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The effects of levosimendan and OR-1896 on isolated hearts, myocyte-sized preparations and phosphodiesterase enzymes of the guinea pig

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Abstract

The concentration dependences of the Ca^{2+}-sensitizing and the phosphodiesterase-inhibitory effects of levosimendan (the (−) enantiomer of {[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]hydrazono}propanedinitrile) and its active metabolite, OR-1896 (the (−) enantiomer of *N*-[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl] acetamide), were compared with their positive inotropic effects to reveal their mechanisms of action in guinea pig hearts. In Langendorff-perfused hearts, left ventricular $+dP/dt_{max}$ increased by 26 ± 4% and 25 ± 3% (mean ± S.E.M.), with EC_{50} values of 15 ± 2 and 25 ± 1 nM for levosimendan and OR-1896, respectively. In permeabilized myocyte-sized preparations, levosimendan and OR-1896 both increased isometric force production via Ca^{2+} sensitization (at pCa 6.2), by 51 ± 7% and 52 ± 6%, with EC_{50} values of 8 ± 1 and 36 ± 7 nM ($P<0.05$), respectively. Thus, the two molecules could be defined as Ca^{2+} sensitizers and positive inotropes with very similar concentration dependences. However, major differences appeared when the phosphodiesterase-inhibitory effects of levosimendan and OR-1896 were probed on the two phosphodiesterase isoforms (phosphodiesterases III and IV) dominant in the left ventricular cardiac tissue. Levosimendan was a 40-fold more potent and a 3-fold more selective phosphodiesterase III inhibitor (IC_{50} for phosphodiesterase III = 2.5 nM, and IC_{50} for phosphodiesterase IV = 25 μM, selectivity factor ≈ 10000) than OR-1896 (IC_{50} for phosphodiesterase III = 94 nM, and IC_{50} for phosphodiesterase IV = 286 μM, selectivity factor ≈ 3000). Hence, our data support the hypothesis that levosimendan and OR-1896 both exert positive inotropy via a Ca^{2+}-sensitizing mechanism and not via simultaneous inhibition of the phosphodiesterases III and IV isozymes in the myocardium at their maximal free plasma concentrations.

Keywords: Ca^{2+}-sensitization; Phosphodiesterase inhibition; Phosphodiesterase subtype; Positive inotropy; Levosimendan; OR-1896

1. Introduction

Levosimendan (the (−) enantiomer of {[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]hydrazono}-propanedinitrile) is a positive inotrope drug that proved to be effective during the decompensation of chronic heart failure and acute myocardial infarction (Follath et al., 2002; Moiseyev et al., 2002). OR-1896 (the (−) enantiomer of *N*-[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl] acetamide) is the biologically active metabolite of levosimendan with a relatively long half-life (75–78 h). It is presumable, therefore, that levosimendan and OR-1896 are both involved in the cardiovascular effects that develop following levosimendan administration (Kivikko et al., 2002). Besides increasing the strength of cardiac contractions, levosimendan induces coronary and peripheral vasodilatation through the opening of ATP-dependent K^+ channels (Kaheinen et al., 2001; Pataricza et al., 2000; Yokoshiki et al., 1997). For the positive inotropic action of levosimendan, a Ca^{2+}-sensitizing mechanism has been postulated through an interaction between levosimendan and the Ca^{2+}-saturated form of the cardiac troponin C

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molecule (Pollesello et al., 1994; Sorsa et al., 2001, 2003). Similarly to levosimendan, OR-1896 has been reported to be a Ca^{2+} sensitizer and a positive inotropic drug both in vitro and in vivo in cardiac preparations of the dog (Takahashi et al., 2000). Additionally, both molecules display structural similarities with a family of inhibitors of phosphodiesterases. This leads to an alternative hypothesis for the cardiotonic effects of levosimendan and OR-1896 that signifies a possible phosphodiesterase-inhibitory action (Ajiro et al., 2002; Boknik et al., 1997; Takahashi et al., 2000; Zimmermann et al., 1998). Phosphodiesterase inhibition would increase the intracellular cAMP level and thus the amplitude of the intracellular Ca^{2+} transient, similarly to the effects of β-adrenoreceptor agonists (Hasenfuss et al., 1994). However, a clear dose-effect relation between levosimendan-induced positive inotropy and the intracellular cAMP concentration ([cAMP]) elevation could not be established (Boknik et al., 1997; Edes et al., 1995). Moreover, several reports have stated that levosimendan either did not increase the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) at all (Brixius et al., 2002; Hasenfuss et al., 1998; Lancaster and Cook, 1997), or not to levels high enough to explain its positive inotropic effect (Hasenfuss et al., 1998; Sato et al., 1998).

To elucidate the positive inotropic mechanism of levosimendan, we set out to perform a sequence of tests in which we characterized the concentration dependences of the putative subcellular effects of levosimendan and its metabolite OR-1896. The Ca^{2+}-sensitizing potentials of levosimendan and OR-1896 were determined in permeabilized myocyte-sized preparations at a steady $[Ca^{2+}]$ in order to avoid interference with the phosphodiesterase isoenzymes and ionic channels. Since simultaneous inhibition of two phosphodiesterase isoenzymes (i.e. phosphodiesterases III and IV) has been shown to be a prerequisite of a biologically effective elevation in intracellular [cAMP] in the myocardium (Shahid and Nicholson, 1990; Verde et al., 1999), the IC_{50} values for the inhibitory activities of levosimendan and OR-1896 on the isolated phosphodiesterases III and IV isoforms were also determined. Additionally, we assessed the potencies and efficacies of the two molecules on the left ventricular performance in Langendorff-perfused isolated guinea pig hearts at constant heart rate and coronary flow. We found well-pronounced similarities in the concentration dependences of the Ca^{2+}-sensitizing and positive inotropic actions of levosimendan and OR-1896. However, these agents failed to evoke parallel inhibition of phosphodiesterases III and IV in the same concentration range. In particular, the inhibition of phosphodiesterase IV required significantly higher concentrations of levosimendan and OR-1896 than their maximal free (i.e. not protein bound) plasma concentrations (6 or 12 nM, respectively) following the therapeutic administration of levosimendan (0.2 μg/kg/min for 24 hours; Kivikko et al., 2002). This finding may well explain why the myoplasmic [cAMP] might remain unaltered during the development of levosimendan-induced positive inotropy.

2. Materials and methods

2.1. Chemicals

Levosimendan [CAS registry number 141505-33-1] and OR-1896 [CAS registry number 139052-02-1] (Fig. 1) were synthesized at Orion Pharma, Espoo, Finland. Concentrated stock solutions (concentration 10 mM) of levosimendan or OR-1896 were prepared with dimethyl sulfoxide (DMSO) as solvent and subsequently stored at 4 °C. Test concentrations of levosimendan or OR-1896 between 1 nM and 10 μM were obtained by dissolving appropriate volumes of stock solution in the different test solutions. All drug-containing test solutions were freshly prepared on each experimental day. The final concentration of DMSO never exceeded 0.1%. All other chemicals were obtained from Merck (Darmstadt, Germany) or Sigma (St. Louis, USA).

2.2. Animals

Adult guinea pigs of either sex (Duntley Hartley, Mollegaard Breeding Center, Denmark), weighing 300–350 g were used. The study was conducted with the permission of the Animal Ethic Committee of Orion Pharma, in accordance with Finnish law and government regulations complying with the European Community guidelines for the use of experimental animals. In addition, all the experiments involving animals were performed according to the rules and institutional guidelines of the University of Debrecen. Before interventions, the guinea pigs were anesthetized with an intraperitoneal injection of a cocktail containing ketamine (54 mg kg^{-1}), acepromazine (1.8 mg kg^{-1}), xylazine (10.9 mg kg^{-1}) and heparin 1500 (U kg^{-1}), thereafter hearts were excised for Langendorff experiments or for the isolated myocyte study.

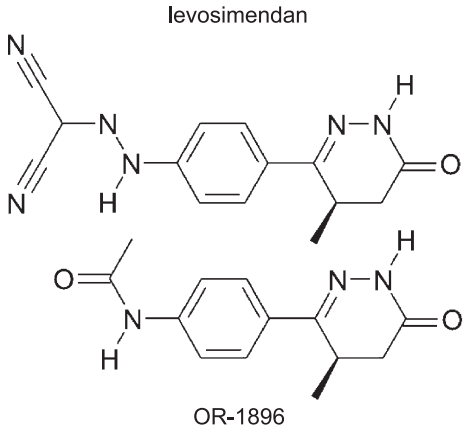

Fig. 1. The chemical structures of levosimendan, which is the (−) enantiomer of {[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]hydrazono}propanedinitrile, and of its active metabolite, OR-1896, which is the (−) enantiomer of *N*-[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl] acetamide.

2.3. Force measurements in permeabilized myocyte-sized preparations

The isolation technique of myocyte-sized preparations has been described elsewhere (Papp et al., 2002). Briefly, left ventricular myocyte-sized preparations of the guinea pig were isolated in relaxing solution (pH 7.0; in mM: Mg^{2+} 1.0, KCl 145.0, EGTA 2.0, ATP 4.0, imidazole 10.0) using a tissue homogenizer at 4 °C. The resultant suspension of small clumps of myocytes, single myocyte-sized preparations and cell fragments was permeabilized with 0.5% Triton X-100 (5 min) at room temperature. Triton X-100 removed all membranous structures and allowed the study of myofibrillar contractile properties under standardized conditions (i.e. as concerns the composition of the intracellular medium and the sarcomere length) without disturbing factors being present in the intact heart (i.e. hormonal factors and variable $[Ca^{2+}]$). To eliminate Triton-X-100, preparations were washed twice and subsequently kept in relaxing solution at 0 °C for 6–24 h. The compositions of relaxing and activating solutions used during force measurements were calculated as described by Fabiato and Fabiato (1979). The pCa, i.e. $-\log[Ca^{2+}]$, of the relaxing and activating solution (pH 7.2) was 10 and 4.75, respectively. Solutions with intermediate free $[Ca^{2+}]$ levels were obtained by mixing activating and relaxing solutions. All the solutions for force measurements contained (in mM): Mg^{2+} 1.0, MgATP 5.0, phosphocreatine, 15.0 and *N*,*N*-bis[2-Hydroxyethyl]-2-aminoethanesulfonic acid (BES) 100.0. The ionic equivalent was adjusted to 150.0 by KCl at an ionic strength of 186.0.

A single myocyte-sized preparation was attached with silicone adhesive (100% silicone, Dow Corning, Aquarium sealant, Midland, USA) to two thin stainless steel needles while viewed by means of an inverted microscope. One needle was connected to a force transducer (SensoNor, Horten, Norway) and the other to an electromagnetic motor (Aurora Scientific, Aurora, Canada), both maneuvered by joystick-controlled micromanipulators. The average sarcomere length of the preparation was determined by means of a spatial Fourier transform as described previously (Fan et al., 1997) in relaxing solution. The diameters of the preparations were measured microscopically, in two perpendicular directions. Cross-sectional area was calculated by assuming an elliptical cross-section. Isometric force measurements were performed at 15 °C and a sarcomere length of 2.2 μm. Force development was followed after the preparation had been transferred from a droplet of relaxing to a droplet of activating solution by moving laterally the stage of the microscope. The active force was taken as the difference between the peak force and the passive force levels. Zero force level and the passive force component were determined via a rapid length change in activating solution and a slow one in relaxing solution, respectively. The sampling rate during the experiments was 20 Hz, while during rapid length changes 1 kHz.

2.4. Langendorff-perfused hearts

The heart was rapidly excised and rinsed at room temperature in oxygenated perfusion buffer. A cannula was inserted into the aorta and retrograde perfusion of the heart began as soon as the heart was placed in the thermostatically controlled moist chamber of the Langendorff apparatus (Hugo Sachs Elektronik, Germany). Modified Tyrode solution (37 °C), equilibrated with carbogen (95% O_2 and 5% CO_2) in the temperature-controlled bulb oxygenator, was used as perfusion buffer. The composition of the Tyrode solution was (in mM): NaCl 135.0, $MgCl_2$ 1.0, KCl 5.0, $CaCl_2$ 2.0, $NaHCO_3$ 15.0, Na_2HPO_4 1.0, glucose 10.0, pH 7.3–7.4. The experiments were carried out in the presence of constant coronary flow (10.6 ml/min) and heart rate (280 beats/min). After a short prestabilization period (10 min), a latex balloon (size 4) was placed into the left ventricle through the left pulmonary vein and the left atrium. The latex balloon was attached to a stainless-steel cannula coupled to a pressure transducer by which the isovolumetric left ventricular pressure was recorded. The pressure signal was amplified by using a two-channel bridge amplifier (Type 301, Hugo Sachs Elektronik). The digitized signals were collected by using the Acquisition v3.1 program (Orion Pharma). At the beginning of the experiment, the volume of the balloon was adjusted so as to obtain a diastolic pressure of approximately 5 mm Hg. Samples recorded for a period of 3 s from the pressure signal were digitized and stored every 20 s. For each measure, the samples recorded over 3 min were averaged. The left ventricular systolic pressure of the heart was obtained from the digitized (frequency 1 kHz) pressure signals. The left ventricular pressure signals were used to calculate the positive peak of the first derivative of left ventricular pressure as a function of time ($+dP/dt_{max}$).

The hearts were allowed to stabilize for 30–50 min in order to reach a steady state in the coronary flow, which was thereafter fixed to its maximal value (in average 10.6 ml/min). After 15-min further stabilization, the hearts were paced at 280 beat/min and allowed to stabilize for additional 15 min. Thereafter, levosimendan or OR-1896 was added to the buffer and the response curves for the concentration range 0.01–1 μM were obtained by increasing their concentrations at 15-min intervals.

2.5. Inhibition of phosphodiesterase isoenzymes

Phosphodiesterases III and IV were isolated from guinea pig hearts and from a human myeloid leukemia promonocytic cell line (U-937), respectively, to achieve high level phosphodiesterase isoenzyme purity. The isolation procedure of phosphodiesterase isoenzymes and the determination of their activities were described earlier (Frodsham and Jones, 1992; Torphy et al., 1992). Briefly, tissue homogenates were applied to DEAE-cellulose columns and subsequently eluted with a linear gradient of sodium acetate

buffers. Collected fractions were subsequently assayed for cAMP phosphodiesterase activity. The reaction mixture containing [^{3}H]cAMP (0.1 μM) and cAMP (0.1 μM) as substrates with or without levosimendan or OR-1896 was incubated at 30 °C for 30 min in the presence of isolated phosphodiesterase III or phosphodiesterase IV isoforms. The [^{3}H]5'AMP product was measured by means of a liquid scintillation detection method. Milrinone (IC_{50}: 0.4 μM) and rolipram (IC_{50}: 0.39 μM) were employed as reference compounds for phosphodiesterases III and IV, respectively, in order to verify the precision of the assays. Phosphodiesterase-inhibitory potential determinations were performed in duplicate and the results are given as the means of the data from the individual test runs.

2.6. Statistical analyses

Statistical significance was calculated by analysis of variance (ANOVA, repeated measures) followed by Dunnet's two-tailed test. Values are given as means ± S.E.M. The number of experiments in each group was five or more unless indicated otherwise. Statistical significance was accepted at $P<0.05$.

3. Results

3.1. Positive inotropic effects of levosimendan and OR-1896 in paced Langendorff-perfused hearts with constant coronary flow

The cardiotonic effects of levosimendan and OR-1896 were tested in Langendorff-perfused guinea pig hearts. The positive peak of the first derivative of the left ventricular pressure signal ($+dP/dt_{max}$) was regarded as a measure of the contractile state of the myocardium. This parameter revealed highly similar concentration dependences for levosimendan and OR-1896. Fig. 2 depicts the concentration responses of $+dP/dt_{max}$ on levosimendan or OR-1896 application, based on experiments in 10 different guinea pig hearts for the two groups (5 in each group). The maximal drug-induced increase in left ventricular $+dP/dt_{max}$ corresponded to relative E_{MAX} values of 26 ± 4% and 25 ± 3% over the baseline ($P<0.05$), with a slightly lower ($P<0.05$) EC_{50} value (15 ± 2 nM) for levosimendan than that for OR-1896 (25 ± 1 nM).

3.2. Ca^{2+}-sensitizing properties of levosimendan and OR-1896 in isolated permeabilized myocyte-sized preparations

The maximal Ca^{2+}-activated force production (P_o) of permeabilized myocyte-sized preparations was determined at pCa 4.75 under drug-free conditions. P_o normalized to the cross-sectional area was 27 ± 6 kN m^{-2} (as calculated from 20 myocyte-sized preparations). This value is comparable to that found elsewhere (Van der Velden et al., 1998).

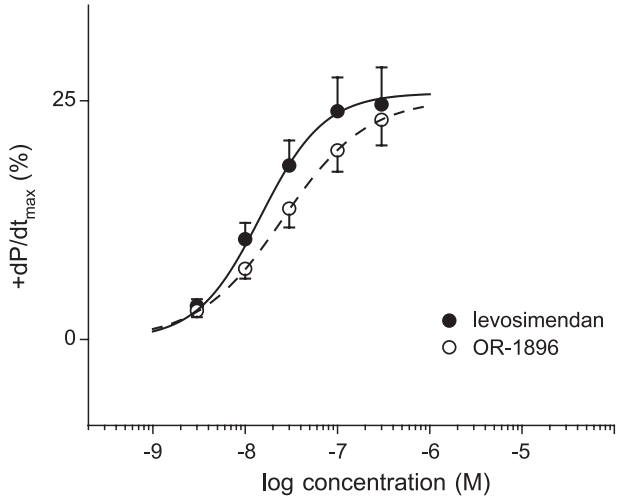

Fig. 2. Positive inotropic effects of levosimendan or OR-1896 in Langendorff-perfused hearts at constant flow and constant heart rate. Values are expressed as percentage increases from the drug-free mean values (100%) (baseline values of $+dP/dt_{max}$ were 1129 ± 85 mm Hg s^{-1} for the levosimendan group and 1239 ± 67 mm Hg s^{-1} for the OR-1896 group). The means were fitted to a dose–response equation for assessment of the maximal increases in the measured parameters and for determination of the drug concentrations at half-maximal effects (EC_{50}) (see text for numerical values). Continuous and dashed lines depict the results of concentration–response fittings for levosimendan and OR-1896, respectively.

On the basis of the previous experience (Edes et al., 1995; Haikala et al., 1995), the concentration dependences of levosimendan- or OR-1896-induced Ca^{2+} sensitization were assessed during repeated activations at a single steady submaximal [Ca^{2+}] (at pCa 6.2 in this case). The drug-free isometric force was 5.2 ± 1 kN m^{-2} (19 ± 4% of P_o) at pCa 6.2. Fig. 3A illustrates two sequences of repeated force recordings in the presence of increasing concentrations of levosimendan (upper panel) or OR-1896 (lower panel). The amplitude of the isometric Ca^{2+} contractures increased with the concentration of both levosimendan and OR-1896. Levosimendan of 1 μM increased the peak isometric force by 72% relative to the drug-free value, while 5 μM OR-1896 induced a 56% increase in the example shown in Fig. 3A.

To obtain a more detailed picture of the Ca^{2+}-sensitizing effects of levosimendan and its metabolite, isometric force production was also measured at maximal (pCa 4.75), and submaximal [Ca^{2+}] (pCa 5.8, pCa 6, pCa 6.2 and pCa 7) ($n=7–10$ myocyte-sized preparations), using a supramaximal concentration (10 μM) of levosimendan or OR-1896 (Fig. 3B). Neither 10 μM levosimendan nor 10 μM OR-1896 affected the maximal Ca^{2+}-activated force (P_o, measured at pCa 4.75) ($P>0.05$). Moreover, these compounds did not increase the force at the lowest applied [Ca^{2+}] either (i.e. at pCa 7). The lack of effect at pCa 7 is in agreement with the previous observations suggesting unaltered cross-bridge dissociation at the diastolic [Ca^{2+}]$_i$ (Haikala et al., 1995). However, levosimendan and OR-1896 both increased the isometric force production at intermediate [Ca^{2+}] (at pCa 5.8, pCa 6 and pCa 6.2) ($P<0.05$). The

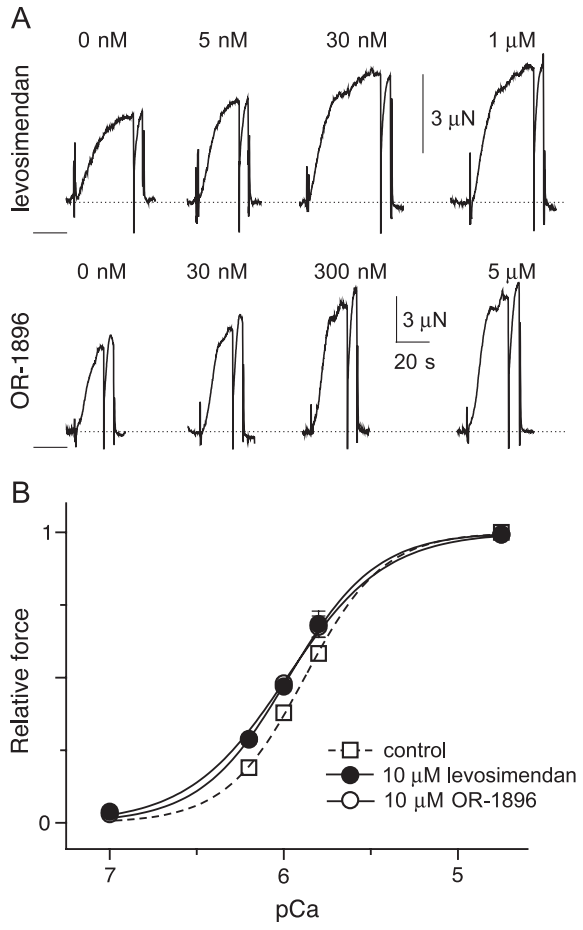

Fig. 3. Determination of the Ca^{2+}-sensitizing effects of levosimendan and of OR-1896 in permeabilized myocyte-sized preparations. (A) Force development during Ca^{2+} contractures of single myocyte-sized preparations at a submaximal [Ca^{2+}] (pCa 6.2) in the absence and presence of increasing concentrations of levosimendan (upper panel) or OR-1896 (lower panel). Drug concentrations are depicted above the force traces. The transfers of the preparation from the relaxing solution (pCa 10) to the activating solution and back were accompanied by brief vertical artifacts in the force recordings. Rapid length changes applied after the attainment of maximal force levels served for the determination of the zero and total force levels at each drug concentration. Horizontal dashes and doted lines indicate zero and passive force levels, respectively. (B) Determination of Ca^{2+}–relative force relationships based on force measurements at various [Ca^{2+}] in the absence (control) and presence of 10 μM levosimendan or 10 μM OR-1896. Force values were normalized to the drug-free P_o. Peak isometric forces at pCa 5.8, 6.0 and 6.2 in the presence of 10 μM levosimendan or 10 μM OR-1896 were significantly higher than those for the respective drug-free controls due to Ca^{2+} sensitization. Dashed and continuous lines illustrate the results of Hill fits to the means of force values measured under identical experimental conditions. Symbols illustrate means ± S.E.M.

Ca^{2+}–force relationships (obtained by fitting the Hill equation to the mean relative force levels (Papp et al., 2002) in the absence or presence of 10 μM levosimendan or 10 μM OR-1896 (Fig. 3B) revealed identical Ca^{2+}-sensitizing characteristics for levosimendan and its metabolite. The [Ca^{2+}] required to induce half-maximal force development (i.e. pCa_{50}) corresponded to pCa 5.88 ± 0.01 under control conditions, while it was increased ($P < 0.05$) identically by 10 μM levosimendan or 10 μM OR-1896 (to pCa 5.98 ± 0.03 or pCa 5.98 ± 0.02, respectively).

3.3. Concentration-dependent Ca^{2+}-sensitizing and phosphodiesterase-inhibitory functions of levosimendan and OR-1896

To assess the concentration dependences of the Ca^{2+}-sensitizing effects of levosimendan and OR-1896, measurements similar to those illustrated in Fig. 3A were repeated in 20 isolated permeabilized myocyte-sized preparations, and the resultant force values were expressed in relative terms (Fig. 4A). On the basis of this approach, the Ca^{2+}-sensitizing potential of levosimendan could be illustrated by an EC_{50} value of 8 ± 1 nM and by a maximal increase in the isometric force production (E_{MAX}) of 51 ± 7%. In comparison, the OR-1896-induced increase in force reached half-maximal effect (EC_{50}) at a concentration of 36 ± 7 nM,

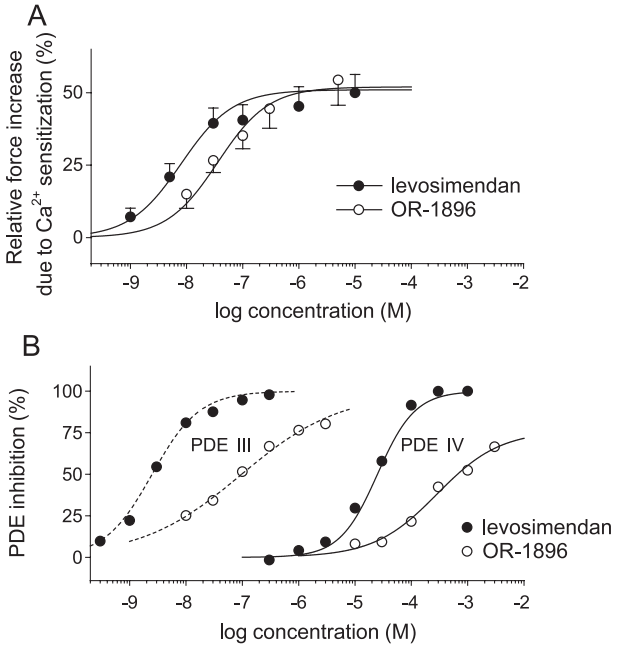

Fig. 4. Concentration dependences of levosimendan- or OR 1896-induced Ca^{2+} sensitization and phosphodiesterase inhibition. (A) Ca^{2+} sensitization was assessed through the relative force increases in response to increasing concentrations of levosimendan or OR-1896 in permeabilized myocyte-sized preparations at pCa 6.2. (B) The inhibitory potentials of levosimendan or OR-1896 on phosphodiesterase III or phosphodiesterase IV were determined from the reductions in the phosphodiesterase activities of purified phosphodiesterase isozymes at various test concentrations of levosimendan or its metabolite. Means in panels A and B were fitted to a concentration–response equation for assessment of the maximal increases in the measured parameters and for determination of the drug concentrations at half-maximal effects (EC_{50} for Ca^{2+}-sensitization and IC_{50} for phosphodiesterase inhibition) (see text for numerical values). Continuous and dashed lines in B depict the results of concentration–response fittings for levosimendan- or OR-1896-induced phosphodiesterase IV or phosphodiesterase III inhibition, respectively.

while the maximal force increase corresponded to a relative value of 52 ± 6%.

The phosphodiesterase-inhibitory potentials of levosimendan and its metabolite were tested on purified phosphodiesterase isoforms. Fig. 4B shows the results of biochemical assays on the phosphodiesterases III and IV activities in the presence of various test concentrations of levosimendan or OR-1896. Increasing concentrations of levosimendan or OR-1896 decreased the phosphodiesterase III activity in a dose-dependent manner. The half-maximal inhibition of phosphodiesterase III was achieved at a concentration (IC_{50}) of 2.5 nM by levosimendan, while in the case of OR-1896, the IC_{50} was 94 nM. Similarly to the results with phosphodiesterase III, increasing concentrations of levosimendan or OR-1896 also progressively decreased the phosphodiesterase IV activity. However, to attain half-maximal inhibition of phosphodiesterase IV, significantly higher drug concentrations were required. The IC_{50} value for levosimendan was 25 μM, while that for OR-1896 was 286 μM.

4. Discussion

This study compares the concentration dependences of the positive inotropic effects of levosimendan and OR-1896 with those of their possible underlying mechanisms. Myocardial contractile state was assessed in Langendorff hearts at constant preload, heart rate and coronary flow. Under these conditions, myocardial contractility could be increased either by an increase in the Ca^{2+}-sensitivity of the contractile system and/or by an increase in the amplitude of the Ca^{2+} transient due to phosphodiesterase inhibition. Using permeabilized myocyte-sized preparations and isolated phosphodiesterase isoforms, the effects on Ca^{2+}-sensitivity and phosphodiesterase inhibition could be dissected. Though the contractile system was somewhat more sensitive to levosimendan than to OR-1896, both molecules proved to be positive inotropes, with almost identical potentials in Langendorff hearts. Hence, the similarity in the inotropic potentials of levosimendan and OR-1896 could be theoretically explained by antagonistic differences in their effects on Ca^{2+}-handling due to phosphodiesterase-inhibition or in tissue affinities/accumulation properties (Kivikko et al., 2002). In view of the higher IC_{50} values of OR-1896 for phosphodiesterases III and IV than those of levosimendan, the former hypothesis is, however, unlikely.

Several earlier experimental efforts furnished evidence in full agreement with the idea that levosimendan-induced positive inotropy does not require phosphodiesterase inhibition and hence the activation of the β-adrenergic intracellular pathway. The protein kinase A inhibitor KT 5720 did not decrease the levosimendan induced positive inotropy (Haikala et al., 1997) in Langendorff-perfused guinea pig hearts. Levosimendan did not increase the L-type Ca^{2+} current in enzymatically dissociated myocytes of rat and rabbit hearts at concentrations lower than 1 μM (Yokoshiki et al., 1997; Virag et al., 1996). We wish, moreover, to highlight that in enzymatically dissociated guinea-pig myocytes levosimendan increased cell shortening without changes in $[Ca^{2+}]_i$ with an EC_{50} value of 9 nM (Lancaster and Cook, 1997). This data matches very well with our value (EC_{50} = 8 nM) on the effect of levosimendan in permeabilized myocyte-sized preparations. Hence, the increase in force of isolated cells with intact membranes was presumably due primarily, if not exclusively, to Ca^{2+}-sensitization.

Of the four different phosphodiesterase isoenzymes (phosphodiesterases I, II, III and IV) of the human myocardium (Reeves et al., 1987), phosphodiesterase III is frequently cited as a mediator of the positive inotropic effect. This assumption is based on experiments with hypothetically phosphodiesterase III-selective compounds (e.g. milrinone) (Katano and Endoh, 1992), a concept of intracellular compartmentation of the G protein-coupled signaling pathways (Steinberg and Brunton, 2001) and/or the apparent K_m value of phosphodiesterase III for cAMP (Muller et al., 1992). However, it has also been demonstrated that phosphodiesterase inhibition decreases the predicted intracellular compartmentation of cAMP (Jurevicius and Fischmeister, 1996) and this may allow effective interactions between the different phosphodiesterase isoforms. Indeed, the experimental evidence suggested that a marked increase in L-type Ca^{2+} current or positive inotropism through cAMP signalling could be achieved only if at least two phosphodiesterase subtypes were inhibited simultaneously in rat or rabbit myocardial cells (Shahid and Nicholson, 1990; Verde et al., 1999). In particular, dual inhibition of low K_m cAMP phosphodiesterases (i.e. phosphodiesterases III and IV) was shown to be a prerequisite for the development of a phosphodiesterase-dependent elevation in the intracellular cAMP concentration (Kelso et al., 1995), and of positive inotropy (Shahid and Nicholson, 1990). When only one phosphodiesterase isoenzyme was inhibited, cAMP was presumably metabolized by other phosphodiesterase isoenzymes, thereby preventing significant increase in cAMP content and consequently the activation of cAMP-dependent protein kinase A. Conversely, phosphodiesterase inhibitors with poor phosphodiesterase-selectivity were more potent elevators of intracellular cAMP levels in cardiomyocytes (Cone et al., 1999; Shakur et al., 2002). The claim that phosphodiesterase inhibition may not contribute to the positive inotropic effects of levosimendan and its metabolite is therefore supported by their phosphodiesterase isoenzyme selectivity being higher than most other phosphodiesterase inhibitors. In our in vitro enzyme inhibition tests, the selectivity factors (phosphodiesterase III over IV) for levosimendan and OR-1896 were 10 000 and 3000, respectively. The corresponding value for milrinone has been found to be only 14 (De Cheffoy de Courcelles et al., 1992). To our best knowledge, the phosphodiesterase selectivity factor of levosimendan reported in this study is the highest among all known phosphodiesterase inhibitors. This makes it difficult to reproduce the selective phosphodiesterase III inhibitory effect of levosimendan with other drugs in cardiac

preparations. Additionally, we found a poor correlation between the concentration dependencies of OR-1896 induced positive inotropy and phosphodiesterase III inhibition. Hence, despite its somewhat smaller phosphodiesterase selectivity factor than that of levosimendan, Ca^{2+}-sensitization rather than phosphodiesterase III inhibition explained the positive inotropic effect of OR-1896.

In human atrial cardiomyocytes, Ajiro et al. (2002) suggested that phosphodiesterase III inhibition is responsible for the increase in L-type Ca^{2+} current at lower than 1 μM of levosimendan concentrations. An increase in L-type Ca^{2+} current via phosphodiesterase inhibition would, however, also increase the amplitude of the intracellular Ca^{2+} transient. In contrast, levosimendan did not increase $[Ca^{2+}]_i$ in ventricular muscle strips from end-stage failing human hearts (Brixius et al., 2002; Hasenfuss et al., 1998). Additionally, levosimendan opposed the worsening of acute heart failure during clinical conditions when cardiac remodeling was more (Slawsky et al., 2000; Nieminen et al., 2000; Follath et al., 2002) or less (Moiseyev et al., 2002) likely. Further, the hemodynamic effects of levosimendan in chronically failing human hearts were inconsistent with those of sympathetic activation (Slawsky et al., 2000) and of the sympathomimetic agent dobutamine (Follath et al., 2002; Nieminen et al., 2000). Hence, the levosimendan-induced increase in cardiac output was better explained by stronger systolic contractions due to a Ca^{2+}-sensitizing mechanism than by alterations in the sympathetic controlled $[Ca^{2+}]_i$ and heart rate (Slawsky et al., 2000). These data are in accord with the idea that the phosphodiesterase IV isoform is also present and functional in both healthy and failing human ventricular myocytes (Reeves et al., 1987). The activity of phosphodiesterase IV, or other phosphodiesterase subtypes, may therefore possibly prevent intracellular cAMP accumulation following levosimendan application in the human myocardium.

The results of our model investigation in cardiac preparations of healthy guinea pigs may explain in part the controversy regarding the mechanism of levosimendan-induced inotropy. We suggest that, despite the phosphodiesterase III-inhibitory potential of levosimendan and OR-1896, these agents may leave the intracellular cAMP-dependent signalling unaltered because they do not inhibit phosphodiesterase IV at their therapeutic plasma concentrations. However, the expression levels and intracellular localization may possibly influence the interactions between the different phosphodiesterase subtypes. Accordingly, alterations in these parameters may give rise to a certain degree of variability in the consequences of selective phosphodiesterase inhibition during health and disease.

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