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Potassium-specific effects of levosimendan on heart mitochondria

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Abstract

In this study, we evaluated levosimendan, a new drug developed for the treatment of acute and decompensated heart failure, as a potential activator of ATP-sensitive potassium flux to the matrix of cardiac mitochondria. We estimated the K_{ATP} channel openers-induced increase in mitochondrial inner membrane permeability for potassium by registering changes in membrane potential of heart mitochondria, oxidizing endogenous substrates. We compared the effect of levosimendan with the effects of the known K_{ATP} channel openers diazoxide and pinacidil. Levosimendan (1 μM) accelerated potassium-specific $\Delta\Psi$ decrease by 0.15%/s, whereas 50 μM diazoxide by 0.10%/s, and 50 μM pinacidil by 0.08%/s, respectively. These results were confirmed by swelling experiments of non-respiring mitochondria in potassium nitrate medium. We found that levosimendan with an EC_{50} of 0.83 ± 0.24 μM activates potassium flux to the mitochondrial matrix. This effect is discussed as a possible explanation of the anti-ischemic action of levosimendan.

Keywords: Levosimendan; K_{ATP} channel; Cardiac mitochondria; Ischemia; Diazoxide; Pinacidil

1. Introduction

Levosimendan is a cardiovascular drug for the treatment of acute and decompensated heart failure. It has positive inotropic and anti-stunning effects mediated by calcium sensitization of the contractile proteins [1,2] and vasodilatory and anti-ischemic effects mediated by opening of ATP-sensitive potassium (K_{ATP}) channels in vascular smooth muscle cells [3,4]. The vasodilatory effect of levosimendan, which is antagonized by glibenclamide (a potassium channel antagonist), has been demonstrated both in arterial [5], and venous [6] vascular beds, as well as in the coronary arteries [7]. The vasodilatory effect of the drug cause a reduction of both preload and afterload, and an improvement in oxygen supply to the myocardium. Opening of K_{ATP} channels has been observed also in ventricular myocytes [8], which could be related to the findings that levosimendan protects ischemic myocardium [4] and decreases the infarct size [9] in coronary-ligated animals. Recently levosimendan was shown to open also the mitochondrial K_{ATP} channel in preparations of liver mitochondria [10]. However, the mitochondrial K_{ATP} channel opening activity of levosimendan has not yet been confirmed on cardiac mitochondria.

K_{ATP} channel openers have been demonstrated to protect myocardium against damage from ischemia-reperfusion [11–14]. These findings have led to the reclassification of many antihypertensive compounds as K_{ATP} channel openers [13,15,16] and to the development of new pharmacological agents, which stimulate ATP-sensitive potassium flux. Extensive studies have revealed that changes in potassium conductance of the mitochondrial inner membrane (for recent reviews, see [12,17,18]) as well as direct modulation of mitochondrial functions [19–23] might be responsible for the cardioprotective action of K_{ATP} channel openers. Mitochondria are the main sites of cellular energy supply, therefore the modulation of their functional activity may be very important for preserving cell viability during metabolic stress [20].

Abbreviations: $\Delta\Psi$, mitochondrial transmembrane potential; FCCP, carbonyl cyanide *p*(tri-fluoromethoxy)phenyl-hydrazone; K_{ATP} channel, ATP-sensitive potassium channel; PN, pyridine nucleotides

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In isolated energized mitochondria, the rate of generation of membrane potential ($\Delta\Psi$) by respiration on exogenous substrates may approach the rate of $\Delta\Psi$ dissipation due to increased inner membrane leak, therefore, under these conditions K_{ATP} channel openers have little effect on $\Delta\Psi$ [24]. Recently, a new sensitive method was developed to assess K_{ATP} channel openers-induced increase in mitochondrial inner membrane permeability for potassium by registering changes in $\Delta\Psi$ of liver [10] and heart mitochondria [24], respiring on endogenous substrates alone.

The aim of our work was to investigate potassium-dependent effects of levosimendan on isolated rat heart mitochondria and to discuss if these effects could be of relevance to the effect of the drug in ischemia-reperfusion models and, generally, to its therapeutic potency.

2. Materials and methods

2.1. Reagents

Levosimendan was provided by Orion Pharma (Espoo, Finland). All other chemicals were from Sigma-Aldrich (St. Louis, MO, USA), bovine serum albumin (BSA) was fraction V, A4503). Levosimendan, diazoxide and pinacidil were dissolved in dimethyl sulfoxide. In all experiments, the final dimethyl sulfoxide concentration was below 0.5%.

2.2. Preparation of rat heart mitochondria

The experiments were carried out on mitochondria isolated from male Wistar rat hearts using a differential centrifugation procedure. After decapitation hearts were excised and rinsed in ice-cold isolation medium, containing 210 mM mannitol, 70 mM sucrose and 10 mM HEPES (pH 7.4, adjusted with Trizma base; 2 °C). Mitochondria were isolated in the same medium supplemented with 2 mg/ml bovine serum albumin (fraction V, A4503, Sigma) and 1 mM EGTA. The homogenate was centrifuged at 750 × *g* for 5 min, then the supernatant was recentrifuged at 10 000 × *g* for 10 min and the pellet was washed once (10 min at 10 000 × *g*) in the isolation medium without BSA and EGTA, suspended in it and kept on ice. Mitochondrial protein concentration was determined by the biuret method using BSA as standard. The final mitochondrial protein concentration in all experiments was 0.5 mg/ml.

2.3. Measurement of $\Delta\Psi$

The final mitochondrial protein concentration in all experiments was 0.5 mg/ml. $\Delta\Psi$ was measured with rhodamine 123 (final concentration 0.1 μM) as a fluorescent probe [25] with the Hitachi F4000 fluorometer with excitation wavelength 503 nm and emission at 527 nm. $\Delta\Psi$ of mitochondria, respiring on endogenous substrates in the presence of 1 μg oligomycin/mg mitochondrial protein, was determined at 25 °C in KCl medium (120 mM KCl, 5 mM KH_2PO_4, 10 mM HEPES and 1 mM $MgCl_2$; pH 7.4, adjusted with Trizma base, 25 °C) or choline chloride medium (120 mM choline chloride, 5 mM NaH_2PO_4, 10 mM HEPES and 1 mM $MgCl_2$; pH 7.4, adjusted with Trizma base, 25 °C). The fluorescent traces were digitalized with Un-Scan-It software (v.5.1, Silk Scientific Inc., USA). The difference in fluorescence between mitochondria after addition of the uncoupling agent carbonyl-cyanide-*p*(trifluoromethoxy)phenyl hydrazone (FCCP) (0.4 μM) and without it was taken as 100%, and decrease in the $\Delta\Psi$ was expressed in percent of the effect of FCCP.

2.4. Mitochondrial swelling

Swelling of non-respiring mitochondria was spectrophotometrically recorded as decreased light scattering at 520 nm in KNO_3 medium (120 mM KNO_3, 10 mM HEPES, 3 μM rotenone, 1 μg oligomycin/mg mitochondrial protein; pH 7.4, adjusted with Trizma base, 25 °C), supplemented with 200 μM ATP. Addition of 1 μg valinomycin/mg mitochondrial protein was used as a control.

3. Results

3.1. Measurement of K_{ATP} channel opening in rat heart mitochondria induced by channel openers

The ability of K_{ATP} channel openers to significantly affect $\Delta\Psi$ depends on electron transport capability and the permeability of the inner membrane to protons [24]. In energized mitochondria respiring on added substrates, $\Delta\Psi$ dissipation due to inner membrane leakiness or ATP-sensitive K^+ flux may be fully compensated by electron transport in the respiratory chain [24]. In liver mitochondria, respiring on endogenous substrates only, $\Delta\Psi$ could be stable for up to 30 min [10]. However, heart mitochondria have less endogenous substrates than liver mitochondria, and $\Delta\Psi$ decreases with time [24]. Nevertheless, it is possible to overcome this limitation by parallel measurements of $\Delta\Psi$ in the potassium-containing medium and in a medium devoid of potassium. If the tested compound increases potassium flux to the mitochondrial matrix, $\Delta\Psi$ decreases faster in the potassium-containing medium than when control medium is used.

Fig. 1A shows that in KCl medium in the presence of 1 μg oligomycin/mg mitochondrial protein, ATP reduced the spontaneous decrease in $\Delta\Psi$ of cardiac mitochondria, respiring only on endogenous substrates. Under our experimental conditions ATP-sensitive potassium flux was

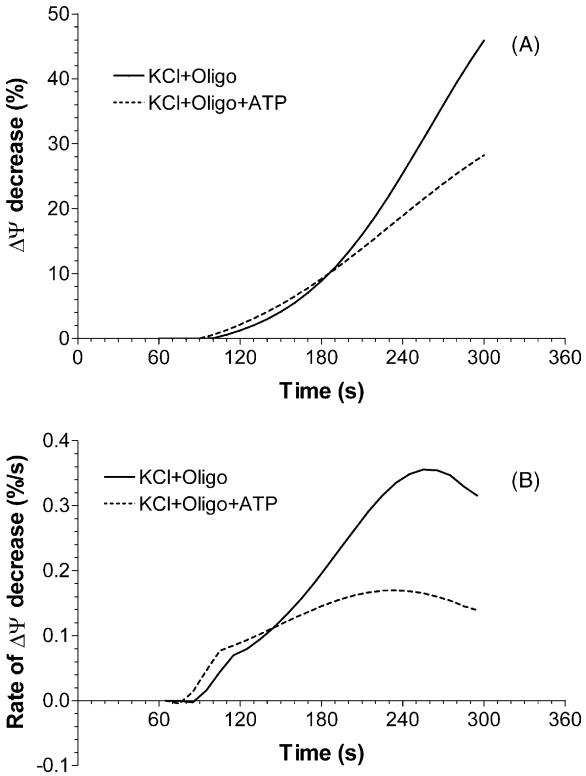

Fig. 1. ATP-reduced spontaneous decrease in $\Delta\Psi$ of rat heart mitochondria respiring on endogenous substrates in the presence of 1 μg oligomycin/mg mitochondrial protein. (A) Original traces, (B) their derivatives. Experiments were performed in potassium chloride medium (KCl) ± 200 μM ATP. Similar curves were obtained in three independent experiments.

capable to decrease $\Delta\Psi$ at the maximal rate of ~0.2%/s (Fig. 1B).

The potassium-specific $\Delta\Psi$ decrease was obtained by subtracting from the $\Delta\Psi$ data in potassium-containing medium those obtained using the control medium (Fig. 2A), and re-plotting the obtained curves (Fig. 2B). It should be pointed out that measurements always were performed in parallel—in KCl and choline chloride media; however, the position of corresponding traces on the time axis could not be used to describe the properties of investigated compounds. The leftward shift of curves was evoked by spontaneous decrease in endogenous substrates in the suspension of isolated heart mitochondria with time. Therefore, in order to quantify the obtained data, we calculated the first derivatives of the potassium-specific $\Delta\Psi$ decrease curves (Fig. 2C), and used their maximal values to characterize the ability of the tested compounds to increase potassium flux through the inner membrane of isolated heart mitochondria. The maximal rate of $\Delta\Psi$ dissipation was not dependent on the amount of endogenous substrates in isolated heart mitochondria, and was similar during all experiments, which were performed in 3 h after isolation of mitochondria. Thus, the increase in the $\Delta\Psi$ dissipation rate induced by levosimendan and the known K_{ATP} channel openers diazoxide and pinacidil was

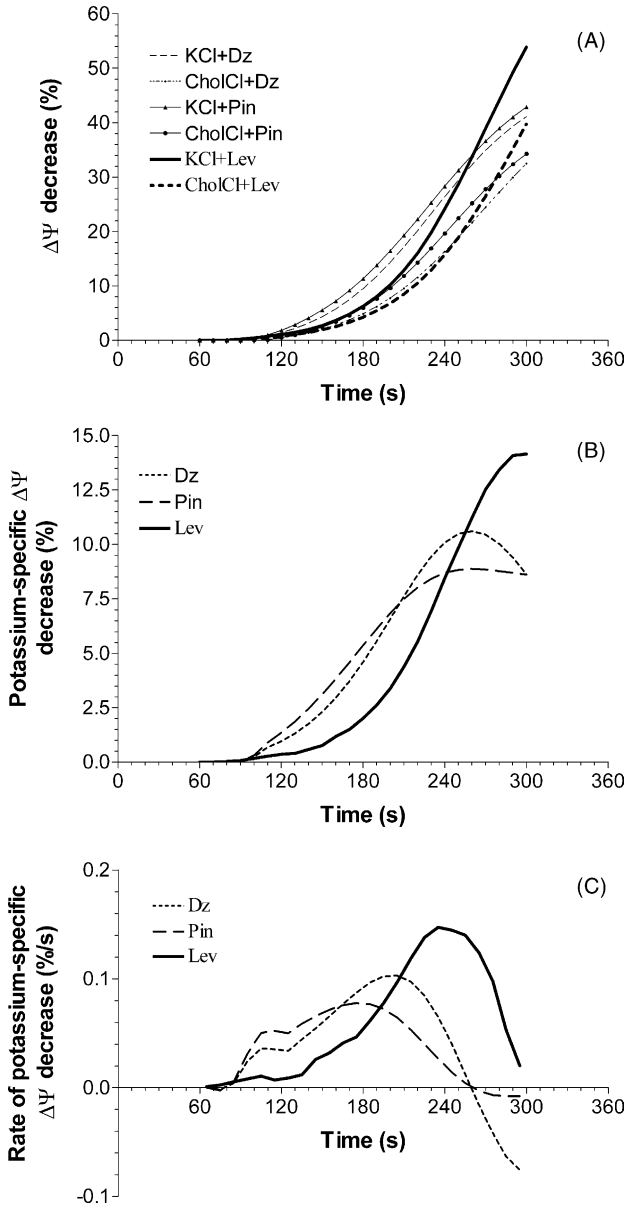

Fig. 2. Effect of diazoxide (Dz, 50 μM), pinacidil (Pin, 50 μM) and levosimendan (Lev, 1 μM) on $\Delta\Psi$ of rat heart mitochondria, respiring on endogenous substrates, in the presence of 200 μM ATP and 1 μg oligomycin/mg mitochondrial protein. Experiments were performed in both potassium chloride medium (KCl) and choline chloride medium (CholCl). (A) Original traces of typical experiments, (B) potassium-specific depolarization curves obtained after calculation of the differences between $\Delta\Psi$ curves in potassium chloride medium and the corresponding curves in choline chloride medium, (C) derivatives of potassium-specific depolarization curves.

higher in the KCl than in the choline chloride medium in cardiac mitochondria, respiring on endogenous substrates in the presence of 1 μg oligomycin/mg mitochondrial protein and 200 μM ATP. These results indicate that all the tested compounds are able to increase potassium flux to the mitochondrial matrix. Moreover, levosimendan at low concentration—1 μM—increased the potassium-specific $\Delta\Psi$ dissipation rate to a larger extent than diazoxide and pinacidil at their maximal concentration (50 μM),

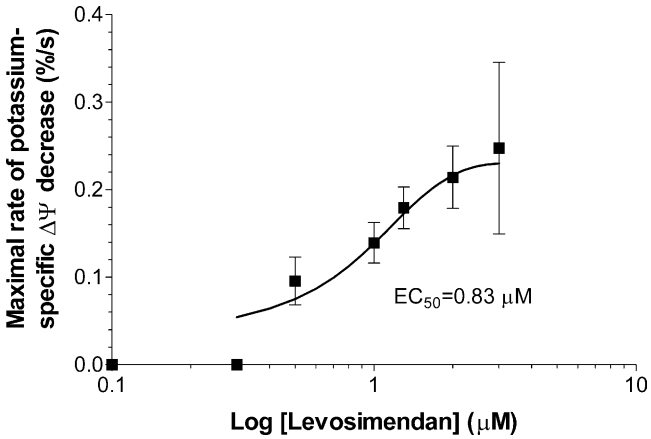

Fig. 3. Concentration-dependent effect of levosimendan on the maximal potassium-specific $\Delta\Psi$ decrease rate in rat heart mitochondria, respiring on endogenous substrates. The maximal derivatives of the differences of $\Delta\Psi$ decrease in potassium chloride and choline chloride media, supplemented with 200 μM ATP and 1 μg oligomycin/mg mitochondrial protein, are presented as mean ± S.D., $n = 3$.

which was used for activation of ATP-sensitive K^+ flux (Fig. 2B, see also swelling experiments, inset in Fig. 4).

3.2. Effect of channel openers on potassium influx in rat heart mitochondria

Fig. 3 shows that levosimendan in a concentration-dependent manner accelerated potassium-specific $\Delta\Psi$ decrease with an EC_{50} of 0.83 ± 0.24 μM. The ability of levosimendan to increase potassium flux to the mitochondrial matrix was confirmed by swelling experiments

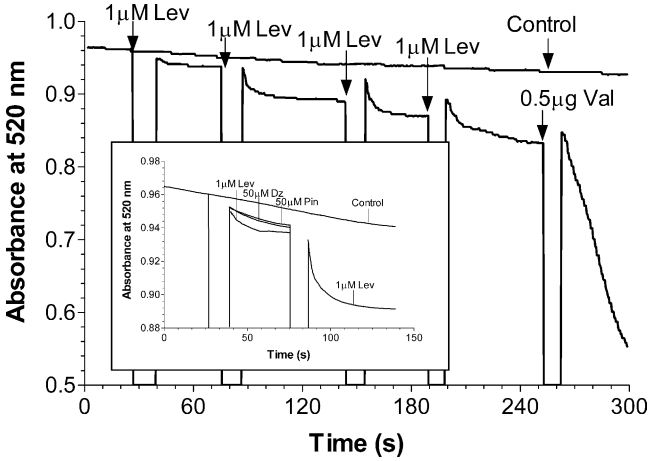

Fig. 4. Levosimendan (Lev)-supported swelling of non-respiring rat heart mitochondria in KNO_3 medium. Swelling was spectrophotometrically recorded as decreased light scattering at 520 nm in KNO_3 medium (120 mM KNO_3, 10 mM HEPES, 3 μM rotenone, 1 μg oligomycin/mg mitochondrial protein; pH 7.4, adjusted with Trizma base, 25 °C), supplemented with 200 μM ATP. Addition of 1 μg valinomycin (Val)/mg mitochondrial protein was used as a control. Similar curves were obtained in five independent experiments. Inset figure represents data obtained with low levosimendan (Lev) concentrations (1 + 1 μM), diazoxide (Dz; 50 μM) and pinacidil (Pin; 50 μM) at expanded absorbance scale.

in the KNO_3 medium. Since NO_3^- freely permeates membranes, the swelling rate of non-respiring mitochondria (respiration is blocked by rotenone, and phosphorylation or ATP hydrolysis—by oligomycin) is limited by the potassium permeability of the mitochondrial inner membrane under these conditions. Fig. 4 shows that levosimendan up to 4 μM increased mitochondrial swelling. Thus, our results indicate that levosimendan activates ATP-sensitive potassium flux through the inner membrane of cardiac mitochondria.

4. Discussion

The ability of K_{ATP} channel openers to mimic natural endogenous cardioprotective mechanism—ischemic preconditioning (for reviews, see [14,17,18,26]) promoted detailed investigations in this field, including also the development of new pharmacological agents designed for protection of the ischemic heart against injuries [27]. Since many pharmacological agents, which open cell membrane K_{ATP} channels, also may open mitochondrial K_{ATP} channels, we suggest that the anti-ischemic action of levosimendan could, at least partly, be due to stimulation of ATP-sensitive potassium flux to the matrix of cardiac mitochondria.

Potassium conductance of the mitochondrial inner membrane plays a significant role in mitochondrial physiology [28–30]. During ischemia, increased ATP-sensitive potassium flux to mitochondria prevents contraction of the mitochondrial matrix and expansion of the intermembrane space [17,31–34]. Matrix swelling could improve the rate of oxidative metabolism by stimulation of electron transfer, fatty acid oxidation and ATP production [28,35]. Potassium influx to mitochondria may optimize energy production or minimize energy loss during ischemia or reperfusion [26].

In this study, we evaluated levosimendan as a potential stimulator of ATP-sensitive potassium flux to mitochondrial matrix. For this purpose, we applied a recently introduced method for estimation of K_{ATP} channel openers-induced increase in mitochondrial inner membrane permeability for potassium by recording changes in $\Delta\Psi$ of heart mitochondria, respiring on endogenous substrates [24]. Under our experimental conditions activated potassium flux could decrease $\Delta\Psi$ at the maximal rate of ~0.2%/s (Fig. 1).

The ability of levosimendan to increase ATP-sensitive potassium flux to the mitochondrial matrix was previously demonstrated in liver mitochondria [10]. In the present report, we confirm these findings on cardiac mitochondria (Figs. 2–4). We compared the effect of levosimendan with the effects of the known K_{ATP} channel openers diazoxide and pinacidil (Fig. 2). Our results showed that 1 μM levosimendan accelerated potassium specific $\Delta\Psi$ decrease by ~0.15%/s, whereas 50 μM diazoxide—by ~0.10%/s,

and 50 μM pinacidil—by ~0.08%/s, respectively. Thus, our results imply that levosimendan, with an EC_{50} of 0.83 ± 0.24 μM (Fig. 3), is a more powerful activator of potassium flux to mitochondrial matrix than diazoxide or pinacidil. We have confirmed the ability of levosimendan to increase potassium flux to the mitochondrial matrix by swelling experiments in a KNO_3 medium, in which the swelling rate of non-respiring mitochondria is limited by the potassium permeability of the mitochondrial inner membrane (Fig. 4). Thus, by two independent methods we have demonstrated that levosimendan activates ATP-sensitive potassium flux through the inner membrane of cardiac mitochondria.

Our data could explain in part the reduction of infarct size seen in coronary-ligated dogs [9] and rabbits [36], where however the action of the drug as a vasodilator (by opening the K_{ATP} channels on the sarcolemma of vascular smooth muscle cells) and as cardioprotector (by opening mitochondrial K_{ATP} channels in cardiomyocytes) remains untangable since the drug was administered before the ischemic event.

The data presented also suggest possible alternative use of levosimendan in therapeutical fields where potassium channel openers which can elicit a preconditioning effect could be beneficial, such as the reduction of pain related to the first intra-aortic baloon inflation in coronary angioplasty [37,38] or the reduction of ischemic adverse events after coronary arthery by-pass grafting [39,40].

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