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Dual regulation of myofilament Ca^{2+} sensitivity by levosimendan in normal and acidotic conditions in aequorin-loaded canine ventricular myocardium

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1 Experiments were carried out in canine ventricular trabeculae loaded with aequorin to investigate the effects of levosimendan {(*R*)-([4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]-hydrazono)-propanedinitrile} on contractile force and Ca^{2+} transients in normal and acidotic conditions.

2 The concentration–response curve for the positive inotropic effect (PIE) of levosimendan was bell-shaped, that is, it declined markedly at 10^{-4} M after achieving the maximum at 10^{-5} M in normal ($pH_o = 7.4$) and acidotic conditions ($pH_o = 6.6$).

3 The positive inotropic effect (PIE) of levosimendan up to 10^{-5} M was associated with an increase in Ca^{2+} transients and a shift of the relationship of Ca^{2+} transients and force to the left of that of elevation of $[Ca^{2+}]_o$.

4 Levosimendan at 10^{-4} M elicited a negative inotropic effect (NIE) in association with a further increase in Ca^{2+} transients, and during washout Ca^{2+} transients increased further, while the force was abolished before both signals recovered to the control.

5 In acidotic conditions, the relationship of Ca^{2+} transients and force during the application of levosimendan in normal conditions was essentially unaltered, whereas the PIE was suppressed due to attenuation of the increase in Ca^{2+} transients.

6 In summary, in intact canine ventricular myocardium, levosimendan elicits a dual inotropic effect: at lower concentrations, it induces a PIE by a combination of increases in Ca^{2+} transients and Ca^{2+} sensitivity, while at higher concentrations it elicits an NIE due to a decrease in Ca^{2+} sensitivity. Acidosis inhibits the PIE of levosimendan due to suppression of the increase in Ca^{2+} transients in response to the compound.
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Abbreviations: ANOVA, analysis of variance; cAMP, cyclic adenosine 3′,5′-monophosphate; DMSO, dimethyl sulfoxide; ISO, isoproterenol; ISO_{max}, the maximal response to isoproterenol; L_{max}, muscle length at which the maximal contractile force is achieved; LVS, levosimendan; NIE, negative inotropic effect; PDE, phosphodiesterase III; PIE, positive inotropic effect

Introduction

Cardiotonic agents are inevitable for the treatment of contractile dysfunction in congestive heart failure. Agents such as digitalis, catecholamines, and phosphodiesterase (PDE) III inhibitors act through the upstream mechanism (Blinks & Endoh, 1986) by an increase in intracellular Ca^{2+} mobilization (Ca^{2+} mobilizers) (Farah *et al*., 1984; Scholz & Meyer, 1986; Endoh, 1995). Ca^{2+} sensitizers, on the other hand, are capable of inducing the positive inotropic effect (PIE) *via* the central and/or downstream mechanisms, acting on troponin C, thin filament complex, and/or crossbridge (Blinks & Endoh, 1986; Endoh, 1995; 1998; 2003; Lee & Allen, 1997). Research interests have currently been focused more on Ca^{2+} sensitizers as they overcome the disadvantages associated with Ca^{2+} mobilizers: they do not increase the activation energy, are not associated with potential risks of inducing arrhythmias, cell injury, and death, and have the potential to reverse contractile dysfunction under pathological conditions, such as acidosis, myocardial stunning, and heart failure (Lee *et al*., 1993; Watanabe *et al*., 1996).

Acidosis is a major component of cardiac ischemia, markedly suppressing contractile function (Bountra & Vaughan-Jones, 1989) by affecting cardiac E-C coupling, including Ca^{2+} mobilization and Ca^{2+} sensitivity (Orchard & Kentish, 1990; Hulme *et al*., 1997). Acidosis either decreases (Hulme & Orchard, 1998) or increases (Bountra & Vaughan-Jones, 1989; Orchard & Kentish, 1990) Ca^{2+} transients, but it consistently decreases Ca^{2+} sensitivity (Allen & Orchard, 1983; Orchard & Kentish, 1990; Orchard *et al*., 1991) due to a decrease in the affinity of troponin C for Ca^{2+} (Palmer & Kentish, 1994) and a direct depression of crossbridge cycling (Hulme & Orchard, 1998).

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Levosimendan {(*R*)-([4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]-hydrazono)-propanedinitrile} has been discovered as an agent that binds selectively to troponin C (Haikala *et al*., 1995) and possesses a Ca^{2+} sensitizing action on cardiac muscle (Edes *et al*., 1995; Hasenfuss *et al*., 1998; Sato *et al*., 1998). It is clinically available in some European countries and beneficial for congestive heart failure (Innes & Wagstaff, 2003; Cleland *et al*., 2004). While levosimendan may increase Ca^{2+} sensitivity by binding to an amino-terminal region of troponin C (Pollesello *et al*., 1994; Haikala *et al*., 1995), it also inhibits PDE III activity *in vitro* with the potency equivalent to milrinone (Haikala *et al*., 1997). The important issue how these biochemical effects contribute to cardiac contractile regulation in *intact* myocardium is still controversial. We revealed that levosimendan increased Ca^{2+} transients and Ca^{2+} sensitivity over a concentration range in rabbit ventricular muscle (Sato *et al*., 1998), but it has been reported that levosimendan elicits a PIE without an increase in Ca^{2+} transients at lower concentrations and by Ca^{2+} mobilization due to PDE III inhibition first at higher concentrations in guinea-pig and human hearts (Edes *et al*., 1995; Hasenfuss *et al*., 1998).

The present experiments were carried out to examine the concentration-dependent effects of levosimendan on the relationship of Ca^{2+} transients and contractile force in detail in canine ventricular trabeculae loaded with aequorin in normal and acidotic conditions. It elicited a dual action on the Ca^{2+} sensitivity, and up to 10^{-5} M it increased the Ca^{2+} sensitivity even in acidotic conditions, although the PIE of levosimendan was attenuated by acidosis due to a reduction of the increase in Ca^{2+} transients induced by the compound.

Methods

The study was conducted in accordance with Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society and with the Guidance for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication no. 85-23, revised 1996). Approval for the animal experiments was obtained from the Committee for Animal Experimentation of Yamagata University School of Medicine prior to the experiments, and the study was carried out also in accordance with the Declaration of Helsinki.

Preparation of trabeculae isolated from canine right ventricle

Mongrel dogs of either sex (8–12 kg) were used in the present study. Animals were anesthetized by intravenous administration of pentobarbital sodium (30 mg kg^{-1}). Hearts were rapidly excised and free-running trabeculae (< 1 mm in diameter) were dissected from the free wall of the right ventricle. Muscles were mounted in 20-ml organ baths containing Krebs–Henseleit solution. The composition of the solution was as follows (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 24.9, KH_2PO_4 1.2, and glucose 11.1 (with 0.057 mM ascorbic acid and 0.027 mM EDTA added to prevent autoxidation of the compounds examined). The solution was bubbled with 95% O_2–5% CO_2 and maintained at pH 7.4.

The muscle preparations were stimulated electrically by square-wave pulses of 5-ms duration at a voltage about 20% above the threshold, at a frequency of 0.5 Hz, through bipolar platinum electrodes. The force of contraction was recorded continuously on a thermal pen-writing oscillograph (Recti-Horiz-8K; NEC Sanei Instruments Ltd, Tokyo, Japan) by means of force–displacement transducers (Shinkoh UL 10 GR; Minebea Co. Ltd, Tokyo, Japan). In most preparations, after an equilibration period of 1 h, the resting tension and contractile force of the muscle was stable during the course of experiments, which lasted for several hours. During the equilibration period, the muscles were stretched initially at a resting tension of 5 mN, and the length was later adjusted to give 90% of L_{max} (muscle length at which the maximal contractile force is achieved). Preparations in which the resting tension increased progressively during the equilibration period were discarded.

At the beginning of each experiment, the responsiveness and stability of individual preparations were checked by successive administration (at least twice or three times) of isoproterenol (ISO) at 10^{-7} M. Only those preparations that produced a consistent and reproducible increase in contractile force in response to the successive administration of ISO were used for the experiments. At the end of each experiment with levosimendan in acidotic conditions, the maximal response to ISO (ISO_{max}) in control conditions was determined in each muscle by cumulative administration of ISO up to 10^{-5} M or 3×10^{-5} M after washout of the drug for 2 h. Acidosis (pH 6.6) was induced by replacing about 80% of HCO_3^- with Cl^- in Krebs–Henseleit solution. The composition of the acidotic solution was as follows (mM): NaCl 138, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 4.9, KH_2PO_4 1.2, and glucose 11.1 (with 0.057 mM ascorbic acid and 0.027 mM EDTA). The solution was bubbled with 95% O_2 and 5% CO_2 so that the influence of hypoxia or anoxia would be negligible.

Preparation of aequorin-loaded trabeculae

For simultaneous detection of the contractile force and Ca^{2+} transients, Ca^{2+}-sensitive bioluminescent protein aequorin was loaded by a modified macroinjection technique, as detailed elsewhere (Watanabe *et al*., 1996; Sato *et al*., 1998; Takahashi *et al*., 2000a, b).

Briefly, a muscle preparation isolated from right ventricular free wall was mounted horizontally in a 12-ml organ bath that contained nominally Ca^{2+}-free modified Krebs–Henseleit solution at 4°C. Aequorin was dissolved at concentrations of approximately 1.0 μg ml^{-1} in a solution of 150 mM KCl and 0.1 mM EDTA-2Na. After immersion of the preparations in nominally Ca^{2+}-free solution for 5 min, 3–4 μl of aequorin solution was gently injected just beneath the endocardium through a fine-tipped glass micropipette. Then the aequorin-loaded preparation was transferred to a 50-ml organ bath that contained modified Krebs–Henseleit solution. The concentration of Ca^{2+} was gradually raised stepwise to 0.025, 0.25, 1.25, and finally to 2.5 mM at intervals of 15 min. Simultaneously, the temperature was gradually raised to 37°C.

A 50-ml organ bath specially designed for the simultaneous high-efficacy detection of light from aequorin and for the minimization of motion artifacts due to isometric contractions was used for the experiments (Blinks & Endoh, 1986). Aequorin light signals were detected with a photomultiplier

(9789A, Thorn EMI Electron Tubes, Ruislip, U.K.) and light signals were smoothed by a low-pass filter. The isometric contractile force was recorded simultaneously with the transducer mentioned above. Both signals were recorded on digital audio tape (PC-108M, Sony Magnescale, Tokyo, Japan) for subsequent analysis.

The muscle was electrically stimulated by square-wave pulses of 5-ms duration at a voltage about 20% above the threshold, at a frequency of 0.5 Hz, through bipolar platinum electrodes. The aequorin-loaded preparation was equilibrated for about 120 min, meanwhile the bioluminescence declined to a steady low level. During the equilibration period, the length of the muscle was adjusted to L_{max}. Only preparations with a baseline contractile force of $>4\,mN/mm^2$ and with stable bioluminescence signals and contraction amplitudes during the course of experiments were used for the analysis of the action of the drug in acidotic conditions.

In total, 50–150 signals of Ca^{2+} transients and isometric contractions were averaged to improve the signal-to-noise ratio by means of data analysis software (Visual Designer; Intelligent Instrumentation, Tucson, AZ, U.S.A.) on a PC/AT personal computer (FMV-Deskpower S13; Fujitsu, Tokyo, Japan). The number of signals to be averaged was determined so as to obtain a sufficient signal-to-noise ratio in each preparation. The 2.5th root of the peak amplitude of aequorin signals was calculated as an indicator of the peak $[Ca^{2+}]_i$, because the strength of the bioluminescence of aequorin varies approximately in proportion to the 2.5th power of the concentration of Ca^{2+} within a range of physiological values of $[Ca^{2+}]_i$ (Blinks *et al*., 1982).

In a series of experiments to determine the concentration–response curve for levosimendan in acidotic conditions, the drug was administrated in a cumulative manner in steps of 0.5 log units. When a steady contractile force had been achieved, levosimendan was added to achieve the next higher concentration. All experiments with aequorin-loaded preparations were carried out in the presence of 3×10^{-7} M (±)-bupranolol to avoid modulation of the drug's action by β-adrenoceptor stimulation induced by norepinephrine released by electrical stimulation or by the drug itself.

Solutions of levosimendan were yellowish. During spectrophotometry, the absorbance of levosimendan in solution at 469 nm, the peak wavelength of the aequorin light signal, was negligibly low even at the highest concentration used in this study. To determine whether levosimendan had any direct effect on aequorin bioluminescence, we performed a cuvette test *in vitro* according to procedures originally developed by Blinks *et al*. (1978). The fractional luminescence of aequorin light signals at various concentrations of Ca^{2+} in the presence of 10^{-5} M levosimendan fell on the curve obtained without levosimendan. Thus, light emission from aequorin was not influenced by levosimendan itself.

Levosimendan was dissolved in dimethyl sulfoxide (DMSO). In preliminary experiments, DMSO at 0.1, 0.19, 0.27, 0.37 and

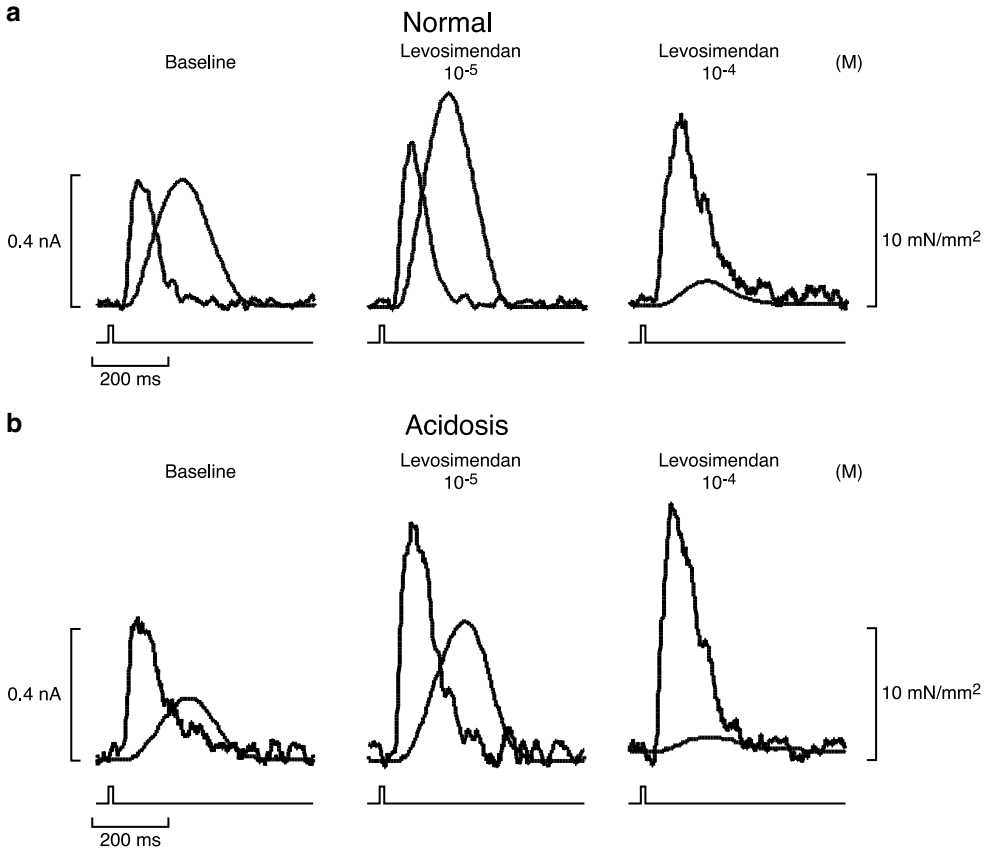

Figure 1 Concentration-dependent effects of levosimendan on aequorin light transients (noisy recordings) and isometric contractions in the presence of 3×10^{-7} M (±)-bupranolol in a canine right ventricular trabecula in normal conditions. (a) Normal conditions; (b) acidotic conditions. Each tracing represents signal-averaged recordings of 100 successive signals; the horizontal line below each tracing represents the recording of stimulus pulse.

up to 0.46% in the bathing solution did not influence the baseline contractile force or the aequorin light signals ($n=3$). The highest concentration of DMSO employed in the present study was 0.27%.

In each preparation, ISO_{max} was determined at the end of the experiments after washing out (±)-bupranolol and levosimendan for more than 2 h, and changing from an acidotic to a control solution. The PIE and the increase in the amplitude of Ca^{2+} transients induced by levosimendan or the elevation of $[Ca^{2+}]_o$ were expressed as a percentage of ISO_{max}.

Chemicals

The drugs used were as follows: levosimendan (Orion-Farmos, Espoo, Finland); (−)-isoproterenol hydrochloride (Sigma Chemical Co., St Louis, MO, U.S.A.); (±)-bupranolol hydrochloride (Kaken Pharmaceutical Co. Ltd, Tokyo, Japan); and pentobarbital sodium (Tokyo Kasei Kogyo Co. Ltd, Tokyo, Japan). Aequorin was purchased from Friday Harbor Photoproteins, Friday Harbor, WA, U.S.A.

Statistical analysis

Data are expressed as means ± standard error of the mean. For analysis of multiple measurements obtained from a single preparation, we used one-way analysis of variance (ANOVA) for repeated measures with Bonferroni's test. A *P*-value smaller than 0.05 was considered to indicate a statistically significant difference.

Results

Effects of elevation of $[Ca^{2+}]_o$ in normal and acidotic conditions

First, we investigated the influence of acidosis on the effects of the elevation of $[Ca^{2+}]_o$. At 2.5 mM $[Ca^{2+}]_o$, acidosis (pH 6.6) produced a pronounced depression of the contractile force (by 37.0 ± 4.24% of the level at pH 7.4) and a significant increase in Ca^{2+} transients (by 5.63 ± 3.81% of the level at pH 7.4; $n=7$ each) in association with the prolongation of Ca^{2+} transients. Elevation of $[Ca^{2+}]_o$ increased the contractile force even under acidotic conditions, but to a lesser extent compared with the control at pH 7.4. In normal conditions, the increase in contractile force at 4.0 mM $[Ca^{2+}]_o$ was 31.2 ± 3.53% of ISO_{max} and was associated with an increase in Ca^{2+} transients by 26.5 ± 5.57% of ISO_{max} ($n=5$). During acidosis, the increase in the contractile force at 4.0 mM $[Ca^{2+}]_o$ was 19.6 ± 4.94% of ISO_{max}, which was approximately half of the increase in normal conditions and was associated with an increase in Ca^{2+} transients by 28.1 ± 4.56% of ISO_{max} ($n=5$), which was not significantly different to the control. In acidotic conditions, the total duration of Ca^{2+} transients was prolonged significantly to 122.3 ± 4.15%, while the duration of isometric contractions showed a tendency to shorten (86.4 ± 4.95%, not significantly different from the control). The results of the influence of acidosis on the effects of elevation of $[Ca^{2+}]_o$ in this series of experiments were consistent with those in the previous study (Takahashi *et al.*, 2001).

Effects of levosimendan in normal and acidotic conditions

Next, the concentration-dependent effects of levosimendan on Ca^{2+} transients and contractions were determined in control (Figure 1a) and acidotic (Figure 1b) conditions. In both conditions, levosimendan up to 10^{-5} M elicited a PIE in association with a definite increase in Ca^{2+} transients, but at 10^{-4} M it elicited an NIE that was accompanied by a further increase and prolongation of Ca^{2+} transients (Figures 1a and b).

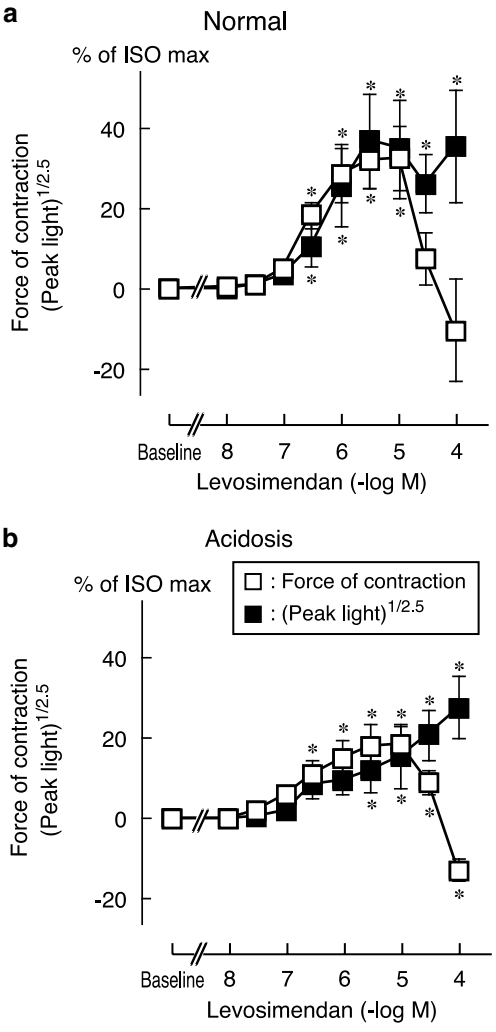

Figure 2 The concentration–response curve for the increase in Ca^{2+} transients and the force of contraction induced by levosimendan in the presence of 3×10^{-7} M (±)-bupranolol in isolated canine right ventricular trabeculae electrically driven at 0.5 Hz at 37°C in control ($n=7$) (a) and acidotic conditions ($n=5$) (b). Concentration–response curves for increases in Ca^{2+} transients and the force of contraction induced by levosimendan administered in a cumulative manner are presented. Ordinate: the increase in Ca^{2+} transients and the force of contraction induced by levosimendan expressed as a percentage of ISO_{max} in each preparation; abscissa: the concentration of levosimendan expressed as −log M. Symbols with vertical bars represent means ± s.e.m. Asterisks indicate significant differences from the corresponding baseline values ($P<0.05$). Actual values of peak light transients and basal force of contraction: 0.34 ± 0.18 nA and 6.12 ± 2.33 mN/mm^2 ($n=7$, each); and ISO_{max}: 3.15 ± 1.89 nA and 48.6 ± 13.9 mN/mm^2 ($n=7$, each), respectively, in (a); actual values of the peaks of light transients and the basal force of contraction: 0.52 ± 0.18 nA and 4.63 ± 2.93 mN/mm^2 ($n=5$, each); and ISO_{max}: 2.38 ± 1.92 nA and 47.5 ± 15.3 mN/mm^2 ($n=5$, each), respectively in (b).

The concentration–response curves for alterations in the contractile force and Ca^{2+} transients induced by levosimendan in normal (a) and acidotic (b) conditions are presented in Figure 2. Levosimendan induced a PIE in association with an increase in Ca^{2+} transients up to 10^{-5} M, but at concentrations of 3×10^{-5} M and higher it elicited a pronounced NIE with insignificant alteration of the amplitude of Ca^{2+} transients under both normal and acidotic conditions. In normal conditions, the maximum response to levosimendan was achieved at 10^{-5} M, amounting to $32.7 \pm 7.77\%$ (the percentage presented here from is % of ISO_{max}) and was associated with an increase in Ca^{2+} transients by $35.6 \pm 12.3\%$ ($n = 7$, each). At 10^{-4} M in normal conditions, the contractile force was $-10.1 \pm 12.8\%$, and the Ca^{2+} transients were $35.7 \pm 13.8\%$ ($n = 5$, each). Under acidosis, the maximum response to levosimendan was achieved at 10^{-5} M: the PIE at 10^{-5} M was 18.9 ± 4.90 and was associated with an increase in Ca^{2+} transients by $15.6 \pm 7.68\%$ ($n = 5$). At 10^{-4} M in acidotic conditions, the contractile force was $-12.6 \pm 2.69\%$, and Ca^{2+} transients were $27.9 \pm 7.80\%$ ($n = 5$, each).

Superimposed tracings of Ca^{2+} transients and contractions are presented, which were normalized to facilitate the comparison of the time courses of both signals, in the presence of 10^{-5} M levosimendan in control (Figure 3a) and acidotic (Figure 3c) conditions; and the effects from 10^{-5} to 10^{-4} M in control (Figure 3b) and acidotic (Figure 3d) conditions.

In control conditions, levosimendan at 10^{-5} M abbreviated the duration of contractions with little alteration of duration of Ca^{2+} transients (Figure 3a), which was abolished in acidotic conditions (Figure 3c). Levosimendan at 10^{-4} M elicited a pronounced dissociation of amplitude (Figure 1) and time course of isometric contractions from that of Ca^{2+} transients; in control conditions, the duration of Ca^{2+} transients was prolonged but that of isometric contractions was scarcely affected (Figure 3b); in acidotic conditions, time to peak contraction was abbreviated with little change in duration of Ca^{2+} transients (Figure 3d).

Figure 4 shows the concentration-dependent effects of levosimendan on the time course of isometric contractions in normal (a) and acidotic (b) conditions. In normal conditions, levosimendan up to 10^{-5} M abbreviated significantly the duration of contraction mainly due to decrease in relaxation time (Figure 4a), which is abolished in acidotic conditions (Figure 4b). While in normal conditions, levosimendan at 10^{-4} M prolonged the relaxation time resulting in a prolonged total duration of contraction (Figure 4a), under acidosis,

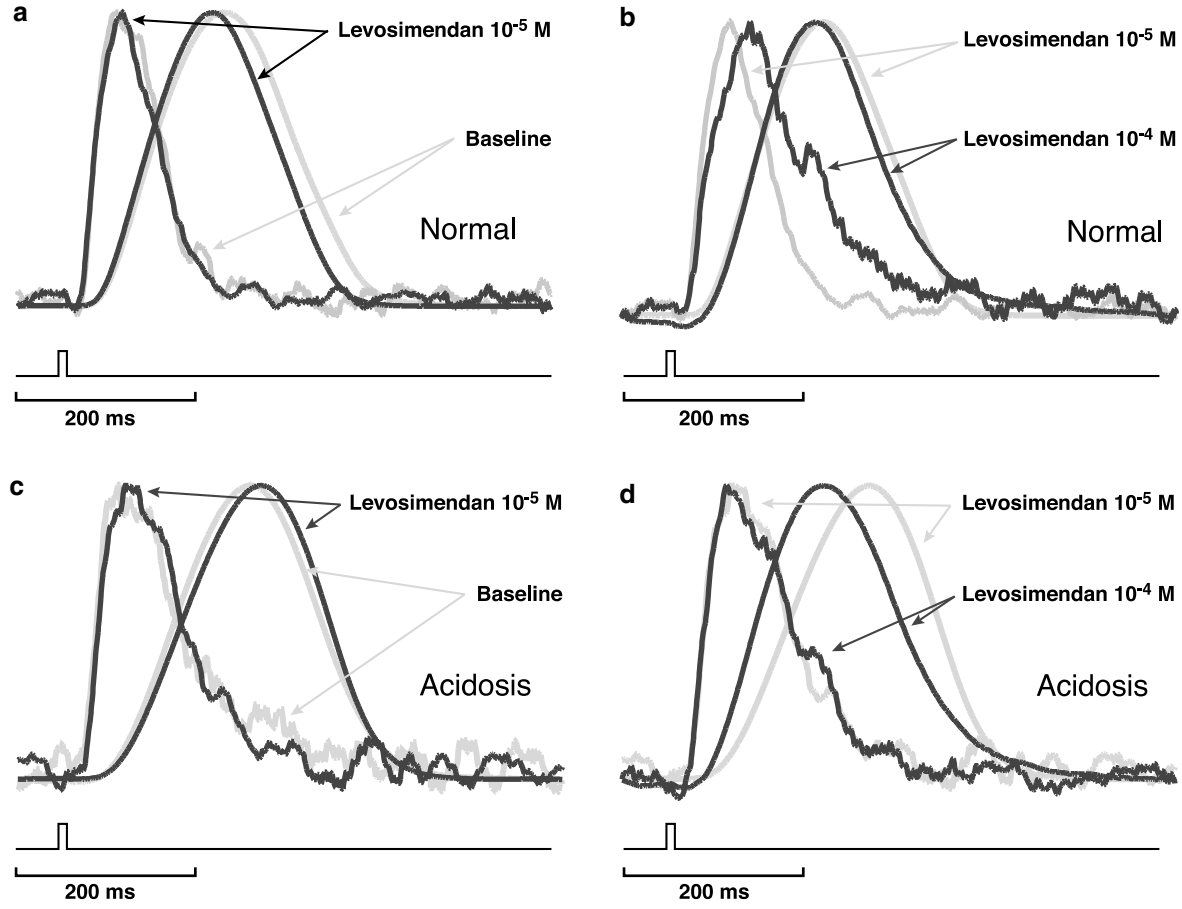

Figure 3 Superimposed tracings of the effects of levosimendan on aequorin light transients (noisy recordings) and isometric contractions in the presence of 3×10^{-7} M (±)-bupranolol in a canine right ventricular trabecula in normal (a, b) and acidotic (c, d) conditions. Amplitudes of the peaks of light transients and isometric contractions were normalized and superimposed to facilitate a comparison of the time courses of both signals. Superimposed tracings recorded before and during administration of levosimendan at 10^{-5} M (a, c) and during administration of levosimendan at 10^{-5} and 10^{-4} M (b, d). Each tracing represents signal-averaged recordings of 100 successive signals; the horizontal line below each tracing represents the recording of stimulus pulse.

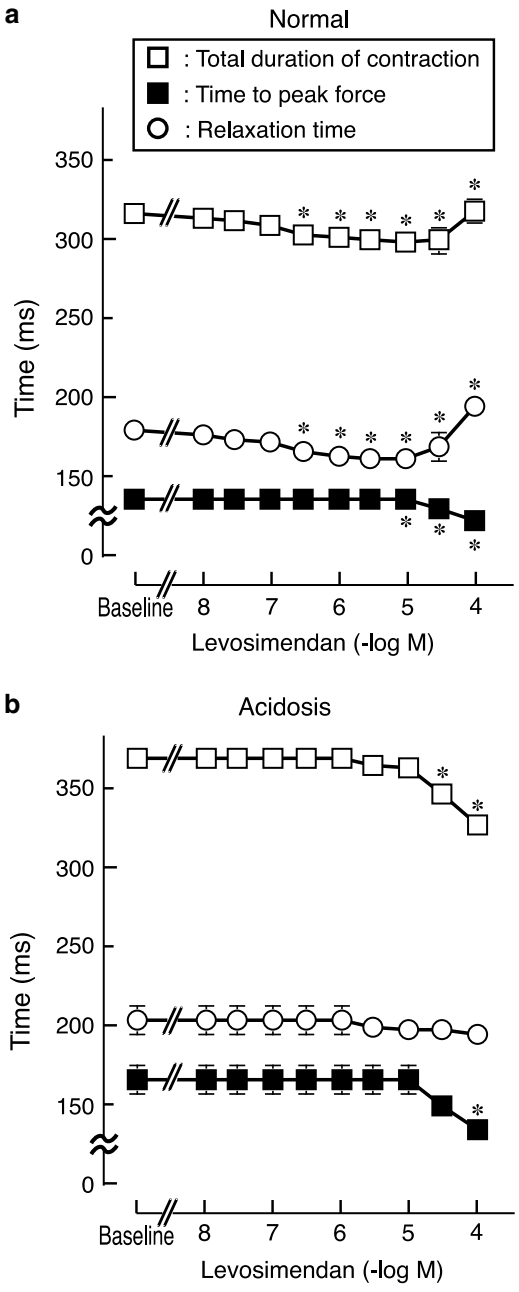

Figure 4 Concentration–response curves for the time courses of isometric contractions induced by levosimendan administered in a cumulative manner in normal (a) and acidotic (b) conditions. Ordinate: the time course induced by levosimendan expressed as ms in each preparation; abscissa: the concentration of levosimendan expressed as $-\log$ M. Symbols with vertical bars represent means ± s.e.m. Asterisks indicate significant differences from the corresponding baseline values ($P<0.05$).

levosimendan at 10^{-4} M abbreviated the time to peak force, resulting in a shortened total duration of contraction (Figure 4b).

Alteration of Ca^{2+} sensitivity by levosimendan up to 10^{-5} M

The relationship between Ca^{2+} transients and contractile force during application of levosimendan up to 10^{-5} M in normal and acidotic conditions are examined. In normal conditions, levosimendan up to 10^{-5} M shifted the relationship to the left and upwards compared with that for the elevation of $[Ca^{2+}]_o$, while the relationship for isoproterenol was shifted to the right of that for the elevation of $[Ca^{2+}]_o$ (Figure 5a).

The relationship for the elevation of $[Ca^{2+}]_o$ in acidotic conditions was shifted to the right and downward compared with that in normal conditions, however, the relationship during application of levosimendan up to 10^{-5} M was shifted to the left of that for $[Ca^{2+}]_o$ even under acidotic conditions, while the maximal force achieved by levosimendan was markedly suppressed in acidotic conditions (Figure 6).

Alteration of Ca^{2+} sensitivity by levosimendan at higher concentrations ($>10^{-5}$ M)

Figure 5b shows the relationship between Ca^{2+} transients and contractile force during application of levosimendan at 3×10^{-5} and 10^{-4} M in normal conditions. The relationship for the elevation of $[Ca^{2+}]_o$ was also presented for reference. The amplitude of isometric contractions was suppressed without a significant alteration of Ca^{2+} transients, which is an indication that the compound elicits a decrease in Ca^{2+} sensitivity at higher concentrations. In acidotic conditions, levosimendan produced essentially a similar effect on the relationship: the amplitude of isometric contractions was decreased, whereas the peak of Ca^{2+} transients was increased further (Figure 6).

Figure 7 shows the effects of 10^{-4} M levosimendan and the influence of the washout on isometric contractions and Ca^{2+} transients under acidotic conditions. After administration of 10^{-4} M levosimendan, the contractile force was markedly decreased and abolished, whereas Ca^{2+} transients were increased further (a in Figure 7). During washout, the amplitude of Ca^{2+} transients was remarkably elevated in the absence of the contractile force (b in Figure 7). After the repetitive washout with the drug-free solution, the contractile force and Ca^{2+} transients returned to their respective levels prior to the application of levosimendan (c in Figure 7). In normal conditions, the alterations of isometric contractions and Ca^{2+} transients induced by 10^{-4} M levosimendan and washout are essentially similar to those observed in acidotic conditions (data not shown).

Discussion

The important findings with levosimendan in *intact* canine ventricular myocardium are that: (1) it elicited a PIE due to combined increases in Ca^{2+} transients and Ca^{2+} sensitivity over the same concentration range ($<10^{-5}$ M); (2) Ca^{2+} sensitivity increased up to 10^{-5} M, but decreased at higher concentration range; and (3) under acidosis, the increase in Ca^{2+} sensitivity was not suppressed, but the PIE was inhibited due to attenuation of the increase in Ca^{2+} transients.

The potency of levosimendan to increase Ca^{2+} transients and Ca^{2+} sensitivity ($<10^{-5}$ M) in nonfailing *intact* canine ventricular myocardium accords well with that of the compound to shift the *p*Ca-tension relationship to the left in skinned cardiac fibers (Edes *et al*., 1995), to inhibit PDE III activity *in vitro* (Haikala *et al*., 1995), and to enhance the PIE induced *via* β-adrenoceptors (Boknik *et al*., 1997). The increase

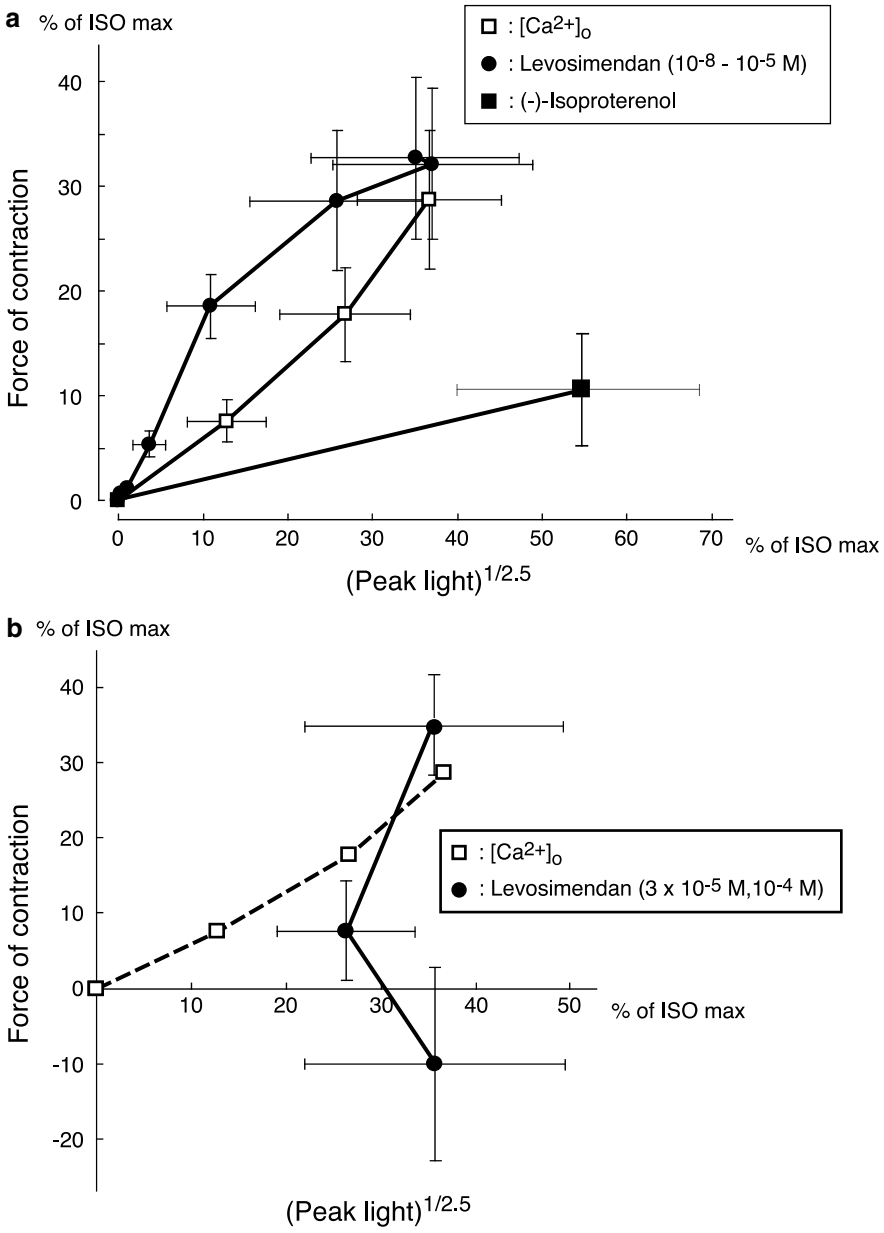

Figure 5 The relationship between the peaks of Ca^{2+} transients and the force of contraction during applications of different inotropic interventions, including elevation of $[Ca^{2+}]_o$, levosimendan, and isoproterenol in normal conditions in isolated canine right ventricular trabeculae. (a) Elevation of $[Ca^{2+}]_o$; levosimendan 10^{-8}–10^{-5} M and isoproterenol 10^{-8} M. (b) Levosimendan 10^{-5}–10^{-4} M. The relationship with elevation of $[Ca^{2+}]_o$ was presented for reference. Numbers in parentheses indicate the numbers of experiments. Ordinate: the changes in the force of contraction induced by these interventions expressed as a percentage of ISO_{max} in each preparation; abscissa: the increase in Ca^{2+} transients. Symbols with vertical and horizontal bars represent means ± s.e.m.

in Ca^{2+} transients and acceleration of relaxation induced by levosimendan may be due to cAMP accumulation induced by PDE III inhibition; and they are inhibited by carbachol (Boknik *et al*., 1997; Sato *et al*., 1998). Levosimendan never impaired relaxation in normal and acidotic conditions, which may be in part due to cAMP accumulation. In failing heart, however, the cAMP-mediated signaling is aggravated (Feldman *et al*., 1987); therefore, it is probable that the increase in Ca^{2+} sensitivity could be more pronounced than the PDE III inhibitory action in failing ventricular myocardium (Hasenfuss *et al*., 1998). Furthermore, under acidosis, where cAMP loses the effectiveness, lack of impairment of relaxation may be mainly due to the Ca^{2+}-dependent nature of the binding of levosimendan to troponin C (Haikala *et al*., 1995).

It has been well documented that the concentration–response curve for the PIE of levosimendan in *intact* myocardium is bell-shaped (e.g., Boknik *et al*., 1997; Sato *et al*., 1998), but the subcellular mechanism has not been addressed in previous studies. The findings in the current study imply that the suppression of cardiac contractility induced by levosimendan at high concentrations ($>10^{-5}$ M) is due to a reversible decrease in Ca^{2+} sensitivity. While the decrease in

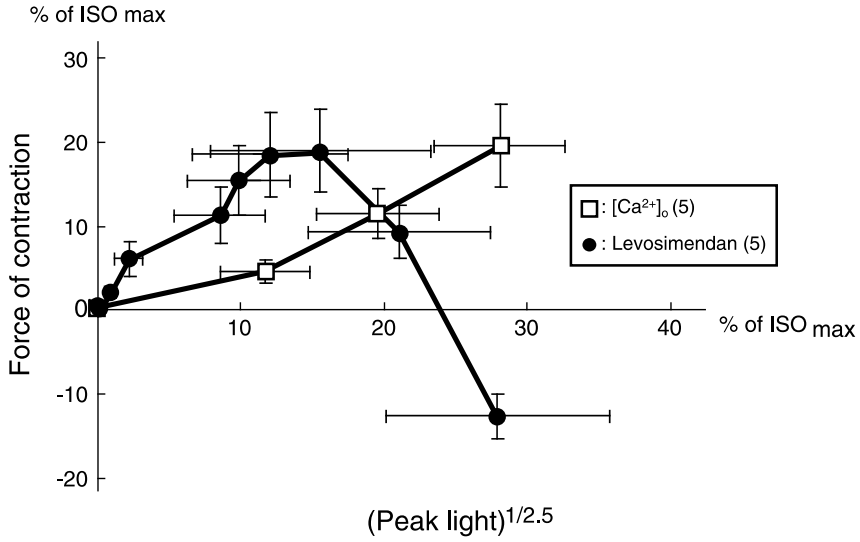

Figure 6 The relationship between the peaks of Ca^{2+} transients and the force of contraction during applications of different inotropic interventions, including elevation of $[Ca^{2+}]_o$ and levosimendan in acidotic conditions in isolated canine right ventricular trabeculae. Numbers in parentheses indicate the numbers of experiments. Ordinate: the changes in the force of contraction induced by these interventions expressed as a percentage of ISO_{max} in each preparation; abscissa: the increase in Ca^{2+} transients. Symbols with vertical and horizontal bars represent means ± s.e.m.

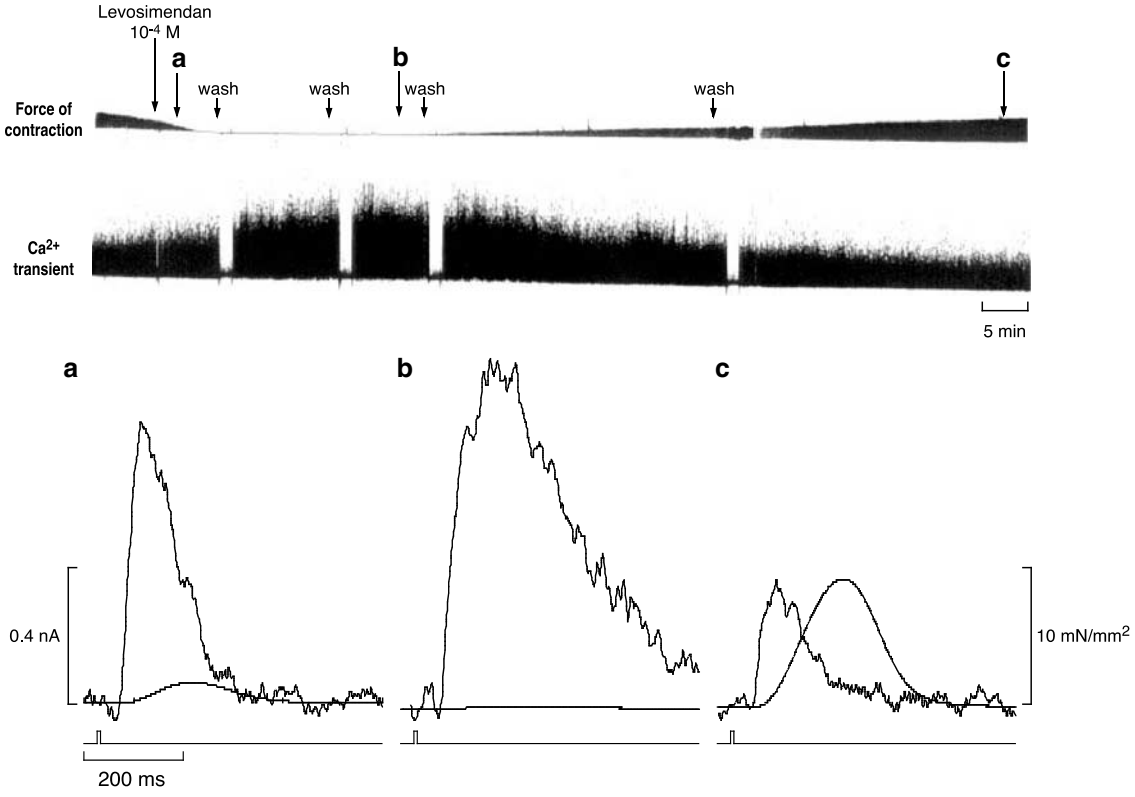

Figure 7 Influence of 10^{-4} M levosimendan on isometric contractions and aequorin light transients in the presence of 3×10^{-7} M (±)-bupranolol, and alterations of these parameters during washout with drug-free solution in a canine right ventricular trabecula electrically driven at 0.5 Hz at 37°C in acidotic conditions. Representative tracings (a, b, c in the lower panel) of aequorin light transients (noisy recordings) and isometric contractions were recorded at the times corresponding to a, b, c in the upper panel (force of contraction and Ca^{2+} transients). Signal-averaged recordings of one hundred successive signals are presented.

Ca^{2+} sensitivity may have less clinical relevance because of the high concentrations at which this action is produced, this finding provides evidence for the mechanism of the bell-shaped feature, and suggests the low risks of untoward effects from levosimendan, namely an elevation of diastolic tension, which is associated with some Ca^{2+} sensitizers, such as EMD 57033

and Org 30029 (Lee & Allen, 1991; Hajjar *et al*., 1997). While the mechanism of the decrease in Ca^{2+} sensitivity is unknown, the findings *in vitro*, including the activation of protein phosphatase (Zimmermann *et al*., 1996; Boknik *et al*., 1997) and inhibition of the cAMP-dependent protein kinase (Earl *et al*., 1986) induced by high concentrations of the compound, may contribute to the NIE of levosimendan.

The PIE of levosimendan was attenuated approximately by 50% in acidotic conditions due to a reduction of the increase in Ca^{2+} transients, whereas the increase in Ca^{2+} sensitivity was less susceptible to acidosis. This is in strong contrast to the influence of acidosis on the PIE of another class of the Ca^{2+} sensitizer pimobendan and its active metabolite UD-CG 212 Cl: acidosis inhibited the increase in Ca^{2+} sensitivity, but did not suppress Ca^{2+} transients induced by these agents (Takahashi *et al*., 2001; Endoh, 2002). Influence of acidosis on the levosimendan-induced PIE was similar to that of its active metabolite OR 1896 in that attenuation of the PIE was due to a decrease in Ca^{2+} transients (Takahashi & Endoh, 2002). These observations imply that acidosis affects the PIE of Ca^{2+} sensitizers differentially but probably *via* the common process depending on the family of individual Ca^{2+} sensitizers. The limitation of the present experimental procedure, which is suitable for clarifying the mechanism of action in relation to Ca^{2+} signaling in intact myocardium, is that it is unable to provide the molecular mechanism of action. Nevertheless, the resistance of Ca^{2+} sensitization to acidosis provides some insight in the action mechanism: the effect of agents such as Org 30029 and EMD 57033 that act primarily *via* the downstream mechanism is resistant to acidosis (Lee *et al*., 1993; Watanabe *et al*., 1996), while the Ca^{2+} binding to troponin C and the effect *via* the central mechanism is most susceptible to acidosis (Westfall *et al*., 1997, 2000; Takahashi *et al*., 2001). In accordance with these observations it has been suggested that levosimendan may act *via* the downstream mechanism (Haikala & Linden, 1995; Endoh, 2002).

In conclusion, levosimendan up to 10^{-5} M elicited a PIE by a combination of increases in Ca^{2+} transients and Ca^{2+} sensitivity, but at higher concentrations ($>10^{-5}$ M) it induced an NIE by a decrease in Ca^{2+} sensitivity. Acidosis suppressed the PIE of levosimendan due to an attenuation of the increase in Ca^{2+} transients, whereas levosimendan increased Ca^{2+} sensitivity even in acidotic conditions.

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