Potassium channel openers are uncoupling protonophores: implication in cardioprotection

Ekhson L. Holmuhamedov[a], Arshad Jahangir[a], Andrew Oberlin[a], Alexander Komarov[b,1], Marco Colombini[b], Andre Terzic[a,*]

[a] *Division of Cardiovascular Diseases, Departments of Medicine, Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic College of Medicine, Guggenheim 7, Rochester, MN 55905, USA*
[b] *Department of Biology, University of Maryland, College Park, MD 20742, USA*

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Abstract Excessive build-up of mitochondrial protonic potential is harmful to cellular homeostasis, and modulation of inner membrane permeability a proposed countermeasure. Here, we demonstrate that structurally distinct potassium channel openers, diazoxide and pinacidil, facilitated transmembrane proton translocation generating H^+-selective current through planar phospholipid membrane. Both openers depolarized mitochondria, activated state 4 respiration and reduced oxidative phosphorylation, recapitulating the signature of mitochondrial uncoupling. This effect was maintained in K^+-free conditions and shared with the prototypic protonophore 2,4-dinitrophenol. Diazoxide, pinacidil and 2,4-dinitrophenol, but not 2,4-dinitrotoluene lacking protonophoric properties, preserved functional recovery of ischemic heart. The identified protonophoric property of potassium channel openers, thus, implicates a previously unrecognized component in their mechanism of cardioprotection.

Keywords: Uncoupling; Mitochondrion; Diazoxide; Pinacidil; Cardioprotection; Ischemia-reperfusion; Heart

1. Introduction

Coupling of aerobic oxidation with mitochondrial ATP production proceeds through respiration-dependent generation of electrochemical H^+ gradient ($\Delta\mu H$), the driving force of ADP phosphorylation [1,2]. While $\Delta\mu H$ is a prerequisite for oxidative phosphorylation, high $\Delta\mu H$ is associated with production of reactive oxygen species, a potentially detrimental byproduct of mitochondrial electron transfer [3,4]. Recent findings suggest that a regulated increase in H^+ permeability of the inner mitochondrial membrane could serve to offset excessive build-up of protonic potential [5]. In fact, the inner mitochondrial membrane harbors uncoupling proteins that facilitate H^+ translocation and contribute to the overall regulation of proton fluxes in mitochondria [6]. Genetic deletion of uncoupling proteins produces aberrant phenotypes with augmented reactive oxygen species generation, while overexpression reduces free radical formation and confers increased protection against metabolic challenge [7–11]. These emerging evidences implicate regulation of the mitochondrial protonic potential in cellular tolerance to injury, yet little is known on cytoprotective agents capable to modulate mitochondrial H^+ permeability.

In this regard, a class of cardioprotective potassium channel openers, exemplified by diazoxide and pinacidil, has been recently shown to target and depolarize the mitochondrial inner membrane, and to preserve mitochondrial structural and functional integrity under stress [12–15]. Indeed, hearts treated with these agents demonstrate a favorable energetic profile with limited damage following stress challenge [16,17]. Here, we identify a previously unrecognized ability of potassium channel openers to promote transmembrane H^+-translocation underscoring a role for protonophoric uncoupling in protection against injury.

2. Materials and methods

2.1. Mitochondrial function

Intact mitochondria were isolated from pentobarbital-anesthetized rat hearts. Respiration, membrane potential and oxidative phosphorylation were measured by polarography, potentiometry and chromatography, respectively, as described [18,19]. NADH-O_2 oxidoreductase activity in permeabilized mitochondria was monitored from NADH absorbance and verified using sodium cyanide, an inhibitor of cytochrome *c* oxidase.

2.2. Proton transfer

Proton translocation was monitored from ^3H flux in a Pressman's U-tube in the presence of charge-compensating tetraphenyl borate/tertramethyl ammonia [20,21] and from H^+-selective current across a voltage-clamped planar phospholipid membrane [22].

2.3. Cardioprotection

Hearts isolated from anesthetized (75 mg/kg) and heparinized (500 units/animal) rats were retrogradely perfused with Krebs–Henseleit buffer on a Langendorff apparatus. A fluid-filled balloon-tipped pressure transducer (Harvard Apparatus) was used to record left ventricular developed pressure [23]. Hearts were subjected to 45 min of global non-flow ischemia and re-perfused for an additional 45 min.

2.4. Drugs and chemicals

Diazoxide and pinacidil were from RBI (Natick, MA), ^3H_2O from Amersham Biosciences (Piscataway, NJ), sodium tetraphenyl borate and tertramethylammonium chloride from ICN (Costa Mesa, CA).

* Corresponding author. Fax: +1-507-284-9111.
E-mail address: terzic.andre@mayo.edu (A. Terzic).

[1] Present address: St. Petersburg Nuclear Physics Institute, Gatchina 188350, Russia.

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2,4-Dinitrophenol and 2,4-dinitrotoluene were from Sigma (St. Louis, MO).

2.5. Data analysis

Data are expressed as means ± S.E.M. and comparison between groups is performed using the Student's *t*-test or ANOVA with a post hoc correction procedure. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Potassium channel openers uncouple mitochondria

Diazoxide (100 μM; $n = 21$) and pinacidil (100 μM; $n = 12$) accelerated mitochondrial state 4 respiration from 43 ± 1 to 70 ± 2 ng-atoms O_2/min/mg protein and from 39 ± 3 to 75 ± 4 ng-atoms O_2/min/mg protein, respectively ($P < 0.05$; Fig. 1A). Concomitantly, diazoxide and pinacidil reduced mitochondrial membrane potential (from −201 ± 1 to −194 ± 2 mV and from −200 ± 2 to −189 ± 2 mV, $P < 0.05$; Fig. 1B) and diminished ATP production (from 487 ± 15 to 419 ± 12 nmoles ATP/min/mg protein and from 495 ± 27 to 273 ± 9 nmoles ATP/min/mg protein, $P < 0.05$; Fig. 1C). In this way, potassium channel openers recapitulated typical features of mitochondrial uncoupling established with 2,4-dinitrophenol (5 μM), the conventional mitochondrial uncoupler (Fig. 1A–C).

Opener-induced uncoupling was independent from the respiratory substrate or the activity of the electron transporting chain and required an intact mitochondrial membrane (Fig. 1D–F). Although only diazoxide inhibited succinate oxidation, both openers depolarized mitochondria oxidizing either succinate or pyruvate/malate (Fig. 1D and E *insets*). Moreover, both pinacidil and diazoxide activated pyruvate/malate supported respiration in intact mitochondria (Fig. 1D and E), but inhibited NADH oxidation in permeabilized mitochondria (Fig. 1F).

Modulation of mitochondrial K^+ permeability [24–28] was not a prerequisite for opener-induced uncoupling. Substitution of KCl with NaCl or LiCl partially reduced, but did not abolish activation of respiration, mitochondrial depolarization and reduction of ATP synthesis in the presence of diazoxide or pinacidil (Fig. 1G–I). Diazoxide (100 μM) increased state 4 respiration by 25 ± 3 and 21 ± 4 ng-atoms O_2/min/mg protein in NaCl ($n = 5$) and LiCl ($n = 6$), respectively (Fig. 1G). Pinacidil (100 μM) increased respiration by 29 ± 4 and 30 ± 2 ng-atoms O_2/min/mg protein in NaCl ($n = 6$) and LiCl ($n = 10$), respectively (Fig. 1H). The depolarizing effect was 7 ± 1 and 6 ± 1 mV for diazoxide, and 12 ± 1 and 10 ± 1 mV for pinacidil (Fig. 1H), in NaCl and LiCl, respectively. ATP synthesis in diazoxide- and pinacidil-treated mitochondria was reduced by 13% and 11% in NaCl, and by 37% and 39% in LiCl ($n = 3$; Fig. 1I). In contrast, valinomycin, a K^+ ionophore, lost its ability to activate respiration, depolarize mitochondria, and reduce ATP synthesis upon substitution of KCl with NaCl or LiCl (not illustrated). Moreover, 5-hydroxydecanoic acid

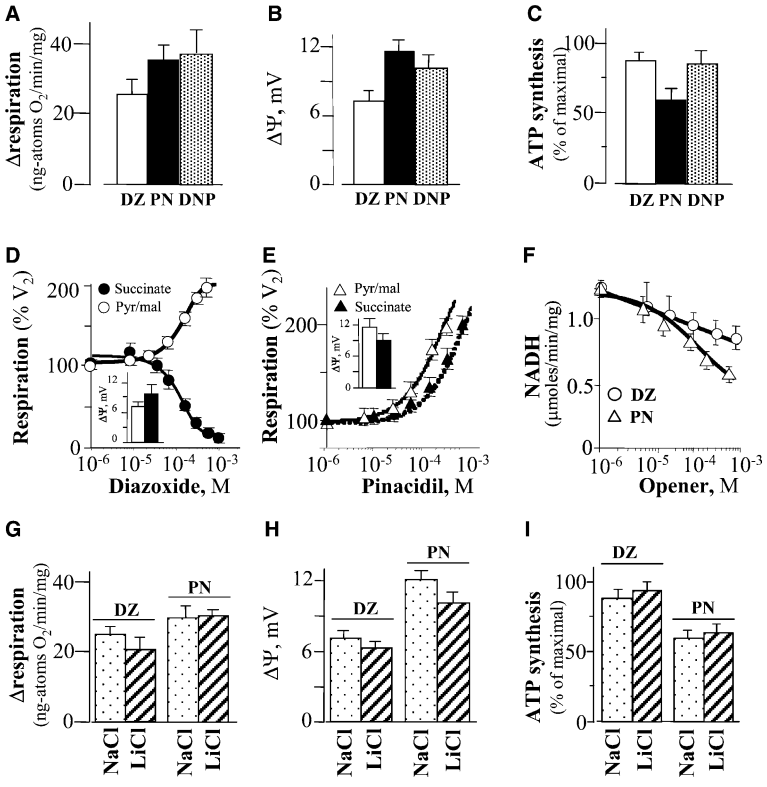

Fig. 1. Potassium channel openers induce mitochondrial uncoupling. (A–C) Diazoxide (100 μM, DZ), pinacidil (100 μM, PN) and 2,4-dinitrophenol (5 μM, DNP) activated state 4 respiration (A), depolarized mitochondria (B) and reduced ATP synthesis (C). (D–F) Pyruvate/malate- (*open*) or succinate-supported (*closed*) respiration in the presence of diazoxide (D) or pinacidil (E). *Insets*: Opener-induced depolarization of mitochondria oxidizing pyruvate/malate (*open*) or succinate (*filled*). (F) Effect of diazoxide (*circles*) and pinacidil (*triangles*) on NADH oxidation in permeabilized mitochondria. (G–I) Maintained opener-induced activation of respiration (G), membrane potential (H) and ATP synthesis (I) in intact mitochondria oxidizing pyruvate/malate following KCl substitution with NaCl or LiCl.

(5-HD), a blocker of the mitochondrial K_{ATP} channel [12,13,27,28], had no effect on opener-mediated uncoupling (not illustrated). Thus, diazoxide- and pinacidil-induced mitochondrial uncoupling was K^+-independent.

3.2. Diazoxide and pinacidil are protonophores

Diazoxide (100 μM) and pinacidil (100 μM) increased ^3H flux across chloroform layers, from a baseline of 2 ± 2 $^3H/h/cm^2$ to 10 ± 2 and 13 ± 2 $^3H/h/cm^2$, respectively ($n = 6$ each, $P < 0.01$), comparable to the protonophoric action of 2,4-dinitrophenol (Fig. 2A and B). The ability of openers to facilitate ^3H transfer was further confirmed by the generation of H^+-selective current in planar phospholipid membranes (Fig. 2C and D). In the absence of openers, no current was recorded across membranes separating media with different pH (Fig. 2C). Addition of pinacidil (Fig. 2D, $n = 6$) and diazoxide (not shown, $n = 7$) induced current typical of carrier-mediated transport [22], with a reversal potential at 52.6 ± 0.2 mV (Fig. 2D, $n = 6$) and 51.5 ± 0.1 mV ($n = 7$), respectively. These values closely approximate the theoretical reversal potential of 56.2 mV calculated for the utilized H^+ gradient (pH 7.02 versus 7.98 on *cis* and *trans* sides, Fig. 2C). Opener-induced current was not observed in the absence of H^+ gradient and showed a H^+/K^+ selectivity $>10^5$ (not shown). Thus, pinacidil and diazoxide facilitate H^+ transfer, thereby dissipating transmembrane proton gradient and fulfilling criteria for protonophores.

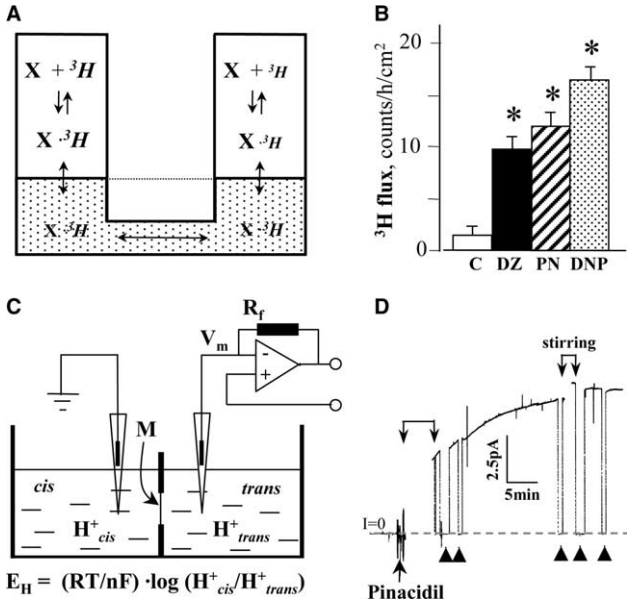

Fig. 2. Potassium channel openers are protonophores. (A) Drug (X)-facilitated diffusion of ^3H across the chloroform-layer (*dotted*) separating aqueous arms in Pressman's U-tube. (B) ^3H flux in the absence (C) and presence of diazoxide (DZ, 100 μM), pinacidil (PN, 100 μM) or DNP (DNP, 5 μM). Stars: $P < 0.05$. (C) Set-up used to measure H^+ current across planar phospholipid membranes (M) with *cis* and *trans* sides buffered at different pH (H^+_{cis} and H^+_{trans}). Reversal membrane potential (E^+_H) expressed by Nernst equation; V_m – applied voltage; R_f – feedback resistor. (D) Transmembrane current prior and following addition of pinacidil (upward arrow). Arrowheads indicate time-points used to determine reversal potential measured as the voltage that nullified opener-mediated current. Opener-induced increase in transmembrane conductance from baseline ($I = 0$) was determined under continuous stirring at a given pH gradient. Voltage was held at 0 mV except at reversal potential measurements.

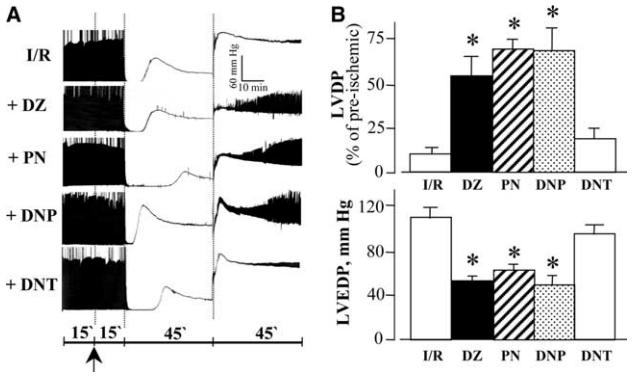

Fig. 3. Protonophores protect ischemic heart. (A) Performance in untreated (I/R), diazoxide- (DZ), pinacidil- (PN), 2,4-dinitrophenol (DNP) or 2,4-dinitrotoluene (DNT)-treated (arrow) hearts after ischemia-reperfusion. (B) Post-ischemic left ventricular developed pressure (LVDP) and left ventricular end-diastolic pressure (LVEDP). Stars: $P < 0.05$.

3.3. Protonophoric uncouplers improve cardiac recovery

Pretreatment with diazoxide, pinacidil or 2,4-dinitrophenol promoted functional recovery and attenuated post-ischemic contracture of hearts at reperfusion (Fig. 3A). In contrast to poor recovery observed in the absence of drug treatment ($<10 \pm 4\%$ of pre-ischemic levels, $n = 6$), left ventricular developed pressure recovered to $53 \pm 12\%$, $68 \pm 5\%$ and $67 \pm 17\%$ in diazoxide-, pinacidil- and dinitrophenol-treated hearts, respectively (Fig. 3B; $n = 6$ each). In parallel, following ischemia/reperfusion left ventricular end-diastolic pressure was 107 ± 5 mmHg in untreated hearts, and was significantly improved to 51 ± 3, 61 ± 4 and 48 ± 7 mmHg in diazoxide-, pinacidil- and dinitrophenol-treated hearts, respectively (Fig. 3B). In contrast, 2,4-dinitrotoluene, a chemical analog of 2,4-dinitrophenol that lacks protonophoric activity due to replacement of the proton-translocating hydroxyl-moiety with a methyl group [29], did not induce myocardial protection (Fig. 3A). In dinitrotoluene-treated hearts ($n = 3$) following ischemia, left ventricular developed (20 ± 5 mmHg) and left ventricular end-diastolic (95 ± 6 mmHg) pressures were not significantly different from untreated controls (Fig. 3A and B). Thus, cardioprotection is associated with agents that manifest properties of protonophoric uncoupling.

4. Discussion

The present study demonstrates that diazoxide and pinacidil, two structurally distinct potassium channel openers, facilitate proton translocation and generate H^+-selective current across membranes. Typical for protonophoric uncouplers, diazoxide and pinacidil activated mitochondrial respiration, depolarized mitochondrial membrane and reduced oxidative phosphorylation. Underscoring the potential benefit of mitochondrial uncoupling, potassium channel openers, along with the prototypic protonophore dinitrophenol, protected ventricular function under ischemia. Demonstrating that potassium channel openers act as protonophores identifies a novel mechanism of action that could contribute to mitochondria-mediated cardioprotection.

While the pharmacodynamics of potassium channel openers could involve multiple targets, including the previously defined

ATP-sensitive K^+ channels [12–15,24–28,30,31] and succinate dehydrogenase [32,33], the present identification of H^+ translocating properties provides a possibly additional component of their action. Diazoxide and pinacidil contain an amide or N-cyanoguanidine moiety, respectively, that can be reversibly protonated/deprotonated in a broad pH range [34,35] offering a physico-chemical basis for protonophoric action. Accordingly, their uncoupling property mandated an intact inner membrane with an inherently low permeability for H^+, and was lost following mitochondrial permeabilization. Opener-induced uncoupling [28,36,37] was insensitive to 5-HD and was maintained in nominally K^+-free medium [38], in line with recently described K^+-independent effects of potassium channel openers on free radical production under metabolic challenge [39] and maintenance of mitochondrial Ca^{2+} homeostasis [40,41]. Indeed, moderate mitochondrial uncoupling prevents excessive reactive oxygen species generation and abnormal mitochondrial Ca^{2+} loading, critical events in ischemic injury [3,10,42]. Protection afforded here by diazoxide and pinacidil, and mimicked by the mitochondrial uncoupler 2,4-dinitrophenol [43] could be in part associated with attenuation of mitochondrial damage following sudden build-up of protonic potential at reperfusion. Thus, cardioprotective potassium channel openers act as protonophores and produce mitochondrial uncoupling providing a novel pharmacological approach aimed at manipulating mitochondria-associated functions through modulation of membrane proton permeability.

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