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Levosimendan is a mitochondrial K_{ATP} channel opener

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Abstract

Levosimendan, a new inodilator developed for the treatment of heart failure has been shown to have a vasodilatory effect via opening of K_{ATP} channels in the plasma membrane of vascular smooth muscle cells. In this study, we investigated the effects of levosimendan on the mitochondrial K_{ATP} channel. This compound did not influence mitochondrial transmembrane potential ($\Delta\Psi$), and at up to 2.2 μM had no effect on the respiration rate of rat liver mitochondria, respiring on 5 mM succinate (+5 μM rotenone). A sensitive method was developed for assessing K_{ATP} channel opening activity employing rat liver mitochondria, respiring only on endogenous substrates in the presence of 400 μM ATP and 1 μg oligomycin/mg mitochondrial protein. In this model, levosimendan (0.7–2.6 μM) decreased $\Delta\Psi$ by 6.5–40.4% ($n = 3$, incubation time 15 min). This effect was dependent on the K^+ concentration in the incubation medium and was abolished by the selective blocker of the mitochondrial K_{ATP} channel-5-hydroxydecanoate (200 μM). Our results indicate that levosimendan opens mitochondrial K_{ATP} channels. © 2001 Published by Elsevier Science B.V.

Keywords: K_{ATP} channel opener; Levosimendan; Mitochondrion

1. Introduction

Levosimendan, a novel agent developed for the treatment of heart failure, exerts positive inotropic action and peripheral vasodilatory effects (Lochner et al., 2000). The mechanism of action of levosimendan as a vasorelaxant is the opening of ATP-sensitive potassium channels in the plasma membrane. A direct evidence of this was obtained by recording K^+ currents and membrane potential ($\Delta\Psi$) in rat arterial myocytes (Yokoshiki et al., 1997). However, the effect was seen also as an increase in myocardial perfusion in coronary-occluded dogs (Kersten et al., 2000), an increase in coronary flow in isolated guinea pig heart (Du Toit et al., 1999; Kaheinen et al., 2001), a relaxation of noradrenaline-precontracted human portal vein (Pataricza et al., 2000) and a decrease in intracellular $[Ca^{2+}]$ in porcine coronary arteries (Bowman et al., 1999). All these effects were antagonized by glibenclamide (an inhibitor of K_{ATP}-channel) or by increasing $[K^+]_o$.

Many compounds which open K_{ATP} channels in the cell plasma membrane have been shown to affect also K_{ATP} channels in mitochondria (for recent reviews, see Grover and Garlid, 2000; Gross, 2000; Terzic et al., 2000; Szewczyk and Marban, 1999).

Mitochondrial K_{ATP} channels together with the K^+/H^+ antiporter are believed to maintain K^+ homeostasis within the mitochondrion and thereby to control mitochondrial volume (Garlid, 1996). The second putative functional role of mitochondrial K_{ATP} channels is enabling the formation of ΔpH along with membrane potential ($\Delta\Psi$) (Czyz et al., 1995). Opening of mitochondrial K_{ATP} channels increases swelling of the mitochondrial matrix, depolarizes the inner membrane of mitochondria, stimulates respiration, reduces Ca^{2+} uptake into and releases Ca^{2+} from the mitochondrial matrix (Szewczyk and Marban, 1999).

The use of the pharmacological agents, which stimulate K^+ flux through mitochondrial K_{ATP} channels, provides a new way to maintain cellular energy homeostasis and to protect mitochondria from oxidative injury and excessive volume changes (Terzic et al., 2000). The aim of our work

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was to investigate the effects of levosimendan on mitochondrial K_{ATP} channels. Our results indicate that this compound has a potent channel-opening capability.

2. Materials and methods

2.1. Preparation of mitochondria

The experiments were carried out on mitochondria isolated from male Wistar rat liver by differential centrifugation procedure. After decapitation, the liver was excised and rinsed in ice-cold isolation medium, containing 210 mM mannitol, 70 mM sucrose, 10 mM Hepes (pH 7.4 at room temperature with Trizma base). Mitochondria were isolated in the same medium supplemented with 1 mM EGTA and 5 mg/ml bovine serum albumin with two washings in the isolation medium without EGTA and bovine serum albumin. The final pellet was suspended in the isolation medium and kept on ice.

2.2. Assays

The mitochondrial protein concentration was determined by a modified biuret method (Gornall et al., 1949). The final mitochondrial protein concentration in all experiments was 1 mg/ml.

$\Delta\Psi$ of mitochondria was measured with rhodamine 123 as a fluorescent probe using the excitation at 503 nm and emission at 527 nm (Emaus et al., 1986) at 25 °C with the Hitachi F4000 fluorometer in a high KCl medium (100 mM KCl, 2 mM KH_2PO_4, 10 mM HEPES, 1 mM $MgCl_2$, pH 7.4 with TRIZMA base); a low KCl medium (200 mM sucrose, 20 mM KCl, 2 mM KH_2PO_4, 10 mM HEPES, 1 mM $MgCl_2$, pH 7.4 with TRIZMA base) or in a choline chloride (100 mM) medium (100 mM choline chloride, 2 mM NaH_2PO_4, 10 mM HEPES, 1 mM $MgCl_2$, pH 7.4 with TRIZMA base). The difference in fluorescence between mitochondria after addition of the uncoupling agent carbonyl-cyanide-*p*-trifluoromethoxy phenylhydrazone (FCCP) (0.4 μM) and without it was taken as 100%, and decrease in the $\Delta\Psi$ by the tested compounds was expressed in % of the FCCP effect.

Mitochondrial oxygen consumption was recorded at 25 °C by means of the Clark-type electrode system. The solubility of oxygen was taken to be 211 nmol O_2/ml. Respiration rates were expressed as nmol O_2/min/mg mitochondrial protein.

2.3. Reagents

All reagents were of the highest purity. Levosimendan was provided by Orion Pharma (Espoo, Finland). 5-hydroxydecanoate (5-HD) was purchased from RBI (Natick, MA, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.4. Statistical methods

The results are presented as means ± S.E. of three to six independent experiments. The results of three groups were analysed with one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test. $P < 0.05$ was taken as the level of significance. Where missing, error bars were smaller than the symbol size.

3. Results

Fig. 1 shows the effects of levosimendan on oxygen consumption rate and $\Delta\Psi$ of rat liver mitochondria, respiring on 5 mM of succinate (plus 5 μM rotenone) in the high KCl medium. This compound had no effect upon $\Delta\Psi$, and up to 2.2 μM had no effects on the respiration rate in State 2 (V_2). However, at 2.6 μM levosimendan slightly increased V_2.

In respiring mitochondria, the decrease in $\Delta\Psi$ due to the K_{ATP} channel opening is compensated by an increased respiration rate. In addition, when mitochondria respire only on endogenous substrates and oxidative phosphorylation is blocked by oligomycin, the respiration rate is sufficient to generate a high $\Delta\Psi$. In this case, however, the opening of K_{ATP} channels leads to a decrease in $\Delta\Psi$, which could not be compensated by increased respiration rate. In such a model, opening of K_{ATP} channels is unmasked, and can thus be recorded. We applied this model to test if opening could be induced by levosimendan (Figs. 2–4).

Both in high KCl and choline chloride media, the $\Delta\Psi$ was stable for 30 min (the longest time tested). The decrease in $\Delta\Psi$ was obtained after prolonged incubation (up to 15 min) of levosimendan with mitochondria in the KCl medium, supplemented with 400 μM ATP and 1 μg oligomycin/mg mitochondrial protein (Fig. 2), suggest-

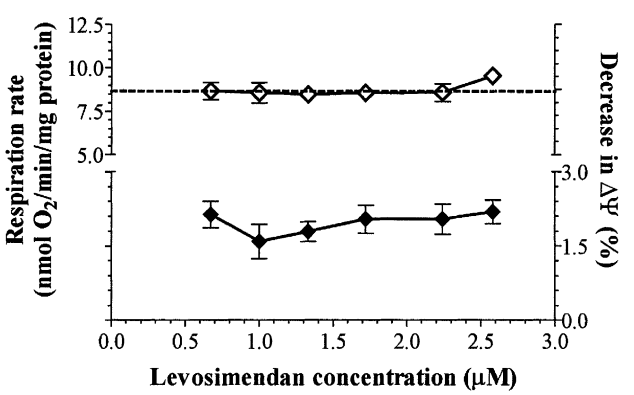

Fig. 1. Effect of levosimendan on the respiration rate in State 2 (open symbols) and $\Delta\Psi$ (closed symbols) of rat liver mitochondria, respiring on succinate + rotenone in high KCl medium; $n = 3$. Dotted line indicates level of the averaged State 2 respiration rate. For experimental conditions, see Materials and methods.

ing that the effect was cumulative. This assumption is strengthened by our finding that the effect of both compounds was more pronounced when more diluted mitochondrial suspensions were used (data not shown). Levosimendan, depending on its concentration, decreased the $\Delta\Psi$ of rat liver mitochondria, respiring only on endogenous substrates in the high KCl medium (Fig. 4), but did not significantly change the $\Delta\Psi$ in the choline chloride medium (Figs. 2 and 4). This decrease was not sensitive to the blocker of mitochondrial permeability transition pore-cyclosporin A. Moreover, 5-hydroxydecanoate (5-HD), the selective blocker of mitochondrial K_{ATP} channels, abolished the effect of levosimendan (Fig. 2). The decrease in $\Delta\Psi$ was smaller in the low KCl than in the high one (Fig. 3), indicating that this effect depends on the K^+ concentration in the medium. These results show that levosimendan

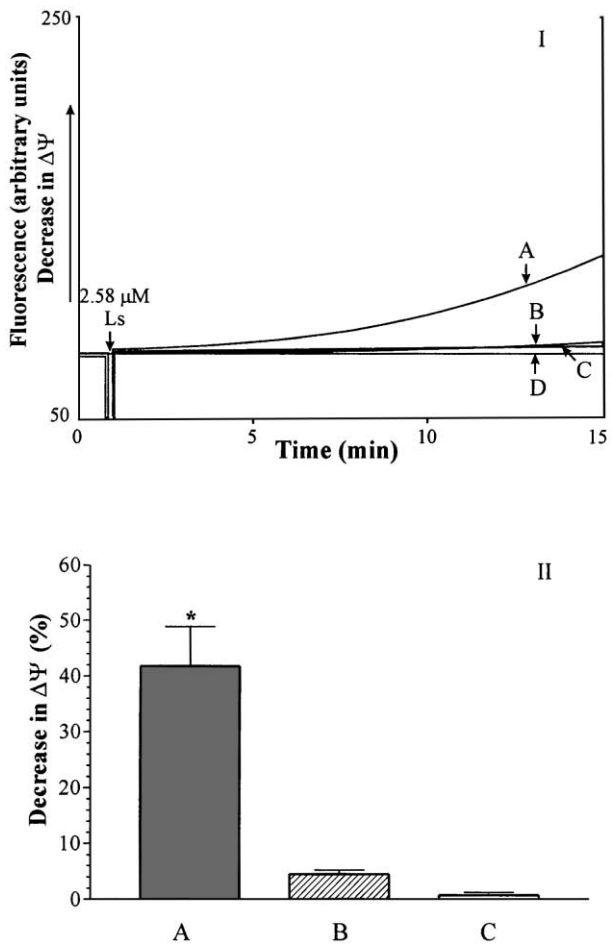

Fig. 2. Effect of levosimendan (Ls) on $\Delta\Psi$ of rat liver mitochondria, respiring only on endogenous substrates, in the presence of 400 μM ATP and 1 μg oligomycin/mg mitochondrial protein. (A) High KCl (100 mM) medium; (B) choline chloride (100 mM) medium; (C) high KCl medium supplemented with 200 μM 5-HD; (D) control, no additions. I, original traces; II, statistical analysis of the data. $^{*}p < 0.01$; statistically significant effect of 2.58 μM levosimendan in high KCl medium compared to two controls: choline chloride (100 mM) medium and high KCl medium, supplemented with 200 μM 5-HD, $n = 6$.

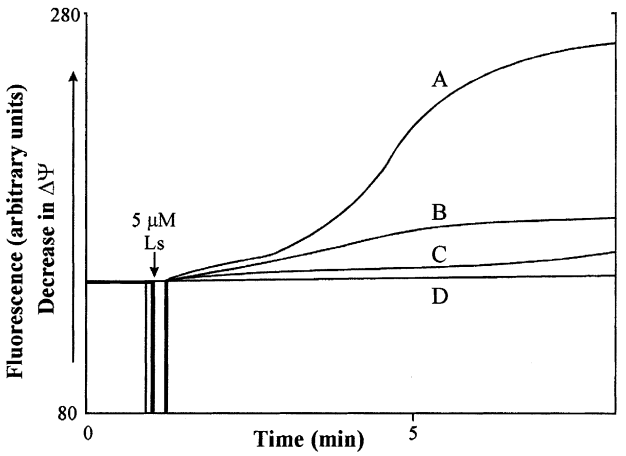

Fig. 3. Effect of levosimendan (Ls) on $\Delta\Psi$ of rat liver mitochondria, respiring only on endogenous substrates, in the presence of 1 μg oligomycin/mg mitochondrial protein. (A) High KCl (100 mM) medium; (B) low KCl (20 mM) medium; (C) choline chloride (100 mM) medium; (D) control, no additions.

decreases the $\Delta\Psi$ of mitochondria, respiring only on endogenous substrates, due to the opening of the mitochondrial K_{ATP} channel.

4. Discussion

Mitochondrial K_{ATP} channel activity has been directly followed by patch-clamp single-channel recordings in the inner membrane of rat liver mitochondria (Inoue et al., 1991). Therefore, we have chosen rat liver mitochondria as the object for levosimendan investigations. The results show that the new Ca^{2+}-sensitising positive inotropic agent levosimendan opens mitochondrial K_{ATP} channels.

Although the physiological role of mitochondrial K_{ATP} channels remains unclear, it was proposed that the opening of K^+-selective ion channels in the inner mitochondrial membrane could to some extent dissipate the $\Delta\Psi$ established by the proton pumps (Garlid, 1996). This would stimulate electron transfer by the respiratory chain (Liu et

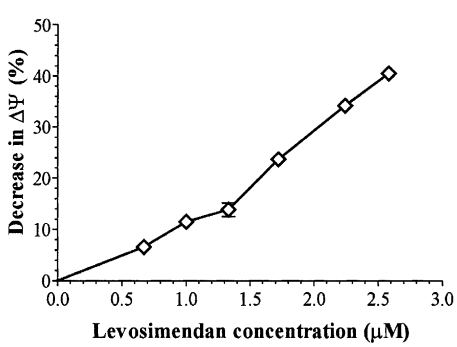

Fig. 4. Effect of different levosimendan (Ls) concentrations on $\Delta\Psi$ of rat liver mitochondria, respiring only on endogenous substrates, in high KCl (100 mM) medium supplemented with 400 μM ATP and 1 μg oligomycin/mg mitochondrial protein.

al., 1998), leading to an increase in the mitochondrial respiration rate. It was shown that the K^+ flux through the newly opened mitochondrial K_{ATP} channels increases the respiration rate by no more than 5% and decreases the $\Delta\Psi$ by 2–4 mV (Garlid, 2000; Kowaltowski et al., 2001). Taking all this into consideration, the slight increase in V_2 obtained by 2.6 μM of levosimendan (Fig. 1) could be explained as being due to the opening of mitochondrial K_{ATP} channels.

In Figs. 2–4, our results show that levosimendan, in a concentration-dependent manner, decreased the $\Delta\Psi$ in rat liver mitochondria respiring only on endogenous substrates. This effect was time-dependent that could be due to the enrichment in the membranes of the hydrophobic compounds, and dependent also on the K^+ concentration in the medium. We demonstrated that the selective blocker of mitochondrial K_{ATP} channel—5-HD (200 μM)—abolished the effect of levosimendan (Fig. 2); furthermore, the compound had no significant effect in the choline chloride medium (Figs. 2–4). These results show that the decrease in the $\Delta\Psi$ is a consequence of mitochondrial K_{ATP} channel opening. 5-HD blocked the effect of levosimendan only in the presence of 400 μM ATP. This is in good agreement with data (Jaburek et al., 1998) showing that K_{ATP} channel blockers can close K_{ATP} channels only under conditions when the channels are blocked by ATP and opened by pharmacological or physiological openers.

Thus, our results demonstrate that a novel Ca^{2+}-sensitising and positive inotropic agent levosimendan also has mitochondrial K_{ATP} channel opening activity.

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