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Sarcolemmal and mitochondrial effects of a K_{ATP} opener, P-1075, in "polarized" and "depolarized" Langendorff-perfused rat hearts

Olga Jilkina[a], Bozena Kuzio[a], Gary J. Grover[b], Clifford D.L. Folmes[c], Hee-Jeong Kong[d], Valery V. Kupriyanov[a,d,*]

[a] *Institute for Biodiagnostics, National Research Council of Canada, Winnipeg, MB, Canada*
[b] *Cardiovascular and Metabolic Disease Drug Discovery, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ, USA*
[c] *University of Winnipeg, Winnipeg, MB, Canada*
[d] *Department of Biochemistry and Medical Genetics, University of Manitoba, Winnipeg, MB, Canada*

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Abstract

We investigated consequences of cardiac arrest on sarcolemmal and mitochondrial effects of ATP-sensitive potassium channel (K_{ATP}) opener, P-1075, in Langendorff-perfused rat hearts. Depolarised cardiac arrest (24.7 mM KCl) did not affect glibenclamide-sensitive twofold activation of rubidium efflux by P-1075 (5 μM) from rubidium-loaded hearts, but eliminated uncoupling produced by P-1075 in beating hearts: 40% depletion of phosphocreatine and ATP, 50% increase in oxygen consumption, and reduction of cytochrome *c* oxidase. Depolarized cardiac arrest by calcium channel blocker, verapamil (5 μM), also prevented uncoupling. Lack of P-1075 mitochondrial effects in depolarized hearts was not due to changes in phosphorylation potential, because 2,4-dintrophenol (10 μM) reversed the [PCr]/[Cr] increase and P_i decrease, characteristic of KCl-arrest, but did not restore uncoupling. In agreement with this conclusion, pyruvate (5 mM) increased [PCr]/[Cr] and decreased P_i, but did not prevent uncoupling in beating hearts. A decrease in mean $[Ca^{2+}]$ in KCl-arrested hearts could not account for lack of P-1075 mitochondrial effects, because calcium channel opener, *S*-(−)-Bay K8644 (50 nM), and beta-agonist, isoproterenol (0.5 μM), did not facilitate uncoupling. In contrast, in adenosine (1 mM)-arrested hearts (polarized arrest), P-1075 caused 40% phosphocreatine and ATP depletion. In isolated rat liver mitochondria, P-1075 (20 μM) decreased mitochondrial membrane potential ($\Delta\Psi$) by approximately 14 mV (demonstrated by redistribution of $\Delta\Psi$-sensitive dye, rhodamine 800) in a glibenclamide-sensitive manner. We concluded that cell membrane depolarization does not prevent activation of sarcolemmal K_{ATP} by P-1075, but it plays a role in mitochondrial uncoupling effects of P-1075.

Keywords: ATP-sensitive potassium channel; K_{ATP} opener; P-1075; Oxidative phosphorylation; Cytochrome *c* oxidase; Oxygen consumption; Sarcolemmal polarization; Rb^+/K^+ efflux

1. Introduction

Activation of sarcolemmal ATP-sensitive potassium channels (s-K_{ATP}) results in stimulation of K^+ efflux from the cardiomyocytes [1] and sarcolemmal membrane hyperpolarization, when membrane potential is more negative than resting potential; this reduces Ca^{2+} entry and may cause cardiac arrest [2]. It is thought that cardiac arrest in this state preserves intracellular PCr and ATP because of reduced energy turnover [2–6].

Based on patch-clamp experiments in giant fused mitoplasts, it has been proposed that a second type of K_{ATP} is present in the inner mitochondrial membrane [7]. It is believed that activation of mitochondrial K_{ATP} channels (m-K_{ATP}) would result in K^+ influx into mitochondrial matrix, its swelling, activation of K^+/H^+ exchanger, partial mitochondrial membrane potential ($\Delta\Psi$) depolarization and, correspondingly, a decrease in ATP synthesis. Up to now, molecular correlate of m-K_{ATP} has not been determined. However, m-K_{ATP} existence was indirectly confirmed in a number of models measuring: (1) K^+-dependent fluorescence in mitochondrial membrane frac-

* Corresponding author. Institute for Biodiagnostics, National Research Council, 435 Ellice Avenue, Winnipeg, MB, Canada R3B 1Y6. Tel.: +1-204-984-6620; fax: +1-204-984-7036.
E-mail address: Kupriyanov@ibd.nrc-cnrc.gc.ca (V.V. Kupriyanov).

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tion reconstituted in proteoliposomes [8], (2) currents in mitochondrial membrane-enriched lipid bilayers [8–10], (3) mitochondrial swelling [11], (4) uncoupling induced by K_{ATP} openers in isolated mitochondria [12,13] and cardiomyocytes [14], and (5) binding of a K_{ATP} blocker, glibenclamide, to inner mitochondrial membrane [15], although validity of some of the indirect methods has recently been questioned [16–19]. Recently, a sulfonylurea-binding subunit (SUR2) and pore-forming subunits (Kir6.1 and Kir6.2) of s-K_{ATP} were co-localized with mitochondria in rat ventricular myocytes using anti-s-K_{ATP} subunit-specific antibodies [20].

A pinacidil derivative, P-1075 [21], activates recombinant s-K_{ATP} [22,23]. P-1075 also binds with high affinity to myocardial membrane preparations (containing sarcolemmal and mitochondrial membranes) in MgATP-dependent and glibenclamide-sensitive manner [24]. Recently we have demonstrated that P-1075 (5 μM) stimulated efflux of a K^+ congener, rubidium (Rb^+), from Rb^+-preloaded Langendorff-perfused beating rat hearts more than twofold and induced cardiac arrest [25]. However, P-1075 also induced depletion of PCr and ATP, stimulation of respiration, and reduction of cytochrome *c* oxidase, indicating uncoupling of oxidative phosphorylation [25,26]. These sarcolemmal and metabolic effects of P-1075 were glibenclamide- and HMR 1098- (a cardioselective derivative of glibenclamide) [25,26] sensitive. Metabolic uncoupling induced by P-1075 is consistent with activation of putative m-K_{ATP}. However, there is a controversy surrounding intracellular specificity of P-1075. In quiescent rabbit cardiomyocytes, P-1075 did not cause uncoupling, assessed by monitoring oxidation of mitochondrial flavoproteins and, thus, was proposed to have sarcolemmal specificity [27,28]. However, cardiomyocytes in cell culture and in situ are not equivalent in several aspects. In non-beating cardiomyocytes, sarcolemmal membrane potential as well as cytosolic and mitochondrial calcium concentrations do not fluctuate [29], and energetic demands are drastically diminished, resulting in resistance to metabolic inhibition.

We arrested rat hearts to investigate how cessation of heartbeats may affect sarcolemmal and mitochondrial effects of P-1075. Surprisingly, we found that cardiac arrest in a depolarized form (induced by hyperkalemia or verapamil) eliminated mitochondrial effects induced by P-1075, while cardiac arrest in a polarized form (induced adenosine) did not. However, hyperkalemic arrest did not affect activation of Rb^+ efflux by P-1075, implying that s-K_{ATP} were activated in KCl-arrested hearts without having an effect on mitochondrial uncoupling. In addition, in isolated rat liver mitochondria, P-1075 (20 μM) slightly, but significantly, decreased $\Delta\Psi$ by approximately 14 mV (demonstrated by redistribution of a $\Delta\Psi$-sensitive dye, rhodamine 800). This decrease was glibenclamide-sensitive, which is consistent with activation of putative m-K_{ATP}.

2. Methods

The investigation conforms with the "Guide to the Care and Use of Experimental Animals" published by the Canadian Council on Animal Care (Ottawa, ON, 1993).

2.1. Reagents

Glibenclamide, dimethylsulfoxide (DMSO), 2,4-dinitrophenol (DNP), ethylene glycol bis-(β-aminoethyl ether) *N*,*N*,*N*′*N*′-tetraacetic acid (EGTA), DL-glutamic acid, K-gluconate, carbonyl cyanide 4-trifluoromethoxyphenyl hydrazone (FCCP), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), isoproterenol, Mg-ATP, RbCl, sodium pyruvate, succinic acid, and verapamil were purchased from Sigma (St. Louis, MO, USA). Adenosine and *S*-(−)-Bay K8644 were purchased from Research Biochemicals International (Natick, MA, USA). P-1075 was synthesized in Bristol-Myers Squibb (BMS) Pharmaceutical Research Institute laboratories to be used for internal and collaborative use by BMS. Stock solutions of P-1075 and glibenclamide were prepared in DMSO and further diluted in water. Rhodamine 800 (also known as MitoFluor Far Red 680) was purchased from Molecular Probes (Eugene, OR, USA).

2.2. Heart perfusion

Male Sprague–Dawley rats (320–370 g) were anesthetized with pentobarbital solution (120 mg/kg) intraperitoneally. The hearts (1.4–1.7 g) were quickly removed and perfused in a Langendorff mode with phosphate-free Krebs–Henseleit buffer (KHB) containing (in mM): 25 $NaHCO_3$, 118 NaCl, 4.7 KCl, 1.75 $CaCl_2$, 1.2 $MgSO_4$, 0.5 EDTA, and 11 glucose aerated with a mixture of 95% O_2 and 5% CO_2 to keep pO_2 at 500–600 mm Hg and pH at 7.4 at 36 °C. A group of hearts was perfused with high-potassium KHB, in which 20 mM KCl was added. KHB-Rb had the same composition as KHB, except for K^+, which was substituted with Rb^+ by 50%.

Following the placement of a left ventricular apical drain, a latex balloon was inserted through the mitral valve into the left ventricular cavity and filled with H_2O. The balloon was connected to a Statham P23Db pressure transducer and to a Digi-Med Model-210 heart performance analyser (Micro-Med, Louisville, KY, USA) to monitor heart rate (HR), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), and perfusion pressure (PP). Pressure-rate-product (PRP), calculated as developed pressure (LVSP minus LVEDP) multiplied by HR, was used as an index of mechanical work. The coronary flow rate was monitored using an ultrasonic blood flow meter (Transonic Systems Inc. Ithaca, NY, USA), and PP was measured continuously through the catheter connecting the aortic line and the second pressure transducer. Following stabilization period, the hearts were perfused at a

constant flow of 13–15 ml/min to provide the desired concentration of drugs during infusion. Typical baseline parameters were: HR, 270 beats/min (no pacing); LVSP, 100; LVEDP, 7; PP, 70 mm Hg.

2.3. Experimental protocols

The protocols are presented as schematic diagrams on Fig. 1. In all ^{31}P-NMR protocols, three initial 4-min ^{31}P spectra were acquired and used as a baseline. After that, in beating hearts, P-1075 (5 μM) was infused for 20 min (five 4-min ^{31}P-NMR spectra acquired), followed by a 16-min recovery period (four additional ^{31}P-NMR spectra). In the groups of beating hearts treated with P-1075 in combination with other drugs, the drugs (5 μM verapamil, 5 mM pyruvate, 1 mM adenosine) were infused for 8 min prior to P-1075 and then for additional 20 min, simultaneously with P-1075. In experiments involving potassium-arrested hearts, following the acquisition of three baseline spectra, the perfusion was switched to high-potassium KHB and two 4-min spectra were acquired before drug treatment. The drugs (5 μM P-1075, 10 or 50 μM DNP, 50 nM *S*-(−)-Bay K8644, 0.5 μM isoproterenol, or 1 mM adenosine) were infused for 40 min (Fig. 1). No drugs were infused in control hearts.

In ^{87}Rb experiments, all hearts were loaded with Rb^+ by perfusing with KHB-Rb for 30 min. Rb^+ efflux was initiated by switching to a Rb^+-free normokalemic or high-potassium KHB, containing P-1075 (5 μM) or no drugs (Fig. 1).

In optical spectroscopy and oxygen consumption experiments with P-1075, the protocols were similar to those used in ^{31}P-NMR experiments (Fig. 1).

2.4. NMR spectroscopy

NMR experiments were performed using a Bruker AM-360 WB spectrometer equipped with a 20-mm Morris Instruments broadband probe placed in a wide bore vertical 8.4-T magnet. The ^{23}Na signal (95.25 MHz) from the heart and surrounding bath was used for shimming. ^{31}P-NMR spectra were acquired at 145.8 MHz using a 35-μs pulse length (60° flip angle), 1.8-s recycle time and 4-min resolution time. The sweep width was 10 kHz, memory size, 4 K data points, line broadening factor, 20 Hz. Baseline correction and peak amplitude measurements were done using 1D WINNMR (Bruker) computer program. The heights of the PCr and ATP peaks prior to interventions were set as 100%. A capillary containing 10 μl of 1 M solution of phenylphosphonic acid was used as a reference in ^{31}P-NMR protocols. ^{87}Rb-NMR spectra were acquired at 117.8 MHz every 2 min using a spectral sweep width of 18 kHz, a recycle time of 10 ms, and a pulse duration of 55 μs (90° flip angle). Memory size was 512 data points. Line broadening factor was 150 Hz. To minimize the signal from the extracardiac and extracellular ^{87}Rb, a suction line was placed at the bottom of the NMR tube. A 10-μl capillary containing 1 M RbCl and 5 M KI was used as a reference in ^{87}Rb-NMR protocols.

2.5. Kinetics of Rb^+ efflux

Rb^+ efflux rate constant (k) was calculated as described previously [30], as a slope from a linear portion of a semilogarithmic plot (natural logarithm of Rb^+ peak intensity, a.u., versus time, min) using a linear regression method.

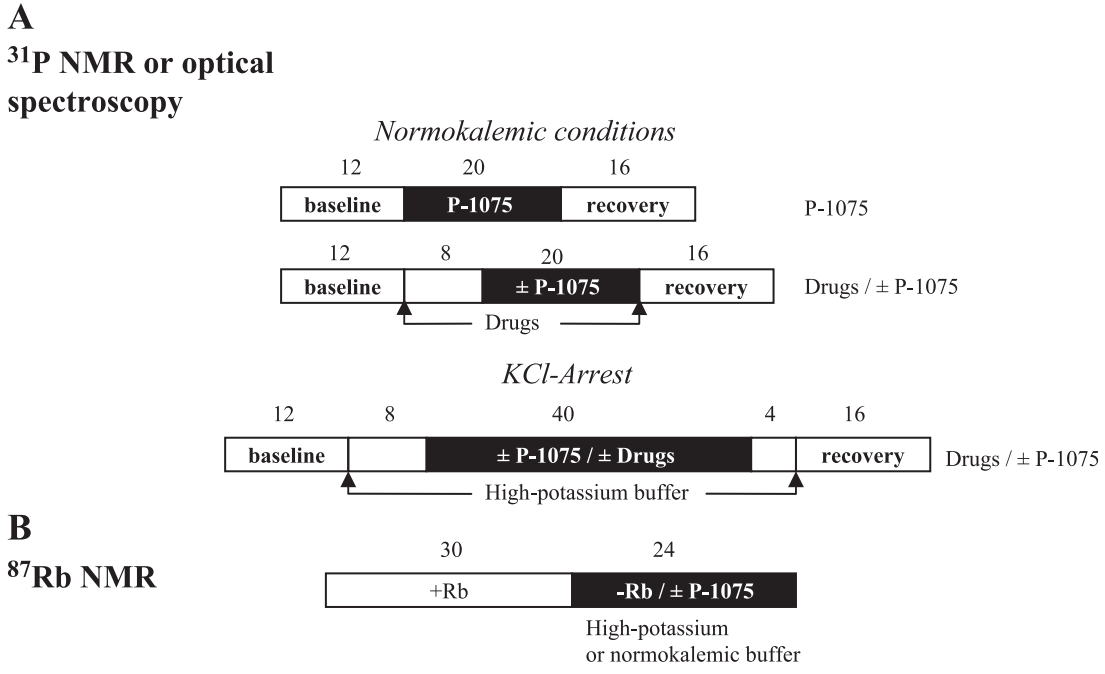

Fig. 1. Perfusion protocols used for ^{31}P-NMR and optical spectroscopy (A) and ^{87}Rb-NMR spectroscopy (B). Values above the bars represent time in minutes.

Data points corresponding to the extracellular Rb^+ efflux (first 4 min of the efflux) were discarded.

2.6. Myocardial absorbance measurements

Optical absorbance was measured using a spectrometer built by Control Development (South Bend, IN, USA) and equipped with Optical Spectrograph Card Instrument Driver Software, version 3.0 F, produced by the same company. The spectrometer was equipped with a bifurcated fiber-optic cable. One end of the cable was connected to a source of white light (Fiber Optic Illuminator, model 77501, Oriel Instruments, Stratford, CT, USA), while a second end terminated at the detector. The individual fibers of the cable were combined into a common probe tip that was in a direct contact with a left ventricle. Non-gated spectra were acquired in the range 400–1000 nm every 30 s (60 scans). Spectral data were processed using Grams/32 Version 4.11 computer program (Galactic Industries Corp, USA).

2.7. Oxygen consumption

An aliquot of the perfusate entering a heart was taken as an arterial sample. Venous effluent was collected from the cannulated right atrium. The oxygen content (pO_2, mm Hg) in the samples was measured at approximately 7-min intervals using Novastatprofile-Plus-9 (Nova Biomedical, MA, USA). The rate of O_2 consumption (VO_2, μmol min^{-1} g^{-1}) was calculated as a product of the difference in pO_2 between the arterial and venous samples, and the coronary flow, normalized to the wet heart weight.

2.8. Measurements of membrane potential (ΔΨ) in rat liver mitochondria

Mitochondria were isolated as described elsewhere [31]. Mitochondrial preparation was suspended in the medium containing 20 mM HEPES-Na (pH 7.2), 120 mM KCl and 1 mM EGTA to bring the concentration to 30–50 mg of protein/ml and stored on ice. Mitochondrial protein was determined by bicinchoninic acid assay using Sigma kit BCA-1.

Mitochondrial oxygen consumption was measured using Clark-type oxygen electrode (Yellow Springs Instruments, OH, USA) at 30 °C in the medium containing 20 mM HEPES-Na (pH 7.2), 20 mM KCl, 100 mM K-gluconate and 1 mM EGTA. Glutamate-Na (5 mM) + 5 mM malate-Na or 5 mM succinate-Na was used as oxidizable substrates. Following stabilization of state 2 respiration, 0.3 mM MgADP was added to stimulate respiration (state 3), which returned to the level close to state 2 (state 4) upon completion of ADP phosphorylation. FCCP (1 μM) was added at the end of the assay to obtain uncoupled respiration rate. The respiration rates were expressed in nmol O_2/min mg and the respiratory control index (RCI) as a ratio of state 3 to state 2 respiration rate. RCI was 6.9 ± 1.11 (n = 5) with glutamate + malate and 4.52 ± 0.49 (n = 4) with succinate.

Membrane potential was estimated using an optical potentiometric probe, rhodamine 800. Mitochondria (0.2 mg/ml) were incubated in 1 ml of aerated medium containing 20 mM HEPES-Na (pH 7.2), 20 mM KCl, 100 mM K-gluconate, 1 mM $MgSO_4$ and 1 mM EGTA at 30 °C with 5 μM rhodamine 800 for 5 min in the presence of 5 mM MgATP and oxidizable substrates (5 mM glutamate-Na + 5 mM succinate-Na). FCCP (1–10 μM), P-1075 (20 μM), and glibenclamide (5 μM) were added simultaneously with rhodamine 800. The reaction mixture in open scintillation vials (25-mm diameter) was continuously shaken to provide adequate aeration. Following 5-min incubation, the mixture was rapidly cooled and centrifuged for 5 min at 10,000 × g. Mitochondrial pellet was extracted by 1-ml 95% EtOH and rhodamine 800 content was measured in the supernatant and extract spectrophotometrically (Beckman DU 650 spectrophotometer, Beckman Instruments (Canada) Inc, Mississauga, ON, Canada) at 680–685 nm (ε = 99 mM^{-1} cm^{-1}). We assumed that the equilibrium distribution of the dye between the external space (e) and matrix (m) obeys the Nernst law with the correction for membrane binding [32]: Mitochondrial dye content (MDC),

$$\mathrm{MDC}/[C]_e = (V_{matrix} + P \times V_{membr}) \times \exp(-\Delta\Psi \times F/RT), \quad (1)$$

where $[C]_e$ is an equilibrium supernatant concentration (C), V_{matrix} and V_{membr} are matrix and membrane volumes, respectively, P is a partition coefficient between the matrix and membrane, F, Faraday constant, R, gas constant and T, an absolute temperature.

In the presence of a modulator X (where X is FCCP, or P-1075, or glibenclamide)

$$(\mathrm{MDC}/[C]_e)_x = (V_{matrix} + P \times V_{membr}) \times \exp(-\Delta\Psi_x \times F/RT) \quad (2)$$

Thus

$$(\Delta\Psi_x - \Delta\Psi_{control}) = 59[\log(\mathrm{MDC}/[C]_e)_{control} - \log(\mathrm{MDC}/[C]_e)_x] \text{ (in mV)} \quad (3)$$

assuming that the matrix volume and dye partition coefficient do not change significantly in the presence of modulators.

2.9. Calculations of [ATP]/[ADP] and [PCr]/[Cr]

These parameters were calculated as described previously [33] according to the following formula

$$([\mathrm{ATP}]/[\mathrm{ADP}])/([\mathrm{ATP}]/[\mathrm{ADP}])_0 = ([\mathrm{PCr}]/[\mathrm{Cr}])/([\mathrm{PCr}]/[\mathrm{Cr}])_0 \times \text{antilog } \Delta pH_i \quad (4)$$

where subscript $_0$ denotes initial values.

Assuming that $[PCr]_0=[Cr]_0$ in glucose-perfused hearts, ratio of [PCr]/[Cr] was calculated from the following formulas, where the levels of high-energy phosphate metabolites were expressed as % of the initial values

$$[PCr] + [Cr] = [PCr]_0 + [Cr]_0 = 100\% \quad (5)$$

$$[PCr]/[Cr] = [PCr]/(100 - ([PCr] - [PCr]_0)) \quad (6)$$

Assuming that pH_i did not change considerably, a relative change in [ATP]/[ADP] could be substituted with [PCr]/[Cr].

2.10. Statistics

ANOVA (single factor) was used for data comparison. Differences were considered statistically significant when $P<0.05$. Data are presented as means ± S.E.

3. Results

3.1. Activation of Rb^+/K^+ efflux by P-1075 in beating and KCl-arrested rat hearts

Elevated (24.7 mM) potassium induced sarcolemmal membrane depolarization resulting in cardiac arrest [2]. P-1075 (5 μM) stimulated the Rb^+/K^+ efflux in beating as well as KCl-arrested rat hearts (Fig. 2). At a lower concentration (1 μM), P-1075 also stimulated the Rb^+ efflux marginally; however, the change did not reach statistical significance [25]. The degree of stimulation did not depend on the sarcolemmal polarization state, as the rate constant of the Rb^+ efflux in the beating hearts was similar to that in the KCl-arrested hearts: $k_{beating}=0.087\pm0.005$ ($n=4$), $k_{arrested}=0.082\pm0.002$ ($n=5$) min^{-1}. In drug-untreated hearts, $k=0.040\pm0.002$ ($n=3$) in the beating hearts, and 0.048 ± 0.002 ($n=4$) min^{-1} in the KCl-arrested hearts. Glibenclamide (5 μM) blocked Rb^+ efflux stimulation by P-1075 in beating [25] and KCl-arrested ($k=0.049\pm0.001$, $n=3$) hearts, which is consistent with activation of s-K_{ATP} by P-1075.

3.2. Effects of P-1075 on high-energy phosphates and cardiac function in beating and KCl-arrested hearts

Treatment of beating rat hearts with 5 μM P-1075 caused cardiac arrest after, on average, 9 min, probably due to sarcolemmal membrane hyperpolarization. After 20 min of treatment, diastolic pressure increased from 5 to 60 mm Hg; PCr and ATP were depleted by 40% (demonstrated by ^{31}P-NMR) (Table 1). The damage was irreversible, recovery was poor; the hearts were fibrillating and trembling.

KCl-arrest resulted in an approximately 25% increase in the PCr peak (~ 80% average increase in [PCr]/[Cr] for all KCl-arrested hearts, Table 2), and a decrease in the P_i peak to the level that was not discernible with this method (Fig. 3). The [PCr]/[Cr] increase corresponds to a similar relative increase in [ATP]/[ADP] under the conditions of KCl arrest,

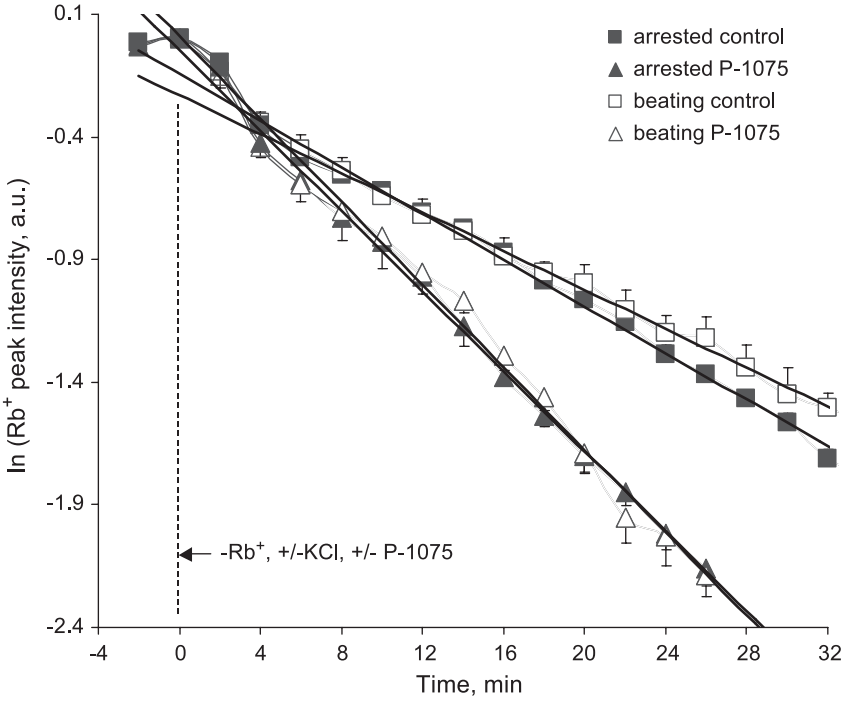

Fig. 2. Activation of Rb^+ efflux by P-1075 from beating and potassium-arrested perfused rat hearts. The hearts were loaded with Rb^+ by perfusion with KHB-Rb normokalemic buffer for 30 min. Rb^+ washout was initiated by switching perfusion to Rb^+-free KHB in beating (open symbols) and potassium (24.7 mM)-arrested (closed symbols) rat hearts. Rb^+ efflux measured in the presence (triangles) or absence (squires) of P-1075 (5 μM). Data are presented as means ± S.E.

Table 1
Modulation of P-1075 effects by verapamil, pyruvate, and adenosine under normokalemic conditions

Group	[PCr]/[Cr] prior to P-1075 infusion	PCr, %	ATP, %	LVEDP, mm Hg	PP, mm Hg	PRP, %
(1) Control, $n=5$	1	93.2 ± 2.8	96.6 ± 3.9	5.6 ± 4.7	80.0 ± 6.0	101.5 ± 6.9
(2) P-1075, $n=7$	1	60.3 ± 2.2	59.7 ± 4.6	60.7 ± 7.5	80.0 ± 4.6	0
(3) Verapamil/P-1075, $n=3$	1.44 ± 0.17	96.7 ± 1.2	80.3 ± 5.0	27.5 ± 3.7	84.1 ± 6.0	0, except for one heart: ~ 7%
(4) Pyruvate/P-1075, $n=3$	1.44 ± 0.24	57.0 ± 3.9	47.5 ± 8.9	70.9 ± 1.3	93.7 ± 3.2	0
(5) Adenosine, $n=3$	1.20 ± 0.12	96.3 ± 5.6	100.7 ± 2.5	17.7 ± 3.6	65.3 ± 4.3[a]	3.5 ± 2.2
(6) Adenosine/P-1075, $n=3$	1.09 ± 0.23	63.4 ± 8.5	62.7 ± 3.7	61.8 ± 1.1	73.4 ± 1.5	0

P-1075 (5 μM) was infused for 20 min. Infusion of verapamil (5 μM), pyruvate (5 mM), or adenosine (1 mM) started 8 min prior to the infusion of P-1075 and continued for additional 20 min, simultaneously with P-1075 (see protocols on Fig. 1A). The metabolic and functional parameters correspond to 16–20-min perfusion with P-1075 (groups 2, 3, 4, and 6) or to the respective time point in the groups without P-1075 (groups 1 and 5). [PCr]/[Cr] values correspond to 4–8-min treatment with verapamil (group 3), pyruvate (group 4), or adenosine (group 5 and 6). In groups 1 and 2 [PCr]=[Cr] before any treatment, by assumption (see Methods). Means ± S.E. are presented.

[a] PP transitory declined from 70 to 60, then gradually increased to 65 mm Hg.

when pH_i changes only slightly (increases by ~ 0.05 units [34]). Hyperkalemia on its own had very little adverse effects on the hearts: after 52 min of high-potassium perfusion, recovery was nearly complete.

To analyse mitochondrial effects of P-1075 under the conditions of KCl-arrest, the hearts were perfused with a high-potassium buffer for 8 min prior to P-1075 infusion, to allow new equilibrium parameters be reached. P-1075 (5 μM) treatment time increased to 40 min to detect smaller (if any) metabolic effects of P-1075, since energy expenditures were drastically reduced in the arrested hearts. Addition of P-1075 did not alter PCr and ATP levels in comparison to drug-untreated KCl-arrested hearts (Fig. 3, Table 1). In contrast, a classic mitochondrial uncoupler, DNP (50 μM), that uncouples oxidative phosphorylation due to its properties as a protonophore [35] retained its ability to do so in the KCl-arrested hearts (Table 2).

3.3. Effects of P-1075 on cytochrome c oxidase redox state in beating and KCl-arrested hearts

Monitoring of myocardial 603-nm absorbance allows in situ measurements of cytochrome *c* oxidase redox state that reflects changes in the rate of movement of redox equivalents to cytochrome *c* oxidase [34]. Under normal metabolic conditions when respiration is controlled by ADP availability, level of reduction of electron carriers decreases from the dehydrogenase (flavins) to the oxygen end (cytochrome *c* oxidase). Uncouplers of oxidative phosphorylation (DNP) release acceptor control accelerating electron flux from the dehydrogenases to the cytochrome *c* oxidase; thus, oxidizing flavins [14] and reducing cytochrome *c* oxidase (Fig. 4A). Indeed, in beating hearts, on treatment with DNP [26] and P-1075 (Fig. 4A), an increase in 603-nm absorbance was observed indicating that cytochrome *c* oxidase became reduced. The increase was 30% of the level detected during anoxia induced by complete cessation of perfusion (Fig. 4B). Absorbance at the neutral wavelength did not change, indicating a lack of artefacts associated with the possible changes in light scattering [26]. The P-1075-induced increase was transient, probably reflecting changes in kinetic properties of cytochrome *c* oxidase and Krebs cycle following approximately 10-min exposure to P-1075 (Fig. 4B and C). In contrast, in potassium-arrested hearts, 603-nm absorbance decreased, reflecting oxidation of cytochrome *c* oxidase due to increased oxygen accessibility. This decrease

Table 2
Effects of P-1075 and other drugs in KCl-arrested hearts

Group	[PCr]/[Cr] prior to drug infusion	Metabolic and functional parameters corresponding to 36–40 min of drug treatment				
		PCr, %	ATP, %	LVEDP, mm Hg	PP, mm Hg	PRP, %
(1) Control, $n=3$	1.80 ± 0.49	118.6 ± 9.6	93.0 ± 5.0	31.5 ± 3.5	94.1 ± 2.0	0
(2) P-1075, $n=3$	2.18 ± 0.24	127.0 ± 5.4	92.8 ± 2.6	36.9 ± 9.2	97.6 ± 6.5	0
(3) DNP (50 μM), $n=3$	2.04 ± 0.43	35.7 ± 12.5	40.8 ± 9.0	68.7 ± 7.6	97.9 ± 0.9	0
(4) DNP (10 μM), $n=3$	1.41 ± 0.06	84.5 ± 4.4	92.6 ± 6.9	46.1 ± 6.2	114.4 ± 12.2	0
(5) DNP (10 μM)/P-1075, $n=3$	1.87 ± 0.63	83.8 ± 9.2	82.9 ± 2.2	57.3 ± 15.5	115.9 ± 11.8	0
(6) Bay K[a], $n=3$	1.57 ± 0.11	85.4 ± 4.1	82.4 ± 3.6	29.8 ± 14.2	168.8 ± 9.7	0
(7) Bay K/P-1075, $n=4$	1.98 ± 0.32	88.8 ± 9.2	82.9 ± 4.6	49.5 ± 8.7	160.1 ± 13.3	0
(8) Isoproterenol/P-1075, $n=4$	1.54 ± 0.23	95.0 ± 5.6	87.9 ± 2.2	3.7 ± 4.7	64.5 ± 4.2	30.9 ± 11.5
(9) Adenosine/P-1075, $n=3$	1.70 ± 0.33	111.8 ± 3.9	98.9 ± 7.1	33.9 ± 1.6	91.8 ± 7.8	0

Hearts were arrested with KHB containing 24.7 mM KCl. Eight minutes after perfusion with high-potassium buffer, P-1075 (5 μM), DNP (10 or 50 μM), *S*-(−)-Bay K8644 (50 nM), isoproterenol (500 nM), or adenosine (1 mM), alone or in the combinations as described in the Table, was infused for 40 min (see protocols on Fig. 1A). [PCr]/[Cr] was determined at 4–8 min of KCl treatment. Means ± S.E. are presented.

[a] *S*-(−)-Bay K8644 denoted as Bay K.

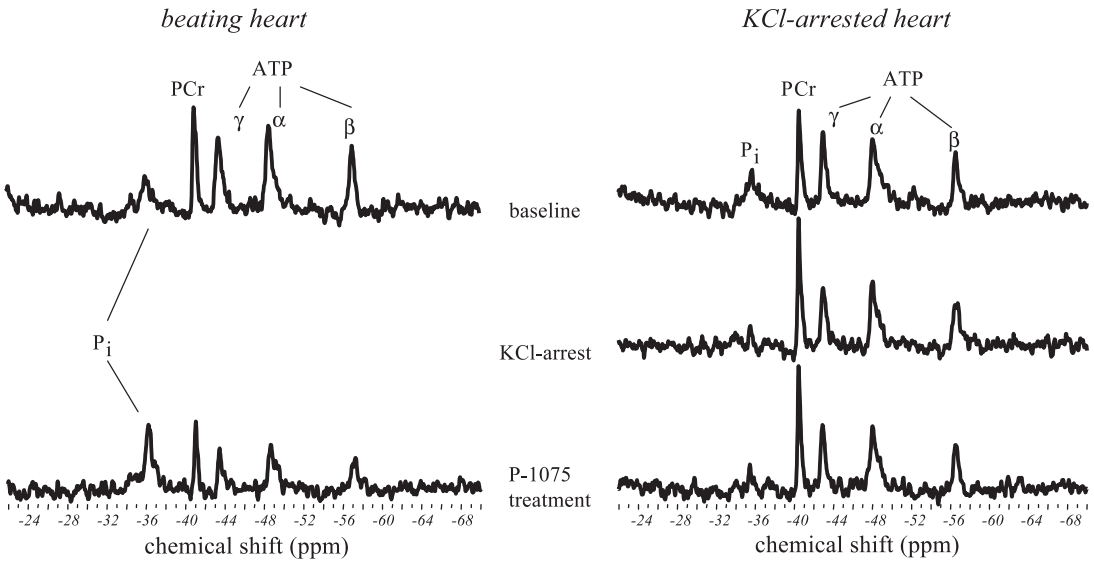

Fig. 3. Inhibition of P-1075-induced mitochondria uncoupling by potassium arrest in perfused rat hearts. Representative ^{31}P-NMR spectra of a beating heart and KCl-arrested rat heart, both of them being treated with P-1075 (5 μM). The spectra are the sum of 116 acquisitions collected over 4-min periods before any treatment (baseline), 4–8 min of KCl-arrest, and 16–20-min of P-1075 infusion. P_i, inorganic phosphate.

was reversed on return to normokalemic conditions and starting of heartbeats (Fig. 4B and C). Addition of P-1075 to the KCl-arrested hearts did not increase myocardial absorbance at 603 nm (Fig. 4B and C), thus confirming that no uncoupling by P-1075 took place under the conditions of KCl-arrest.

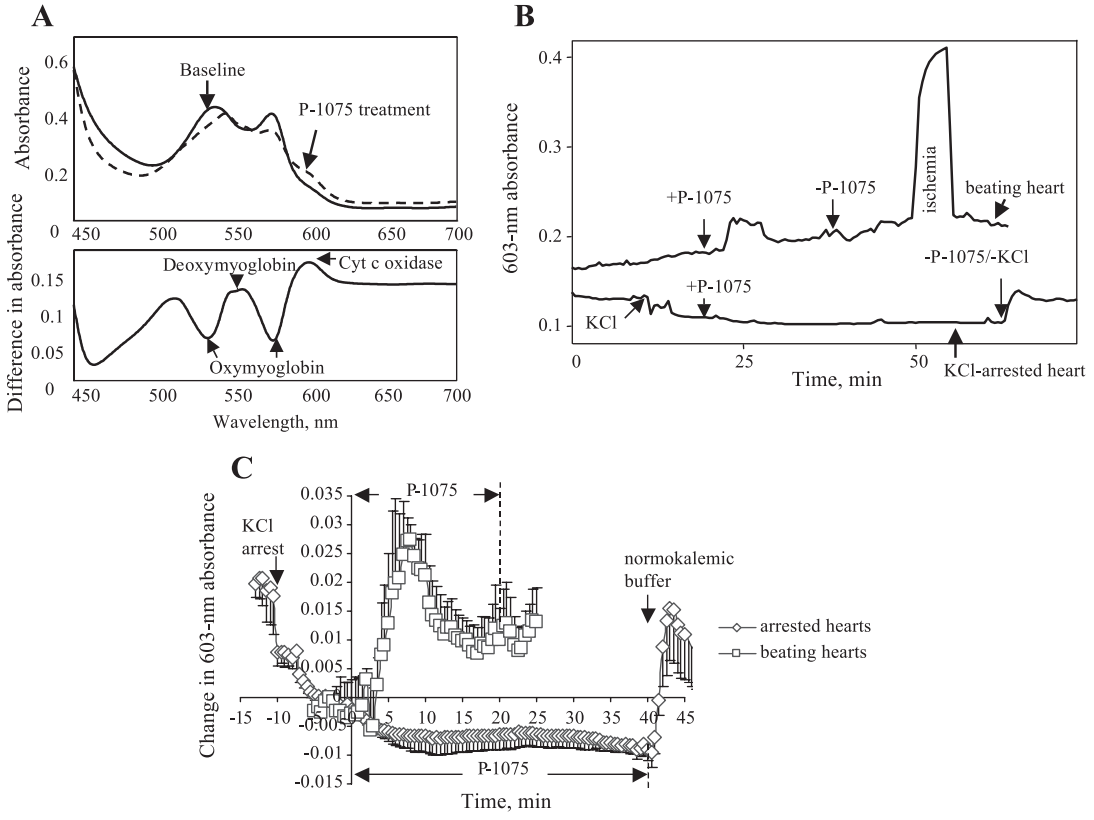

Fig. 4. Effects of P-1075 on redox state of cytochrome *c* oxidase in beating and potassium-arrested rat hearts. (A) Top: myocardial absorbance from a representative rat heart before (solid line) and during (dash line) treatment with P-1075 (5 μM). Bottom: difference between the spectra. (B) Time course of 603-nm absorbance from a beating (top trace) and a potassium-arrested (bottom trace) rat heart treated with P-1075. The beating rat heart was subjected to global no-flow ischemia between 50 and 55 min of the protocol to calibrate the changes caused by P-1075. (C) Pooled data for the beating (squares, $n=3$) and potassium-arrested (diamonds, $n=4$) rat hearts treated with P-1075 (5 μM). Data are presented as means ± S.E.

3.4. Effects of P-1075 on cellular respiration in beating and KCl-arrested hearts

We demonstrated earlier that oxygen uptake was elevated by 50% in the P-1075-treated beating hearts [25]. KCl arrest resulted in an almost twofold increase in the venous oxygen concentration (Fig. 5A). Myocardial oxygen consumption fell to approximately 30% of the baseline, due to a cessation of heart beats and a decrease in ATP turnover (Fig. 5B). On infusion of P-1075, venous oxygen concentration did not decrease, and the rate of oxygen consumption did not increase (Fig. 5).

3.5. Effects of changes in phosphorylation potential on P-1075-induced uncoupling

We investigated if changes in phosphorylation potential were responsible for the lack of mitochondrial effects of P-1075 in KCl-arrested hearts. To reverse these changes, we infused 10 μM DNP into the KCl-arrested hearts to slightly uncouple oxidative phosphorylation and stimulate ATP futile cycles and respiration. Following DNP infusion, [PCr]/[Cr] ratio fell from nearly 2 to approximately 1, becoming similar to that in beating hearts (not shown). However, this change did not restore the uncoupling by P-1075, as there was no difference in the PCr and ATP levels between the potassium-arrested hearts treated with DNP + P-1075 or DNP-alone (Table 2). Additional confirmation came from the experiments in beating hearts, where infusion of pyruvate (5 mM) significantly increased [PCr]/[Cr] and decreased P_i to an undetectable level, but did not prevent depletion of high-energy-phosphates by P-1075 (Table 1).

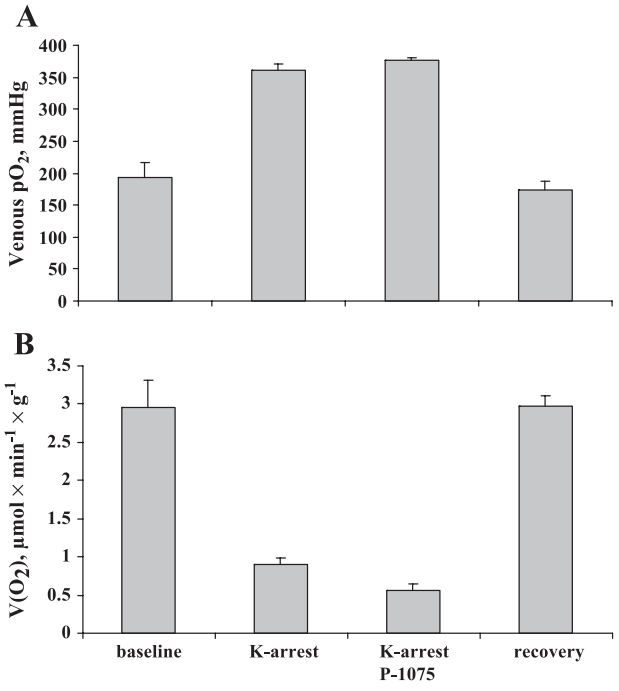

Fig. 5. The effects P-1075 (5 μM, $n = 4$) on venous oxygen pressure (A) and oxygen consumption (VO_2) rate (B) in the potassium-arrested rat hearts. Venous effluent was collected from the cannulated right atrium, an arterial sample was taken from the perfusion line close to the heart. Data are presented as means ± S.E.

3.6. Effects of variations in intracellular calcium on P-1075-induced uncoupling

A calcium channel blocker, verapamil [36] (5 μM), similarly to high potassium, stopped the hearts in a depolarized state [2], increased [PCr]/[Cr] and prevented depletion of PCr and ATP by P-1075 (Table 1). This could imply that a certain level of intracellular $[Ca^{2+}]$ is required for the uncoupling. However, the effects of verapamil were not due to a decrease in mean cytosolic $[Ca^{2+}]$, because an L-type calcium channel opener, *S*-(−)-Bay K8644 [37] (50 nM), which increases $[Ca^{2+}]_i$, did not facilitate the uncoupling effect of P-1075 in potassium-arrested hearts (Table 2). Elevated PP in *S*-(−)-Bay K8644-treated hearts (Table 2) was a manifestation of $[Ca^{2+}]$ increase in vascular smooth muscle cells. Similarly, a structurally unrelated calcium channel activator, FPL 64176 [37] (500 nM), did not facilitate uncoupling by P-1075 in potassium-arrested hearts (not shown).

To confirm that increased intracellular $[Ca^{2+}]$ was not a critical factor in mitochondrial uncoupling by P-1075, we infused a β-adrenergic agonist, isoproterenol (0.5 μM) into KCl-arrested hearts. Isoproterenol not only increases intracellular $[Ca^{2+}]$, but also stimulates signal transduction pathways leading to activation of extracellular signal-regulated protein kinases (ERKs) and protein kinase A [38,39]. Isoproterenol induced heartbeats and restored mechanical function of potassium-treated hearts to approximately 30% (Table 2). However, P-1075 did not cause a decrease in PCr and ATP in KCl-arrested/isoproterenol-treated hearts (Table 2).

3.7. Metabolic effects of P-1075 in a hyperpolarized cardiac arrest

Since P-1075-induced uncoupling disappeared in KCl- and verapamil-arrested hearts, we investigated the effects of other forms of cardiac arrest. Adenosine (1 mM) induced almost complete and reversible cardiac arrest presumably in a polarized or hyperpolarized state [40] (Table 1). Occasional escape heartbeats were observed and mechanical function (PRP) was maintained at about 3% of baseline (Table 1). PCr and ATP levels in adenosine-arrested hearts were comparable to those in potassium- and verapamil-arrested hearts (Tables 1 and 2). However, adenosine-arrest did not prevent depletion of PCr and ATP caused by P-1075 treatment (Table 1). Yet, P-1075 did not induce uncoupling in KCl-arrested + adenosine-treated hearts (Table 2), thus eliminating the possibility that adenosine-initiated signal transduction cascades were critical for the uncoupling.

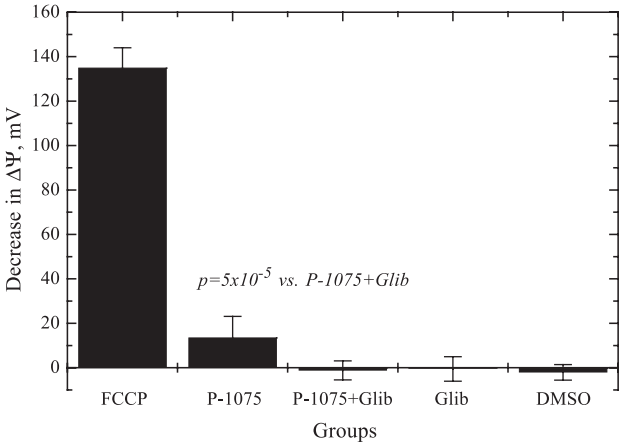

Fig. 6. Effect of P-1075 on membrane potential in isolated liver mitochondria. Mitochondria were incubated for 5 min in high K^+ medium at 30 °C with 5 μM rhodamine 800 in the presence of 1 μM FCCP ($n=11$), 5 μM P-1075 ($n=11$), 5 μM glibenclamide ($n=9$), 5 μM P-1075 + 5 μM glibenclamide ($n=8$) and 0.3% DMSO ($n=9$). Changes in the membrane potential relative to the control are shown. Data are presented as means ± S.D.

3.8. Effect of P-1075 on mitochondrial ΔΨ in isolated rat liver mitochondria

Cardiac and liver mitochondria have similar biochemical characteristics. Presence of mito-K_{ATP} was demonstrated in both heart and liver mitochondria [7,8]. Therefore, we used liver mitochondria to investigate the mitochondrial effects of P-1075. Incubation of 5 μM rhodamine 800 with liver mitochondria energized by ATP hydrolysis + respiration (glutamate + succinate) resulted in a drop of rhodamine 800 concentration in extramitochondrial medium to 0.13 ± 0.02 μM due to strong binding of the dye to mitochondria. Mitochondria accumulated 26.8 ± 2.4 ($n=7$) nmol of rhodamine 800/mg protein. FCCP reduced mitochondrial dye content 10-fold to 2.7 ± 0.04 nmol/mg ($n=7$) and increased rhodamine 800 concentration in the external medium to 3.1 ± 0.32 μM (24-fold). These changes in rhodamine 800 binding induced by FCCP corresponded to depolarization of $\Delta\Psi$ by 135 ± 9 mV (Fig. 6). P-1075 (20 μM) slightly decreased $\Delta\Psi$ by 13.6 ± 9.5 mV ($n=7$). Glibenclamide (5 μM) completely reversed the P-1075 effect, while glibenclamide alone did not change $\Delta\Psi$. DMSO (0.3%) that was used for preparation of drug solutions had no effect on $\Delta\Psi$ (Fig. 6).

4. Discussion

4.1. Methodological considerations for in situ measurements

We used three different noninvasive techniques to detect mitochondrial uncoupling in intact rat hearts: (1) ^{31}P-NMR, (2) optical redox changes in cytochrome *c* oxidase, and (3) oxygen uptake measurements. ^{31}P-NMR provides information on inhibition of ATP synthesis/activation of ATP hydrolysis; however, this method cannot distinguish between uncoupling and inhibition of electron transport/Krebs cycle/glycolysis. Cytochrome *c* oxidase becomes reduced during both hypoxia and uncoupling. However, stimulation of VO_2 indicated that P-1075-induced changes were due to activation of mitochondrial electron transport rather than other O_2 consuming pathways.

Oxygen uptake rate is a crucial parameter, which increases due to uncoupling or activation of cellular ATPases, and decreases in a response to inhibition of electron transport (e.g., hypoxia, blockade of dehydrogenases) or deactivation of cellular ATPases (KCl-arrest). P-1075 depressed cardiac contractility, which was initially associated with indirect inhibition of myofibrillar ATPase activity, probably due to reduced Ca^{2+} entry through hyperpolarized sarcolemma. However, P-1075 could indirectly activate Na^+/K^+ ATPase by increasing K^+ turnover rate, estimated as $k_{Rb\ efflux} \times [K^+]_{intracell} \times V_{intracell}$ (where, $[K^+]_{intracell} = 140$ μmol/ml, $V_{intracell}$ is intracellular volume per gram of tissue = 0.5 ml/g), from 2.8 to 6.0 μmol K^+/min/g in beating hearts. Therefore, the rate of ATP utilization by Na^+/K^+ ATPase increased from 1.4 to 3.0 μmol/min/g (stoichiometry of K^+ transport per ATP used is $ATP/K^+ = 0.5$), which is only 8–17% of the basal ATP turnover rate of 18 μmol/min/g ($= 6V(O_2)$). However, glibenclamide-sensitive activation by P-1075 of other ATPases, such as Ca^{2+} ATPase of sarcoplasmic reticulum and sarcolemma, was unlikely. Furthermore, if there was no uncoupling, P-1075-stimulated rate of oxygen uptake (3.8 μmol/min/g [25]) and, therefore, the rate of ATP synthesis ($6V(O_2) \approx 23$ μmol/min/g) would not be limited by O_2 supply since $pO_{2venous}$ was 110 mm Hg [25], which is still in the range of arterial pO_2 in blood. In addition, this rate of ATP synthesis was well below the maximal rate of that observed in glucose-perfused hearts under normal physiological conditions (>50 μmol/min/g wet weight [41]). Therefore, it is unlikely that P-1075 effects were mediated by increased cellular ATPase activity without mitochondrial uncoupling.

Thus, taken together, these three parameters provided unequivocal evidence of mitochondrial uncoupling. Because P-1075-induced uncoupling was reversed by sulfonylureas [25,26] any possible non-K_{ATP}-mediated effects of P-1075 were negligible. The question is whether the uncoupling effects of P-1075 resulted from activation of sarcolemmal or mitochondrial K_{ATP} or both.

4.2. Activation of s-K_{ATP} by P-1075 in situ

In rat hearts, P-1075 can activate s-K_{ATP} directly by binding to sulfonylurea receptor of the channel and indirectly by a decrease in [ATP]/[ADP] resulting from mitochondrial uncoupling [22,23,42]. The observation that the rate of Rb^+ efflux from KCl-arrested hearts, where P-1075

had no uncoupling effect, was the same as that in the beating hearts is consistent with full activation of s-K_{ATP} by 5 μM P-1075. This degree of activation makes P-1075 one of the most potent K_{ATP} openers available today. A closely related compound, pinacidil [21], taken at 60-fold higher concentration, activated the Rb^+ efflux by only 20% (Jilkina et al., unpublished observation).

It is possible that activation of s-K_{ATP} can contribute towards the metabolic effects by P-1075. However, the link between the activation of s-K_{ATP} and uncoupling of oxidative phosphorylation is not currently understood. Elevated cytoplasmic Ca^{2+} can impair ATP production. K_{ATP} openers hyperpolarize cardiomyocyte sarcolemma and, therefore, decrease Ca^{2+} entry in isolated cells. However, in beating hearts the situation is more complicated. First, cytosolic [Ca^{2+}] fluctuates, and relations between membrane polarization, calcium oscillations, and cardioenergetics are not clear. Second, for a long time it was recognised by electrophysiologists that there are clefts in the extracellular space (e.g., t-tubules) where K^+ concentration can be different from the average extracellular one (for review see Ref. [43]). In beating hearts, significant activation of K^+ efflux by P-1075 may result in accumulation of K^+ in the extracellular clefts, local depolarization of cell membrane and increased Ca^{2+} entry. In the KCl-arrested hearts, the extracellular K^+ is already high (24.7 mM) and, therefore, it buffers K^+ increase caused by activation of s-K_{ATP} channels. However, there are no data to support this hypothesis. Interestingly, P-1075 is known to have higher potency of activating SUR2B/Kir6.2 channels as opposite to SUR 2A/Kir6.2 [22], and it is the SUR 2B subunits that were shown to co-localize strongly with the t-tubules [20].

Hyperpolarized/polarized cardiac arrest per se should not, theoretically, induce metabolic uncoupling [2–6]. Indeed, a similar hyperpolarized arrest induced by adenosine did not produce any deleterious effects on the cardiac metabolism (Table 1). For these reasons, strictly the sarcolemmal origin of metabolic effects of P-1075 seems to be unlikely.

4.3. Activation of m-K_{ATP} by P-1075 in situ

The metabolic effects of P-1075 can be readily explained by its interaction with putative m-K_{ATP}. Indeed, in KCl-arrested hearts, s-K_{ATP} were activated by P-1075, yet this had no metabolic consequences. However, the factors that either promoted interaction of P-1075 with m-K_{ATP} in polarized hearts or blocked this interaction in depolarized hearts remained elusive. KCl arrest causes a number of metabolic and ionic changes: (1) an increase in the cytoplasmic [ATP]/[ADP] and phosphorylation potential, (2) a decrease in mean cytoplasmic [Ca^{2+}], (3) oxidation of mitochondrial cytochrome *c* oxidase and reduction of NAD^+ and dehydrogenases [34,44]. The hypothesis that the uncoupling effect was sensitive to different energetic status ([ATP]/[ADP]) of cardiomyocytes was tested in experiments involving heart treatment with KCl/P-1075/DNP or P-1075/pyruvate. However, we found no evidence to support this hypothesis. Another attractive possibility was a role of [Ca^{2+}] as a critical factor. In a beating heart, [Ca^{2+}] fluctuates between 10^{-7} M in diastole and 10^{-5} M in a systolic peak [45]. In mitochondria, [Ca^{2+}] fluctuations follow changes of [Ca^{2+}] in the surrounding cytoplasm, although they are never greater than in cytoplasm [29]. Therefore, it is plausible that high systolic cytosolic or mitochondrial [Ca^{2+}] promotes the uncoupling effect of P-1075. Potassium arrest and calcium channel blockers prevented [Ca^{2+}] transients, decreased mean [Ca^{2+}] in intact rat hearts [44] and eliminated P-1075-induced uncoupling. However, lack of the effects of calcium channel openers and isoproterenol in the KCl-arrested hearts argues against [Ca^{2+}] being a crucial factor in the P-1075 interaction with m-K_{ATP}.

4.4. Effects P-1075 on $\Delta\Psi$ in isolated liver mitochondria

Direct mitochondrial effect of P-1075 was demonstrated in isolated rat liver mitochondria. P-1075 depolarized $\Delta\Psi$ by approximately 14 mV in the presence of K^+ and MgATP, while glibenclamide completely reversed this effect. Although the effect was relatively small, it was of a magnitude similar to that observed for other K_{ATP} openers on isolated rat liver and heart mitochondria [12,13,17] and demonstrated the possibility of mitochondrial uncoupling by P-1075. In agreement with our data, Oldenburg et al. recently demonstrated that P-1075 activated K^+ fluxes, with a 10-fold higher potency than diazoxide, in liposomes containing reconstituted mitochondrial fraction, and increased reactive oxygen species-dependent fluorescence in isolated rabbit cardiomyocytes, which is consistent with ability of P-1075 to activate m-K_{ATP} [46].

5. Conclusions

A drastic difference in metabolic effects produced by P-1075 in depolarized and polarized/hyperpolarized hearts has a great implication to the interpretation of the results obtained in non-beating cardiomyocytes and isolated mitochondria. This implies that metabolic consequences of activation of sarcolemmal and putative mitochondrial K_{ATP} may be different, depending on the polarization state of cardiomyocyte plasma membrane. Furthermore, we cannot exclude that depolarization of the endothelial cells and/or nerve endings is also important because it may change release of endogenous norepinephrine, nitric oxide, endothelins and other factors.

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