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K_{ATP} Channel Openers Have Opposite Effects on Mitochondrial Respiration under Different Energetic Conditions

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Abstract: Mitochondrial (m) K_{ATP} channel opening has been implicated in triggering cardiac preconditioning. Its consequence on mitochondrial respiration, however, remains unclear. We investigated the effects of two different K_{ATP} channel openers and antagonists on mitochondrial respiration under two different energetic conditions. Oxygen consumption was measured for complex I (pyruvate/malate) or complex II (succinate with rotenone) substrates in mitochondria from fresh guinea pig hearts. One of two mK_{ATP} channel openers, pinacidil or diazoxide, was given before adenosine diphosphate in the absence or presence of an mK_{ATP} channel antagonist, glibenclamide or 5-hydroxydecanoate. Without ATP synthase inhibition, both mK_{ATP} channel openers differentially attenuated mitochondrial respiration. Neither mK_{ATP} channel antagonist abolished these effects. When ATP synthase was inhibited by oligomycin to decrease [ATP], both mK_{ATP} channel openers accelerated respiration for both substrate groups. This was abolished by mK_{ATP} channel blockade. Thus, under energetically more physiological conditions, the main effect of mK_{ATP} channel openers on mitochondrial respiration is differential inhibition independent of mK_{ATP} channel opening. In contrast, under energetically less physiological conditions, mK_{ATP} channel opening can be evidenced by accelerated respiration and blockade by antagonists. Therefore, the effects of mK_{ATP} channel openers on mitochondrial function likely depend on the experimental conditions and the cell's underlying energetic state.

Keywords: heart, mitochondria, cardiac preconditioning, ion channels, state, ischemia

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Introduction

Opening of mitochondrial (m) adenosine triphosphate (ATP) sensitive K^+ channels has been postulated to be a key component of the signaling mechanism of ischemic and pharmacologic preconditioning of the myocardium.[1–3] This is primarily based on observations that transient administration of mK_{ATP} channel openers, such as diazoxide, elicits a memory effect that lasts beyond their elimination and attenuates subsequent ischemia/reperfusion (IR) injury in different models. Moreover, the nonspecific K_{ATP} channel antagonist glibenclamide, as well as 5-hydroxydecanoic acid (5-HD), a putative mK_{ATP} channel antagonist, abolish ischemic and pharmacologic preconditioning with different agents.[4–6]

In most of these studies, conclusions are derived from the assessment of IR injury on reperfusion and, therefore, solely rely on the specificity for the mK_{ATP} channel of the drugs given before ischemia. However, not only have these specificities recently been more and more questioned,[7–11] there is also an ongoing debate as to the putative effect of mK_{ATP} channel opening on mitochondrial function in general. Liu et al,[12] for example, argue that mK_{ATP} channel opening leads to accelerated electron transport and, therefore, a net oxidation of the mitochondrial electron transport chain (ETC); this was shown by increased fluorescence of oxidized flavoprotein in resting myocytes by the mK_{ATP} channel opener diazoxide. However, using a more physiological, intact beating heart model, we were unable to reproduce these findings; in fact, we observed decreased rather than increased oxidation with different known and putative mK_{ATP} channel openers.[13–15] This is supported by Garlid et al,[2] who oppose the idea of mild uncoupling by mK_{ATP} channel opening and state that the critical effect of mK_{ATP} channel opening is the regulation and maintenance of mitochondrial matrix volume during ischemia.

We hypothesized that these opposing results and seemingly mutually exclusive theories in the literature could possibly be unified and explained by their different underlying experimental conditions, that is, the energetic state of the cells and their mitochondria. The objective of this study was, therefore, to assess the effects of commonly used K_{ATP} channel openers on mitochondrial respiration, but under different energetic conditions within the same model. To test our

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hypothesis, we compared the effects of different mK_{ATP} channel openers and blockers on the rate of O_2 consumption in isolated cardiac mitochondria under energetically more physiological conditions versus those under energetically less physiological conditions produced by ATP synthase inhibition.

Methods

All investigations conformed to the Guide for the Care and Use of Laboratory Animals (U.S. National Institutes of Health no. 85-23, revised 1996) and were approved by the institutional animal care and use committee (Medical College of Wisconsin, Milwaukee, Wisc). Thirty milligrams of ketamine and 1000 units of heparin were injected intraperitoneally into 20 albino English short-haired guinea pigs (250–300 g). Animals were decapitated 15 minutes later, when unresponsive to noxious stimulation. After thoracotomy, the heart was immediately taken out and immersed in 4°C cold isolation buffer[15,16]: 200 mM mannitol, 50 mM sucrose, 5 mM KH_2PO_4, 1 mM EGTA, 5 mM MOPS, and 0.1% bovine serum albumin; pH 7.15 adjusted with KOH. The atria were discarded, and the ventricles were minced into 1-mm pieces. The tissue was rinsed, transferred to a glass Potter–Elvehjem homogenizing vessel on ice, and gently homogenized with a Teflon pestle (DuPont, Wilmington, Del) for 30 seconds in the presence of 1 mg/mL of protease. This was followed by another 30 seconds of homogenization after 10-fold dilution of the protease. Mitochondria were then isolated by differential centrifugation at 4°C.[17] The tissue suspension was centrifuged at 8000g for 10 minutes to remove the protease. The resulting pellet was then resuspended in 28-mL isolation buffer, and the suspension was centrifuged at 700g for 10 minutes to remove cellular debris. The supernatant containing the mitochondrial fraction was further centrifuged at 8000 g for 10 minutes. The pellet was resuspended in 7-mL isolation buffer without EGTA and was centrifuged at 8000 g for 10 minutes. The final mitochondrial pellet was resuspended in 500-μL cold isolation buffer without EGTA. Total protein concentration was determined[18] with bovine serum albumin as a standard. Anatomic integrity of isolated mitochondria was verified by electron microscopy in random studies.

Measurement of Mitochondrial Oxygen Consumption

The 500-μL mitochondrial suspension was kept at 4°C. Immediately before each experiment, an aliquot of the concentrated mitochondria was added to 27°C respiration buffer[15,16,19] to yield 500 μL with a concentration of 500 μg of protein per milliliter. The buffer contained 110 mM KCl, 5 mM $K_2HPO_4 \cdot 3H_2O$, 10 mM MOPS, 10 mM Mg-acetate, 1 mM EDTA, 1 μmM tetrasodium pyrophosphate, and 0.1% bovine serum albumin; pH 7.15 adjusted with KOH. The low concentration of acetate was added to improve mitochondrial function and facilitate K^+ transport and matrix volume adjustments.[20] Although it may serve as a potential mitochondrial substrate,[21] it does not cause uncoupling at this concentration.[20]

Mitochondria from one heart were sufficient for approximately 15 experiments on average. [O_2] was measured polargraphically with a Clark-type oxygen electrode (model 1302, Strathkelvin Instruments, Glasgow, Scotland) in a water-jacketed 500-μl chamber (Model MT200A, Strathkelvin Instruments) equipped with a Teflon-coated magnetic stirring bar and monitored by an oxygen meter (Model 782, Strathkelvin Instruments). The oxygen electrode was calibrated with air-saturated water ($pO_2 \approx 150$ mm Hg) and sodium sulphite (Na_2SO_3) solution to achieve near-zero pO_2 at the same temperature as the buffer to be used. Rate of mitochondrial respiration was determined as the maximum rate of [O_2] decrease after addition of substrate and adenosine diphosphate (ADP) to initiate oxidative phosphorylation.[22] Data were stored online on a computer using the manufacturer's software (Strathkelvin Instruments). Microsoft Excel (Microsoft Corporation, Redmond, Wash) software was used for later analysis.

Experimental Protocol

After sealing the chamber with a plexiglass plug (time t = 0 minutes), drugs, substrates, and ADP (5 μL each) were subsequently injected into the chamber according to the protocol displayed in Figure 1. The time intervals in the experimental protocol have been successfully used in previous studies[15,23] and were carefully chosen after extensive preliminary experiments to ensure sufficient time for each drug to exhibit its full effect. All final drug concentrations are provided in Table 1. To test for possible antagonism, the mK_{ATP} channel blocker 5-HD,[4] the nonspecific K_{ATP} channel blocker

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glibenclamide,[5] or their vehicle with or without the ATP synthase inhibitor oligomycin (see below) were added at t = 1 minute. Pyruvate and malate, or succinate with the complex I blocker rotenone to prevent reverse electron flow,[24] were added at t = 2 minutes as substrates for complex I or for complex II of the ETC, respectively. The mK_{ATP} channel opener pinacidil or diazoxide or their vehicle was added at t = 3 minutes to test for drug-induced alterations of mitochondrial respiration. In additional experiments, 2,4-dinitrophenol (DNP) as an uncoupler, or antimycin A as a blocker of complex III of the ETC, were given at t = 3 minutes to verify mitochondrial function and to assess the degree of maximal uncoupling and maximal blockade of mitochondrial respiration in our model (Fig. 2). ADP was added at t = 4 minutes. All drugs were purchased from Sigma (St. Louis, Mo). Chamber $[O_2]$ in micromoles per liter was monitored for up to 12 minutes or until it approached zero. All experiments were performed at 27°C. Experiments with mitochondria from the same heart were randomized to one of the above treatment groups with at least three control experiments interspersed. All respiration rates from experiments of one heart were normalized and expressed as percent change compared with the average of control experiments from the same heart.

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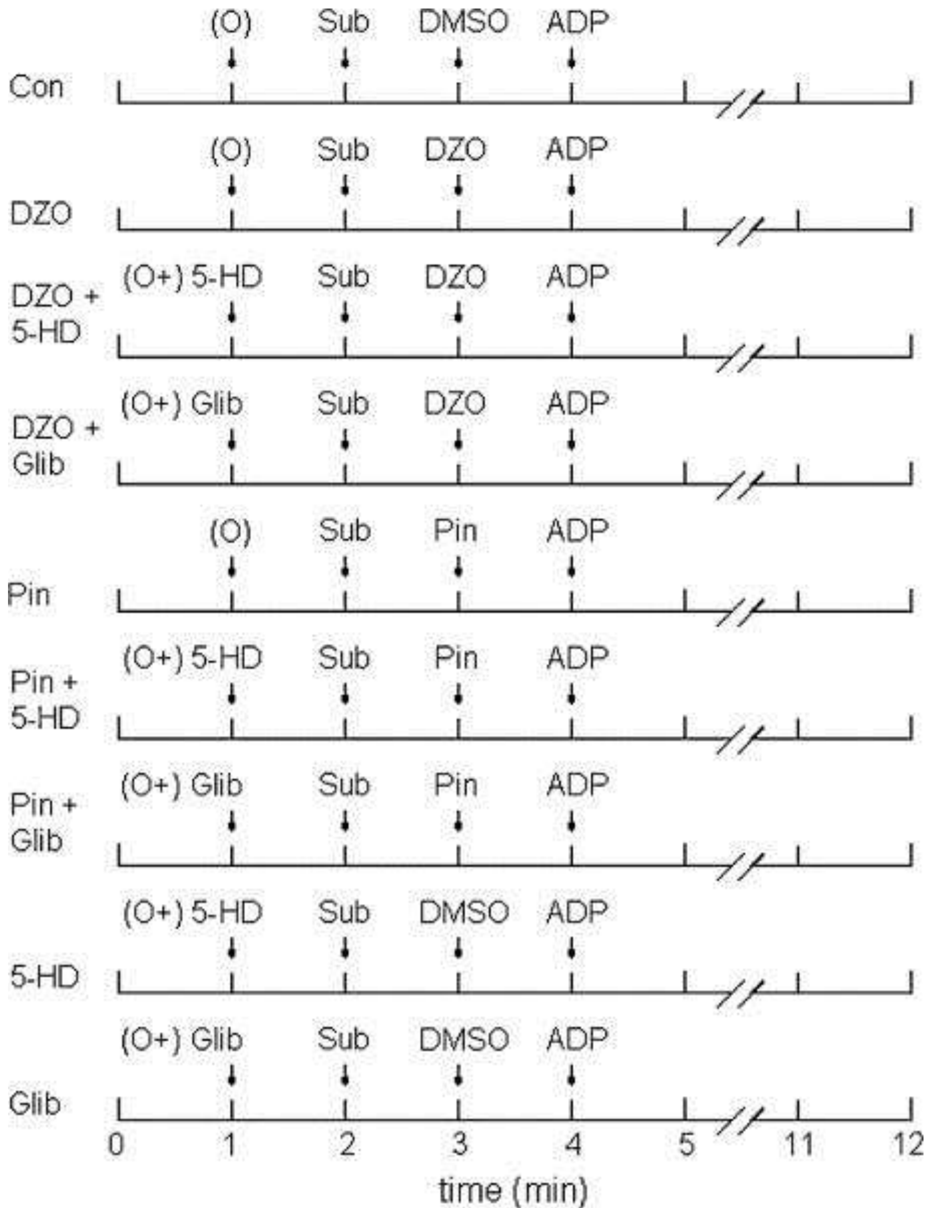

FIGURE 1 Experimental protocol. After stabilization of the mitochondrial suspension for 1 minute, the mitochondrial K_{ATP} channel inhibitor 5-hydroxydecanoic acid (5-HD), the non-specific K_{ATP} channel inhibitor glibenclamide (Glib), or their respective vehicle (buffer) was injected into the chamber with or without the ATP synthase inhibitor oligomycin (O). Substrate (Sub) for complex I (pyruvate and malate) or for complex II (succinate with rotenone to block complex I) was added at t = 2 minutes. The K_{ATP} channel openers diazoxide (DZO) or pinacidil (Pin) or their vehicle (dimethyl sulfoxide, DMSO) were given at t = 3 minutes. Adenosine diphosphate (ADP) was added at t = 4 minutes. Each drug or substrate was given as a 5-μL bolus into the 500-μL chamber to yield the final concentrations given in Table 1. Chamber $[O_2]$ in micromoles was monitored for up to 12 minutes or until it approached zero. All experiments were performed at 27°C.

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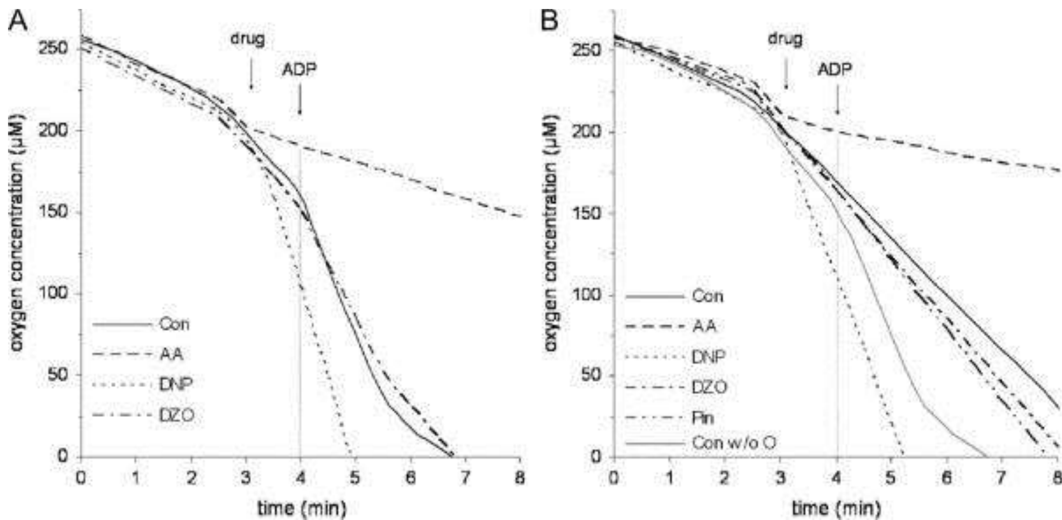

FIGURE 2 Original sample tracings of oxygen consumption when succinate was given as substrate for complex II in the presence of rotenone to block complex I. The complex III blocker antimycin A (AA), the uncoupler 2,4-dinitrophenol (DNP), the mK_{ATP} channel opener diazoxide (DZO), or vehicle (Con; plain DMSO) were given at t = 3 minutes (drug) before ADP was added at t = 4 minutes (dotted vertical line). Note the difference in slope (ie, mitochondrial respiration) after t = 4 minutes between blockade by antimycin A and uncoupling by DNP regardless of ADP addition. A, Diazoxide caused a slightly slower respiration after ADP addition compared with control in the absence of ATP synthase inhibition with oligomycin. B, In addition to control, antimycin A, DNP, and diazoxide, a tracing of the mK_{ATP} channel opener pinacidil (Pin) at t = 3 minutes (drug) is shown in the presence of the ATP synthase inhibitor oligomycin (O). Note the decreased rate of respiration in the control run compared with a control without (w/o) oligomycin after t = 4 minutes as well as the lack of a slowing of respiration secondary to complete phosphorylation of ADP to ATP in the absence of oligomycin after t = 5 minutes. Both pinacidil and diazoxide caused a slight increase in respiration compared with control after t = 4 minutes. All drug concentrations are listed in Table 1.

TABLE 1. Substrates and Drugs

	Abbreviation	Concentration	Dissolved in	Function
Adenosine diphosphate	ADP	250 µM	Buffer	ATP synthase substrate
Antimycin A	AA	100 µM	DMSO	Complex III blocker
Diazoxide	DZO	30 µM	DMSO	K_{ATP} channel opener
Dinitrophenol	DNP	100 µM	DMSO	Uncoupler
Glibenclamide	Glib	2 µM	Buffer	K_{ATP} channel antagonist
5-Hydroxydecanoate	5-HD	300 µM	Buffer	K_{ATP} channel antagonist
Malate	M	10 mM	Buffer	Complex I substrate
Oligomycin	O	100 µM	DMSO	ATP synthase blocker
Pinacidil	Pin	100 µM	DMSO	K_{ATP} channel opener
Pyruvate	P	10 mM	Buffer	Complex I substrate
Rotenone	R	10 µM	DMSO	Complex I blocker
Succinate	S	10 mM	Buffer	Complex II substrate

DMSO, dimethyl sulfoxide.

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State 3 Versus State 4 Respiration

Under energetically more physiological conditions, that is, in the absence of ATP synthase inhibition by oligomycin, addition of ADP initiates the transition to so-called "state 3" respiration.[22] In short, the energy from mitochondrial electron transport along the ETC is used to actively pump protons against their gradient into the intermembrane space, which contributes to the mitochondrial membrane potential ($\Delta\psi_m$) that is then used by the ATP synthase to actively phosphorylate ADP to ATP. Mitochondrial respiration, $\Delta\psi_m$, and phosphorylation are coupled and in a steady state (↔, Table 2). When ADP is completely phosphorylated to ATP (so-called "state 4" respiration[22]), ATP synthase activity is decreased because of a lack of ADP as substrate. This increases $\Delta\psi_m$ and attenuates respiration indirectly. However, we chose to induce "state 4" conditions by pharmacological inhibition with oligomycin rather than to use regular "state 4" respiration by ADP depletion, because this approach allowed us to selectively block ATP synthase and while comparing respiration rates at the same time intervals under otherwise similar experimental conditions, that is, oxygen concentrations, equilibration times, etc. In addition, this approach enabled us to keep the ATP/ADP ratio low (< 0.3 in our model as assessed with HPLC) and, in that way, mimic an energetically less physiological state better than a regular "state 4" with its higher (>50) ATP/ADP ratio. "State 3" and the two different "state 4" conditions are compared in Table 2.

TABLE 2. Comparison of Different Mitochondrial Respiration States

	State 3	State 4 *Without* ATP Synthase Inhibition	State 4 *With* ATP Synthase Inhibition
ATP/ADP ratio	↔	↑↑	↓
Membrane potential	↔	↑	↑
Electron transport	↔	↓	↓

↔ Steady state; ↑ increase compared with state 3; ↓ decrease compared with state 3.

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Statistical Analysis

All data were expressed as means ± standard errors of the means (SEM). Group data were compared by analysis of variance to determine significance (Super ANOVA 1.11 software for Macintosh from Abacus Concepts, Berkeley, Calif). If F values ($P < 0.05$) were significant, post hoc comparisons of means tests (Student–Newman–Keuls) were used to compare the groups. Differences among means were considered statistically significant when $P < 0.05$ (two tailed). Statistical symbols used were * versus Con, † versus DZO, # versus DZO + 5-HD, § versus DZO + Glib, and ‡ versus Pin.

Results

Control experiments without ATP synthase inhibition revealed functionally intact mitochondria with "state 3" O_2 consumptions (nmol $O_2 \cdot mg^{-1}$ protein$\cdot min^{-1}$) of 107.8 ± 12.8 and 193.6 ± 12.0 and with respiratory control indices of 3.2 ± 0.2 and 2.4 ± 0.1 for complex I and complex II substrates, respectively. Original sample tracings of O_2 chamber concentrations with complex II substrate are shown in Figure 2. Panel A depicts typical O_2 tracings after addition of the complex III blocker antimycin A, the uncoupler DNP, or the K_{ATP} channel opener diazoxide compared with a control experiment without ATP synthase inhibition. In contrast, panel B shows antimycin A, DNP, diazoxide, and the K_{ATP} channel opener pinacidil compared with a control experiment after ATP synthase inhibition with oligomycin, and one control experiment without ATP synthase inhibition.

In the absence of oligomycin to inhibit ATP synthase, the mK_{ATP} channel antagonists 5-HD and glibenclamide had no effect on respiration for either complex I or complex II substrates when given alone (Fig. 3A and B). Diazoxide did not alter respiration when complex I substrates (pyruvate and malate; panel A) were given, but it decreased respiration by about 10% when succinate with rotenone was given as a substrate for complex II (panel B). In contrast, pinacidil decreased respiration by about 20% when complex I substrates were given (panel A), but it had no effect when complex II substrate was given (panel B). Neither of these effects was prevented by mK_{ATP} channel blockade (panels A and B). In comparison, antimycin

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A decreased respiration by 50.2 ± 3.5%* and 78.8 ± 3.5%* for complex I and II substrates, respectively, whereas DNP increased respiration by 35.6 ± 18.4%* and 28.9 ± 11.5%*, respectively.

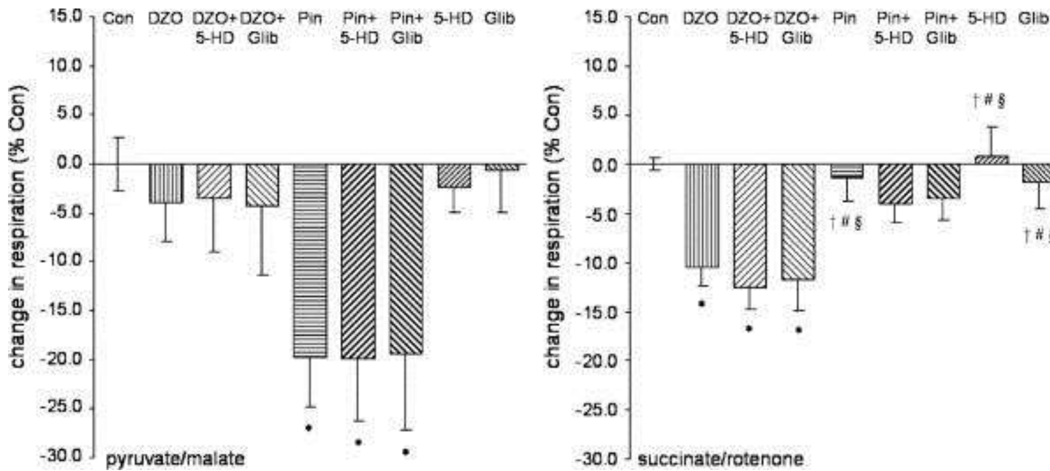

FIGURE 3 Percent change in mitochondrial respiration from control levels (Con) by the K_{ATP} channel openers diazoxide (DZO) and pinacidil (Pin) and by the K_{ATP} channel inhibitors 5-hydroxydecanoic acid (5-HD) and glibenclamide (Glib) when pyruvate and malate were given as substrate for complex I (panel A) or when succinate (with rotenone to block complex I) was given as substrate for complex II (panel B). in absence of the ATP synthase inhibition. All values are given as means and SEM; $P <$ 0.05 *versus Con, †versus DZO, #versus DZO+5-HD, §versus DZO+Glib, and ‡versus Pin; n = 7 experiments per experimental group. All drug concentrations are listed in Table 1.

At the selected concentration, the ATP synthase inhibitor oligomycin attenuated, but did not completely inhibit, mitochondrial respiration, for both complex I and complex II substrates: control experiments with oligomycin exhibited a 16.0 ± 4.6%* lower respiration rate for pyruvate/malate and a 9.5 ± 2.8%* lower rate for succinate/rotenone. In the presence of the ATP synthase inhibitor, both K_{ATP} channel openers increased respiration for either substrate group by 7% to 10% (Fig. 4A and B). For complex I substrates, both mK_{ATP} channel antagonists reversed both K_{ATP} channel agonist–induced increases in respiration back to control levels (panel A). For complex II substrate, both mK_{ATP} channel antagonists reversed the pinacidil-induced increase back to control levels, whereas in the presence of diazoxide, glibenclamide led to a decrease even below control levels (panel B). In comparison, DNP increased respiration by 54.5 ± 15.1%* and 79.7 ± 15.9%* for complex I and II substrates, respectively,

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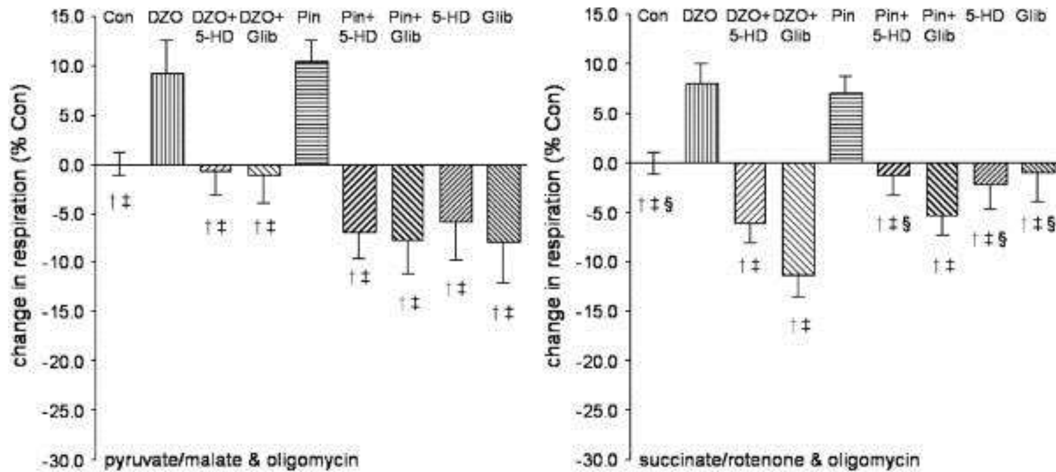
whereas antimycin A decreased respiration by 85.9 ± 1.6%* and 85.1 ± 1.3%*, respectively, under these conditions.



DZO + Glib, and ‡versus Pin; n = 7 experiments per experimental group. All drug

FIGURE 4 Percent change in mitochondrial respiration from control levels (Con) by the K_{ATP} channel openers diazoxide (DZO) and pinacidil (Pin) and by the K_{ATP} channel inhibitors 5-hydroxydecanoic acid (5-HD) and glibenclamide (Glib) when pyruvate and malate were given as substrate for complex I (panel A), or when succinate (with rotenone to block complex I) was given as substrate for complex II (panel B). in the *presence* of the ATP synthase inhibitor oligomycin. All values are given as means and SEM; $P < 0.05$ *versus Con, †versus DZO, #versus DZO+5-HD, § versus DZO + Glib, and ‡versus Pin; n = 7 experiments per experimental group. All drug concentrations are listed in Table 1.

Discussion

Results from this study in isolated cardiac mitochondria indicate that (a) K_{ATP} channel openers produce differential effects on mitochondrial function, and (b) these effects depend on the mitochondrial energy state. Under energetically more physiological conditions, the K_{ATP} channel openers diazoxide and pinacidil attenuated mitochondrial respiration: pinacidil inhibited complex I, whereas diazoxide inhibited complex II. These inhibitory effects were independent of mK_{ATP} channel opening. In contrast, under energetically less physiological conditions, that is, when ATP synthase was pharmacologically inhibited, both K_{ATP} channel openers accelerated mitochondrial respiration, which seemed to be mediated by mK_{ATP} channel opening.

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mK_{ATP} Channel Opening and Cardioprotection

K_{ATP} channels were first identified in 1983 by Noma[25] in membrane patches prepared from guinea pig myocytes. Since then, they have also been shown to exist in various other tissues and seem to consist of several subtypes. K_{ATP} channels are composed of two distinct proteins, an inwardly rectifying K^+ channel and a sulfonylurea receptor, which may have a regulatory role as well as a function in modulating the sensitivity of the channel to ATP, other nucleotides, and pharmacological agonists and antagonists.[26] Two types of K_{ATP} channels have been postulated to exist in the cell, a sarcolemmal (s) channel, whose structure has been delineated, and a putative channel in the inner mitochondrial membrane, the mK_{ATP} channel.[27] Although the mK_{ATP} channel has been characterized pharmacologically in cells and in isolated lipid bilayers, it has not been cloned, and its exact molecular structure has not been fully elucidated.[28] In fact, the very existence of the mK_{ATP} channel has been questioned[8,29] and, thus, is a matter of considerable controversy.

Cardioprotection by drugs believed to be K_{ATP} channel openers is well established. Nineteen years ago, cromakalim and pinacidil,[30] and subsequently other K_{ATP} channel openers,[31] were found to be protective in perfused rat hearts. Initially, it was believed that sK_{ATP} channel opening was responsible for this cardioprotection because it shortened the action potential duration, thereby reducing Ca^{2+} entry to the cytosol. However, it was shown later that cardioprotection was preserved in conditions without shortening of the action potential duration[32] and that selective pharmacological sK_{ATP} channel inhibition had no effect on infarct size after IR or on preconditioning.[33]

Garlid et al[34] provided the first evidence to support a role for the mK_{ATP} channel in cardioprotection. They found that mK_{ATP} channels in lipid bilayers were 1000 to 2000 times more sensitive to diazoxide than were sK_{ATP} channels. Furthermore, diazoxide, at low concentrations that did not activate the sK_{ATP} channel, had a pronounced cardioprotective effect in isolated hearts. This effect was abolished by 5-HD and glibenclamide, suggesting that the mK_{ATP} channel, rather than the sK_{ATP} channel, may be responsible for this cardioprotection.

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However, it is still unclear whether mK_{ATP} channel opening acts as a trigger or a distal effector in pharmacologic preconditioning, or both. As a trigger, mK_{ATP} channels would have to open under physiological conditions before ischemia and lead to activation of downstream signaling pathways of preconditioning. In contrast, if mK_{ATP} channel opening was an effector of preconditioning, these signaling pathways would contribute to mK_{ATP} channel opening during energetically less physiological conditions such as IR and, thus, afford protection.

IR impairs mitochondrial function through an alteration of $\Delta\psi_m$, electron transport, and increased ROS production. Pharmacological K_{ATP} channel opening inhibited ischemia-induced depletion of high-energy phosphates, which was abolished by glibenclamide; it was proposed that mK_{ATP} channel opening may partially restore the $\Delta\psi_m$, allowing further extrusion of H^+, forming a more favorable electrochemical gradient for ATP synthesis.[35]

How Does mK_{ATP} Channel Opening Affect Mitochondrial Function?

Despite all this evidence, there is considerable disagreement as to the exact mechanism by which mK_{ATP} channel opening alters mitochondrial function. On one side, Marban and colleagues have argued that opening of any mitochondrial K^+ channel in the inner mitochondrial membrane, including the mK_{ATP} channel, would tend to dissipate $\Delta\psi_m$ established by the proton pump.[3,12] This dissipation would accelerate electron transfer by the ETC, lead to a net oxidation in the mitochondrial matrix, and "uncouple" oxidative phosphorylation. Consequently, autofluorescent measurements of the mitochondrial redox state have become an increasingly popular tool to assess mK_{ATP} channel opening in isolated myocytes.[12,36–38]

Alternatively, the concept of uncoupling by mK_{ATP} channel opening is opposed by Garlid and colleagues,[2] who contend that the critical effect resulting from mK_{ATP} channel opening is the regulation and maintenance of mitochondrial matrix volume.[39] Decreased $\Delta\psi_m$, for instance, during ischemia, would lead to decreased mitochondrial matrix volume, resulting in decreased and less efficient electron

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transport and ATP synthesis.[40] Increased K^+ conductance by mK_{ATP} channel opening and concomitant uptake of weak acids and water by osmotic forces[34] would counteract this volume decrease and help maintain a constant matrix volume, permitting a more efficient energy transfer between mitochondria and cellular ATPases.[41,42]

Garlid and colleagues[2] further argue that a K^+ influx sufficient to cause significant uncoupling would cause massive matrix swelling and rupture the mitochondrial inner membrane under physiological conditions. Therefore, at least under energetically more physiological conditions, uncoupling by mK_{ATP} channel opening would not occur, and the fact that accelerated electron transport and net oxidation by mK_{ATP} channel openers was observed in several studies may merely be attributable to artificial study conditions.[2,43] Our findings agree with those of Garlid et al[2]: experiments in intact beating hearts[14] have revealed dose-dependent increases in reduced nicotinamide adenine dinucleotide fluorescence by pinacidil and decreases in oxidized flavin adenine dinucleotide fluorescence by diazoxide. This reduced mitochondrial redox state could be produced by attenuated electron transport secondary to inhibition of complex I or II of the ETC, respectively, rather than accelerated electron transport, as would have been expected for mK_{ATP} channel opening. Furthermore, these inhibitory effects were not prevented by mK_{ATP} channel blockers.

In the present study, by measuring the rate of O_2 consumption in isolated cardiac mitochondria, we used a different approach to complement and confirm these findings. Under energetically more physiological conditions with sufficient substrate as electron donor, O_2 as electron acceptor, and ADP to allow oxidative phosphorylation, we found diazoxide and pinacidil to differentially attenuate electron transport at complexes I and II, respectively, independent of mK_{ATP} channel opening; in contrast, accelerated respiration as an indication of mK_{ATP} channel opening could not be observed under these conditions (Fig. 3).

Inhibitory Side Effects of K_{ATP} Channel Openers

The selective inhibition of complex I and complex II of the ETC by pinacidil and diazoxide, respectively, confirms earlier[44–46] and more recent[7,8,10,11,39,47] reports of mK_{ATP} channel–independent inhibitory

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effects of these drugs on the ETC in mitochondria of various cell types. In fact, it was known long ago that the hydrophobic sites of the mitochondrial ETC are sensitive to hydrophobic agents[48]; this may offer a relatively simple explanation for the otherwise paradoxical observation of ETC inhibition by putative mK_{ATP} channel openers. Interestingly, we find very similar results for NS1619, a mitochondrial Ca^{2+} sensitive K^+ channel opener, that also causes a mild attenuation of mitochondrial respiration under energetically more physiological conditions, whereas respiration is accelerated under energetically less physiological conditions[23]; similarly, the acceleration, but not attenuation, was blocked by the mK_{Ca} channel blocker paxilline.

Mitochondrial Energetic State is an Important Determinant of K_{ATP} Channel Opening

Energetically less physiological "state 4" conditions impede mitochondrial respiration indirectly by inhibiting protons from reentering the mitochondrial matrix via ATP synthase and, thus, increasing $\Delta\psi_m$, either because of a shortage of ADP or, as achieved in this study, by pharmacological ATP synthase inhibition. Under conditions of increased $\Delta\psi_m$, any form of ion leakage, as with pharmacological uncoupling or mK_{ATP} channel opening, would be expected to result in a robust increase in electron transport and O_2 consumption. Under conditions of pharmacological ATP synthase inhibition, ADP phosphorylation to ATP, and, therefore, the ATP/ADP ratio, are decreased (Table 2), which would be expected to favor opening of the mK_{ATP} channels even more, because they are normally kept closed by a high ATP/ADP ratio.

Indeed, in this isolated mitochondrial model, a mild, indirect attenuation of mitochondrial respiration by ATP synthase inhibition was sufficient to profoundly change the observed effects of the two mK_{ATP} channel openers. Under these conditions, both mK_{ATP} channel openers accelerated mitochondrial respiration. Reversal of their effects by two different K_{ATP} channel antagonists is consistent with mK_{ATP} channel opening. So, within the same model, and under identical conditions except for an altered energetic state, we were able to change the effect of known and putative[49] mK_{ATP} channel openers from differential ETC attenuation and slowed respiration toward accelerated respiration

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by mK_{ATP} channel opening (Fig. 4). These findings emphasize the crucial importance of a lower mitochondrial energy state for mK_{ATP} channels to open, and they help to explain previous observations of increased mitochondrial oxidation, that is, respiration, by mK_{ATP} openers such as diazoxide[12] or volatile anesthetics[37] in isolated resting myocytes that were performed or cultured in substrate-free solutions.[2,43]

Drawing any further conclusions from these findings remains challenging. On the one hand, in favor of a pharmacological approach,[2] one can conclude that mK_{ATP} openers indeed open mK_{ATP} channels under both energetically more physiological and energetically less physiological conditions, and that mK_{ATP} channel opening simply has only a negligible effect on mitochondrial respiration under physiological conditions. As an alternative conclusion, one can conclude that K_{ATP} channel openers are ineffective under physiological conditions if K_{ATP} channel opening is primarily a function of the cell's energetic state, that is, its ATP/ADP ratio, especially in the vicinity of the channels, and mK_{ATP} channel openers may act, for instance, by reducing the ATP affinity of the channel.

Although we cannot safely rule out such possibilities as the presence of restricted spaces preventing instantaneous equilibration of ATP, ADP, or mK_{ATP} channel openers near the channels in vivo or in our model, this latter conclusion would go along with the notion of other investigators: the less physiological, the earlier K_{ATP} channels open, and any potential opener[36,50,51] shifts this opening to more physiological states and, in this way, "sensitizes" or "primes" the channels to open earlier and to a greater extent under energetically less physiological conditions such as ischemia.

A Further Lack of Specificity of K_{ATP} Channel Openers and Antagonists

Conclusions about mK_{ATP} channel involvement derived from pharmacological studies are further complicated by recent reports[11,52–54] that diazoxide mildly uncouples respiration even in the absence of K^+, raising the possibility of mK_{ATP} channel–independent iono- and protonophoric effects of putative mK_{ATP} channel openers that may be

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(partly) responsible for their cardioprotective effect. Similar findings have recently been reported for the mK_{Ca} channel opener NS1619.[55]

In addition, the specificities of not only openers, but also of antagonists of mK_{ATP} channels and other intracellular signaling components, for instance, PKCε,[56] are now more and more questioned. For example, it was recently suggested that 5-HD, as a fatty acid, could be converted to 5-HD-CoA in the presence of CoA, ATP, and fatty acyl CoA synthetase.[7–9] 5-HD could then be further metabolized and serve as a substrate that feeds electrons into the ETC at the level of coenzyme Q, thus providing a bypass for ETC sites that are attenuated by lipophilic drugs such as K_{ATP} channel openers[7,39] or volatile anesthetics.[6,57] K_{ATP} channel–independent effects have also been described for glibenclamide. For example, it inhibits carnitine palmitoyltransferase activity[58] and, at higher concentrations, Cl^- channels,[59] whereas permeabilization of the mitochondrial membrane to Cl^- may contribute to mitochondrial depolarization.[60]

Alternative explanations of cardioprotection by K_{ATP} channel openers also include ETC inhibition of complex I[61] or II,[62] and the activation of the adenine nucleotide translocase,[63]possibly even as part of a multiprotein complex that contains complex II and ATP synthase.[64] All of these findings clearly reinforce the notion that any conclusion as to the mechanisms of action of a certain drug has to rely on its pharmacological specificity. In this particular case, an observed effect associated with administration of a putative mK_{ATP} channel opener or its blockade by a potential antagonist does not necessarily furnish direct evidence of mK_{ATP} channel involvement (for a more detailed review, see Hanley and Daut[43]). We need to be aware of the "pleiotropic" character of these drugs, and, ideally, we have to confirm any findings by using more than one model as well as a variety of chemically different drugs with different chemical profiles to strengthen our conclusions.

Conclusion

In summary, the finding of differential effects of mK_{ATP} channel openers on mitochondrial function under different energetic conditions underscores and reemphasizes the importance of the chosen experimental model and its physiological condition when studying

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mitochondria and helps explain some of the contradictory data in literature.

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