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Short communication

The effects of levosimendan on $[Ca^{2+}]_i$ in guinea-pig isolated ventricular myocytes

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Abstract

Ca^{2+}-sensitising agents offer a new approach to the treatment of congestive heart failure. This study examined the effects of the Ca^{2+}-sensitising agent, levosimendan, on contraction and $[Ca^{2+}]_i$ in guinea-pig ventricular myocytes. Levosimendan 100 nM produced an increase in cell shortening without affecting the $[Ca^{2+}]_i$ transient or the Ca^{2+} content of the sarcoplasmic reticulum. 1 μM levosimendan increased the rate of decay of the $[Ca^{2+}]_i$ transient and increased the Ca^{2+} content of the sarcoplasmic reticulum. These results suggest that at therapeutically relevant concentrations levosimendan can produce a significant inotropic effect without affecting $[Ca^{2+}]_i$ but at higher concentrations may also inhibit phosphodiesterase. © 1997 Elsevier Science B.V.

Keywords: Levosimendan; (Guinea pig); Myocyte; $[Ca^{2+}]_i$

1. Introduction

Reduced contractility of cardiac muscle is considered to be the primary defect in patients with congestive heart failure. This has led to the development of positive inotropic compounds that can enhance force production in the failing heart. Cardiac glycosides, such as digitalis, achieve this increase in force by increasing the influx of Ca^{2+} into cardiac cells, resulting in an increased Ca^{2+} load of the sarcoplasmic reticulum. These agents are therefore limited by their narrow therapeutic margin as they have the potential to induce $[Ca^{2+}]_i$ overload which in turn can trigger cardiac arrhythmias. An agent which could produce a similar inotropic response without disturbing $[Ca^{2+}]_i$ would be of important therapeutic value.

During recent years great interest has focused on the development of so-called Ca^{2+}-sensitising agents (Rüegg and Solaro, 1993) that might lead to safe positive inotropic drugs for treating heart failure. Levosimendan ((*R*)-[[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]-hydrazono]propanedinitrile) is a novel positive inotropic drug targeted to increase contractile force of the heart through its binding to troponin C (Pollesello et al., 1994; Haikala et al., 1995). The resulting Ca^{2+}-sensitising effect means that levosimendan, at least in theory, should not induce $[Ca^{2+}]_i$ overload. Thus, the occurrence of arrhythmias due to spontaneous release of Ca^{2+} from an overfilled sarcoplasmic reticulum is unlikely, offering a definite advantage for the treatment of heart failure. However, binding to troponin C may not be the only mechanism of action of levosimendan. A feature of a number of Ca^{2+}-sensitising agents is that they also possess activity as phosphodiesterase inhibitors (Rüegg and Solaro, 1993). This is an undesirable property as phosphodiesterase inhibition will lead to an increase in $[Ca^{2+}]_i$ and the possibility of arrhythmias.

In the present study we have examined the effects of levosimendan on single isolated guinea-pig ventricular myocytes. We have looked for effects on the systolic $[Ca^{2+}]_i$ transient and sarcoplasmic reticulum Ca^{2+} content.

2. Materials and methods

Ventricular myocytes were isolated from guinea-pig hearts using a modification of the method of Grantham and Cannell (1996). Cells were loaded with the acetoxymethyl ester of indo-1 (5 μM) for 5 min. Loading was terminated by dilution. Cells were placed in a bath on the stage of an inverted microscope and superfused with the following solution (mM): NaCl, 134; KCl, 4; $MgCl_2$, 1; HEPES, 10;

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$CaCl_2$, 1; glucose, 10, pH was titrated to 7.4 with NaOH. Fluorescence was excited at 340 nm and collected at 400 and 500 nm. The ratio of emission at 400 and 500 nm gives a measure of $[Ca^{2+}]_i$. Cells were stimulated at 0.5 Hz with field electrodes and cell length measured using a video-based edge detection system. Voltage clamp control was imposed with the amphotericin perforated patch clamp technique. The perforated patch pipette solution was (mM): KCH_3O_3S, 125; KCl, 20; NaCl, 12; HEPES, 10; $MgCl_2$, 5; K_2EGTA, 0.1; titrated to pH 7.2 with KOH. Amphotericin B was added to a final concentration of 240 μg/ml. In voltage clamp experiments the experimental solution was modified with the addition of 5 mM 4-aminopyridine (to inhibit the transient outward current) and 0.1 mM $BaCl_2$ to block K^+ currents. Voltage clamped cells were held at -80 mV and stimulated by 450 ms depolarisations to 0 mV. In these cells the integral of the resulting Na^+/Ca^{2+} exchange current following the rapid application of 10 mM caffeine was used as an estimate of the sarcoplasmic reticulum Ca^{2+} content (Varro et al., 1993; Negretti et al., 1995). The values were converted to total calcium fluxes by correcting for mechanisms other than Na^+/Ca^{2+} exchange which remove Ca^{2+} from the cell. This is done by measuring the rate constant of decay of the caffeine response under control conditions (k_{cont}) and with the Na^+/Ca^{2+} exchange inhibited by 10 mM Ni^{2+} (k_{ni}). Multiplying the measured flux value by $k_{cont}/(k_{cont}-k_{ni})$ gives the corrected flux. In guinea-pig cells Na^+/Ca^{2+} exchange is responsible for 84% of Ca^{2+} removal (unpublished observations of Choi and Eisner). These values are then related to cell volume. This is calculated from the cell membrane surface area (obtained from the membrane capacitance) and then converted to volume taking a value of 0.5 μm^{-1} for the surface to volume ratio (Page, 1978). All experiments were carried out at 25°C. Levosimendan (Orion Pharma) was added to the basic perfusate from a stock made in ethanol. The amount of ethanol did not exceed 0.01% in any of the experiments. Levosimendan was added in increasing concentrations at 10 min intervals. Data are expressed as mean ± standard error of the mean. Statistical differences were assessed using one-factor ANOVA (repeated measures) followed by Dunnett's test to compare the effects of different concentrations of levosimendan with control values. The null hypothesis was rejected if $P < 0.05$.

3. Results

3.1. Effect of levosimendan on cell shortening and the $[Ca^{2+}]_i$ transient amplitude

Levosimendan (0.1 nM–1 μM) caused a concentration-dependent increase in cell shortening (Fig. 1A; EC_{50} 9.19 ± 2.42 nM). In many cases increasing the concentration of levosimendan beyond 1 μM led to the development of spontaneous oscillations (i.e. unstimulated

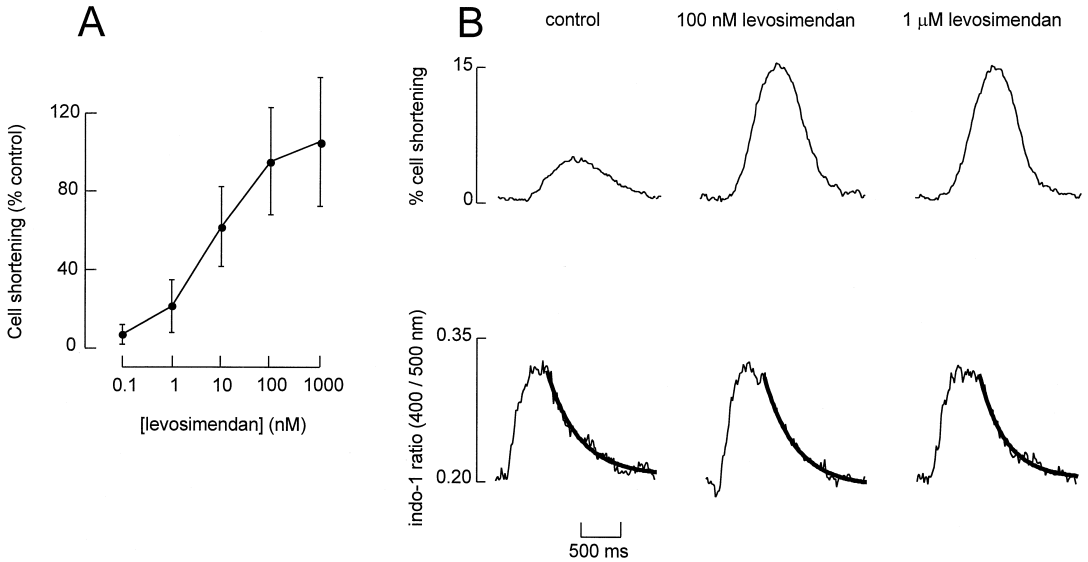

Fig. 1. (A) Concentration–response curve for the effect of levosimendan on cell shortening in guinea-pig isolated ventricular myocytes. An EC_{50} of 9.19 ± 2.42 nM was calculated from these data assuming 1 μM levosimendan gave the maximum response. (B) Typical traces from a single guinea-pig ventricular myocyte illustrating the effect of 100 nM and 1 μM levosimendan on percentage cell shortening (upper panel) and $[Ca^{2+}]_i$ transient expressed as the indo-1 fluorescence ratio (lower panel). 100 nM levosimendan produced an increase in cell shortening yet clearly the amplitude of the $[Ca^{2+}]_i$ transient is not affected. 1 μM levosimendan had no further effect on cell shortening and also did not affect the $[Ca^{2+}]_i$ transient amplitude. The rate constants for the decay of the $[Ca^{2+}]_i$ transients were calculated from single exponential decay curves fitted to the data as shown by the smooth lines in bold. The rate constants for the data shown were 2.55 s^{-1} for the control transient and 2.56 s^{-1} in the presence of 100 nM levosimendan. 1 μM levosimendan increased the rate constant to 3.01 s^{-1}.

contractions) so that any further changes in cell shortening could not be measured.

In cells loaded with indo-1, levosimendan 100 nM and 1 μM significantly increased cell shortening without affecting the $[Ca^{2+}]_i$ transient amplitude (Fig. 1B). In 7 cells levosimendan 100 nM and 1 μM increased cell shortening by an average of 61 and 63% above control, respectively ($P < 0.05$). The mean $[Ca^{2+}]_i$ transient amplitudes (400/500 nm) were 0.41 ± 0.13 for control conditions, 0.41 ± 0.11 in the presence of 100 nM levosimendan and 0.39 ± 0.10 in the presence of 1 μM levosimendan. In control conditions the rate constant for the decay of the $[Ca^{2+}]_i$ transient was 3.1 ± 0.6 s^{-1}. Levosimendan (100 nM) did not significantly affect the rate of decay of the $[Ca^{2+}]_i$ transient (3.5 ± 0.7 s^{-1}). However, levosimendan (1 μM) caused a significant increase in the rate of decay of the $[Ca^{2+}]_i$ transient (4.6 ± 0.7 s^{-1}; $P < 0.05$).

3.2. Effect of levosimendan on sarcoplasmic reticulum Ca^{2+} content

In cells voltage clamped using the amphotericin perforated patch technique the current evoked by the rapid application of caffeine was used to estimate sarcoplasmic reticulum Ca^{2+} content. Levosimedan (100 nM) had no effect on sarcoplasmic reticulum Ca^{2+} content (Fig. 2). However, levosimendan (1 μM) caused a significant increase in sarcoplasmic reticulum Ca^{2+} content (Fig. 2). In 5 cells the mean sarcoplasmic reticulum Ca^{2+} content was 26.5 ± 6.2 μmol l^{-1} under control conditions, 28.2 ± 6.7 μmol l^{-1} in the presence of 100 nM levosimendan and 36.1 ± 6.7 μmol l^{-1} in the presence of 1 μM levosimendan ($P < 0.05$).

4. Discussion

The present results show that in guinea-pig ventricular myocytes levosimendan (100 nM) can cause a substantial increase in cell shortening without affecting either the $[Ca^{2+}]_i$ transient or the sarcoplasmic reticulum Ca^{2+} content (Figs. 1 and 2). This is the first study which provides direct evidence that $[Ca^{2+}]_i$ is not altered by levosimendan at concentrations up to 100 nM and supports the idea that the effect of levosimendan (≤ 100 nM) results from a direct action on the myofilaments, presumably binding to troponin C (Haikala et al., 1995), rather than an indirect effect, such as inhibition of phosphodiesterase. At the higher concentration (1 μM) which has little additional effect on cell shortening our findings support the idea that levosimendan may also act as a phosphodiesterase inhibitor. This is reflected in an increase in sarcoplasmic reticulum Ca^{2+} content (Fig. 2) and in an increased rate of decay of the $[Ca^{2+}]_i$ transient. We have also confirmed (data not shown) the observation of Virág et al. (1996) that levosimendan 1 μM significantly increases the inward Ca^{2+} current in isolated guinea-pig myocytes, another feature of phosphodiesterase inhibition. Haikala et al. (1997) have recently shown that at 22°C there is no cAMP-dependent component of the levosimendan effect up to a concentration of 300 nM supporting the present results. However, Edes et al. (1995) have previously shown that at 37°C levosimendan (100 nM) can produce a significant increase in cAMP in guinea-pig perfused hearts, raising the possibility of temperature-dependency of the phosphodiesterase inhibiting properties of levosimendan.

One slightly surprising observation was that despite increasing sarcoplasmic reticulum Ca^{2+} content and the

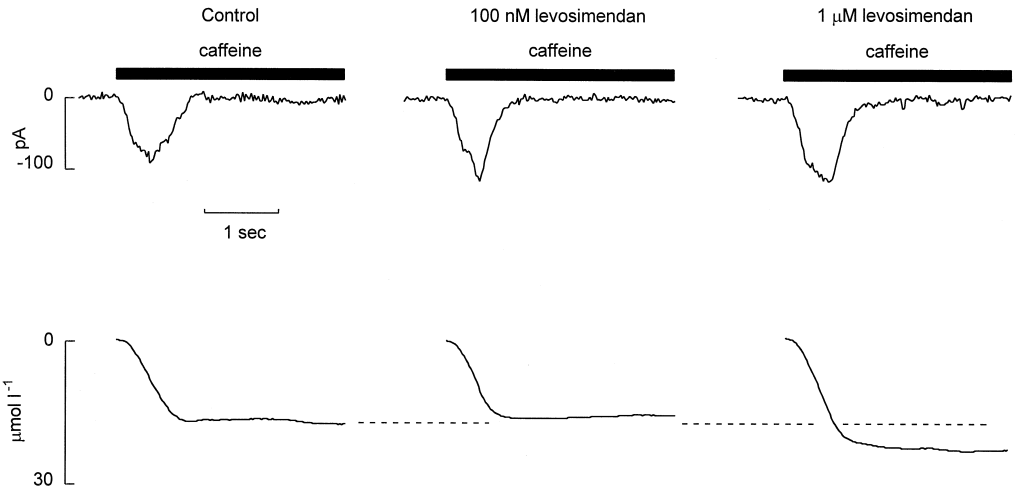

Fig. 2. The effect of levosimendan on the sarcoplasmic reticulum Ca^{2+} content in a single guinea-pig ventricular myocyte. During the period indicated by the solid bars caffeine was applied to the cell releasing the Ca^{2+} content of the sarcoplasmic reticulum which leaves the cell via the Na^+/Ca^{2+} exchanger producing the inward current shown in the upper panel. The lower panel shows the integral of this current corrected for other Ca^{2+} fluxes and expressed as a Ca^{2+} concentration per liter of cell volume as derived from cell capacitance measurements. At a concentration of 100 nM, levosimendan did not significantly alter the sarcoplasmic reticulum Ca^{2+} content. However, at a concentration of 1 μM the sarcoplasmic reticulum Ca^{2+} content of this cell increased by 40%.

Ca^{2+} current, 1 μM levosimendan failed to increase the $[Ca^{2+}]_i$ transient amplitude. This may indicate an effect of levosimendan on other components of Ca^{2+}-induced Ca^{2+} release and warrants further investigation.

In heart failure patients 95–98% of levosimendan is bound to plasma proteins and therefore at therapeutic doses its active free plasma concentration is less than 20 nM (Sandell et al., 1995). Our results suggest that at therapeutically relevant concentrations levosimendan, whilst providing significant inotropic effect, is not likely to increase the influx of Ca^{2+} into cardiac cells. The Ca^{2+} load of the sarcoplasmic reticulum should not be altered and therefore, unlike conventional inotropic therapies, levosimendan should have little potential to trigger cardiac arrhythmias.

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