria is similar to that one *in vivo* [23]. However, there is very few data about the influence of K_{ATP} channel openers on the functions of mitochondria *in situ*, respiring on physiological NAD-dependent substrates [27]. Therefore in this study we investigated the effects of pinacidil and diazoxide on skinned cardiac fibers, oxidizing pyruvate and malate.

Influence of physiological salt solution components on the effects of K_{ATP} channel openers

The respiration rate of skinned cardiac fibers is measured in physiological salt solution, that to the great extent mimics the ionic contents of the muscle cell cytoplasm [23, 25, 27]. The components of physiological salt solution did not influence the uncoupling effect of diazoxide (data not shown). However, BSA at the concentration of 2 mg/ml significantly decreased the uncoupling effect of diazoxide (Fig. 1). This is important to note since in many studies BSA is commonly used as a supplement of mitochondrial incubation medium to achieve higher respiratory control indexes [28, 29].

Uncoupling of oxidative phosphorylation by K_{ATP} channel openers

Both pinacidil and diazoxide (60–1250 μM) in a concentration-dependent manner increased pyruvate-malate supported State 2 respiration rate of skinned

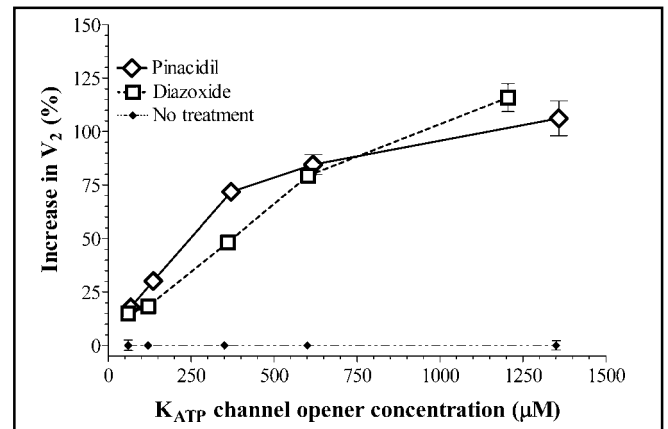


Fig. 2. Effect of K_{ATP} channel openers on the respiration rate of skinned cardiac fibers in the State 2. Effect of pinacidil and diazoxide on the State 2 respiration rate (V_2) was expressed in % of initial respiration rate, which was 59.1 ± 5.1 nmol O/min/mg fiber dry weight (RCI 2.6 ± 0.2). Experiments were performed in physiological salt solution at 37°C , substrate-pyruvate and malate (6+6 mM), $n=4$.

cardiac fibers by 15–120% (Fig. 2). However, it was reported in one study that 100 μM diazoxide had no effect on glutamate-malate supported respiration rate of mitochondria *in situ* [27]. A more detailed analysis suggest that the authors could not notice the effect of diazoxide due to the presence of 2 mg/ml BSA [27], capable to bind diazoxide [30]. Regardless of the results of their study, we clearly demonstrated that K_{ATP} channel openers pinacidil and diazoxide starting from 60 μM uncouple oxidative phosphorylation of mitochondria *in situ* respiring on physiological NAD-dependent substrates pyruvate-malate (Fig. 2) in a K^+ -independent way [21]. Furthermore, this uncoupling was not abolished by the K_{ATP} channel blocker 5-hydroxydecanoate (5–500 μM) which had no effects on the pyruvate-malate supported State 2 respiration rate of skinned cardiac fibers neither under control conditions nor in the presence of the tested K_{ATP} channel openers (data not shown).

In the State 2 mitochondrial respiration rate to the great extent depends on the passive proton flux through the mitochondrial inner membrane [31]. Based on the principles of linear nonequilibrium thermodynamics, it was proposed that the optimal efficiency of oxidative phosphorylation could be reached when mitochondria are slightly uncoupled [32]. Moreover, small decrease in mitochondrial membrane potential can prevent ROS formation in mitochondria [33]. Enhanced fatty acid transport and mild mitochondrial uncoupling due to activation of the adenine nucleotide translocator occurred

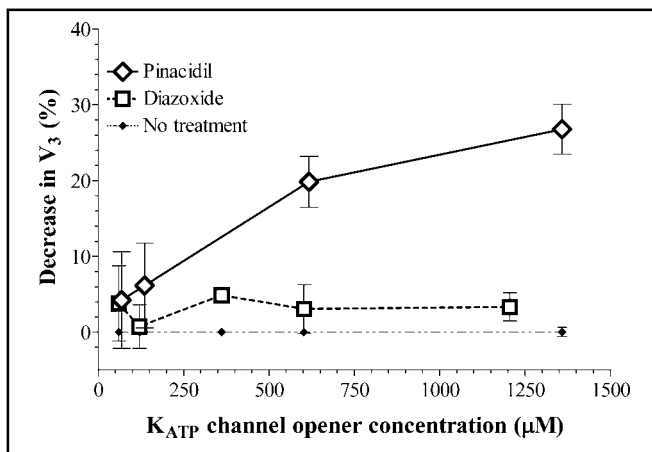


Fig. 3. Effect of K_{ATP} channel openers on the respiration rate of skinned cardiac fibers in the State 3. Effect of pinacidil and diazoxide on the State 3 respiration rate (V_3) was expressed in % of maximal respiration rate, which was 116.6 ± 13.6 nmol O/min/mg fiber dry weight (RCI 2.3 ± 0.2). Experiments were performed in physiological salt solution at 37°C , substrate-pyruvate and malate (6+6 mM) + 1 mM ADP, $n=4$.

during ischemic preconditioning [34]. Furthermore, pharmacological uncoupling of mitochondrial oxidation from phosphorylation was shown to be cardioprotective in the isolated rat heart [35, 36] and in isolated mitochondria [9, 22]. Since mitochondria *in situ* resemble to the great extent mitochondria *in vivo* [23], our results suggest that the cardioprotection by K_{ATP} channel openers *in vivo* could be mediated by the uncoupling of oxidative phosphorylation.

Effects of K_{ATP} channel openers on mitochondrial respiratory chain

Pyruvate and malate oxidation rate in the State 3 mainly depends on the activity of mitochondrial respiratory chain and ATP synthase [37]. Diazoxide did not affect, whereas pinacidil (60–1250 μM) decreased the State 3 respiration rate of skinned cardiac fibers by 4–27% (Fig. 3). For the detailed investigation of this inhibition we have tested the effect of pinacidil on the State 3 respiration rate of isolated rat heart mitochondria oxidizing pyruvate and malate in potassium chloride medium and choline chloride medium without K^+ . The results showed that the effect of pinacidil remained the same in the medium without K^+ (Fig. 4). Thus, the decrease of mitochondrial respiration rate in the State 3 by pinacidil is not related to pinacidil-mediated K^+ flux to mitochondrial matrix, and could be due to inhibition of mitochondrial respiratory chain.

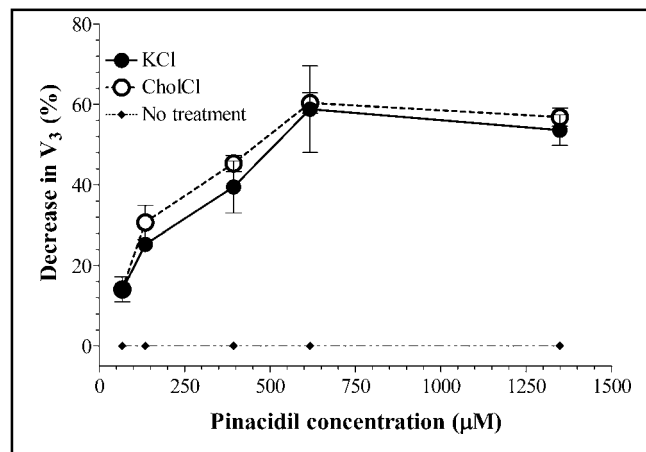


Fig. 4. Effect of pinacidil on the state 3 respiration rate of isolated rat heart mitochondria. Effect of pinacidil on the State 3 respiration rate (V_3) was expressed in % of maximal respiration rate, which was 482.3 ± 55.5 nmol O/min/mg protein (RCI 7.5 ± 0.8) in KCl medium and 476.7 ± 33.7 nmol O/min/mg protein (RCI 5.7 ± 0.2) in choline chloride medium; $n=4$.

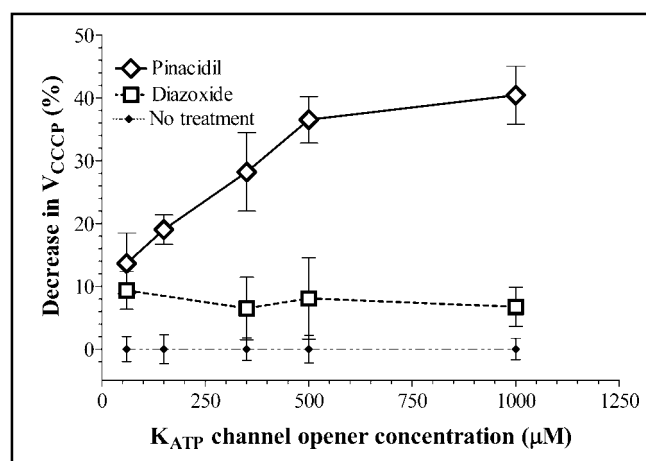


Fig. 5. Effect of K_{ATP} channel openers on the uncoupled respiration rate of isolated rat heart mitochondria. Effect of pinacidil and diazoxide on the uncoupled respiration rate (V_{CCCP}) was expressed in % of uncoupled respiration rate, which was 307.3 ± 30.0 nmol O/min/mg protein (RCI 8.8 ± 0.3). Experiments were performed in KCl medium at 37°C , substrate-pyruvate and malate (6+6 mM) + 0.3 μM CCCP, $n=4$.

Respiratory inhibition was also proposed as one of the possible mechanisms of cardioprotection by K_{ATP} channel openers [8, 10]. Since in the presence of uncoupler mitochondrial respiration rate completely depends on the activity of the respiratory chain, we tested the effect of pinacidil and diazoxide on the carbonyl-cyanide-m-chlorophenylhydrazone (CCCP) uncoupled pyruvate-malate supported respiration rate of isolated rat heart mitochondria. Although diazoxide inhibits succinate-

supported mitochondrial respiration [8, 10, 20, 27, 38], it did not influence uncoupled mitochondrial respiration rate (Fig. 5). In contrast, pinacidil in a concentration-dependent manner (starting from 60 μ M) decreased uncoupled pyruvate-malate supported respiration rate of isolated rat heart mitochondria. These results are in line with findings that pinacidil inhibits pyruvate and malate-induced ATP production in mitochondria isolated from cardiomyocytes and pancreatic β -cells [39] and oxidation of external NADH in submitochondrial particles [8].

Physiological implications

Many compounds which open K_{ATP} channels in the cell plasma membrane have been shown to increase ATP-sensitive K^+ flux through mitochondrial inner membrane [5, 40, 41]. Although molecular identity of mitochondrial K_{ATP} channels has not been entirely determined yet, recent results have shown that a purified inner mitochondrial membrane fraction containing five proteins: succinate dehydrogenase, ATP-synthase, phosphate carrier, mitochondrial ATP-binding cassette protein 1 and adenine nucleotide translocase (ANT) confers mitochondrial K_{ATP} channel activity [42]. Furthermore, the K_{ATP} channel openers were demonstrated to have direct effects on the oxidative phosphorylation in mitochondria [7, 18, 27, 43, 44]. Several investigations have also shown that pharmacological uncoupling of mitochondrial oxidation from phosphorylation [35, 36], as well as mitochondrial respiratory inhibition-triggered ROS signaling [10] promotes preconditioning-like cardioprotection in the heart. Altogether these results imply that the K_{ATP} channel opener-induced cardioprotection could occur via mitochondrial pathway and effects exerted by K_{ATP} channel openers could have

impact on the total cellular energy turnover.

Our results show that in contrast to diazoxide, pinacidil not only uncouples pyruvate and malate oxidizing mitochondria *in situ*, but also inhibits the mitochondrial respiratory chain. These effects could be detected both in isolated mitochondria and in skinned cardiac fibers, and did not depend on the presence of potassium ions in the incubation medium.

Since moderate mitochondrial uncoupling prevents excessive ROS generation [33] and Ca^{2+} overload [45], and mitochondria *in situ* resemble to the great extent mitochondria *in vivo* [23], our results suggest that the cardioprotection by K_{ATP} channel openers *in vivo* could be mediated by the uncoupling of oxidative phosphorylation and/or by inhibition of mitochondrial respiratory chain.

Abbreviations

BSA (bovine serum albumin); MES (2-(N-Morpholino)ethanesulfonic acid); TES (N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid); RCI (respiratory control index); CCCP (carbonyl-cyanide-m-chlorophenylhydrazone).

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