ELSEVIER

European Journal of Pharmacology 435 (2002) 27–33

ejp

www.elsevier.com/locate/ejphar

Levosimendan increases L-type Ca^{2+} current via phosphodiesterase-3 inhibition in human cardiac myocytes

Youichi Ajiro [a], Nobuhisa Hagiwara [a,*], Yasuhiro Katsube [a], Nicholas Sperelakis [b], Hiroshi Kasanuki [a]

[a] *The Heart Institute of Japan, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku, Tokyo 162-8666, Japan*
[b] *Department of Molecular and Cellular Physiology, College of Medicine, University of Cincinnati, Cincinnati, OH, USA*

Received 8 October 2001; received in revised form 26 November 2001; accepted 30 November 2001

Abstract

To evaluate the potency of levosimendan, a newly developed cardiotonic agent, as a phosphodiesterase-3 inhibitor, we examined its effects on the L-type Ca^{2+} current ($I_{Ca,L}$) in single human atrial cells using the whole-cell voltage-clamp method. Levosimendan significantly increased $I_{Ca,L}$ in a concentration-dependent manner (E_{max}, 139.0 ± 1.8%; EC_{50}, 54 ± 3.6 nM). The increase in $I_{Ca,L}$ induced by 1 μM levosimendan was significantly greater in human atrial cells (136.7 ± 11.0%, $n=8$) than in rabbit atrial cells (23.5 ± 3.5%, $n=6$) (depolarization to +10 mV in each case). In rat atrial and ventricular cells, $I_{Ca,L}$ was unaffected by 1–10 μM levosimendan. These results indicate that the selective phosphodiesterase-3 inhibitor levosimendan increases cardiac-cell $I_{Ca,L}$ significantly more strongly in human than in rabbit and rat. It seems likely that the positive inotropic effect of levosimendan on the human myocardium depends on an increase in $I_{Ca,L}$ that is modulated by adenosine 3′ 5′ -cyclic monophosphate (cAMP)-dependent phosphorylation.

Keywords: Phosphodiesterase inhibitor; Atrial cell, human; Atrial cell, rabbit; Ca^{2+} current, L-type; Levosimendan

1. Introduction

(*R*)-([4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]-hydrazono)-propanedinitrile (Levosimendan) (for review, see Pagel et al., 1996) was originally identified as a compound that binds selectively to cardiac troponin C during high-performance liquid chromatography on a troponin-C-coupled affinity column (Haikala et al., 1995a). It has been shown that levosimendan has Ca^{2+}-sensitizing activity in preparations isolated from guinea-pig (Edes et al., 1995; Haikala et al., 1995b) and human (Hasenfuss et al., 1995) hearts. It has been proposed that levosimendan binds to the amino-terminal region of troponin C to stabilize the Ca^{2+}-induced change in the conformation of the protein (Pollesello et al., 1994), an action that may be responsible for this drug's positive inotropic action. It has also been reported that levosimendan binds to troponin C in a Ca^{2+}-dependent manner, binding to it at the systolic intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and detaching from it at the diastolic $[Ca^{2+}]_i$ (Haikala et al., 1995a,b). Hence, among Ca^{2+} sensitizers, levosimendan has a unique property: it does not impair cardiac relaxation.

It was shown a decade ago that in vitro, levosimendan inhibits phosphodiesterase-3 activity selectively and more potently than milrinone (Raasmaja et al., 1991). In guinea-pig intact-heart preparations, it has been demonstrated that levosimendan increases the level of 3′ 5′ -cyclic monophosphate (cAMP) as well as the heart rate and the amplitude of the L-type Ca^{2+} current ($I_{Ca,L}$), suggesting the involvement of a cAMP-dependent mechanism of action in ventricular cells in this species (Edes et al., 1995; Bokník et al., 1997). However, $I_{Ca,L}$ was not affected by levosimendan at concentrations between 0.2 and 10 μM in rat ventricular cells (Yokoshiki et al., 1997). Hence, it remains unclear whether levosimendan acts mainly as a Ca^{2+} sensitizer or as a phosphodiesterase-3 inhibitor in exerting a positive inotropic influence on the myocardium (Todaka et al., 1996; Bokník et al., 1997; Haikala et al., 1997). Furthermore, little or no detailed electrophysiological information on the effects of levosimendan on $I_{Ca,L}$ has been published, certainly not in single human cardiac cells. Since the activity of the cardiac $I_{Ca,L}$ is strongly regulated by cAMP-dependent phosphorylation, the measurement of $I_{Ca,L}$ in isolated cardiac myocytes

* Corresponding author. Tel.: +81-3-3353-8111; fax: +81-3-3356-0441.
E-mail address: mhagi@hij.twmu.ac.jp (N. Hagiwara).

0014-2999/02/$ - see front matter
PII: S0014-2999(01)01569-2

can be used as a probe for studying the efficacy of phosphodiesterase inhibitors.

In the present study, to enable us to evaluate the potency of levosimendan as a phosphodiesterase-3 inhibitor, we examined its effects on $I_{Ca,L}$ in single human atrial cells using the whole-cell voltage-clamp method.

2. Material and methods

2.1. Preparation of single cells

The cell-isolation procedure for human atrial myocytes was derived from a technique described previously (Escande et al., 1987; Sakai et al., 1995; Kajimoto et al., 1997; Seki et al., 1999). Small segments of myocardium sampled from right atrial appendages were obtained at the time of open-heart surgery, in accordance with the institutional guidelines for research on human subjects (Sakai et al., 1995). Each patient had a normal right atrial pressure and a normal sinus rhythm and was between 2 and 75 years of age. The clinical diagnosis was congenital heart disease in 21 patients and ischaemic heart disease in one patient. None of the patients were on medications such as Ca^{2+} channel blockers, β-adrenoceptor antagonist or phosphodiesterase inhibitors. In brief, each segment of human myocardium was cut into pieces and incubated in warm Tyrode's solution for 10 min. The strips were then cut into even smaller pieces and left in nominally Ca^{2+}-free Tyrode's solution for 17 min. They were then transferred to a nominally Ca^{2+}-free Tyrode's solution containing 0.8 mg/ml collagenase (Yakult, Tokyo, Japan) with 1 mg/ml protease (Type 27, Sigma, St. Louis, MO, USA) for 70–80 min at 37.0 °C. The collagenase was then washed out by rinsing with high K^+, low Cl^- solution (Isenberg and Klöckner, 1982) and the digested tissue was stored in the same solution. For the rabbit atrium, the method of cell isolation by the use of enzymes was as described elsewhere (Hagiwara et al., 1992a,b). All procedures complied with institutional guidelines governing animal experimentation.

2.2. Electrical recordings

The whole-cell voltage-clamp method used here was the same as one described previously (Hamill et al., 1981; Hagiwara et al., 1992a,b). Patch pipettes were pulled from 1.25 mm borosilicate capillaries (D941-8.5-85 Clinitubes: Radiometer, Copenhagen, Denmark). The resistance of each electrode, when filled with the pipette solution, was in the range 2–3 MΩ. The amplifier (TM-1000; ACT ME Laboratory, Tokyo, Japan) employed a 100-MΩ feedback resistor and series resistance was partially compensated. The current–voltage (I–V) signals were stored on a video recorder (S-6000; Victor, Tokyo, Japan), with a PCM converter system (RP-880; NF Electronic Instruments, Tokyo, Japan) being used for computer analysis (PC 9801 RA; NEC, Tokyo, Japan). The current signals were fed from the video recorder to the computer via a 2.5-kHz, eight-pole Bessel-type low-pass filter. The liquid junction potential (−7.5 mV) between the pipette and the bathing solutions was corrected. For recording $I_{Ca,L}$, the cells were usually depolarized every 10 s from a holding potential of −40 mV to a test potential of +10 mV for 300 ms. This test pulse was based on the voltage corresponding to the peak current in the I–V relationships obtained in both the control situation and after application of levosimendan or 3-isobutyl-1-methylxanthine (IBMX). No leakage correction was applied. To avoid the effects of current run-down, the measurement of $I_{Ca,L}$ during the cumulative application of drugs was performed within a 10–20 min experimental period. Under our experimental conditions, $I_{Ca,L}$ was stable over experimental periods of this length. $I_{Ca,L}$ was measured as the peak inward current. From a holding potential of −40 mV, ramp pulses of amplitude ±90 mV (0.72 V/s) were applied to enable measurement of cell-membrane capacitance (Hagiwara et al., 1992a). The value so obtained was 72.9 ± 25.3 pF (n = 11). Experiments were performed at 37 ± 0.5 °C.

2.3. Solutions

The normal Tyrode's solution contained (in mM): 136.9 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.33 NaH_2PO_4, 5 glucose and 5 HEPES (pH = 7.4 with NaOH). The standard pipette solution contained (in mM): 120 CsOH, 20 CsCl, 100 aspartic acid, 10 EGTA, 2 $MgCl_2$, 5 Mg-ATP, 5 K_2-creatine phosphate, 0.2 Na-GTP and 5 HEPES (pH = 7.4 with CsOH). The standard external solution contained (in mM): 150 NaCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$ and 5 HEPES (pH = 7.4 with NaOH; Kajimoto et al., 1997).

2.4. Drugs

Levosimendan was kindly provided by Orion-Farmos (Espoo, Finland) and dissolved in dimethyl sulphoxide (DMSO) as a 100 mM stock solution. IBMX (Sigma) was dissolved in DMSO and prepared as a 100 mM stock solution. In this study, the concentration of DMSO did not exceed 0.1%; this concentration was found to have no effect on $I_{Ca,L}$.

2.5. Statistical analysis

All statistical data are given as mean ± S.E.M. Paired and unpaired Student's t-tests were used to evaluate the statistical significance of differences between means. Values of $P < 0.05$ were considered to indicate statistical significance.

3. Results

3.1. Effect of levosimendan on $I_{Ca,L}$ in human atrial cells

To evaluate the inhibitory effect of levosimendan on phosphodiesterase-3, we examined its effect on the $I_{Ca,L}$ in

human atrial myocytes. Fig. 1 illustrates these effects at various membrane potentials (from a holding potential of −40 mV). Superfusing the particular cell illustrated in Fig. 1A with 1 μM levosimendan increased $I_{Ca,L}$ from 1030 pA

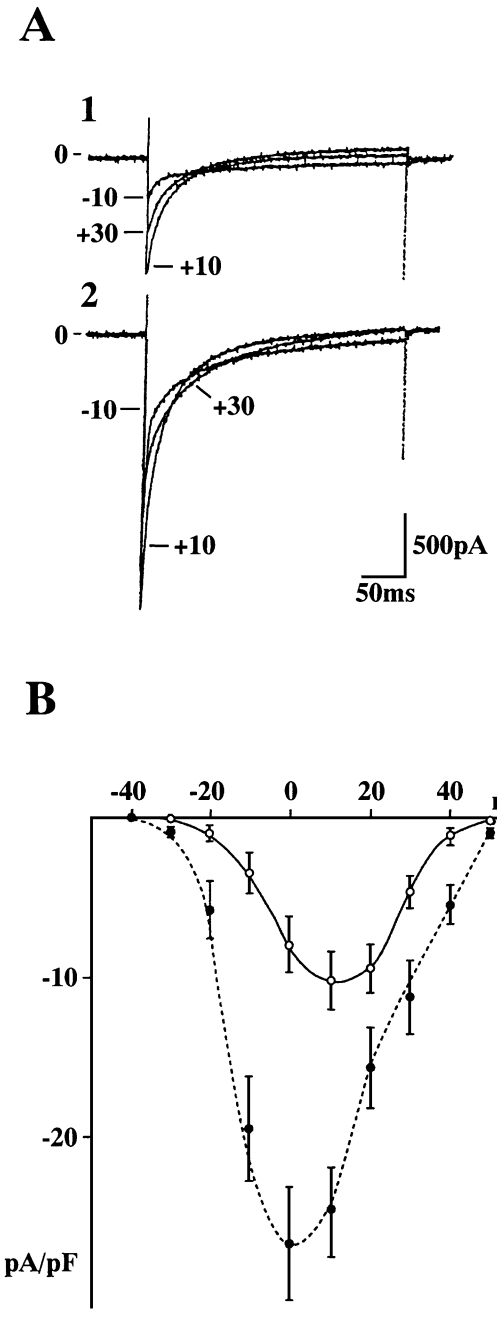

Fig. 1. Current–voltage relationships for $I_{Ca,L}$ before and after application of 1 μM levosimendan in human atrial cells. (A) $I_{Ca,L}$ elicited by depolarizing pulses from a holding potential of −40 mV to −10, +10 and +30 mV for 300 ms in control (A-1) and in the presence of 1 μM levosimendan (A-2). This dose of levosimendan increased $I_{Ca,L}$ from 1030 pA (A-1) to 2213 pA (A-2) on depolarization to +10 mV in this particular cell. (B) $I–V$ relationships for $I_{Ca,L}$ before (open circles) and after application of 1 μM levosimendan (closed circles) in eight human atrial cells. $I_{Ca,L}$ was increased at every membrane potential examined and the $I–V$ relationship was shifted leftwards by this concentration of levosimendan.

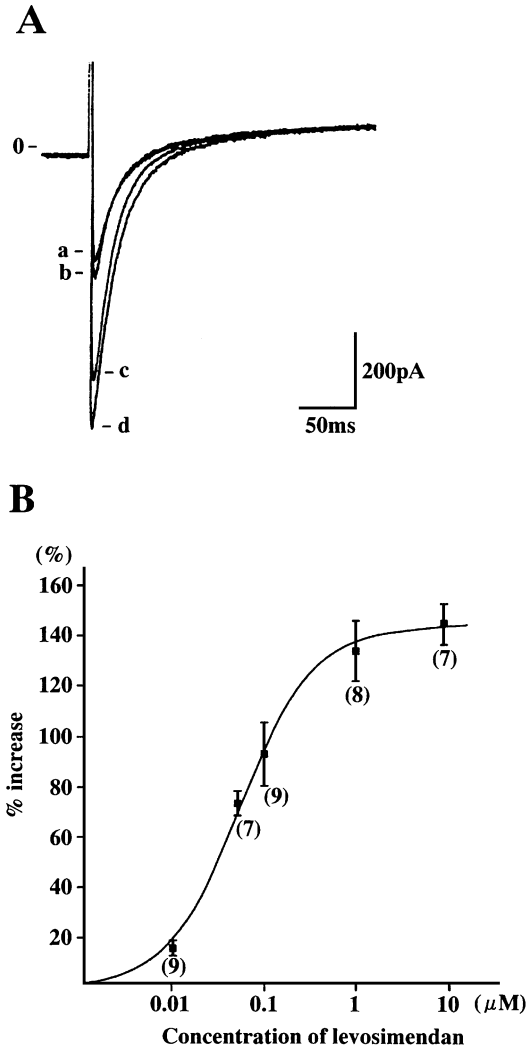

Fig. 2. Dose–response relationship for the effect of levosimendan on $I_{Ca,L}$ in human atrial cells. (A) $I_{Ca,L}$ was increased from 305 pA (a) to 349 pA (b), 651 pA (c) and 785 pA (d) by 10 nM, 100 nM and 1 μM levosimendan, respectively, in this particular cell (in each case, on depolarization to +10 mV from a holding potential of −40 mV). (B) Dose–response relationship derived by a nonlinear least-squares fit of the values for mean percentage increase in $I_{Ca,L}$ to the Hill equation: $\%\text{increase} = E_{max}/[1+(EC_{50}/D)^{n_H}]$, where E_{max} is the maximal effect, D is the concentration of levosimendan, EC_{50} is the concentration of levosimendan that causes a half-maximal current and n_H is the Hill coefficient. Numbers in parenthesis indicate the number of experiments. The values of E_{max} and EC_{50} obtained from this curve were 139.0 ± 1.8% and 54 ± 3.6 nM, respectively. Calculated value of n_H was 1.1 ± 0.1. Data-points show the mean values and vertical bars the S.E.M.

(A-1) to 2213 pA (A-2) at +10 mV within 2 min. The current–voltage ($I–V$) relationships (Fig. 1B) showed that at 1 μM levosimendan, $I_{Ca,L}$ was increased at every membrane potential and that the $I–V$ relationship was shifted leftwards. In eight human atrial cells, 1 μM levosimendan increased $I_{Ca,L}$ by 136.7 ± 11.0% at +10 mV (from 10.4 to 24.7 pA/pF).

The dose–response relationship for the increase in $I_{Ca,L}$ produced by levosimendan is illustrated in Fig. 2. In this experiment, 0.01, 0.1, 1 and 10 μM levosimendan increased

$I_{Ca,L}$ by 14.2 ± 3.0% (n = 9), 90.4 ± 12.0% (n = 9), 136.7 ± 11.0% (n = 8) and 140.0 ± 8.2% (n = 7), respectively, in human atrial cells. The curve (Fig. 2B) was derived by making a nonlinear least-squares fit of the values for mean percentage increase in $I_{Ca,L}$ to the Hill equation. The values obtained by this method for E_{max} and EC_{50} were 139.0 ± 1.8% and 54 ± 3.6 nM, respectively. The increases in $I_{Ca,L}$ induced by levosimendan did not depend on the patient's disease or age.

3.2. Effect of a non-specific phosphodiesterase inhibitor, IBMX, on $I_{Ca,L}$ in human atrial cells

IBMX, a potent phosphodiesterase inhibitor found in the mammalian myocardium, inhibits all four phosphodiesterase isoenzymes (Bethke et al., 1992; Cheffoy et al., 1992). We therefore investigated the effect of IBMX on $I_{Ca,L}$ in human atrial cells to compare it with the effect of levosimendan. Fig. 3A shows the effects of various concentrations of IBMX on $I_{Ca,L}$ at +10 mV (from a holding potential of −40 mV) in a human atrial cell. In this cell, $I_{Ca,L}$ was increased from 337 pA (a) to 399 pA (b), 543 pA (c), 877 pA (d) and 1403 pA (e) at concentrations of 0.1, 1.0, 10 and 100 μM IBMX, respectively.

The dose–response relationship for the increase in $I_{Ca,L}$ produced by IBMX is illustrated in Fig. 3B. In eight human atrial cells, 0.1, 1, 10 and 100 μM IBMX increased $I_{Ca,L}$ by

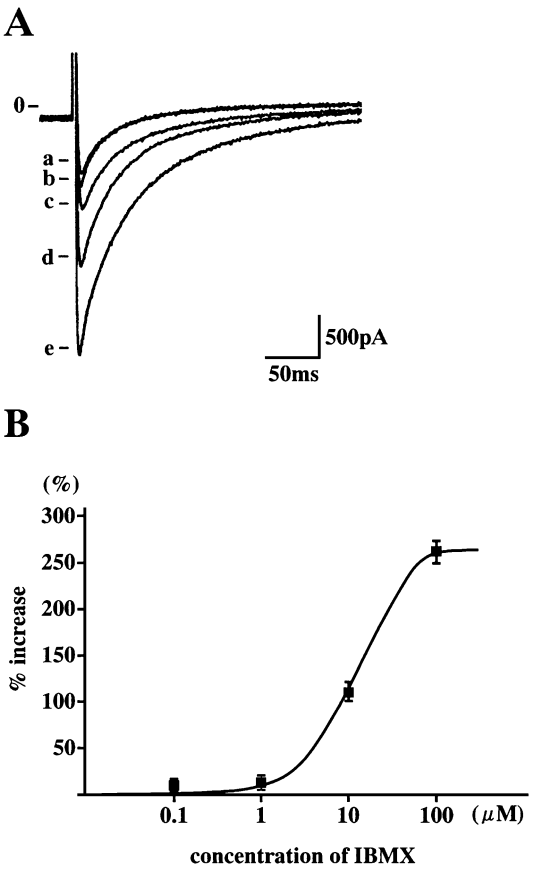

Fig. 3. Dose–response relationship for the effect of IBMX on $I_{Ca,L}$ in human atrial cells. (A) $I_{Ca,L}$ was increased from 337 pA (a) to 877 pA (d) by 10 μM IBMX and to 1403 pA (e) by 100 μM IBMX in this particular cell (in each case, on depolarization to +10 mV from a holding potential of −40 mV). (B) Dose–response relationship derived by a nonlinear least-squares fit of the values for mean percentage increase in $I_{Ca,L}$ to the Hill equation. Ordinate represents percentage increase in $I_{Ca,L}$ over and above the size of the control response. The values of E_{max}, EC_{50} and n_H for IBMX obtained from this curve were 263.7 ± 9.2%, 15.5 ± 1.9 μM and 1.3 ± 0.2, respectively. Datapoints show the mean values and vertical bars the S.E.M. (n = 8).

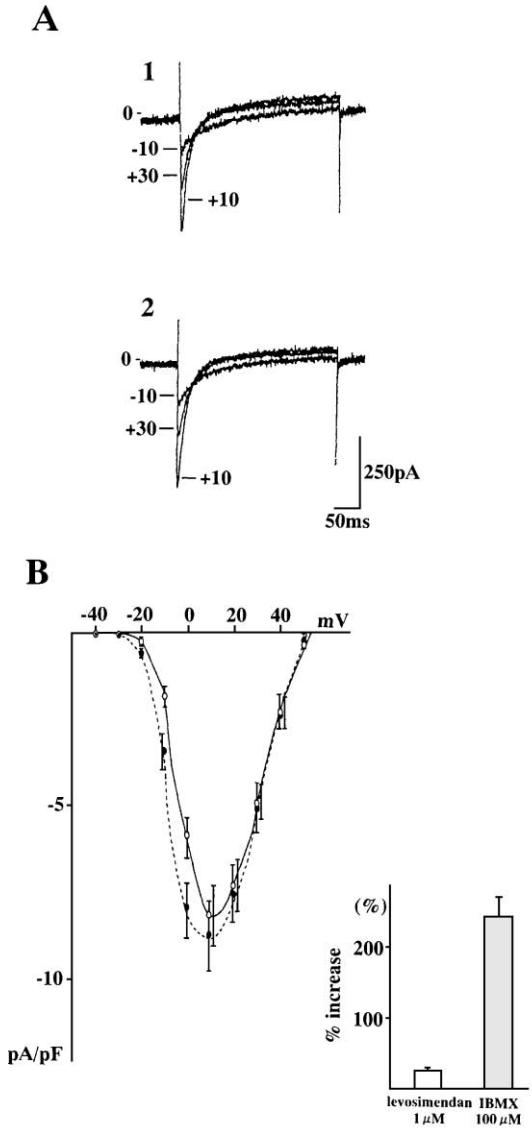

Fig. 4. Current–voltage relationships showing effect of levosimendan on $I_{Ca,L}$ in rabbit atrial cells. (A) $I_{Ca,L}$ elicited by 300 ms depolarizing pulses from a holding potential of −40 mV to −10, +10 and +30 mV in control (A-1) and in the presence of 1 μM levosimendan (A-2). This dose of levosimendan increased $I_{Ca,L}$ from 359 pA (A-1) to 430 pA (A-2) on depolarization to +10 mV. (B) I–V relationships for $I_{Ca,L}$ before (open circles) and after application of 1 μM levosimendan (closed circles) in six rabbit atrial cells. The I–V relationships showed that $I_{Ca,L}$ was increased by 1 μM levosimendan at every membrane potential examined. Inset bar graph shows the percentage increases in $I_{Ca,L}$ induced by 1 μM levosimendan (n = 6) and 100 μM IBMX (n = 10) in rabbit atrial cells.

7.2 ± 2.1%, 21.1 ± 4.8%, 103.3 ± 10.7% and 266.7 ± 20.5%, respectively. The curve was derived by making a nonlinear least-squares fit of the values for mean percentage increase in $I_{Ca,L}$ to the Hill equation. The values obtained by this method for E_{max} and EC_{50} were 263.7 ± 9.2% and 15.5 ± 1.9 μM, respectively, in human atrial cells. These results indicated that the EC_{50} value obtained for the enhancement of $I_{Ca,L}$ by levosimendan was significantly lower than the equivalent for IBMX.

3.3. Effects of levosimendan on $I_{Ca,L}$ in rabbit atrial cells

To compare the inhibitory effects of levosimendan on phosphodiesterase-3 between different species, we also examined its effect on $I_{Ca,L}$ in rabbit atrial myocytes.

Fig. 4 illustrates the effects of levosimendan on $I_{Ca,L}$ at various membrane potentials (from a holding potential of −40 mV) in rabbit atrial cells. Superfusing the particular cell illustrated in Fig. 4A with 1 μM levosimendan increased $I_{Ca,L}$ from 359 pA (Fig. 4A-1) to 430 pA (Fig. 4A-2) at +10 mV within 2 min. In six rabbit atrial cells, 1 μM levosimendan increased $I_{Ca,L}$ by 23.5 ± 3.5% at +10 mV. We next examined the effect of IBMX on $I_{Ca,L}$ in rabbit atrial cells. At 100 μM, IBMX increased $I_{Ca,L}$ by 241.9 ± 28.7% (n = 10, Fig. 4, inset). This enhancement was not significantly different from that seen with the same concentration of 100 μM IBMX in human atrial cells (266.7 ± 20.5%). These results indicated that while the effect of levosimendan in increasing $I_{Ca,L}$ was significantly greater in human than in rabbit atrial cells, the effect of IBMX was not significantly different between these two species.

In a similar experiment on rat ventricular cells (n = 6) and atrial cells (n = 4), we found that 1–10 μM levosimendan had no effect on $I_{Ca,L}$ (results not illustrated), suggesting that the effect of this phosphodiesterase-3 inhibitor on $I_{Ca,L}$ is species-dependent.

4. Discussion

Levosimendan was originally identified as a compound that binds selectively to cardiac troponin C (Haikala et al., 1995a) and soon it was found to show Ca^{2+}-sensitizing activity in preparations isolated from guinea-pig (Edes et al., 1995; Haikala et al., 1995b) and human (Hasenfuss et al., 1995) hearts. Although levosimendan targets troponin C, this may not be its only mechanism of action. Several previous reports suggested the involvement of a cAMP-dependent mechanism in the action of levosimendan. For example, this drug has a positive chronotropic effect on the rabbit right atrium (Sato et al., 1998) and, in isolated rabbit papillary muscles, the positive inotropic effect and the increase in the amplitude of the aequorin-light transients induced by levosimendan were both markedly reduced by 1 μM carbachol (Sato et al., 1998). Similarly, in the guinea-pig levosimendan exerted a positive inotropic effect that was (i) greatly diminished by 10 μM carbachol in the right papillary muscle (Bokník et al., 1997) and (ii) diminished by cAMP-dependent protein kinase inhibitor, (9*R*,10*S*,12*S*)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-fg:3I,2I,1I-kl]pyrrolo[3,4-l][l,6]benzodiazocine-10-carboxylic acid, hexyl ester (KT5720), in the isolated heart (Haikala et al., 1997). It has also been demonstrated that levosimendan increases the cAMP content of guinea-pig ventricular cardiomyocytes (Edes et al., 1995; Bokník et al., 1997; Zimmermann et al., 1998). On this basis, it seemed likely that the positive inotropic effect of levosimendan depends not only on its Ca^{2+}-sensitizing activity but also on an increase in $I_{Ca,L}$ that is modulated by cAMP-dependent phosphorylation. However, little information was available concerning the electrophysiological effects of $I_{Ca,L}$ in cardiac myocytes, especially in human preparations. We therefore decided to examine its effects on $I_{Ca,L}$ in human atrial cells using the whole-cell patch-clamp method.

Levosimendan (1 μM) increased $I_{Ca,L}$ at every membrane potential and that the I–V relationship was shifted in the negative direction in human atrial cells. This finding is in good agreement with results obtained previously for human atrial cells (Ouadid et al., 1991) and for rat ventricular cells (Katsube et al., 1996) that 1 μM isoproterenol shifted the steady-state activation curve of $I_{Ca,L}$ to the negative direction. These results suggest that the increase in $I_{Ca,L}$ due to cAMP-dependent protein kinase is accompanied by a negative shift in the voltage dependence of activation, as has already been reported by Sculptoreanu et al. (1993). In human atrial cells, 1 μM levosimendan increased $I_{Ca,L}$ by 136.7 ± 11.0% at +10 mV (n = 8) with an EC_{50} value of 54 ± 3.6 nM (Fig. 2). This EC_{50} value was close to the IC_{50} value for the effect of levosimendan on cardiac phosphodiesterase-3 purified from guinea-pig hearts (Raasmaja et al., 1992). On the other hand, the same concentration of levosimendan increased $I_{Ca,L}$ by only 23.5 ± 3.5% (n = 6, Fig. 4) at +10 mV in rabbit atrial cells. Similarly, a weak or absent effect has been reported for ventricular cells in the rabbit: viz. 1 μM levosimendan did not affect $I_{Ca,L}$ (1.7 ± 3.1%) at room temperature (Virág et al., 1996). Further, we found that levosimendan did not affect $I_{Ca,L}$ in rat cardiac cells, as also reported by Yokoshiki et al. (1997). In guinea-pig ventricular cells, the effect of levosimendan on $I_{Ca,L}$ seems to be more complicated. In one previous study, 1 μM [[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]hydrazono]propanedinitrile (OR-1259), which is an analogue of levosimendan, decreased $I_{Ca,L}$ by around 20% in isolated guinea-pig cardiomyocytes, probably due to direct binding to Ca^{2+} channels (Raasmaja et al., 1991). However, other reports have described levosimendan-induced increases in $I_{Ca,L}$ of 302 ± 86% at 10 μM (Bokník et al., 1997) or 42.5 ± 12.5% at 5 μM (Virág et al., 1996), again in isolated-guinea pig cardiomyocytes.

Taken together, these results indicate that the selective phosphodiesterase-3 inhibitor, levosimendan, has different effects on $I_{Ca,L}$ in different species. For this reason, we

suggest that the distribution of the phosphodiesterase-3 isozyme may differ between humans and other species. In fact, results consistent with this idea were obtained in the present experiments. We found that levosimendan had little or no effect on $I_{Ca,L}$ in both rabbit and rat myocardium, whereas the non-selective phosphodiesterase inhibitor, 100 μM IBMX, increased $I_{Ca,L}$ to a similar extent in rabbit (241.9 ± 28.7%, Fig. 4) and human (266.7 ± 20.5%, Fig. 3) atrial cells. In rat ventricular cells, selective phosphodiesterase-3 or phosphodiesterase-4 inhibition had no effect on $I_{Ca,L}$, while the combination of a phosphodiesterase-3 inhibitor with a phosphodiesterase-4 inhibitor produced a significant increase in $I_{Ca,L}$ (Verde et al., 1999). These results suggest that in both rabbit and rat myocardium, phosphodiesterase isozymes other than phosphodiesterase-3 may make an important contribution to the regulation of the intracellular cAMP concentration, as already indicated in previous reports (Kajimoto et al., 1997; Shahid and Nicholson, 1990).

In the present experiments on human preparations, levosimendan had an EC_{50} value of 54 ± 3.6 nM for its enhancement of $I_{Ca,L}$, whereas IBMX had an EC_{50} value of 15.5 ± 1.9 μM. In our previous report, another selective phosphodiesterase-3 inhibitor, pimobendan, and its active metabolite, (2-(4-hydroxyphenyl)-5-(5-methyl-3-oxo-4,5-dihydro-2*H*-6-pyridazinyl)benzimidazole·HCl (UD-CG 212 Cl), had EC_{50} values of 1.13 and 1.78 μM, respectively, for their enhancements of $I_{Ca,L}$ (Kajimoto at al., 1997). To judge from these data, levosimendan may be one of the most potent selective phosphodiesterase-3 inhibitors known.

In a previous report on the pharmacokinetics of levosimendan in healthy volunteers, the therapeutic range for the inotropic action of levosimendan in humans was between 50 and 150 ng/ml (around 0.18–0.53 μM; Pagel et al., 1996). We used atrial specimens in the present experiments; however, the effects of phosphodiesterase-3 inhibitor on $I_{Ca,L}$ are almost identical in human ventricular and atrial tissues (Li et al., 1994). This being so, our data suggest that the positive inotropic effect of levosimendan in humans is due at least in part to an increase in $I_{Ca,L}$ achieved via phosphodiesterase-3 inhibition.

Acknowledgements

We thank Drs. Mitsuru Aoki, Toshiharu Shinoka and Kazuhiro Seo (Department of Cardiac Surgery, The Heart Institute of Japan) for providing human atrial specimens. This work was supported by a research grant from the Ministry of Education, Science and Culture of Japan.

References

Bethke, T., Meyer, W., Schmitz, W., Scholz, H., Stein, B., Thomas, K., Wenzlaff, H., 1992. Phosphodiesterase inhibition in ventricular cardiomyocytes from guinea-pig hearts. Br. J. Pharmacol. 107, 127–133.

Bokník, P., Neumann, J., Kaspareit, G., Schmitz, W., Scholz, H., Vahlensieck, U., Zimmermann, N., 1997. Mechanisms of the contractile effects of levosimendan in the mammalian heart. J. Pharmacol. Exp. Ther. 280, 277–283.

Cheffoy, D.D., Loore, D.K., Freyne, E., Janssen, P.A.J., 1992. Inhibition of human cardiac cyclic AMP-phosphodiesterase by R80122, a new selective cyclic AMP-phosphodiesterase 3 inhibitor: a comparison with other cardiotonic compounds. J. Pharmacol. Exp. Ther. 263, 6–14.

Edes, I., Kiss, E., Kitada, Y., Powers, F.M., Papp, J.G., Kranias, E.G., Solaro, R.J., 1995. Effects of levosimendan, a cardiotonic agent targeted to troponin C, on cardiac function and on phosphorylation and Ca^{2+} sensitivity of cardiac myofibrils and sarcoplasmic reticulum in guinea pig heart. Circ. Res. 77, 107–113.

Escande, D., Coulombe, A., Faivre, J.F., Deroubaix, E., Coraboeuf, E., 1987. Two types of transient outward currents in adult human atrial cells. Am. J. Physiol. 252, H142–H148.

Hagiwara, N., Irisawa, H., Kasanuki, H., Hosoda, S., 1992a. Background current in the sino-atrial node cells of the rabbit heart. J. Physiol. 448, 53–72.

Hagiwara, N., Masuda, H., Shoda, M., Irisawa, H., 1992b. Stretch-activated anion currents of rabbit cardiac myocytes. J. Physiol. 456, 285–302.

Haikala, H., Kaivola, J., Nissinen, E., Wall, P., Levijoki, J., Lindén, I.B., 1995a. Cardiac troponin C as a target protein for a novel calcium sensitizing drug, levosimendan. J. Mol. Cell. Cardiol. 27, 1859–1866.

Haikala, H., Nissinen, E., Etemadzadeh, E., Levijoki, J., Lindén, I.B., 1995b. Troponin C-mediated calcium sensitization induced by levosimendan does not impair relaxation. J. Cardiovasc. Pharmacol. 25, 794–801.

Haikala, H., Kaheinen, P., Levijoki, J., Lindén, I.B., 1997. The role of cAMP- and cGMP-dependent protein kinases in the cardiac actions of the new calcium sensitizer, levosimendan. Cardiovasc. Res. 34, 536–546.

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J., 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Arch. 391, 85–100.

Hasenfuss, G., Pieske, B., Kretschmann, B., Holubarsch, C., Alpert, N.R., Just, H., 1995. Effects of calcium sensitizers on intracellular calcium handling and myocardial energetics. J. Cardiovasc. Pharmacol. 26 (Suppl. 1), S45–S51.

Isenberg, G., Klöckner, U., 1982. Calcium tolerant ventricular myocytes prepared by preincubation in a 'KB-medium'. Pflügers Arch. 395, 6–18.

Kajimoto, K., Hagiwara, N., Kasanuki, H., Hosoda, S., 1997. Contribution of phosphodiesterase isozymes to the regulation of the L-type calcium current in human cardiac myocytes. Br. J. Pharmacol. 121, 1549–1556.

Katsube, Y., Yokoshiki, H., Nguyen, L., Sperelakis, N., 1996. Differences in isoproterenol stimulation of Ca^{2+} current of rat ventricular myocytes in neonatal compared to adult. Eur. J. Pharmacol. 317, 391–400.

Li, Q., Himmel, H.M., Ravens, U., 1994. Effects of the new phosphodiesterase-3 inhibitor R80122 on contractility and calcium current in human cardiac tissue. J. Cardiovasc. Pharmacol. 24, 133–143.

Ouadid, H., Séguin, J., Richard, S., Chaptal, P.A., Nargeot, J., 1991. Properties and modulation of Ca channels in adult human atrial cells. J. Mol. Cell. Cardiol. 23, 41–54.

Pagel, P.S., Haikala, H., Pentikäinen, P.J., Toivonen, M.L., Nieminen, M.S., Lehtonen, L., Papp, J.G., Warltier, D.C., 1996. Pharmacology of levosimendan: a new myofilament calcium sensitizer. Cardiovasc. Drug Rev. 14, 286–316.

Pollesello, P., Ovaska, M., Kaivola, J., Tilgmann, C., Lundström, K., Kalkkinen, N., Ulmanen, I., Nissinen, E., Taskinen, J., 1994. Binding of a new Ca^{2+} sensitizer, levosimendan, to recombinant human cardiac troponin C. A molecular modeling, fluorescence probe, and proton nuclear magnetic resonance study. J. Biol. Chem. 269, 28584–28590.

Raasmaja, A., Talo, A., Nissinen, E., Lindén, I.B., Pohto, P., 1991. Biochemical mechanisms of the novel cardiotonic agent OR-1259. J. Mol. Cell. Cardiol. 23 (Suppl. V), S129.

Raasmaja, A., Talo, A., Haikala, H., Nissinen, E., Lindén, I.B., Pohto, P., 1992. Biochemical properties of OR-1259—a positive inotropic and vasodilatory compound with an antiarrhythmic effect. In: Frank, G.B., Bianchi, C.P., Keurs, H.E.D.J. (Eds.), Excitation–Contraction in Skeletal, Cardiac, and Smooth Muscle. Plenum, New York, p. 423.

Sakai, R., Hagiwara, N., Kasanuki, H., Hosoda, S., 1995. Chloride conductance in human atrial cells. J. Mol. Cell. Cardiol. 27, 2403–2408.

Sato, S., Talukder, M.A.H., Sugawara, H., Sawada, H., Endoh, M., 1998. Effects of levosimendan on myocardial contractility and Ca^{2+} transients in aequorin-loaded right-ventricular papillary muscles and Indo-1-loaded single ventricular cardiomyocytes of the rabbit. J. Mol. Cell. Cardiol. 30, 1115–1128.

Sculptoreanu, A., Rotman, E., Takahashi, M., Scheuer, T., Catterall, W.A., 1993. Voltage-dependent potentiation of the activity of cardiac L-type calcium channel β1 subunits due to phosphorylation by cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. U.S.A. 90, 10135–10139.

Seki, A., Hagiwara, N., Kasanuki, H., 1999. Effects of propafenone on K currents in human atrial myocytes. Br. J. Pharmacol. 126, 1153–1162.

Shahid, M., Nicholson, C.D., 1990. Comparison of cyclic nucleotide phosphodiesterase isoenzymes in rat and rabbit ventricular myocardium: positive inotropic and phosphodiesterase inhibitory effects of Org 30029, milrinone and rolipram. Naunyn-Schmiedeberg's Arch. Pharmacol. 342, 698–705.

Todaka, K., Wang, J., Yi, G.H., Stennett, R., Knecht, M., Packer, M., Burkhoff, D., 1996. Effects of levosimendan on myocardial contractility and oxygen consumption. J. Pharmacol. Exp. Ther. 279, 120–127.

Verde, I., Vandecasteele, G., Lezoualc'h, F., Fischmeister, R., 1999. Characterization of the cyclic nucleotide phosphodiesterase subtypes involved in the regulation of the L-type Ca^{2+} current in rat ventricular myocytes. Br. J. Pharmacol. 127, 65–74.

Virág, L., Hála, O., Marton, A., Varró, A., Papp, J.G., 1996. Cardiac electrophysiological effects of levosimendan, a new calcium sensitizer. Gen. Pharmacol. 27, 551–556.

Yokoshiki, H., Katsube, Y., Sunagawa, M., Sperelakis, N., 1997. The novel calcium sensitizer levosimendan activates the ATP-sensitive K^+ channel in rat ventricular cells. J. Pharmacol. Exp. Ther. 283, 375–383.

Zimmermann, N., Bokník, P., Gams, E., Herzig, J.W., Neumann, J., Scholz, H., 1998. Calcium sensitization as new principle of inotropic therapy in end-stage heart failure? Eur. J. Cardiothoracic Surg. 14S, 70–75.