The Cardiotonic Effects of Levosimendan in Guinea Pig Hearts Are Modulated by β-Adrenergic Stimulation

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Abstract. The effects of the Ca^{2+}-sensitiser levosimendan alone or in combination with β-adrenergic stimulation on the contractile function were studied in various guinea pig cardiac preparations. Echocardiography in narcotised animals indicated that a maximal dose of levosimendan (50 $\mu g \cdot kg^{-1}$) increased the left ventricular posterior wall movement velocity during systoles and diastoles by $25 \pm 3\,\%$ (mean ± S.E.M.) and $17 \pm 2\,\%$, respectively. In Langendorff hearts, a saturating concentration of levosimendan (0.3 $\mu mol \cdot l^{-1}$ for 5 min) increased $+dP/dt_{max}$ and $-dP/dt_{max}$ by $28 \pm 3\,\%$ and $14 \pm 2\,\%$, respectively. Further, the Ca^{2+}-sensitising potential of levosimendan in Triton-skinned cardiomyocytes (EC_{50}: 5 ± 3 $nmol \cdot l^{-1}$) was illustrated by a maximal increase in the isometric force production by $51 \pm 5\,\%$ (at pCa 6.2). However, following stimulation by isoproterenol, when the level of troponin I phosphorylation was elevated, no significant additional increase in the contractile parameters could be demonstrated upon levosimendan administration. Moreover, the levosimendan-induced increase in force production in isolated skinned myocytes could be prevented by incubation with the catalytic subunit of protein kinase A (100 $U \cdot ml^{-1}$ for 40 min). These data indicate that thin filament-targeted Ca^{2+}-sensitisation by levosimendan is modulated by phosphorylation of the contractile filaments, an effect that should be considered during combination therapy with levosimendan.

Key words: Contractility — Heart — Cardiomyocytes — Ca^{2+}-sensitisers — Levosimendan

Introduction

In the clinical practice, correction for the diminished contractile function of the failing myocardium is a major therapeutic challenge. Conventional positive inotropic agents increase the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and their application is therefore associated with adverse effects: an increased energy demand and

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arrhythmias. Pharmacological sensitisation of the contractile machinery to Ca^{2+} may alleviate these problems.

One of the most promising Ca^{2+}-sensitiser molecules is levosimendan, which proved effective in recent clinical trials (Nieminen et al. 2000; Slawsky et al. 2000; Follath et al. 2002; Moiseyev et al. 2002). The pharmacological profile of levosimendan is complex, however, as it combines the potentials of Ca^{2+}-sensitisers and ATP-dependent K^+ channel openers (Yokoshiki et al. 1997). Accordingly, we set out to characterise the cardiac effects of levosimendan under various model conditions that either allowed or compromised the development of its dual major actions in the cardiovascular system. To this end, the contractile function of the myocardium was monitored in narcotised guinea pigs and in Langendorff-perfused hearts. The cardiac performance in narcotised guinea pigs is a function of the interaction between the contractile activity of the heart and the vascular system, and this arrangement therefore faithfully mimics *in vivo* conditions. In contrast, the cardiac loading was constant in the Langendorff preparations, and the cardiac effects of levosimendan could therefore be isolated from those depending on the preload and/or afterload. Finally, direct force measurement in single skinned cardiomyocytes allowed the analysis of the effects of levosimendan at the subcellular level.

Stimulation of the β-adrenergic signalling pathway involves an increase in the intracellular cyclic adenosine 3′-5′-monophosphate (cAMP) concentration and consequently in the level of protein phosphorylation through the activation of protein kinase A. In cardiac myocytes, protein kinase A phosphorylates the contractile proteins (Kranias and Solaro 1982) (including cardiac troponin I (cTnI) and myosin binding protein C), and hence decreases the Ca^{2+} sensitivity of force production (Van der Velden et al. 2000). Thus, traditional β-mimetic positive inotropes may possibly interfere with the Ca^{2+}-sensitising mechanism of levosimendan. Indeed, Haikala et al. (1997) suggested that the phosphorylation of cTnI may attenuate the positive inotropy induced by levosimendan. Surprisingly, however, it has recently been documented (Brixius et al. 2002) that isoproterenol pretreatment potentiates the contractile effects of levosimendan in isolated cardiac muscle strips from patients with dilated cardiomyopathy. Additionally, levosimendan, when applied at high concentrations (Edes et al. 1995), may inhibit intracellular phosphodiesterases, elevate the level of intracellular cyclic nucleotides, and therefore stimulate protein phosphorylation *via* protein kinases. In particular, the phosphorylation of phospholamban (Edes et al. 1995) and the L-type Ca^{2+}-channel molecule (Virag et al. 1996) would then interfere with the Ca^{2+} regulation of the contractile system.

In the present study, an attempt was made to reveal the possible interactions between the intracellular effects of β-adrenergic signalling and of levosimendan in the myocardium. This was achieved by comparing the contractile effects of levosimendan in guinea pigs before and after β-receptor stimulation (in narcotised animals and in Langendorff-perfused hearts) or after incubation with protein kinase A (in isolated skinned myocytes).

We report that the levosimendan-induced contractile effects were largely attenuated during intensive sympathetic stimulation, in parallel with the development

of an increased level of intracellular phosphorylation. Hence, the benefit of the Ca^{2+}-sensitiser levosimendan may be negatively influenced by the simultaneous application of β-mimetic agents, an effect depending on the phosphorylation level of the contractile filaments.

Materials and Methods

Echocardiography

Echocardiography was performed with an Acuson Sequoia System (Mountain View, Ca., U.S.A.) in Hartley guinea pigs of either sex (body weight: 450–600 g) following narcosis attained with an intraperitoneal (i.p.) injection of 100 $mg \cdot kg^{-1}$ ketamine. The transducer frequency of the annular array was 7 MHz. Two-dimensional (2D) echocardiography and 2D-guided M-mode imaging were applied. 2D targeted M-mode studies were performed *via* the long-axis view of the left ventricle (LV). To assess the systolic and diastolic contractile functions, the velocities of the LV posterior wall motion were determined during contractions (posterior wall contraction velocity (PW-CV)) and relaxations (posterior wall relaxation velocity (PW-RV)), respectively. The results of the measurements were analysed by two experienced readers over an average of 3 cardiac cycles. All images were recorded on super VHS (sVHS) tapes and on magnetooptical discs.

Langendorff perfusions

Hearts were rapidly excised from Hartley guinea pigs anaesthetised with 30 $mg \cdot kg^{-1}$ sodium pentobarbital and heparinised with 500 $U \cdot kg^{-1}$. The hearts were immediately cannulated through the aorta and prepared for retrograde perfusion with a modified Krebs buffer containing ($mmol \cdot l^{-1}$) NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, Na_2 EDTA 0.5, KH_2PO_4 0.23, and glucose 5.5. The buffer solution was saturated with 95% O_2/5% CO_2, pH 7.4, at 37°C. The hearts were initially perfused at a constant aortic pressure (5 kPa) for 20 to 25 min in a drip-through mode. The perfusion circuit was then switched to a recirculating system containing 200 $cpm \cdot pmol^{-1}$ [^{32}P]orthophosphate in 120 ml Krebs buffer for 30 min. After this labelling period, the circuit was returned to the drip-through mode with nonradioactive buffer for 1 min. The drug of interest (levosimendan for 5 min or isoproterenol for 2 min) was administered into the buffer flow line. The hearts were freeze-clamped with a precooled Wollenberger clamp, powdered and stored under liquid nitrogen, as described previously (Kranias and Solaro 1982; Talosi et al. 1993). The heart rate, LV pressure and the derivative of the mechanical function (dP/dt) were monitored continuously, as described previously (Kristof et al. 1999). dP/dt was derived electronically and stored in a personal computer.

Levosimendan, provided by Orion Pharma (Espoo, Finland), was dissolved in aliquots of isotonic saline or Krebs buffer for the studies with narcotised animals or Langendorff preparations, respectively. For the isolated skinned myocyte study, levosimendan was diluted from a concentrated stock solution (10 $mmol \cdot l^{-1}$

in DMSO). The final concentration of DMSO never exceeded 0.1%. During all animal experiments, institutional guidelines were followed.

Gel electrophoresis and autoradiography

Polyacrylamide gel electrophoresis under denaturating conditions was performed according to Laemmli (1970), using 20% slab gels. After electrophoresis, the gels were fixed, stained with Coomassie blue, destained, sealed in plastic bags and placed into Kodak Lanex regular cassettes loaded with Agfa films for 48–72 hours. The radioactive band corresponding to cTnI was identified and cut from the gel for counting in the scintillation fluid (ScintiVerse, Fisher St. Louis, MO, USA). Phosphate incorporation was quantified by dividing the amount of ^{32}P incorporated into the proteins by the specific activity of [gamma-^{32}P]ATP determined for each heart, and expressed as picomoles phosphate·milligram protein^{-1}. The specific radioactivity of [gamma-^{32}P]ATP was determined from the specific activity of [^{32}P]phosphocreatine at the end of perfusion (Kopp and Barany 1979).

Force measurement in isolated skinned myocytes

Myocytes were isolated mechanically, as described previously (Van der Velden et al. 1998). The composition of the solution used for cell isolation was (mmol·l^{-1}): $MgCl_2$ 1, KCl 100, EGTA 2, Na_2ATP 4, imidazole 10 (pH 7.0, adjusted with KOH). The compositions of the relaxing and activating solutions (Table 1) used during force measurements were calculated similarly as described by Fabiato and Fabiato (1979). The pCa, i.e., $-\log[Ca^{2+}]$, values of the relaxing and activating solutions (pH 7.2) were 10 and 4.75, respectively. Solutions with intermediate $[Ca^{2+}]$ levels were obtained by mixing of the activating and relaxing solutions.

The suspension of myocyte-sized preparations was permeabilised with 0.5% Triton X-100 (Calbiochem, San Diego, USA) (5 min). Triton X-100 removed all

Table 1. Compositions of experimental solutions for the skinned myocyte study

	Relaxing	Activating
pCa	10	4.75
MgATP	5	5
CaEGTA	0	7
EGTA	7	0
Free Mg^{2+}	1	1
Phosphocreatine	15	15
Ionic strength	186	186
Ionic equivalent	150	150

Concentrations are given in mmol·l^{-1}. In addition, all solutions contained N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES) as a pH buffer (100 mmol·l^{-1}). CaEGTA was made by mixing equimolar amounts of $CaCO_3$ and EGTA. The ionic equivalent of the solutions was adjusted with KCl (pH 7.2, with KOH).

membranous structures and enabled free diffusion for Ca^{2+}, and thus the study of the Ca^{2+}-sensitising effect of levosimendan under standardised conditions (i.e., in the presence of controlled $[Ca^{2+}]_i$ and sarcomere length) (Vannier et al. 1997). A single myocyte was attached with silicone adhesive (100 % silicone, Aquarium sealant, Dow Corning, Midland, USA) to two thin stainless steel needles while viewed by means of an inverted microscope. One needle was attached to a force transducer (SensoNor, Horten, Norway) and the other to an electromagnetic motor (Aurora Scientific Inc., Aurora, Canada), both connected to joystick-controlled micromanipulators. The average sarcomere length was determined by means of a spatial Fourier transform, as described previously (Gannier et al. 1993; Fan et al. 1997), and adjusted to 2.2 μm in the relaxing solution. Solution exchange was achieved by transferring the myocyte from a small temperature-controlled well (volume 50 μl) containing the relaxing solution to a similar temperature-controlled well containing the activating solution.

The isometric force was measured after the preparation had been transferred to the activating solution. When the peak force was reached, the length of the myocyte was reduced by 20 % within 1 millisecond (slack test). As a result of this intervention, the force dropped from the peak level to zero and then started to redevelop. About 3 seconds after the onset of force redevelopment, the myocyte was returned to the relaxing solution, where a slack test with a long slack duration (10 seconds) was performed to assess the passive force level. The active isometric force was calculated by subtracting the passive force from the peak isometric force. Force and length signals were monitored by using an analogue pen-recorder and were stored in a personal computer. The sampling rate during experiments was 20 Hz, while during slack tests it was 1000 Hz. The temperature during force measurements was set to 15 °C in order to maintain the mechanical stability of the permeabilised myocyte preparations. Force production at the submaximal level of activation (i.e., at pCa 6.2) in the absence or presence of levosimendan was normalised to an interpolated reference force obtained by averaging the two bracketing values of maximal activation (pCa 4.75) at the beginning and at the end of the experiments.

Data analysis

The relation between force (P) and levosimendan concentration ([levo]) was fitted to a modified Hill equation:

$$P_{\text{levo}} = P_{\text{levo(max)}}/(1 + ([\text{levo}]/\text{EC}_{50})^{n_{\text{Hill}}}) \quad (1)$$

where P_{levo} is the steady-state force at a given concentration of levosimendan, $P_{\text{levo(max)}}$ is the steady isometric force at maximal levosimendan effect, the Hill coefficient (n_{Hill}) is a measure of the steepness of the relationship, and EC_{50} is the mid-point of the relation. The results are given throughout as means ± S.E.M. from 4–22 different preparations. Differences were tested by means of Student's unpaired t-test at a level of significance of 0.05 ($p < 0.05$).

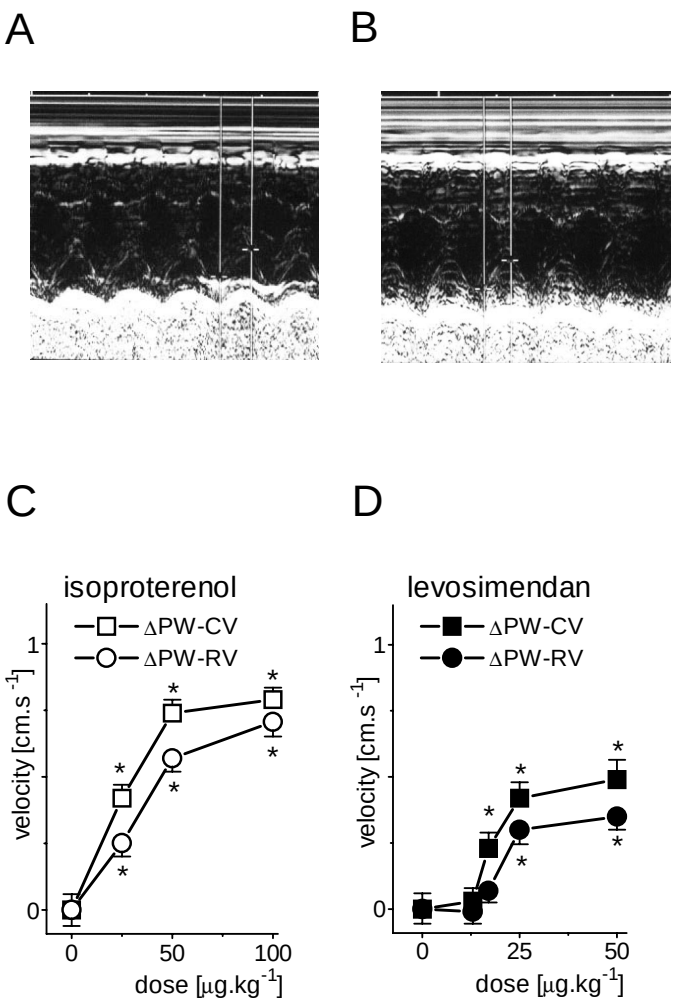

Figure 1. Determination of the velocity of the left ventricular posterior wall motion by M-mode echocardiography in narcotized guinea pigs. **A.** M-mode images (of 1-s duration) were obtained under basal conditions and **B.** 2 min after the i.p. administration of isoproterenol (50 $\mu g \cdot kg^{-1}$). Vertical lines indicate the beginning and the end of systolic wall motion. **C.** The increase (Δ) in the velocity of systolic (PW-CV, □) and diastolic (PW-RV, ○) wall motion as a function of the applied dose of isoproterenol (mean ± S.E.M.). **D.** ΔPW-CV (■) and ΔPW-RV (●) as a function of the applied dose of levosimendan. Asterisks indicate significant differences between values at various drug doses and under drug-free conditions. (PW-CV and PW-RV were determined 2 or 15 min following isoproterenol or levosimendan injections, respectively.)

Results

The motion velocities of the LV posterior wall during systoles (PW-CV) and diastoles (PW-RV) following application of levosimendan and/or isoproterenol in narcotised guinea pigs demonstrated inotropic and lusitropic responses. The cardiac contractility during this experimental arrangement resulted from the interaction between the cardiac and vascular effects of the chemical agents. Figure 1 indicates that both isoproterenol and levosimendan exerted dose-dependent positive inotropic and lusitropic effects when applied independently. PW-CV and PW-RV increased from a baseline level of 2.08 ± 0.06 cm·s^{-1} and 2.28 ± 0.06 cm·s^{-1} to 2.87 ± 0.09 cm·s^{-1} and 2.99 ± 0.06 cm·s^{-1}, respectively, upon i.p. application of 100 μg·kg^{-1} isoproterenol. These changes were accompanied by an increase in the heart rate from 284 ± 11 to 341 ± 10 beats·min^{-1} ($n = 4$). Levosimendan also induced marked elevations in both the contraction and relaxation velocities (from 1.96 ± 0.06 cm·s^{-1} and 2.03 ± 0.055 cm·s^{-1} to 2.45 ± 0.075 cm·s^{-1} and 2.38 ± 0.05 cm·s^{-1}, respectively) at a maximal level of stimulation (50 μg·kg^{-1} i.p). The levosimendan-induced maximal change in the heart rate involved an increase from a baseline value of 278 ± 9 to 307 ± 8 beats·min^{-1} ($n = 4$).

Figure 2 demonstrates the time courses of the cardiac contractile effects during a period of 30 min in two groups of narcotised animals, both treated with a high dose of isoproterenol (50 μg·kg^{-1} i.p.). In the first group of guinea pigs ($n = 6$), isoproterenol was applied alone; while in the second group ($n = 5$), 15 min after the isoproterenol injection levosimendan (50 μg·kg^{-1} i.p.) was also injected. The isoproterenol-evoked contractile responses peaked 2 min after administration and thereafter remained almost constant for the next 28 min. The effect of levosimendan application was judged after 30 min by comparing the contractile parameters observed with and without levosimendan. (About 15 min was required for the cardiotonic effects of levosimendan to develop when it was applied alone under these experimental conditions.) However, the mean PW-CV and PW-RV values in the two groups of animals did not differ significantly at the end of the experiments.

Next, we set out to assess whether the cardiac effects of levosimendan can be mitigated by isoproterenol pretreatment in isolated hearts. To this end, Langendorff guinea pig hearts were first perfused with a modified Krebs solution that contained isoproterenol (0.1 μmol/l, for 2 min) and 15 min later they were perfused in the presence of levosimendan (0.3 μmol/l, for 5 min) (Figure 3). (The concentrations of levosimendan and isoproterenol were selected on the basis of a previous study (Edes et al. 1995) so as to evoke the maximal effects in this preparation.) Isoproterenol or levosimendan applied alone evoked a maximal increase in $+\mathrm{d}P/\mathrm{d}t_{\max}$ (from a baseline value of 2066 ± 43 mmHg·s^{-1} to 4896 ± 415 mmHg·s^{-1} ($n = 8$) or from a baseline value of 2050 ± 39 mmHg·s^{-1} to 2624 ± 83 mmHg·s^{-1} ($n = 8$), respectively) and in $-\mathrm{d}P/\mathrm{d}t_{\max}$ (from a baseline value of 2002 ± 40 mmHg·s^{-1} to 3904 ± 238 mmHg·s^{-1} or from a baseline value of 1990 ± 37 mmHg·s^{-1} to 2269 ± 56 mmHg·s^{-1}, respectively). The isoproterenol-stimulated maximal heart rate corresponded to 273 ± 11 beats·min^{-1}, which developed from a baseline value of 172 ± 16

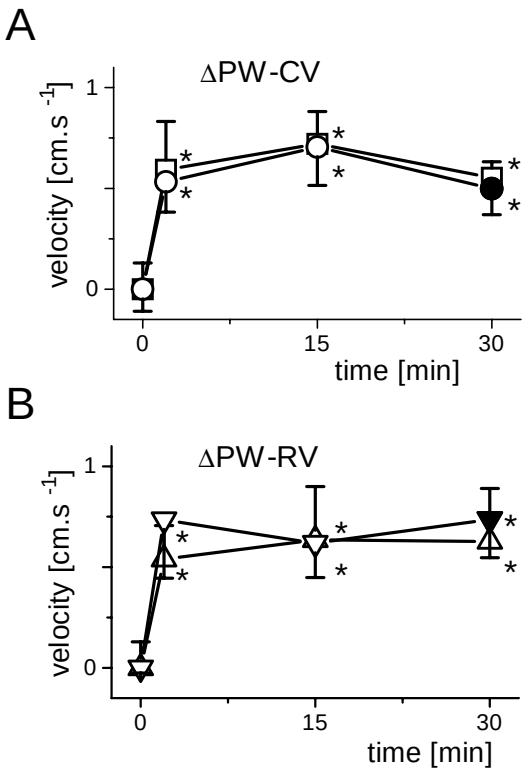

Figure 2. Time courses of ΔPW-CV (A) and ΔPW-RV (B) in groups of narcotised animals subjected either to a single dose of isoproterenol (50 μg·kg^{-1} at min 0) (ΔPW-CV, □ ΔPW-RV, △) or to a single dose of isoproterenol (50 μg·kg^{-1} at min 0) combined with levosimendan (50 μg·kg^{-1} at min 15) (ΔPW-CV, ○; ΔPW-RV, ▽). Despite the combination of isoproterenol with levosimendan, no significant differences were noted between the contractile parameters after 30 min in the presence (ΔPW-CV, ●; ΔPW-RV, ▼) and in the absence of levosimendan (□ or △). Asterisks indicate significant differences between values obtained in the presence of drugs and baseline contractile parameters.

beats·min^{-1}. In comparison, the levosimendan-stimulated maximal heart rate was 201 ± 3 beats·min^{-1}, which developed from a baseline value of 175 ± 4 beats·min^{-1}. In contrast to the narcotised animals, the isoproterenol-induced contractile effects could be washed out rapidly: they had vanished 15 min after isoproterenol application in the Langendorff-perfused hearts. However, as shown in Figure 3A, the contractile parameters were not increased significantly by levosimendan administration 15 min after isoproterenol application. To estimate the phosphorylation levels of the contractile proteins in parallel with the combined isoproterenol and subsequent levosimendan treatment, ^{32}P incorporation into cTnI was determined in the Langendorff-perfused hearts ($n = 4$). Figure 3B and Figure 3C illustrate

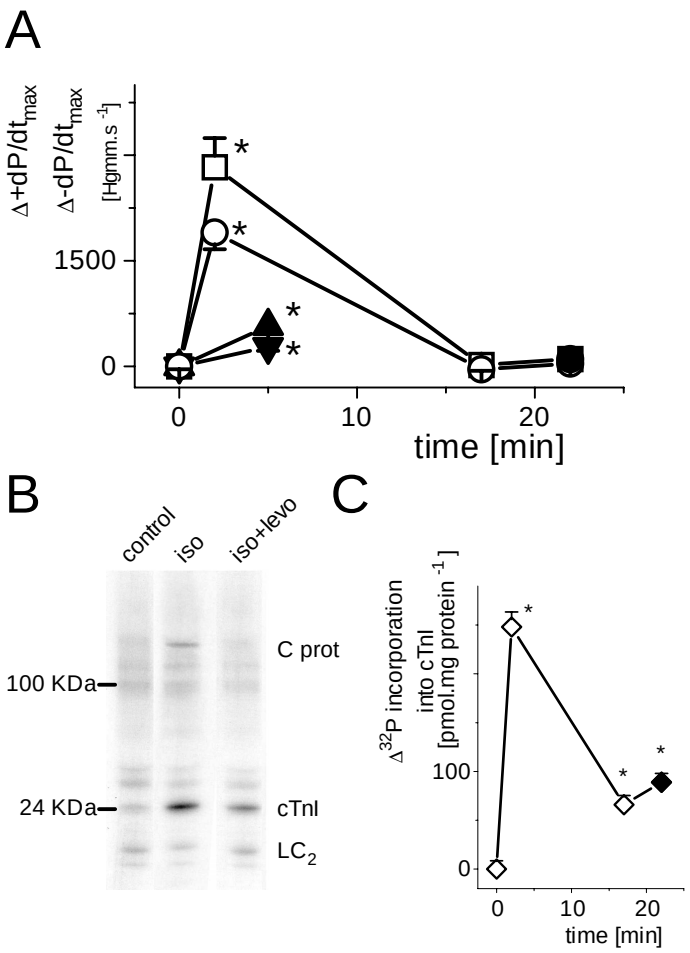

Figure 3. A. Time courses of changes (Δ) in $+\mathrm{d}P/\mathrm{d}t_{\max}$ ($\square$) and $-\mathrm{d}P/\mathrm{d}t_{\max}$ ($\circ$) in Langendorff-perfused guinea pig hearts subjected to perfusion with isoproterenol (0.1 μmol·l^{-1} for 2 min from min 0) followed by levosimendan administration (0.3 μmol·l^{-1} for 5 min from min 17). Contractile effects (on $\Delta + \mathrm{d}P/\mathrm{d}t_{\max}$, $\blacktriangle$ and on $\Delta - \mathrm{d}P/\mathrm{d}t_{\max}$, $\blacktriangledown$) of the application of levosimendan alone (0.3 μmol·l^{-1} for 5 min from time 0) are also depicted. In contrast with the effects of levosimendan applied alone ($\blacktriangle$ and $\blacktriangledown$), no significant increase in the contractile parameters was recorded when the administration of levosimendan was preceded by isoproterenol treatment ($\blacksquare$ and $\bullet$). **B.** The intensities of the autoradiographic bands illustrate the levels of ^{32}P incorporation into myocardial proteins in untreated guinea pig hearts (control) or following 2 min of Langendorff perfusion with 0.1 μmol·l^{-1} isoproterenol (iso) or after the combination of isoproterenol and levosimendan (iso + levo) at the end of the Langendorff perfusions. (C prot, myosin binding protein C; LC_2, myosin light chain 2). **C.** Time course of the change (Δ) in ^{32}P incorporation into cTnI (marker of contractile protein phosphorylation) in response to the application of isoproterenol ($\diamond$) followed by application of levosimendan ($\blacklozenge$) in Langendorff-perfused guinea pig hearts (protocol as in A). Asterisks indicate significant differences between values obtained in the presence of drugs and drug-free baseline conditions (A and C).

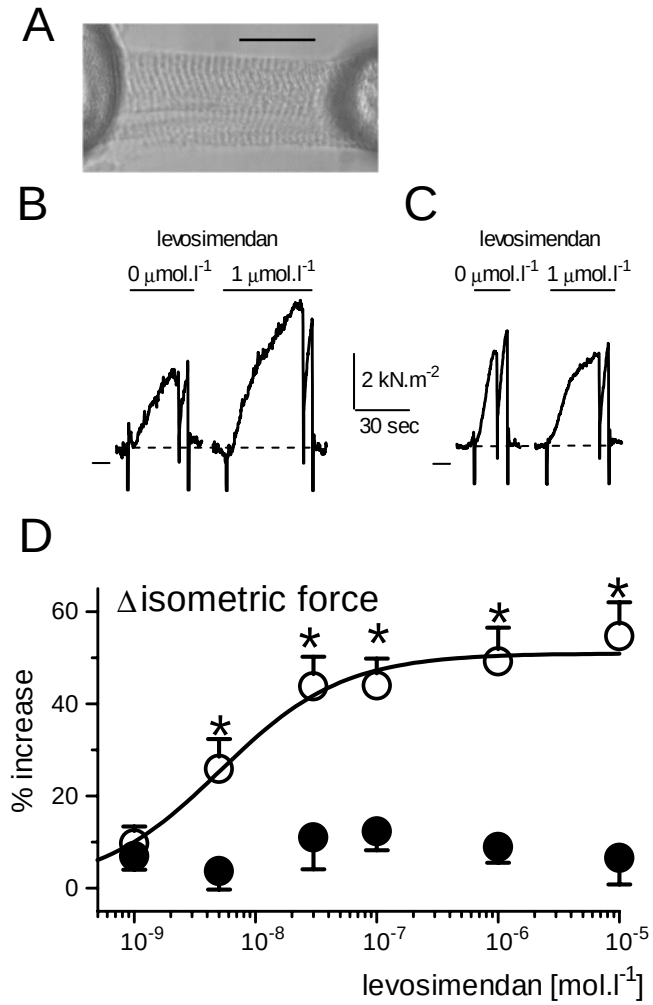

Figure 4. Force measurements in skinned guinea pig myocytes. The experimental arrangement is illustrated in A. A single skinned myocyte was mounted between the force transducer and the electromagnetic motor (sarcomere length: 2.2 μm, calibration line: 20 μm, relaxing solution). Ca^{2+}-evoked contractures in the absence and presence of 1 μmol·l^{-1} levosimendan at pCa 6.2 before (B) and after (C) incubation (40 min) with 100 U·ml^{-1} protein kinase A. Horizontal markers and dashed lines indicate zero and passive force levels, respectively. D. Dose-response relationships of isometric force increase *vs.* levosimendan concentration before (○) and after protein kinase A exposure (●) (100 U·ml^{-1} protein kinase A and 6 mmol·l^{-1} dithiothreitol for 40 min at 20 °C). The superimposed curve illustrates the result of a curve fit using the modified Hill equation (EC_{50} = 5 ± 3 mmol·l^{-1}, n_{Hill} = 0.9 ± 0.3, $P_{levo(max)}$ = 51 ± 5 %). Asterisks indicate significant differences between values at various drug doses and under drug-free conditions.

that isoproterenol induced a major elevation in the level of cTnI phosphorylation and that this parameter remained significantly elevated during the time when levosimendan was administered.

The interaction between levosimendan and the β-adrenergic signalling was further investigated in skinned myocytes ($n = 22$ from 5 guinea pig hearts). This experimental approach allowed the comparison of force values at identical levels of $[Ca^{2+}]$ and protein phosphorylation. Maximal Ca^{2+}-activated force (measured at pCa 4.75) was 22 ± 2 kN·m^{-2} in the myocytes exposed only to levosimendan ($n = 11$) and it was 22 ± 3 kN·m^{-2} ($p < 0.05$) in the myocytes ($n = 11$) treated first with protein kinase A and thereafter with levosimendan. Previous experience (Edes et al. 1995; Haikala et al. 1995a) led to the choice of a single submaximal $[Ca^{2+}]$ (pCa 6.2 in this case) for characterisation of the Ca^{2+}-sensitising effect of levosimendan. At pCa 6.2, the isometric force in the absence of levosimendan was 13 ± 1 % of the maximal isometric force and levosimendan induced a dose-dependent increase from this force level (Figure 4). The maximal increase in force production accounted for an added 51 ± 5 %. However, when the recordings were repeated following 40 min of protein kinase A incubation (100 U·ml^{-1}), the application of increasing concentrations of levosimendan was not accompanied by significant increases in isometric force production. This effect could not be explained by a parallel change in the baseline force (in the absence of levosimendan) at pCa 6.2, because it was also 13 ± 1 % of the maximal isometric force following protein kinase A treatment.

Discussion

This study has revealed that in the presence of intensive β-mimetic stimulation the cardiotonic effects of levosimendan are attenuated. The negative interaction between the action of isoproterenol and levosimendan could be demonstrated in whole animals as well as in Langendorff-perfused hearts, but it was also present in skinned myocytes. This identified the contractile filaments as potential mediators of this relationship. The reduction in the Ca^{2+}-senstitising potential of levosimendan was paralleled by an increase in the level of cTnI phosphorylation, suggesting that the phosphorylation status of the contractile proteins is an important determinant of levosimendan-mediated Ca^{2+}-sensitisation.

The results of echocardiographic examinations identified levosimendan as a potent cardiotonic agent in narcotised guinea pigs. Both the systolic and the diastolic functions underwent dose-dependent enhancements in response to levosimendan administration. In narcotised guinea pigs, the levosimendan-induced maximal increases in the systolic and diastolic functions were comparable to the maximal effects induced by isoproterenol alone. In Langendorff-perfused hearts, however, the maximal effects of levosimendan on $+dP/dt_{max}$ and $-dP/dt_{max}$ were less pronounced when compared to those induced by isoproterenol alone. This suggests that the contractile function of the heart benefits more from levosimendan application when the entire cardiovascular system is exposed to this agent and implies

that Ca^{2+}-sensitised contractile filaments promote the cardiac function to a larger extent in a haemodynamic background modulated by the additional effects of levosimendan. This may involve activation of the ATP-dependent K^+ channels in the vascular smooth muscle cells, with consequent vasodilatation (Nieminen et al. 2000; Slawsky et al. 2000).

In narcotised guinea pigs, the contraction velocity was enhanced at lower doses of levosimendan than was the relaxation velocity. This is most probably a consequence of a pure Ca^{2+}-sensitising effect at lower plasma levels of the drug in the myocardial cells. At the highest concentration of levosimendan, an additional phosphodiesterase-inhibitory effect cannot be excluded. In agreement with this assumption (Edes et al. 1995; Brixius et al. 2002), we observed a pronounced increase in lusitropy (enhancement of the relaxation velocities in narcotised guinea pigs, and an increase in $-\mathrm{d}P/\mathrm{d}t_{\max}$ in Langendorff hearts) at the highest doses of levosimendan. The pure Ca^{2+}-sensitising effect of levosimendan was demonstrated in isolated skinned cardiomyocytes, where Ca^{2+} sequestration and release were prevented by the elimination of the membranous structures. Additionally, the $[Ca^{2+}]$ in the myofibrillar space was maintained at a steady level by the Ca^{2+} buffer applied during the individual activations. Therefore, neither inhibition of phosphodiesterases (Lee et al. 1996) nor ATP-dependent K^+ channel activation could influence force generation in the skinned myocytes. In accordance with previous observations (Edes et al. 1995; Haikala et al. 1995a), levosimendan was able to increase the force at a submaximal level of activation in consequence of its Ca^{2+}-sensitising potential in this preparation. The calculated EC_{50} value for this effect was notably low (5 ± 3 $\mathrm{nmol \cdot l^{-1}}$), which lends further support to the suggested mechanism of action of levosimendan as a Ca^{2+}-sensitiser at low plasma concentrations.

Intensive isoproterenol stimulation prevented the development of the cardiotonic effects of levosimendan in narcotised guinea pigs and in Langendorff hearts. In the Langendorff hearts, the isoproterenol-evoked increases in the contractile parameters had disappeared, and the parameters had returned to the baseline levels before the administration of levosimendan. Nonetheless, no significant effect in response to levosimendan administration could be demonstrated in this phase of the experiments. This suggested that not the contractile reserve, but rather a decrease in the sensitivity for levosimendan, was responsible for the negative interaction following β-stimulation. Isolated skinned myocytes allow direct modulation of the phosphorylation levels of the myofilaments and selective characterisation of the consequences. Previous studies (Van der Velden et al. 2000, 2002) have demonstrated that incubation of skinned myocytes in the presence of the catalytic subunit of protein kinase A is an effective method of inducing marked elevations in the levels of phosphorylation of myofibrillar proteins. Following protein kinase A exposure, levosimendan did not increase the force in isolated guinea pig myocytes. The data on ^{32}P incorporation into the cTnI of Langendorff hearts were in accord with this observation. Hence, as suggested earlier (Haikala et al. 1997), protein kinase A-mediated cTnI phosphorylation may be involved in the reduction of the myofilament sensitivity to levosimendan.

In the failing human myocardium, the β-mimetic responsiveness is reduced, the level of cTnI phosphorylation is presumed to be decreased (Bodor et al. 1997) and the Ca^{2+} sensitivtiy of force production is thought to be increased (Wolf et al. 1996; Van der Velden et al. 2002). Additionally, the amplitude of the $[Ca^{2+}]_i$ transient in the chronically remodeled heart is diminished (Beuckelmann et al. 1992). On the other hand, the binding of levosimendan to cTnC, and therefore, the levosimendan-induced positive inotropic action, are both Ca^{2+}-dependent (Haikala et al. 1995b, 1997). It has been suggested (Brixius et al. 2002) that this Ca^{2+} dependence explains why an increase in the amplitude of the cytosolic Ca^{2+} transient (induced either by isoproterenol or by elevation of the extracellular $[Ca^{2+}]$) augments the Ca^{2+}-sensitising effect of levosimendan in muscle strip preparations from the failing human heart. On the basis of our findings, it may also be argued that the downgraded β-signalling system would probably not allow isoproterenol stimulation to increase the contractile protein phosphorylation to levels high enough to oppose the cardiotonic effects of levosimendan in failing human heart preparations (Brixius et al. 2002).

In summary, the Ca^{2+}-sensitising potential of levosimendan can be negatively influenced by stimulation of the β-adrenergic system. However, the efficiency of levosimendan-induced Ca^{2+}-sensitisation may depend on the interplay between the levels of activating $[Ca^{2+}]_i$ and of contractile protein phosphorylation at the subcellular level. Ca^{2+} handling and β-adrenergic signalling are both impaired during advanced chronic heart failure. This may theoretically generate a more favourable background for the interactions between the β-mimetic agents and levosimendan in the failing human heart as compared with the healthy myocardium.

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