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RESEARCH PAPER

Inhibition of phosphodiesterase-3 by levosimendan is sufficient to account for its inotropic effect in failing human heart

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BACKGROUND AND PURPOSE
Levosimendan is known as a calcium sensitizer, although it is also known to inhibit PDE3. We aimed to isolate each component and estimate their contribution to the increased cardiac contractility induced by levosimendan.

EXPERIMENTAL APPROACH
Contractile force was measured in electrically stimulated ventricular strips from explanted failing human hearts and left ventricular strips from normal male Wistar rats. PDE activity was measured in a two-step PDE activity assay on failing human ventricle.

KEY RESULTS
Levosimendan exerted a positive inotropic effect (PIE) reaching maximum at 10^{-5} M in ventricular strips from failing human hearts. In the presence of the selective PDE3 inhibitor cilostamide, the PIE of levosimendan was abolished. During treatment with a PDE4 inhibitor and a supra-threshold concentration of isoprenaline, levosimendan generated an amplified inotropic response. This effect was reversed by β-adrenoceptor blockade and undetectable in strips pretreated with cilostamide. Levosimendan (10^{-6} M) increased the potency of β-adrenoceptor agonists by 0.5 log units in failing human myocardium, but not in the presence of cilostamide. Every inotropic response to levosimendan was associated with a lusitropic response. Levosimendan did not affect the concentration–response curve to calcium in rat ventricular strips, in contrast to the effects of a known calcium sensitizer, EMD57033 [5-(1-(3,4-dimethoxybenzoyl)-1,2,3,4-tetrahydroquinolin-6-yl)-6-methyl-3,6-dihydro-2H-1,3,4-thiadiazin-2-one]. PDE activity assays confirmed that levosimendan inhibited PDE3 as effectively as cilostamide.

CONCLUSIONS AND IMPLICATIONS
Our results indicate that the PDE3-inhibitory property of levosimendan was enough to account for its inotropic effect, leaving a minor, if any, effect to a calcium-sensitizing component.

Abbreviations
CRC, contraction–relaxation cycles; cTnC, cardiac troponin C; dF/dt_{max}, maximal development of force; F_{max}, maximal developed force; PIE, positive inotropic effect; RT, relaxation time; TPF, time to peak force; TR80, time to 80% relaxation.

Introduction

A powerful mechanism for treatment of acute decompensated heart failure is a cAMP-dependent increase in contractility. However, both β-adrenoceptor stimulation, which induces cAMP production, and PDE3 inhibition, which reduces the breakdown of cAMP, have demonstrated only short-term haemodynamic improvement, at the cost of increased mortality in several clinical trials (Teerlink *et al.*, 2009). The lack of beneficial long-term effects may be due to increased oxygen demand in the cardiomyocytes, mainly because of increased calcium handling, and an increased risk of malignant arrhythmias.

There is therefore a clear need for other ways of providing inotropic support. One such mechanism is sensitization of the myofilaments to calcium ions. Calcium-sensitizing drugs shift the calcium–force relationship to lower calcium concentrations.

Levosimendan is mainly regarded as a calcium sensitizer, but can potentially also cause a positive inotropic effect (PIE) through PDE3 inhibition (Boknik *et al.*, 1997; Hasenfuss *et al.*, 1998; Brixius *et al.*, 2002; Szilagyi *et al.*, 2005). The latter component has been regarded as minor. The calcium-sensitizing component is assumed to involve the binding of levosimendan to cardiac troponin C (cTnC) in a calcium-dependent manner that facilitates systolic interaction, with no observed alteration of the cross-bridge cycling (Haikala *et al.*, 1995a). From the assumption that levosimendan dissociates from cTnC during diastole, which would require sufficiently rapid on–off kinetics, it would not impair relaxation (Haikala *et al.*, 1995b). The relative contributions from calcium sensitization and PDE3 inhibition, respectively, have yet to be clarified with certainty as data on this vary (Boknik *et al.*, 1997; Brixius *et al.*, 2002).

Levosimendan has demonstrated beneficial haemodynamic and symptomatic effects in several clinical trials and also improvements compared with dobutamine have been shown (Moiseyev *et al.*, 2002; Mebazaa *et al.*, 2007). Recently, data from the large REVIVE study also revealed a short-term symptomatic improvement. This came, however, at the expense of a trend towards increased in long-term mortality (Packer *et al.*, 2013). Additionally, from experimental data, it has been suggested that the inotropic effect of levosimendan is the result of a combined effect from both calcium-sensitization and PDE3 inhibition (Takahashi and Endoh, 2005; Endoh, 2008). In light of these studies, it is important to clarify the role of PDE3 inhibition by levosimendan, as PDE3 inhibition, which has known detrimental effects, is potentially the main contributor for the observed cardiac effects of levosimendan. Levosimendan additionally opens ATP-sensitive potassium-channels, causing both arterial and venous vasodilation (Yokoshiki *et al.*, 1997), affecting the haemodynamics of the patient.

The aims of this study were to separate and characterize the components of the inotropic effects of levosimendan in order to evaluate their individual contributions in human and rat myocardium. Levosimendan was compared with known PDE inhibitors and, for the first time, studied in the presence of such inhibitors. Additionally, we compared levosimendan with a classical calcium-sensitizer, EMD57033 [5-(1-(3,4-dimethoxybenzoyl)-1,2,3,4-tetrahydroquinolin-6-yl)-6-methyl-3,6-dihydro-2H-1,3,4-thiadiazin-2-one], in terms of their ability to influence the concentration–response relationship to calcium. We also studied interactions between levosimendan and β-adrenoceptor stimulation. In contrast to previous studies, we found that in both rat and failing human myocardium, the PDE3-inhibitory property of levosimendan was sufficient to account for its inotropic effect.

Methods

Preparation of human trabeculae

The use of human myocardial samples conformed to the principles outlined in the Declaration of Helsinki, and approval was granted from the ethics committee in South-Eastern Norway Regional Health Authority (#S05172). Human left ventricular trabeculae were obtained from 12 explanted hearts from patients with terminal heart failure undergoing heart transplantation at Oslo University Hospital – Rikshospitalet. The patients (12) were, at the time of explantation, currently receiving the following: β-adrenoceptor blockers (9), ACE inhibitors (8), thiazides (4), loop diuretics (11), aldosterone antagonists (7), acetylsalicylate (2), digoxin (6), amiodarone (7), warfarin (11), combined α- and β-adrenoceptor blockers (3), angiotensin II receptor antagonists (2), and clopidogrel (1). Trabeculae from the patients were first placed into relaxing solution containing (mM): NaCl (118.3), KCl (3.0), $CaCl_2$ (0.2), $MgSO_4$ (4.0), KH_2PO_4 (2.4), $NaHCO_3$ (24.9), glucose (10.0) and mannitol (2.2) and kept in this solution until the trabeculae were mounted in organ baths.

Preparation of rat ventricular muscle strips

All animal care complied with the Norwegian Animal Welfare Act, which conforms to the European Convention for the protection of Vertebrate animals used for Experimental and other Scientific Purposes (Council of Europe no. 123, Strasbourg 1985) and experiments were approved by the Norwegian Animal Research Authority. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 118 animals were used in the experiments described here.

Male Wistar rats (Taconic, Ejby, Denmark) of approximately 250–350 g were anaesthetized (2–3% isoflurane in air) and subsequently killed by cervical dislocation. The hearts were excised and prepared for Langendorff perfusion with the relaxing solution described above. The left ventricle was exposed and ventricular strips of approximately 1 mm diameter were excised and mounted in organ baths.

Measurement of ventricular strip contractility

Left ventricular strips (1 mm diameter) from human and rat heart were mounted in organ baths containing the oxygenated relaxing solution (37 and 31°C, respectively) described above. After mounting, the relaxing solution was replaced with a solution of identical composition with the exception of $CaCl_2$ and $MgSO_4$ concentrations (human: 2.5 and 1.2 mM, respectively; rat: 1.8 and 1.2 mM, respectively). The muscles were field stimulated at a frequency of 1 Hz with impulses of 5 ms duration and current about 20% above

individual threshold (10–15 mA, determined in each experiment). The isometrically contracting muscles were stretched to the maximum of their length–tension curve (Skomedal *et al.*, 1997). Maximal developed force (F_{max}), maximal development of force $(dF/dt)_{max}$, time to peak force (TPF), time to 80% relaxation (TR80) and relaxation time (RT, defined as RT = TR80 – TPF) were measured. The measurements were based on averaging 20–30 contraction–relaxation cycles (CRC). Inotropic responses were expressed as increases in $(dF/dt)_{max}$ (Skomedal *et al.*, 1997). The descriptive parameters at the end of the equilibration period were used as basal (control) values. Antagonists (added 90 min before agonist stimulation) of adrenoceptors (prazosin 10^{-7} M, timolol 10^{-6} M) and muscarinic cholinoceptors (atropine 10^{-6} M) were used when indicated. Other inhibitors, when used, were added to the muscles ~45 min before the agonist. Agonists were added cumulatively until the maximal response was obtained (concentration–response curves) or as a single bolus. Following force measurements, muscles were immediately frozen in liquid nitrogen and stored at –70°C.

Measurement of changes in the sensitivity of ventricular strips to extracellular calcium

As rapid on–off kinetics would be required for the assumed mechanism of action of levosimendan, it was essential to study whether levosimendan was able to increase the sensitivity to calcium in electrically stimulated ventricular strips, that is a preparation with continuously repeated CRCs. In this preparation, the contractile force is strictly dependent upon the concentration of extracellular calcium determining the magnitude of the intracellular Ca^{2+} transient elicited by the electrical stimulation. After equilibration of the rat heart ventricular strips in 1.8 mM Ca^{2+} as described earlier, the concentration was reduced to 0.5 mM. When the new steady state was reached, the Ca^{2+} concentration was increased stepwise in a cumulative way to a maximum of 6 mM (Skomedal *et al.*, 1987). This procedure was performed in the absence or presence of levosimendan or the calcium sensitizer EMD57033. The concentration–response curves were constructed as described previously with maximum development of force set to 100%.

PDE assay

A modified standard two-step PDE assay was performed (Marchmont and Houslay, 1980). Frozen tissue from human ventricle was homogenized prior to the addition of known concentrations of radiolabelled and unlabelled cAMP to a final concentration of 10^{-6} M. There was a linear breakdown of cAMP for at least 20 min at 31°C. The reaction was stopped by heating for three minutes at 100°C. The second step of the assay was incubation with snake venom to convert 5′AMP to adenosine, which was not bound by the Dowex added for separation. [^{3}H]adenosine was counted in the supernatant. Dowex binds any unconverted cAMP and possible unconverted 5′AMP. Corrections for the binding ability of Dowex and the conversion efficiency (of [^{14}C]5′-AMP) of snake venom were made.

Data analysis

Data are expressed as mean ± SEM from n animal or human explanted hearts, unless otherwise specified. $P < 0.05$ was considered statistically significant (Student's *t*-test and ANOVA). When appropriate, Bonferroni corrections were made to control for multiple comparisons.

Materials

Levosimendan was purchased from Zhou Xi Fen Pharm Chemical, Shanghai, China. The identity and purity of this levosimendan was validated by HPLC, MS and proton NMR spectroscopy. Dowex anion exchange resin, cAMP, *Crotalus atrox* venom, PMSF, DTT, timolol, isoprenaline, lidocaine, H-89 and atropine were from Sigma-Aldrich (St. Louis, MO, USA). [^{3}H]cAMP and [^{14}C]5′-AMP were from PerkinElmer (Waltham, MA, USA). Cilostamide (10^{-6} M, unless otherwise specified), rolipram (10^{-5} M) and milrinone (10^{-5} M) were from Tocris (Bristol, UK). EMD57033 was graciously donated by Dr. Norbert Beier of Merck KGaA (Darmstadt, Germany). Drug and molecular target nomenclature conforms with British Journal of Pharmacology Guide to Receptors and Channels (Alexander *et al.*, 2013a,b).

Results

The primary aim of the present study was to clarify the mechanism of the inotropic effect of levosimendan in failing human myocardium. Because of the limited availability of human cardiac tissue, the results were complemented with studies on rat myocardium where appropriate.

Effects of levosimendan on failing human myocardium and normal rat myocardium

All experiments were conducted in the presence of complete adrenoceptor and muscarinic cholinoceptor blockade, unless otherwise specified, in order to avoid possible influence of endogenous autonomic agonists on effects of levosimendan. The basal maximal developed force in human ventricular strips was 4.6 ± 0.5 mN. Data on responses are given in % above basal.

Effects of levosimendan alone. In failing human ventricular strips, levosimendan increased contractility in a concentration-dependent manner between 10^{-7} and 10^{-5} M to 40 ± 8% above basal ($n = 5$) (Figures 1A and 2A). Additionally, levosimendan caused a marked lusitropic effect, evident from the shortening of the CRC (Figure 1B).

Levosimendan alone at various concentrations failed to produce any PIE in normal rat myocardium (Figure 1C). Concentrations above 10^{-5} M caused a large reduction in contractile force, an effect previously observed by others to be due to a reversible decrease in calcium sensitivity at high concentrations (Takahashi and Endoh, 2005).

Modulation of levosimendan effects by PDE3 and PDE4 inhibition. In failing human myocardium, PDE3 is the main PDE degrading cAMP that increases contractility, while in rat hearts, this effect is shared by both PDE3 and PDE4. Thus, in human hearts, PDE3 inhibition alone is sufficient to elicit a PIE (Osadchii, 2007) whereas in rats, inhibition of both PDE3 and PDE4 is required (Shahid and Nicholson, 1990; Afzal *et al.*, 2008; Levy, 2013). When given concentration-dependently in the presence of the PDE4 inhibitor rolipram,

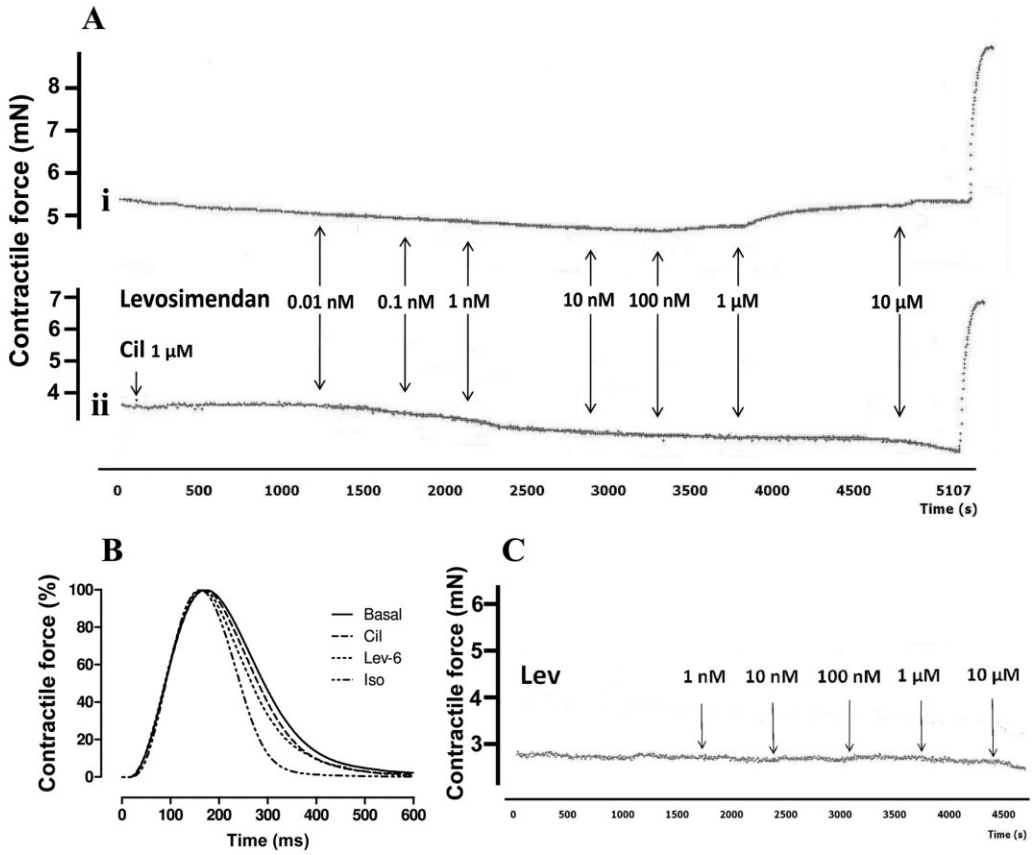

Figure 1

Inotropic and lusitropic effects of levosimendan. (A) Representative tracing of levosimendan on failing human left ventricular myocardial strips, the figure showing changes in contractile force (F_{max}) over time. (i) Levosimendan (Lev) caused a positive inotropic effect (PIE) from about 10^{-7} M whereafter it slowly increased concentration-dependently up to 10^{-5} M. Higher concentrations caused a cardiodepressive effect with a fall in contractile force which eventually terminated the strip (not shown). (ii) Representative tracing showing that in strips pretreated with the PDE3 inhibitor cilostamide (Cil; 10^{-6} M), no additional PIE of levosimendan was observed. The addition of an isoprenaline bolus (10^{-4} M) demonstrates the remaining contractile potential of the myocardium in both situations. The figures in (i) and (ii) show representative tracings, scaled to represent the average basal and maximum force in similar experiments. (B) Representative graph from human myocardium demonstrating averaged, normalized CRCs in the presence of levosimendan (Lev, 10^{-6} M), cilostamide (Cil, 10^{-6} M) and isoprenaline (Iso, 10^{-4} M). All three demonstrate lusitropic effects seen as the shortening of the cycle with earlier onset and increased rate of relaxation when compared with basal. (C) Representative tracing showing lack of PIE of levosimendan (Lev, 10^{-9} M to 10^{-5} M) in normal rat ventricular strips. At the highest concentrations (10^{-5} M) levosimendan caused a cardiodepressive effect.

levosimendan caused a PIE in both failing human ventricular strips and normal rat myocardium (Figure 2A and 2B). Thus, PDE4 inhibition increased the efficiency of levosimendan. The effect was higher in human than in rat myocardium: the maximal PIEs at 10^{-5} M levosimendan were 75 ± 20% and 33 ± 6% above basal respectively.

In failing human myocardium, the selective PDE3 inhibitor cilostamide caused a small inotropic response (7.5 ± 4.0% above basal). In the presence of cilostamide, the PIE of levosimendan could not be observed (Figures 1A and 2A). Similarly, cilostamide removed the PIE of levosimendan in the presence of rolipram (which did not alter baseline) as well. In rat myocardium, the presence of cilostamide did not influence the lack of effect of levosimendan on contractility (Figures 2B, 5A and 5B).

Lusitropic effect of levosimendan. In both humans and rats, the PIE of levosimendan was associated with a lusitropic response (reduction of RT). In the presence of PDE4 inhibition, RT fell concentration-dependently from –1.6 ± 9.0 ms (10^{-8} M levosimendan, n = 3) to –28.8 ± 10.0 ms (10^{-5} M levosimendan, n = 3) in humans, compared with control. This effect was similar in rat myocardium where levosimendan demonstrated a concentration-dependent lusitropic response starting at 10^{-8} M (–0.3 ± 0.9 ms vs. Ctr, n = 5) and reaching a maximum lusitropic response at 10^{-6} M (–11.6 ± 1.1 ms vs. Ctr, n = 5) in the presence of rolipram (see Table 1).

Interaction between levosimendan and β-adrenoceptor stimulation

Effects of levosimendan compared with PDE inhibitors on the concentration–response relationship of β-adrenoceptor stimulation. We studied the concentration–response relationship of the β-adrenoceptor agonist isoprenaline in the absence and presence of levosimendan and PDE3 and PDE4 inhibitors.

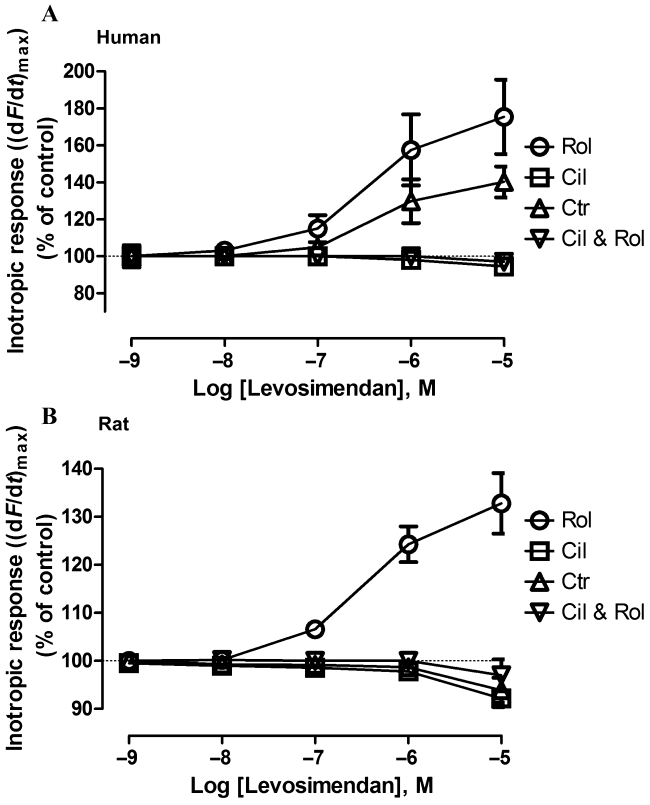

Figure 2

Inotropic response of levosimendan in failing human (A) and normal rat (B) myocardium. PDE4 inhibition enhances, but PDE3 inhibition eliminates the PIE of levosimendan in failing human and normal rat myocardium. (A) PDE4 inhibition by rolipram (Rol; 10^{-5} M) significantly increased the concentration-dependent PIE of levosimendan in myocardial strips from failing human heart ($n = 5$) (maximal effect of levosimendan at 10^{-5} M). In the presence of both PDE3 (cilostamide; Cil, 10^{-5} M) and PDE4 inhibition (Rol, 10^{-5} M), levosimendan did not give a PIE ($n = 5$). [basal developed force values human: Ctr: 5.8 ± 1.2 mN, Cil: 3.8 ± 0.4 mN, Rol: 5.0 ± 0.6 mN, Cil + Rol: 6.0 ± 0.7 mN, data are mean ± SEM from n patients]. (B) Similar experiment in normal rat myocardium ($n = 6$). Maximum PIE with PDE4 inhibition was reached at 10^{-5} M of levosimendan ($n = 5$), whereas no inotropic effect was seen with levosimendan alone ($n = 5$). As with human myocardium, levosimendan did not cause a PIE in the presence of both cilostamide (10^{-5} M) and rolipram ($n = 5$) (basal developed force values rat: Ctr: 3.3 ± 0.4 mN, Cil: 4.0 ± 0.6 mN, Rol: 3.5 ± 0.4 mN, Cil + Rol: 4.4 ± 0.4 mN).

Table 1

Changes in RT and TPF in normal rat myocardial strips ($n = 5$) in ms

	RT	TPF
Control		
Basal	106.1 ± 4.7	104.0 ± 4.0
Lev-8	–1.3 ± 1.4	–0.2 ± 1.1
Lev-6	–0.5 ± 1.7	–1.8 ± 0.7
Lev-5	–0.7 ± 1.9	–2.8 ± 1.1
Cilostamide		
Basal	104.4 ± 4.2	106.4 ± 4.1
Lev-8	–2.4 ± 1.0	–0.4 ± 0.7
Lev-6	–2.2 ± 0.5	–2.6 ± 1.2
Lev-5	–3.4 ± 0.9	–2.2 ± 2.0
Rolipram		
Basal	102.2 ± 5.8	100.3 ± 3.2
Lev-8	–0.3 ± 0.9	–2.0 ± 0.6
Lev-6	–7.5 ± 1.4	–4.2 ± 0.7
Lev-5	–11.6 ± 1.1	–3.0 ± 1.1

Data are mean ± SEM from n animals. Lev-8, 10^{-8} M levosimendan; Lev-6, 10^{-6} M levosimendan; Lev-5, 10^{-5} M levosimendan; Cilostamide was used at 10^{-6} M and rolipram at 10^{-5} M.

In failing human myocardium, levosimendan shifted the concentration–response curve to lower concentrations of isoprenaline, demonstrating a sensitization of the β-adrenoceptor response. This effect started at 10^{-8} M levosimendan and yielded a pEC_{50} for isoprenaline of 6.9 ± 0.1 (n = 6) compared with a pEC_{50} of 6.7 ± 0.1 (n = 6) in the absence of levosimendan. The maximum shift was observed at 10^{-6} M levosimendan (pEC_{50} = 7.1 ± 0.2; n = 6) (Figure 3A).

The PDE3 inhibitor cilostamide shifted the concentration–response curve to lower concentrations of isoprenaline by 0.9 log units in failing human ventricle (pEC_{50} for isoprenaline 7.5 ± 0.2 in the presence of cilostamide, n = 6), and no additional shift was observed when levosimendan was added in the continued presence of cilostamide in either failing human ventricle (Figure 3A) or normal rat ventricle (Figure 3B). In normal rat myocardium, the concentration–response curve to isoprenaline was shifted to lower concentrations by levosimendan [pEC_{50} values for isoprenaline: control: 7.5 ± 0.1 (n = 12); 10^{-8} M levosimendan: 7.6 ± 0.1 (n = 10); 10^{-6} M levosimendan: 7.9 ± 0.1 (n = 10)]. Cilostamide alone caused a sensitization corresponding to 0.6 log units (to pEC_{50} = 8.2 ± 0.1, n = 8; Figure 3B). In the presence of both PDE3 (cilostamide) and PDE4 inhibition (rolipram) a pEC_{50} of 9.4 ± 0.1 (n = 4) for isoprenaline was reached. In this situation, the addition of levosimendan (up to 10^{-6} M) failed to further shift the concentration–response curve to isoprenaline (pEC_{50} 9.4 ± 0.1, n = 3).

When levosimendan (10^{-6} M) was combined with the PDE4 inhibitor rolipram the isoprenaline concentration–response curve in rat myocardium was shifted further to lower concentrations approaching the curve with a combination of PDE3 and PDE4 inhibitors, that is cilostamide and rolipram (Figure 3B).

The lusitropic response to isoprenaline was sensitized by levosimendan in a similar manner to that observed with the PIE (data not shown).

Low-grade β-adrenoceptor stimulation markedly amplifies the inotropic response to levosimendan. Previous studies have demonstrated that a low-grade stimulation of myocardial β-adrenoceptors causes enough sensitization of the system to detect functional effects (increased contractility) of PDE inhibition (Qvigstad *et al.*, 2010). In the absence of

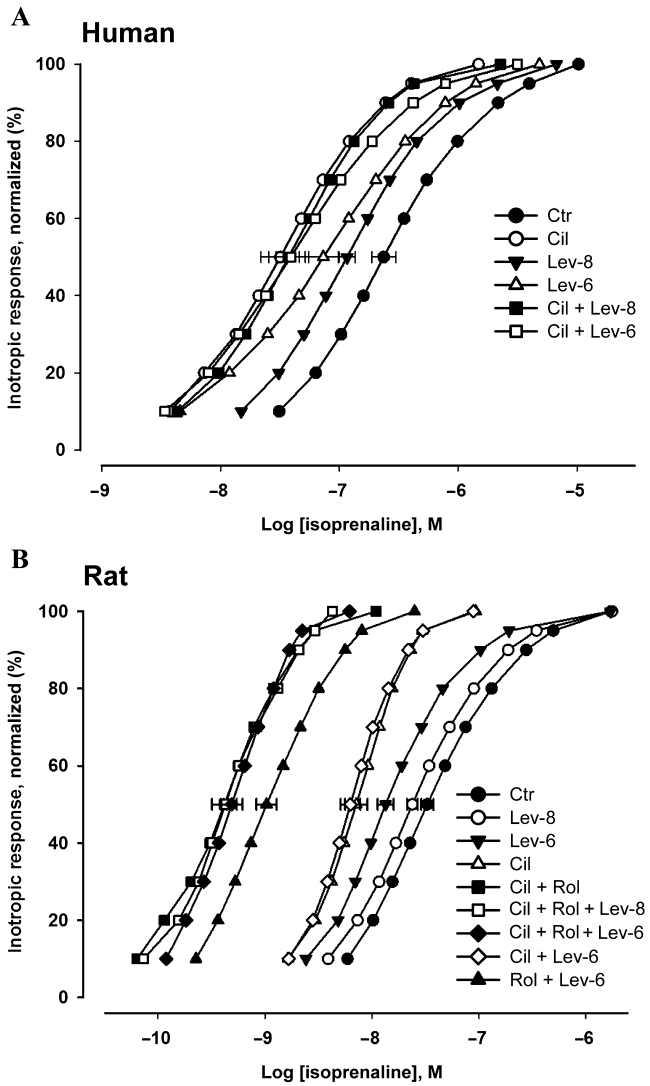

Figure 3

Concentration–response curves demonstrating the effect of β-adrenoceptor stimulation in the presence of levosimendan. Levosimendan potentiates the inotropic effects of a β- adrenoceptor agonist in a similar manner as cilostamide in both failing human and rat myocardium. Data are mean ± SEM from n patients/animals. (A) Concentration–response curves of isoprenaline in failing human myocardial strips in the presence of α- adrenoceptor and muscarinic cholinoceptor blockade. Levosimendan (Lev) sensitized the β-adrenoceptor inotropic effect, seen as a shift of the concentration–response curve to isoprenaline to lower concentrations, already at 10^{-8} M (Lev-8, basal developed force: 5.7 ± 1.4 mN, 5.8 ± 1.4 mN after Lev-8 was added, $n = 5$) and at 10^{-6} M (Lev-6, basal developed force: 3.5 ± 0.8 mN, 4.6 ± 0.8 mN after Lev-6 was added, $n = 5$) with a maximum shift versus control of 0.5 log units (basal developed force: 3.8 ± 0.8 mN, $n = 6$). Cilostamide (Cil; 10^{-6} M, basal developed force: 5.5 ± 1.5 mN, 6.4 ± 1.5 mN after Cil was added, $n = 6$), used for comparison, yielded a shift from control of 0.9 log units. In the presence of both cilostamide and levosimendan, the β-adrenoceptor inotropic effect was not sensitized compared with cilostamide alone (basal developed force: 2.1 ± 0.3 mN, 3.4 ± 0.3 mN after Lev-6 + Cil was added, $n = 3$, 5 strips). (B) Similar experiments conducted on normal rat ventricular strips. Levosimendan demonstrated a sensitization of the β- adrenoceptor inotropic response. Levosimendan (10^{-6} M, $n = 6$) caused a shift versus control of 0.4 log units, whereas cilostamide caused a shift versus control of 0.6 log units ($n = 8$). Combined PDE3 and PDE4 inhibition sensitized the system with 1.8 log units ($n = 4$), and levosimendan did not cause further sensitization ($n = 3$).

β-adrenoceptor blockade and presence of 2.0×10^{-9} M isoprenaline, equivalent to EC_{10}, we conducted concentration–response experiments in failing human ventricle with levosimendan in order to unmask the PDE3-dependent component of levosimendan (Figure 4, i). Levosimendan elicited a concentration-dependent PIE (pEC_{50} of 7.3 ± 0.1; $n = 6$ strips, 3 hearts; Figure 4, inset) and a concentration-dependent shortening of the CRC (basal RT 193 ± 11 ms vs. lowest RT 158 ± 5 ms at 9.5×10^{7} M levosimendan, $n = 6$ strips, 3 hearts, $P < 0.05$). Maximal PIE was obtained at about 10^{-6} M levosimendan and addition of timolol (10^{-5} M) completely reversed the PIE. In the presence of the PDE3 inhibitor cilostamide (10^{-6} M), which caused an inotropic response by itself (30 ± 7% increase above basal), and a correspondingly adjusted EC_{10} for isoprenaline, a complete lack of PIE to levosimendan was demonstrated (Figure 4, ii). Similar results were found in rat myocardium, when the preincubation with rolipram in addition to isoprenaline EC_{10} created conditions comparable to those in the human myocardium.

Similar experiments were performed with milrinone (10^{-5} M; a combined PDE3 and PDE4 inhibitor) and compared with the effects of rolipram (10^{-5} M) or cilostamide (10^{-5} M), followed by the supra-threshold dose of isoprenaline (EC_{10}) and a bolus-dose of levosimendan (10^{-7} M). In the presence of rolipram, levosimendan produced an inotropic response of 54 ± 11% ($n = 4$; Figure 5A and 5B). This effect was eliminated in the presence of either cilostamide or milrinone ($n = 4$, $P < 0.005$). The calcium sensitizer, EMD57033, was added at the end to confirm that a potential calcium-sensitizing component could still be expressed in the muscle preparation (see Figure 5A and 5B).

A cAMP-mediated response can also be influenced downstream of the receptor and PDE activity level. To validate that the observed inotropic response to levosimendan was through this pathway, we conducted experiments in the presence of the PKA inhibitor H89 (2×10^{-5} M). In the presence of rolipram (10^{-5} M) and the supra-threshold dose of isoprenaline (EC_{10}), levosimendan (2×10^{-8} M) gave an inotropic response of 20 ± 6% ($n = 6$), compared with 4 ± 2% ($n = 6$) in the presence of H89 ($P < 0.05$) (Figure 5C). Similar data were obtained in a few human myocardial strips (data not shown).

Levosimendan inhibits PDE enzyme activity in failing human myocardium

To quantify and evaluate the inhibition of PDE by levosimendan, we conducted PDE assays on homogenate from failing human myocardium. In these experiments, levosimendan demonstrated concentration-dependent PDE inhibition. Compared with cilostamide, levosimendan at 10^{-6} M demonstrated very similar efficacy as a PDE-inhibitor [40 ± 3% ($n = 7$) vs. cilostamide 33 ± 2% ($n = 6$) inhibition of total PDE activity; Figure 6B]. We assumed that this was the level

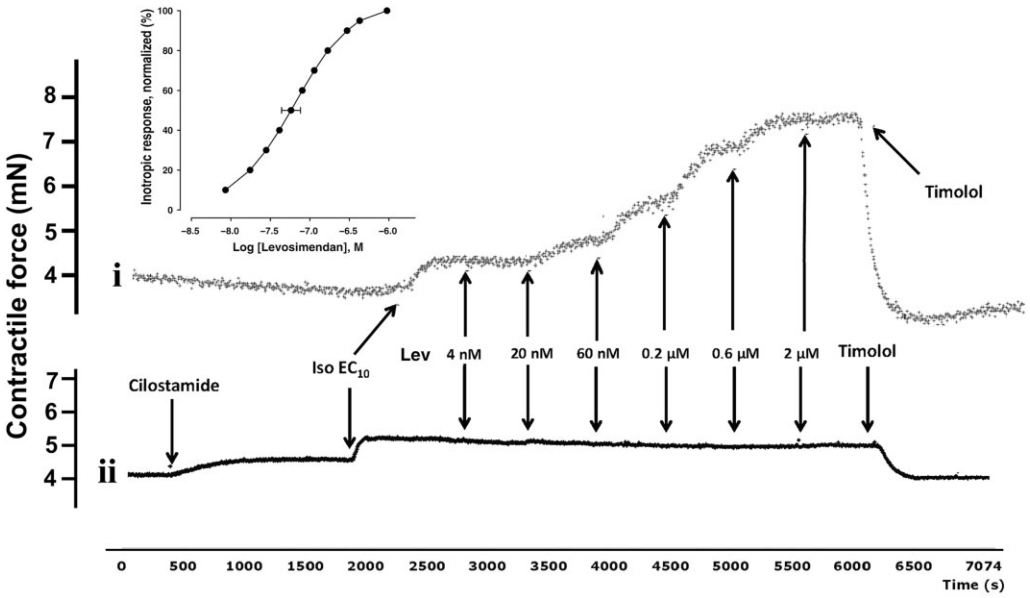

Figure 4

Low-grade β-adrenoceptor stimulation markedly amplifies the inotropic response to levosimendan in failing human ventricular strips. Representative tracing of levosimendan at a low-grade β-adrenoceptor stimulation (2.0×10^{-9} M isoprenaline; Iso) in the presence of α- adrenoceptor and muscarinic cholinoceptor blockade on failing human left ventricular strips. (i) In the absence of cilostamide, levosimendan (Lev) had a clear effect at 2×10^{-8} M, and a $pEC_{50} = 7.3 \pm 0.08$ was calculated (inset). The effect was completely reversed by the addition of timolol 10^{-5} M ($n = 3$, basal force: 3.9 ± 0.8 mN). (ii) In the presence of cilostamide, no PIE of levosimendan was observed under equivalent conditions ($n = 3$, basal force: 4.2 ± 0.8 mN). The figures in (i) and (ii) show representative tracings, scaled to represent the average basal and maximum force in similar experiments. (Inset) Percentile plot illustrating the concentration–response curve of levosimendan ($n = 3$), demonstrating the EC_{50}.

of levosimendan giving maximal PDE3 inhibition, and further increases of concentration would involve different components, such as PDE4 inhibition and possible non-specific effects of levosimendan. Given these conditions, concentration-inhibition experiments revealed a pIC_{50} of 7.7 ± 0.2 for levosimendan as a PDE3 inhibitor, with a Hill slope of –1.06 ± 0.06 (Figure 6A).

Levosimendan revealed only a marginal additional inhibition when combined with cilostamide (41 ± 3%; $n = 6$; Figure 6B). Levosimendan in combination with rolipram caused a further reduction in PDE-activity compared with rolipram alone, very similar to the combination of cilostamide and rolipram (53 ± 4% inhibition; $n = 7$ vs. 46 ± 3% inhibition; $n = 6$). When all three drugs were added together, the combined inhibition in PDE activity was 56 ± 4% ($n = 6$) of total activity.

Levosimendan does not shift the concentration–response curve to calcium

The method used to estimate changes in the calcium sensitivity of rat ventricular strips was validated by studying the effect of the known calcium sensitizer EMD57033 (3×10^{-6} M), which shows a minimal inhibition of PDE3 (Endoh, 2008). This substance changed the EC_{50} of Ca^{2+} from the control $EC_{50} = 1.91 \pm 0.12$ mM Ca^{2+} ($n = 6$) to $EC_{50} = 1.26 \pm 0.03$ mM Ca^{2+} ($n = 6$; $P < 0.005$), demonstrating sensitization to calcium (Figure 7). In the presence of levosimendan (10^{-6} M) the EC_{50} of Ca^{2+} was 1.75 ± 0.09 mM ($n = 6$), which indicated no sensitizing effect (Figure 7). Similarly, in the presence of cilostamide (10^{-6} M) the EC_{50} of calcium was 1.72 ± 0.14 mM ($n = 6$; Figure 7).

Discussion

In the present *ex vivo* study, we demonstrated, in both human and rat myocardium, that (i) no inotropic effect of levosimendan was observed in the presence of the selective PDE3 inhibitor, cilostamide; (ii) the inotropic effect of levosimendan was amplified by low-grade β-adrenoceptor stimulation and attenuated by PKA blockade; (iii) levosimendan sensitized the β-adrenoceptor signalling in the same manner as cilostamide and did not increase the sensitivity to β-adrenoceptor stimulation beyond that caused by PDE3 inhibition; (iv) for every PIE, potentiation or amplification observed when levosimendan was added, a shortening of the CRC (lusitropic effect) was found; (v) levosimendan had a similar PDE inhibitory profile as cilostamide; and (vi) levosimendan, in contrast to the calcium-sensitizer EMD57033, failed to sensitize the response of the electrically stimulated myocardial strips to calcium. Thus, in contrast to others, we demonstrate that PDE3 inhibition alone was enough to account for the observed PIE of levosimendan in human and rat ventricular myocardium, with no detectable additional contribution from a calcium-sensitizing component to the observed PIE.

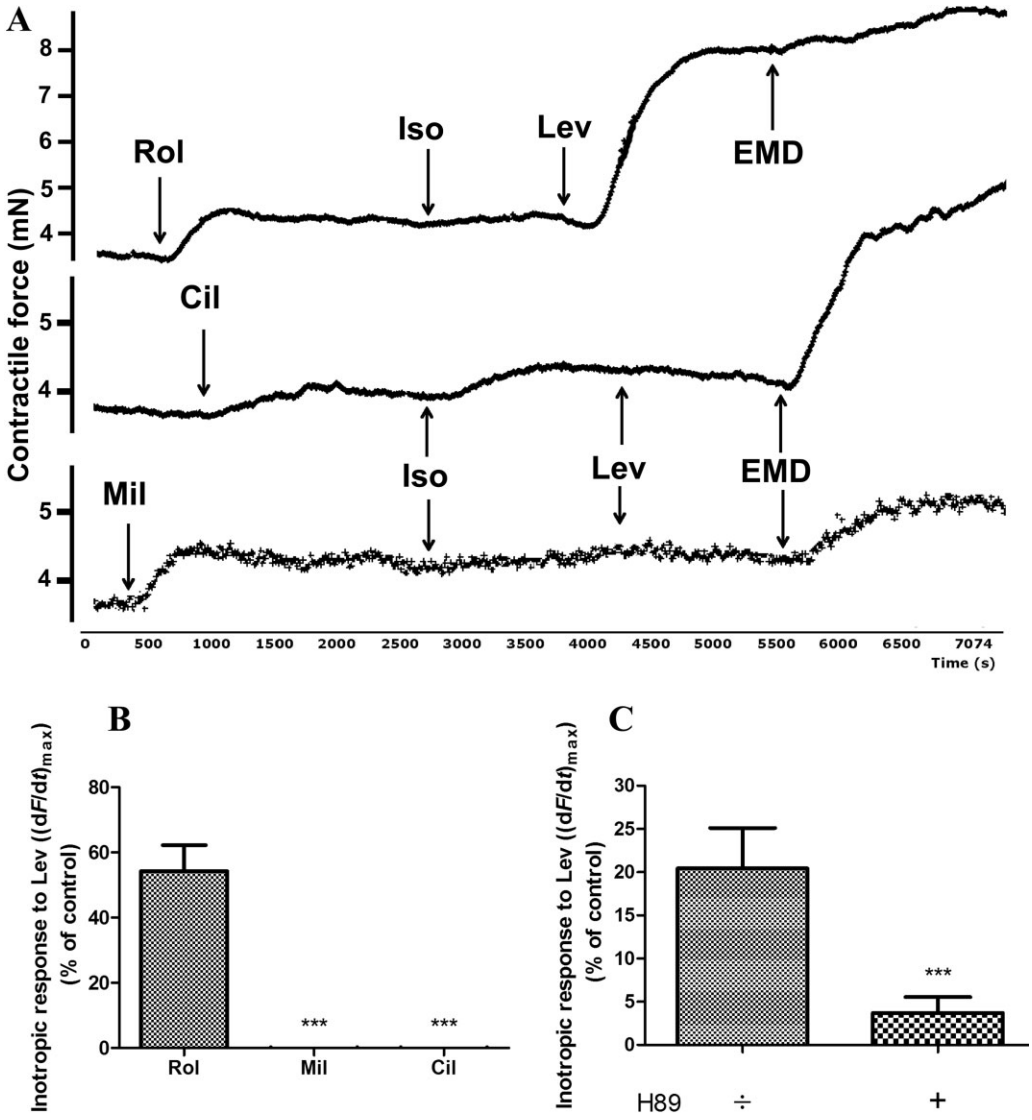

Figure 5

(A) Readout of three experiments showing different PDE-inhibitors and low-grade β-adrenoceptor stimulation (Iso EC_{10}) followed by levosimendan (10^{-7} M) (Lev) in the presence of α-adrenoceptor and muscarinic cholinoceptor blockade in rat myocardium. EMD57033 (EMD) (10^{-5} M) added at the end demonstrates that a possible calcium-sensitizing component is still detectable. The figure shows representative tracings, scaled to represent the average basal and maximum force in similar experiments. (B) Bar graph demonstrating the inotropic effect of levosimendan (10^{-7} M), data are mean ± SEM from *n* animals. In the presence of rolipram (10^{-5} M) (Rol) and an EC_{10} of isoprenaline, levosimendan (10^{-7} M) caused a PIE of 54 ± 11% above basal ($n = 4$, basal developed force: 3.5 ± 0.3 mN). This response was absent in the presence of the PDE3 inhibitors milrinone (Mil) (10^{-5} M, $n = 4$, basal developed force: 3.7 ± 0.3 mN) and cilostamide (Cil) (10^{-5} M, $n = 4$, basal developed force: 3.8 ± 0.4 mN) (***$P < 0.005$ for Mil and Cil vs. control). (C) Bar graph showing the inotropic effect of levosimendan (2×10^{-8} M) in the presence of rolipram and a low-grade β-adrenoceptor stimulation in rat myocardium. Under these conditions levosimendan induced an inotropic response of 20 ± 6% above basal ($n = 6$, basal developed force: 3.7 ± 0.6 mN). This inotropic response was almost completely absent (4 ± 2% above basal, $n = 6$, basal developed force: 4.3 ± 0.4 mN) in the presence of the PKA inhibitor, H89 (***$P < 0.05$).

Levosimendan inhibits PDE3 at therapeutic concentrations

A necessary prerequisite for interpreting the effects of levosimendan as consequences of PDE3 inhibition is to demonstrate directly its ability to inhibit the enzyme activity at therapeutically relevant concentrations. We assayed for PDE-activity at 10^{-6} M cAMP, which is close to the average intracellular concentration. Thus the IC_{50} of 2×10^{-8} M obtained for levosimendan should be representative for conditions *in vivo*. This value corresponds to the therapeutic free plasma concentration of levosimendan given by Haikala *et al.* (1997). Levosimendan showed an inhibitory profile, similar to that of the PDE3 inhibitor cilostamide, both alone and in combination with the PDE4 inhibitor rolipram. Levosimendan did not exert a significant additional effect when added to cilostamide or to the combination of cilostamide and rolipram.

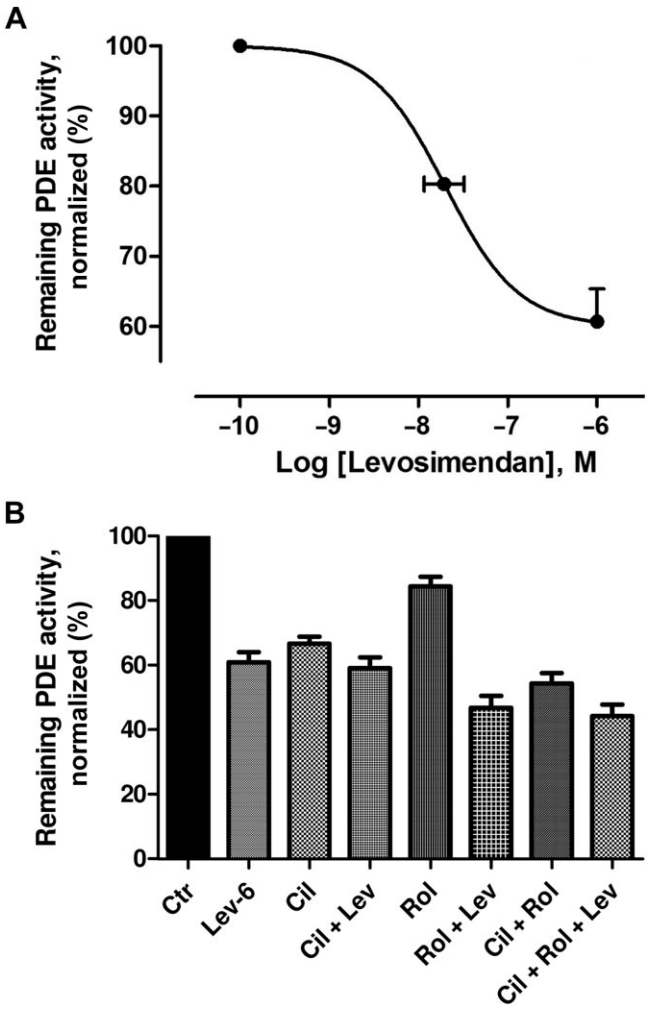

Figure 6

PDE3 inhibition by levosimendan. (A) PDE enzyme assay on failing human myocardium, demonstrating a constructed concentration–response curve of the PDE3 inhibitory ability of levosimendan at varying concentrations based on individual experiments ($n = 7$). (A) log IC_{50} (pIC_{50}) value of 7.7 ± 0.2 was calculated. (B) PDE enzyme assay on failing human myocardium (Total value control: 146 ± 21 pmol mg protein^{-1} min^{-1}, data are mean ± SEM from tissue from n patients) where levosimendan (Lev; 10^{-6} M) was compared with known PDE3 (Cil, 10^{-6} M cilostamide) and PDE4 (Rol, 10^{-5} M rolipram) inhibitors. Levosimendan had similar effects as cilostamide and gave additional inhibition when combined with rolipram, but not with cilostamide, demonstrating that levosimendan is a PDE3 inhibitor at the given concentration.

Thus, the main enzyme inhibited by levosimendan at the concentrations used is PDE3. As the prerequisite of inhibition at therapeutic concentrations is fulfilled, the next aspect to be discussed is whether this inhibition translated to functional effects.

No inotropic response was elicited by levosimendan in the presence of PDE3 inhibition

Our experiments revealed a PIE to stimulation by levosimendan alone in failing human ventricular strips. In order to clarify the role of PDE inhibition in this response we studied, as a novel approach, whether the effect was modified by pretreatment with the PDE3-selective inhibitor cilostamide (Osadchii, 2007). During this PDE3 inhibition, levosimendan did not generate any PIE. We have been unable to find actions of cilostamide, other than PDE3 inhibition, which could explain this observation. However, to eliminate the possibility that unknown effects of cilostamide could confound the results, we confirmed this finding with a different PDE3 inhibitor, milrinone. Thus, levosimendan probably works by inhibiting the same PDE as cilostamide, that is, preferentially, PDE3. Although levosimendan has previously been found to bind *in vitro* to human recombinant cTnC (Pollesello *et al.*, 1994), in the present *ex vivo* model, we did not observe any inotropic effects caused by other mechanisms, such as calcium sensitization, or inhibition of PDEs not inhibited by cilostamide. If such a component of inotropic action were operating, it should have been visible during PDE3 inhibition. Thus, PDE3 inhibition is sufficient to explain the PIE of levosimendan.

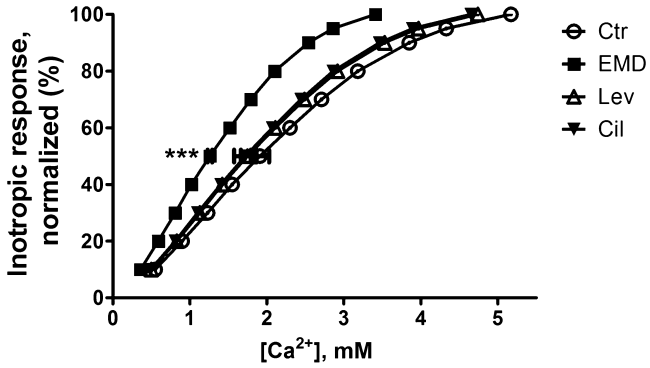

Figure 7

The effect of levosimendan on the calcium concentration–response relationship, data are mean ± SEM from n animals. Levosimendan did not sensitize the concentration–response curve to Ca^{2+}. Levosimendan (Lev; 10^{-6} M, $n = 6$) and cilostamide (Cil; 10^{-6} M, $n = 6$) demonstrated no observable calcium-sensitizing component in comparison to control in normal rat ventricular strips [EC_{50} = 1.75 ± 0.09 mM ($n = 6$) for levosimendan, 1.72 ± 0.14 mM ($n = 6$) for cilostamide vs. 1.91 ± 0.12 mM ($n = 6$) for control]. The classic calcium-sensitizer EMD57033 (EMD; 3×10^{-6} M, $n = 6$) shifted the curve to lower calcium concentrations and significantly decreased the Ca^{2+} EC_{50} value [EC_{50} = 1.26 ± 0.03 ($n = 6$), (***$P < 0.005$)], reflecting Ca^{2+} sensitization.

The inotropic effect of levosimendan is amplified during PDE4 inhibition

In contrast to its abolition in the presence of a PDE3 inhibitor, the PIE of levosimendan was markedly amplified by the presence of a PDE4 inhibitor, both in failing human and in normal rat myocardium. To the best of our knowledge, such a modulation of the effect of levosimendan has not been published previously. This effect strengthens our hypothesis that the effect of levosimendan is mediated by PDE3 inhibition, as PDE4 inhibition will actually amplify the effects of PDE3 inhibition under these conditions (Afzal *et al.*, 2011).

The inotropic effect of levosimendan is amplified by low-grade β-adrenoceptor stimulation

We observed that the presence of low-grade β-adrenoceptor stimulation markedly amplified the PIE of levosimendan. Under these conditions, a small activation of cAMP production through β-adrenoceptor stimulation will increase the consequence of inhibiting cAMP breakdown, thus disclosing the potential of a PDE3 inhibitor (Qvigstad *et al*., 2010). The concentration–response curve revealed a pEC_{50} for levosimendan of 7.3, close to the pIC_{50} (7.7) of levosimendan, demonstrated in the PDE enzyme activity assay. The ability of low-grade β-adrenoceptor stimulation to increase the inotropic effect of levosimendan corresponds with the findings of Haikala *et al*. (1997), who reported that twofold threshold electrical stimulation of guinea-pig papillary muscle, which would release noradrenaline from the adrenergic nerve endings, markedly increased the inotropic effect of levosimendan, an effect also eliminated by β-adrenoceptor blockade.

In our experiments, the complete removal by β-adrenoceptor blockade of the PIE of levosimendan during low-grade β-adrenoceptor stimulation and the markedly reduced inotropic response to levosimendan in the presence of the PKA inhibitor, H89, indicate an inotropic mechanism depending on cAMP. This also corresponds with earlier studies in which the effect of levosimendan was removed by carbachol (Boknik *et al*., 1997; Takahashi *et al*., 2000). On the other hand, a calcium-sensitizing effect of levosimendan should not be affected by β-adrenoceptor blockade, PKA-inhibition or by PDE3 inhibition. Importantly, the PIE of EMD57033 at the end of the experiment in Figure 5 demonstrates that a calcium-sensitizing component can provide an additional inotropic effect under these conditions.

Levosimendan potentiates the response to β-adrenoceptor stimulation

As another approach to study the mechanism of the PIE of levosimendan, we studied the effects of levosimendan and PDE inhibitors on the potency of β-adrenoceptor stimulation. In such experiments, a calcium sensitization should reveal itself as a potentiation of the response, independent of PDE inhibition. As expected with PDE inhibition, cilostamide potentiated the response to β-adrenoceptor stimulation. Importantly, the same effect was seen with levosimendan, with cilostamide being slightly more efficient. This corresponds well with a small study in guinea pigs, demonstrating a small potentiation of the inotropic response to β-adrenoceptor stimulation in the presence of levosimendan (Boknik *et al*., 1997). Our study revealed that the levosimendan-induced sensitization in human myocardium appeared at 10^{-8} M, a concentration below the therapeutic level of levosimendan. However, during PDE3 inhibition with cilostamide, no additional sensitizing effect of levosimendan could be observed, consistent with PDE3 inhibition fully accounting for the effect of levosimendan.

Levosimendan does not potentiate the inotropic effect of increasing concentrations of calcium

The on–off binding of levosimendan to TnC was considered a breakthrough, allowing calcium sensitization without the negative lusitropic effect (slower relaxation) usually seen with this drug type (Endoh, 2008). An unanswered question, however, is whether this on–off rate is rapid enough to follow the CRC in functional myocardium. Although previous studies have studied the relationship between the intracellular calcium and the inotropic effect of levosimendan in canine ventricular trabeculae electrically stimulated at 0.5 Hz (Takahashi and Endoh, 2005), our method is the first to study levosimendan and calcium sensitivity through concentration–response curves to Ca^{2+} on electrically stimulated myocardial strips in a dynamic Ca^{2+}-handling model. Under these conditions, levosimendan should have ample opportunity to sensitize the myocardium through the cyclic binding process to TnC and demonstrate the calcium-sensitizing component of levosimendan, if the process is sufficiently rapid. However, under these conditions, levosimendan did not sensitize the myocardium to calcium. In contrast, EMD57033, which is a known calcium sensitizer with a comparable, but not identical, Ca^{2+}-sensitizing mechanism of action as that considered for levosimendan (Endoh, 2008), did sensitize the myocardium to calcium in the model used. Thus, EMD57033 demonstrated that increased calcium sensitivity by binding to TnC was detectable, by shifting the concentration–response curves to lower concentrations of Ca^{2+}. Thus, our data did not indicate a calcium-sensitizing component of levosimendan in dynamically functioning myocardium, therefore favouring PDE3 inhibition as the dominant and sufficient mechanism involved in the inotropic response to levosimendan.

In canine ventricular trabeculae, Takahashi and Endoh (2005) found that levosimendan shifted the calcium–force relationship to lower calcium concentrations compared with that of elevation of extracellular calcium. Although this is compatible with a calcium sensitization, no inotropic effect occurred unless there was an increase in calcium transients. The apparent discrepancy between these results and ours may be due to different stimulation frequencies (0.5 vs. 1.0 Hz) or different species.

Levosimendan elicits lusitropic effects

A lusitropic response shown as an earlier onset of relaxation (reduction of TPF) and increased rate of relaxation (reduction of RT) associated with an inotropic response, giving a shortening of the CRC, is characteristic of activation of the cAMP/PKA pathway (Skomedal *et al*., 1997). PDE3 inhibition is known to cause such a lusitropic effect (Afzal *et al*., 2011). All our experiments showing an inotropic effect of levosimendan also revealed concomitant concentration-related shortening of the cycle in failing human ventricular strips. This finding corresponds with similar experiments done on normal canine ventricular myocardium (Takahashi and Endoh, 2005). Also, as expected, levosimendan alone did not cause a significant reduction in RT in normal rat ventricular strips, presumably because of the remaining PDE4 activity. However, in the presence of PDE4 inhibition by rolipram, levosimendan revealed a concentration-dependent reduction in RT (see Table 1). Our findings are similar to results previously obtained in guinea pigs (Haikala *et al*., 1995b), where a large lusitropic effect was observed. In that study, the authors emphasized the absence of impairment of relaxation (prolongation of RT) without interpreting the lusitropic effect as a

criterion of cAMP-dependency. As a lusitropic effect associated with an inotropic effect more generally is equivalent to cAMP-dependency, our results indicate that levosimendan causes, or rather enhances, cAMP-dependent inotropic and lusitropic responses, which corresponds to a clear dominating role of PDE3 inhibition as revealed in the present study.

Does levosimendan bind to cTnC in cyclic functioning myocardium?

It is highly relevant to compare the EC_{50} and IC_{50} values found for levosimendan in our study with the binding affinity of levosimendan to cTnC analysed by others. Evidence both for (Pollesello *et al.*, 1994; Sorsa *et al.*, 2001) and against (Kleerekoper and Putkey, 1999) a possible interaction between levosimendan and cTnC have been reported. Recently, Robertson *et al.* (2008) measured the binding affinity of levosimendan to cTnC and found a K_D value of about 0.7 mM in the presence of relevant Ca^{2+} concentration. This K_D value is much higher than the concentrations of levosimendan required to induce PIE both in the present study and in studies by others, in perfused hearts, rabbit ventricular cardiomyocytes (Sato *et al.*, 1998) and human ventricular strips (Hasenfuss *et al.*, 1998; Brixius *et al.*, 2002). PDE3 inhibition, however, is present at therapeutic concentrations, as demonstrated in this study in human myocardium and by others in guinea pigs (Szilagyi *et al.*, 2004; Kaheinen *et al.*, 2006). Thus a binding of levosimendan at therapeutic concentrations to cTnC is expected to be extremely small. The portion of cTnC binding levosimendan can be calculated to be maximally about 0.1%. In any case, if a functional effect of levosimendan through cTnC is to be accepted, a reasonable requirement is that an extremely high efficiency and rapid kinetics of binding must be shown. Our results did not reveal, although indirectly, such properties. On the contrary, we found a good correspondence between PDE3 inhibition (equivalent to binding to the enzyme) and functional data from our and others' experiments, making PDE inhibition the likely mechanism for the PIE of levosimendan.

Other aspects of the clinical effects of levosimendan

In contrast to other studies, our results indicate that PDE3 inhibition is sufficient to account for the inotropic effect of levosimendan. However, it is important to emphasize that levosimendan treatment *in vivo* includes additional mechanisms (such as opening of ATP-dependent potassium channels) involved in the haemodynamic effects on the patient. Other possible effects include mitochondrial protection, anti-inflammatory and anti-apoptotic effects (Antoniades *et al.*, 2007).

Additionally, another important aspect in this regard is the long half-life of the levosimendan metabolite OR-1855 (Antila *et al.*, 1999) (further metabolized to the active form OR-1896). These metabolites have a half-life of up to 70–80 h, inducing and maintaining haemodynamic effects for several days after infusion has been stopped (Kivikko *et al.*, 2002). The present results do not exclude the possibility that the inotropic effects mediated by the active metabolites of levosimendan include calcium sensitization, and the studies demonstrating superiority of levosimendan over other inotropes (primarily short term; Follath *et al.*, 2002; Mebazaa *et al.*, 2007) could open for such a possibility. The inotropic effects of OR-1896 have been studied in detail in rabbit ventricular myocardium (Takahashi *et al.*, 2000), and the conclusion was that a combination of calcium sensitization and PDE inhibition was responsible for the observed PIE.

Finally, this study used tissue from end-stage heart failure patients. It is possible that the calcium-sensitizing effect of levosimendan is more pronounced in earlier stages of heart failure, with less maladaptive changes. Thus our study does not exclude a potential calcium-sensitizing effect revealing itself in earlier or more moderate forms of heart failure. This seems, however, unlikely given the similar results found in non-failing rat ventricular myocardium.

Conclusion

Our data indicate that the observed inotropic effects of levosimendan in human and rat myocardium can be adequately explained by its PDE3 inhibitory properties, leaving very little need for additional mechanisms, such as calcium sensitization.

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Conflict of interest

None.

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