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Stereoselective binding of levosimendan to cardiac troponin C causes Ca^{2+}-sensitization

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Abstract

The effects of the Ca^{2+} sensitizer levosimendan and that of its stereoisomer dextrosimendan on the cardiac contractile apparatus were studied using skinned fibers obtained from guinea pig hearts. Levosimendan was found to be more effective than dextrosimendan in this model. The respective concentrations of levosimendan and dextrosimendan at EC_{50} were 0.3 and 3 μM. In order to explain the difference in efficacy as Ca^{2+} sensitizers, the binding of the two stereoisomers on cardiac troponin C was studied by nuclear magnetic resonance in the absence and presence of two peptides of cardiac troponin I. The two stereoisomers interacted with both domains of cardiac troponin C in the absence of cardiac troponin I. In the presence of cardiac troponin I-(32–79) and cardiac troponin I-(128–180), the binding of both levosimendan and dextrosimendan to the C-terminal domain of cardiac troponin C was blocked and only the binding to the N-terminal domain was observable. Differences in the overall binding behavior of the two isomers to cardiac troponin C were highlighted in order to discuss their structure to activity relation. Our data are consistent with the notion that the action of levosimendan as a Ca^{2+} sensitizer and positive inotrope relates to its stereoselective binding to Ca^{2+}-saturated cardiac troponin C.

Keywords: Cardiac troponin C; Levosimendan; NMR (nuclear magnetic resonance); Drug interaction

1. Introduction

Heart failure is one of the most important cardiovascular syndromes affecting populations of the developed world. Its pathology is characterized by the profound loss of myocardial contractility, which progressively leads to an inability of the heart to pump sufficient blood. The function of the heart can be improved by increasing the cardiac muscle contraction (positive inotropy). It is well known that this can be achieved by increasing intracellular Ca^{2+} in the myocytes. However, exceeding intracellular Ca^{2+} loads can cause arrhythmias resulting from the spontaneous release of Ca^{2+} from an overfilled sarcoplasmic reticulum.

An alternative therapeutic approach was suggested by (Herzig et al., 1980; Rüegg 1987; Solaro and Rüegg 1982). These authors proposed the use of compounds that induce Ca^{2+} sensitization of the contractile apparatus, thus increasing myocardial contractility without a concomitant increase in intracellular Ca^{2+} load. Ca^{2+} sensitizers would thus give rise to a positive inotropic effect devoid of the danger of arrhythmias.

One of the most studied components of the contractile apparatus in the cardiac tissue has been the heterotrimeric troponin complex, which acts as a Ca^{2+}-dependent trigger for the contraction process (Farah and Reinach, 1995). The troponin complex consists of troponin C as the Ca^{2+}-binding component, troponin T as the component that binds to tropomyosin, and troponin I as the inhibitory component. Free intracellular Ca^{2+} binds to troponin C causing structural changes, which eventually enable the formation of cross bridges between actin and myosin ATPase and allow contraction to occur. This protein has been considered a possible target for Ca^{2+} sensitizers since the binding of Ca^{2+} to the active site on the N-domain of cardiac troponin C regulates the contraction–relaxation cycle in heart muscle.

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The use of intravenous levosimendan for the treatment of humans with decompensated heart failure has been approved. Levosimendan is a Ca^{2+} sensitizer that exerts a positive inotropy in cardiac muscle supposedly by binding to the Ca^{2+}-saturated form of cardiac troponin C (Haikala et al., 1995a; Levijoki et al., 2000; Pollesello et al., 1994). It is the (−) enantiomer of {[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]hydrazono}propanedinitrile (R stereoisomer according to Fisher). The (+) enantiomer is dextrosimendan. The aim of this study was to investigate the relationship between the binding of the two stereoisomers to their target and their pharmacological effects on the contractile apparatus. For this purpose, we measured the effects levo- and dextrosimendan on skinned fibers isolated from guinea pig cardiac tissue and we compared their binding on cardiac troponin C and on a model of the cardiac troponin C–troponin I complex by nuclear magnetic resonance spectroscopy.

2. Materials and methods

2.1. Chemicals

Levosimendan and dextrosimendan, the (−) and (+) enantiomers of {[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]hydrazono}propanedinitrile [CAS registry number 141505-33-1], were synthesized at Orion Pharma, Espoo, Finland.

Stock solutions (60 mM) were prepared for the binding experiments by dissolving a weighed amount of the drug in dimethyl sulfoxide. The stock solutions were light-protected and stored at room temperature.

2.2. Animals

Adult guinea pigs of either sex (Duntley Hartley, Mollegaard Breeding Center, Denmark), which weighed between 300 and 350 g, were used. All experiments involving animals were performed according to the rules issued by the regional administration of Southern Finland. Guinea pigs were killed by a blow to the skull, and their hearts were excised for use in the skinned-fiber experiments.

2.3. Skinned fibers

The right ventricular papillary muscles were dissected from the excised heart and rinsed in an ice-cold Tyrode solution. The papillary muscles were immersed in a solution of (in mM): K^+-acetate, 75; EGTA-Na_2, 10; $MgSO_4$, 5.4; ATP-Na_2, 4; dithiolthreitol, 2; 3-(*N*-morpholino)-propane sulfonic acid (MOPS), 20 (pH 7.0) and sonicated at 10 W for 60 s. While still immersed in the same solution, the fibers (<200 μm in diameter) were dissected from the surface of the papillary muscles. The fibers were kept for a further 30 min in the same solution with saponin (250 μg/ml) added. The fibers were then relaxed in a solution of (in mM): EGTA-Na_2, 10; $MgSO_4$, 5.4; ATP-Na_2, 4; MOPS, 20. The pH of the solution was adjusted to 7.0 and the ionic strength to 0.16 M by the addition of KOH and K^+-acetate.

The calculations of the ionic strength and the free Ca^{2+} were as described elsewhere (Fabiato and Fabiato, 1979). The absolute stability constants used in the calculation were as reported by Fabiato (1981). Each fiber was mounted horizontally with glue (cellulose acetate in acetone) between the steel-rod extension of an isometric force transducer (AME-801 strain gauge, Horten Electronics, Norway) and a glass rod attached to a micromanipulator. Then, the fibers were stretched in the relaxing solution until the threshold resting tension was reached. When the Ca^{2+}-induced tension (*p*Ca 6.5) had reached the steady state (which corresponded to about 50% of the maximum developed tension, as ascertained in a preliminary series of Ca^{2+}-titration experiments), compounds were cumulatively added to the solution at 6-min intervals (final concentrations of 0.3, 1, 3, and 10 μM). All the experiments were carried out with fresh fibers (not stored) at room temperature. The skinned fiber experiments were performed according to Haikala et al. (1995b). Only one Ca^{2+} concentration per fiber was used (0.32 μM), and the maximum tension was obtained at the end of the experiment after the test compound was washed out.

2.4. Statistical analysis

The statistical differences of the data were calculated by analysis of variance between groups (ANOVA, repeated measures) followed by Dunnet's two-tailed test. Values are given as means ± S.E.M. The number of

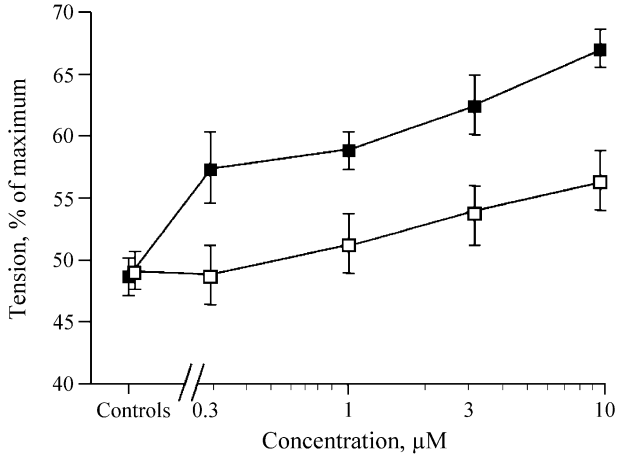

Fig. 1. The Ca^{2+}-sensitizing effects of levosimendan and dextrosimendan in skinned fibers at *p*Ca 6.5. The dose–response curve of the tension (as % of maximum) for levosimendan (■) and dextrosimendan (□) are shown as means ± S.E.M. ($n = 5$).

skinned fiber experiments was 5 for both groups (levosimendan and dextrosimendan treatment) and the tissue for every single experiment was isolated from different hearts.

2.5. Preparation of the protein sample

Uniformly ^{15}N-labelled, full-length chicken recombinant cardiac troponin C with mutation of Cys35 to serine was

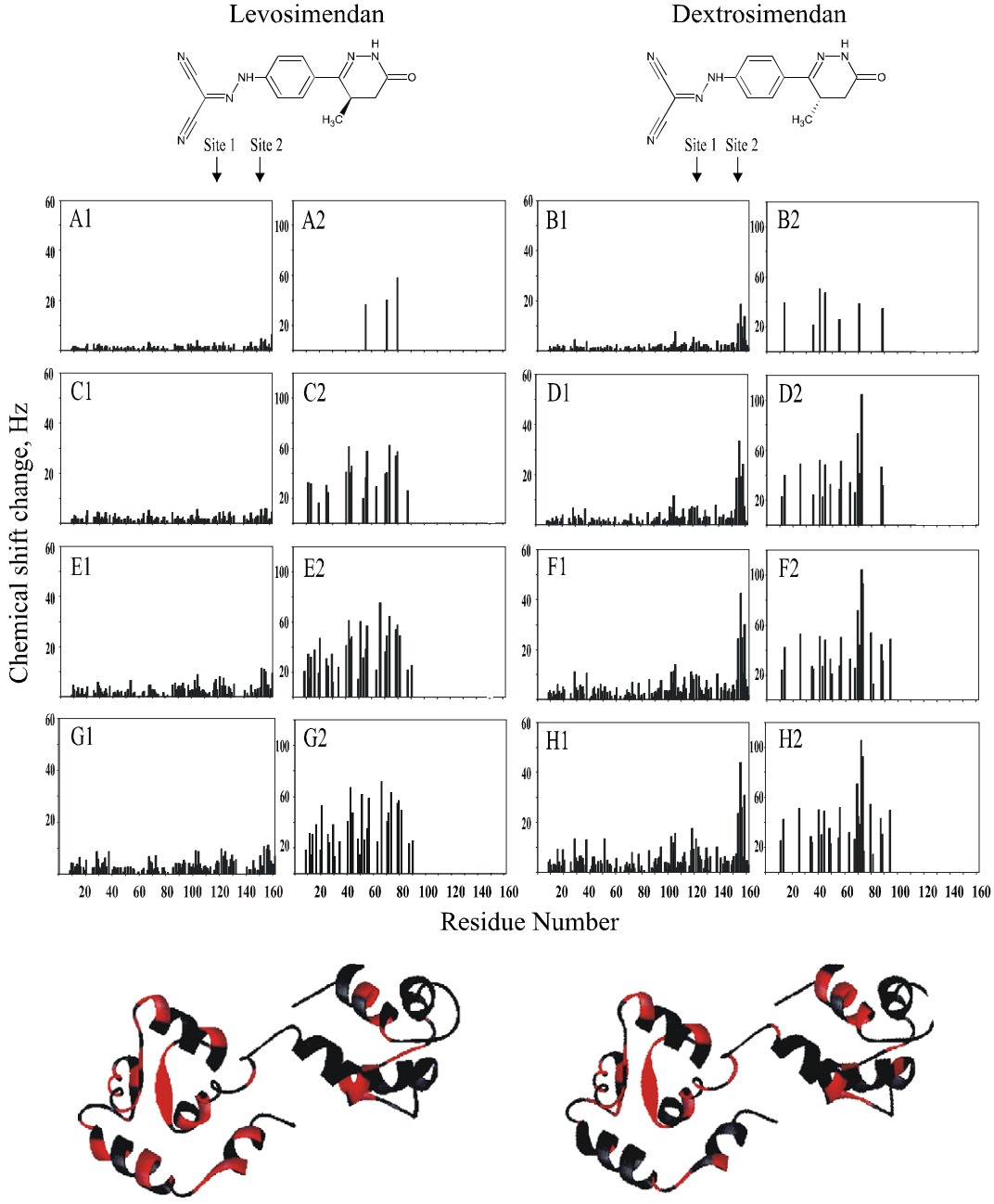

Fig. 2. Simendan binding induced chemical shift changes of full-length Ca^{2+}-saturated cardiac troponin C on $^{1}H-^{15}N$-HSQC spectra at 800 MHz. Chemical structures of levosimendan, the (−) enantiomer, and dextrosimendan, the (+) enantiomer, of simendan are shown at the top of the figure. Below, the titration series of 0.1 mM cardiac troponin C with the respective stereoisomer are shown. Titration of levosimendan or dextrosimendan to Ca^{2+}-saturated cardiac troponin C resulted in small chemical shift changes in fast exchange on the nuclear magnetic resonance time scale (A1 to H1) and larger chemical shift changes in slow exchange. The latter are shown for resonances with a simultaneous decrease in the intensity of the original signal and appearance of a new signal (A2 to H2). Plots show protein to drug ratios 1:0.5 (A and B), 1:1 (C and D), 1:2 (E and F), and 1:3 (G and H) for levosimendan and dextrosimendan, respectively. Each plot shows chemical shift changes in accordance with the cardiac troponin C sequence along the titration. Chemical shift changes are presented as a peak distance (in Hz) from the original peak. Red color on 3D structure of Ca^{2+}-saturated cardiac troponin C (PDB entry 1AJ4) marks residues experiencing the chemical shift changes resulting from the slow exchange and the chemical shift changes larger than the standard deviation of all peaks in fast exchange at the last titration point for both enantiomers.

overexpressed and purified as previously described (Finley et al., 1999; Krudy et al., 1994). Protein solutions of 0.1 to 0.3 mM concentration were prepared. The buffer was 20 mM Bis(2-hydroxyethyl)-imino-tris(hydroxymethyl)methane (Bis-tris) and 5% D_2O, pH 6.8 (not corrected for deuterium effect), containing 10 mM $CaCl_2$. No protease inhibitors, reducing agents, or bacterial inhibitors were added to the samples since we have shown them to be detrimental to the drug molecules (Sorsa et al., 2001). The purity of the protein samples was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), reversed phase chromatography, and matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectroscopy.

2.6. Cardiac troponin I peptides

Human cardiac troponin I-(32–79) and (128–180) peptides (Swiss-Prot ID P19429) were synthesized with an automated solid-phase synthesizer using 9-fluorenylmethoxycarbonyl (Fmoc) amino acids (Fields and Noble, 1990). Peptide purity was confirmed by reversed phase chromatography and MALDI-TOF mass spectrometry. Furthermore, cardiac troponin I-(32–79) was mutated, Cys79Ser, to prevent unwanted interaction between cysteine residues in the protein. Stock solutions (10 mM) of both peptides of cardiac troponin I were prepared by dissolving the lyophilized peptides in pure water (Milli-Q H_2O). The cardiac troponin C/troponin I-(32–79)/troponin I-(128–180) complex was formed as described in Sorsa et al. (2003).

2.7. Nuclear magnetic resonance spectroscopy

The spectra were acquired on a Varian INOVA 800-MHz spectrometer. The nuclear magnetic resonance experiments were all carried out at a temperature of 40 °C. Drug-induced chemical shift changes were measured within two-dimensional $^{1}H-^{15}N$ heteronuclear single-quantum correlation spectra ($^{1}H-^{15}N$-HSQC), as $\Delta\nu_{obs} = \sqrt{\Delta\nu_{1H}^2 + \Delta\nu_{15N}^2}$ in Hz. The number of increments was 128 and the number of transients was either 16 or 32 depending on protein concentration. Spectral width in direct dimension (H) was 11 000 Hz and in indirect dimension (N) 2200 Hz. The nuclear magnetic resonance data was processed using NMRPipe (Delaglio et al., 1995) and NMRView (Johnson and Blevins, 1994) and analyzed using SPARKY (made by Goddard and Kneller, University of California, San Francisco, U.S.A.).

3. Results

3.1. Effects on skinned fibers

The effects of levosimendan and dextrosimendan as Ca^{2+} sensitizers in skinned fibers are shown in Fig. 1. At *p*Ca 6.5 (0.32 μM free Ca^{2+}), a concentration of 0.3 μM levosimendan caused a significant increase in Ca^{2+}-induced tension. The effect of levosimendan increased further at higher concentrations (up to 10 μM, which was the highest concentration used). In contrast, no effect at all could be seen at 0.3 μM for dextrosimendan, whereas a slight increase in tension at higher concentrations occurred. Interestingly, the dose–response curves for the two stereoisomers were somewhat parallel at concentrations above 0.3 μM. The effects of both stereoisomers were completely reversed by washout (data not shown).

3.2. NH chemical shift changes in Ca^{2+}-saturated cardiac troponin C

The results of the titration experiments in which either levosimendan or dextrosimendan were added stepwise to a 0.1-mM solution of full-length cardiac troponin C are summarized in Fig. 2. Both stereoisomers of simendan induced changes in the $^{1}H-^{15}N$–HSQC spectra. Perturbations were detected in both domains of cardiac troponin C.

In the N-domain, both stereoisomers induced similar, but not identical effects. First of all, the interaction resulted in an apparent slow exchange process ($k_{ex} < 50\ s^{-1}$), which resulted in the appearance of new resonances that are only marginally broadened and whose intensities increase with increasing stereoisomer concentration. Normally, such behavior is typical for a strong affinity interaction, where the free protein and the protein–drug complex are detected simultaneously in the $^{1}H-^{15}N$–HSQC spectrum. However, the binding affinity of simendan to the N-terminal domain is only moderate. We were not able to determine accurate K_d values for the binding of either stereoisomer to the N-domain of cardiac troponin C, but the titration only resulted in an equilibrium state (of about equal intensities) of the "old" and "new" resonances at excess concentrations of simendan (up to ~ 9 molar excess of the drug was used). The "new"

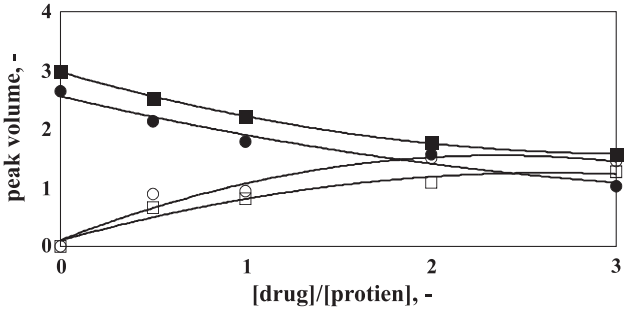

Fig. 3. Titration curves of levo-and dextrosimendan to the N-domain of cardiac troponin C derived from $^{1}H-^{15}N$-HSQC spectra at 800 MHz. The stereoisomers were titrated against 0.1 mM Ca^{2+}-saturated full-length cardiac troponin C solution. The volume of Val44 signal is shown here as a representative of the N-domain binding. The squares (■ for old peaks and □ for new peaks) represent levosimendan data and the spheres represent dextrosimendan (● for old peaks and ○ for new peaks). The number of titration points was limited because of the short lifetime of the drug in the complex.

resonances increase in intensity with increasing simendan concentration and the "old" resonances decrease. As can be seen from Fig. 3, the intensities for the "new" and "old" signals do not level off at the expected values of full intensity and zero. Instead, they level off at an intensity ratio of close to 50:50. However, K_d calculated from the decrease of the volume of the several "old" peaks along the titration gave an estimate of micromolar affinity and slightly larger value for dextrosimendan than levosimendan indicating stronger binding for levosimendan. The slow exchange process displays similar but not identical patterns for the two isomers of simendan (Fig. 4). We observed about 25% fewer "new" peaks for dextrosimendan than for levosimendan. For example, "new" peaks were observed for Leu12, Ile26, and Lys43

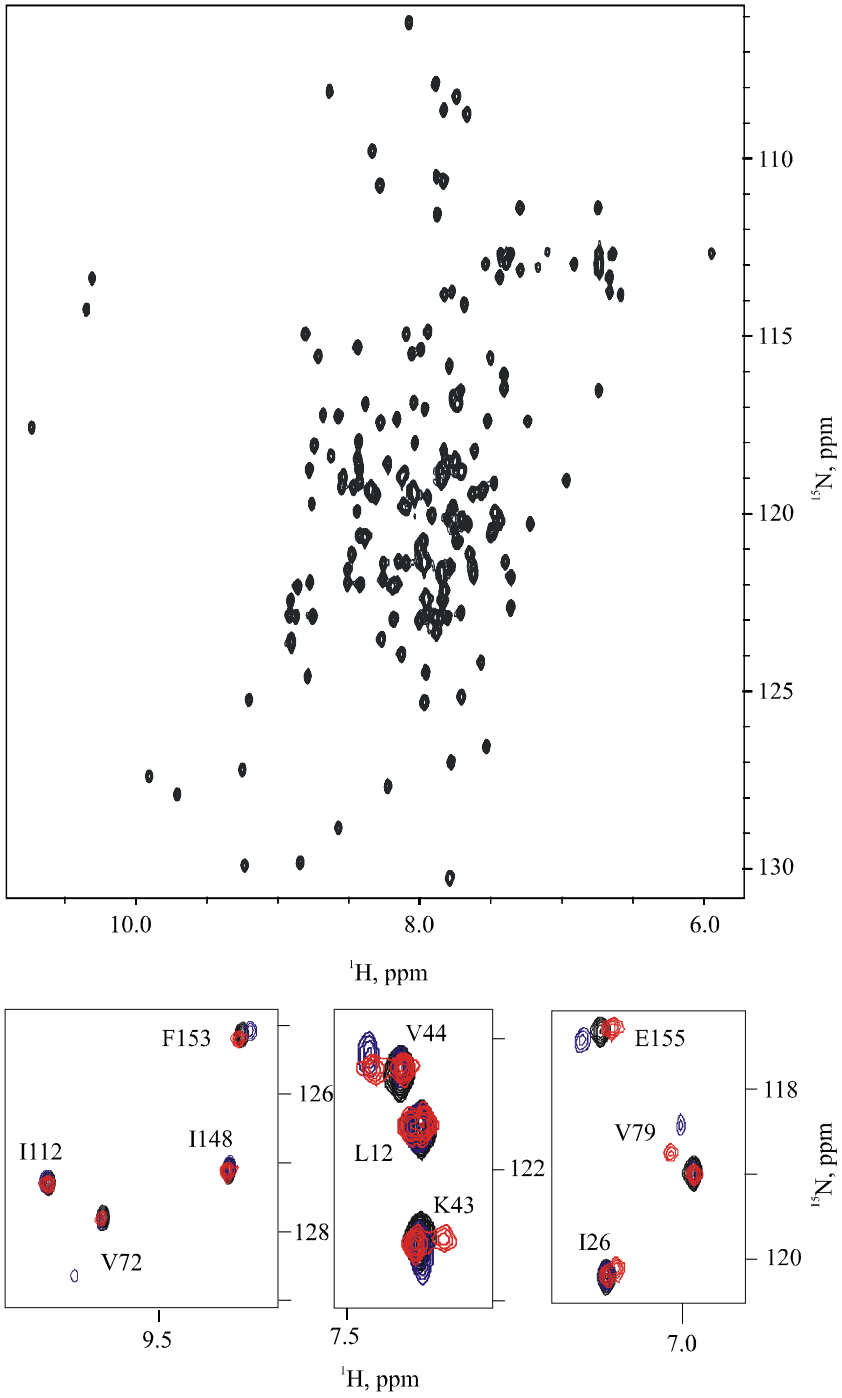

Fig. 4. Binding to Ca^{2+}-saturated cardiac troponin C followed by $^{1}H-^{15}N$-HSQC spectra at 800 MHz. $^{1}H-^{15}N$-HSQC spectra of 0.1 mM cardiac troponin C in the absence of drug (black) and three overlaid extensions in the presence of 0.8 mM levosimendan (red) or 0.8 mM dextrosimendan (blue). The stereoisomers induced chemical shift changes were not identical. A few peaks doubled only in the presence of dextrosimendan (e.g. V72) or levosimendan (e.g. K43 and I26). Moreover, the direction and magnitude of some doubled peaks differed (residues V79, E155).

in the presence of levosimendan but not in the presence of dextrosimendan.

Also, a fast exchange binding (k_{ex}>10^4 s^{-1}) occurred in the N-domain, which resulted in only small chemical shift changes without any noticeable line broadening. However, it is impossible to judge whether these chemical shift changes indicate another binding process or if they are a consequence of the former slow exchange process.

In the C-domain, both stereoisomers of simendan caused chemical shift changes in the fast exchange regime. The patterns are similar for the two isomers though more pronounced for dextrosimendan. In this case (Fig. 2), two patches in the sequence, affected by the binding, could be clearly identified as site 1 (residues 120–130) and site 2 (residues 150–158). According to the three-dimensional structure of troponin C (Sia et al., 1997), they are on opposite sides of the protein structure and therefore can hardly be reconciled as a single binding site. These binding sites are the same sites found for another molecule binding to cardiac troponin C, i.e. EMD-57033 (Wang et al., 2001). In the case of levosimendan, the results are less clear but both sites appear to be occupied by the ligand as well (Sorsa et al., 2001). For the C-domain binding, only the dextrosimendan binding to site 2 could be characterized with a K_d of 7 ± 4 μM, calculated as a mean value of residues of F153, E155, F156, and M157 and assuming two equally strong binding sites for dextrosimendan. The binding of levosimendan to site 2 is too weak to be characterized as are the binding of both stereoisomers to site 1.

One possible explanation for the observation of two types of exchange could be the interdomain communication. This has been proposed for calmodulin (Jaren et al., 2002). However, based on our previous small-angle X-ray scattering (SAXS), results showed that in the presence of levosimendan, the domains of isolated cardiac troponin C were not pulled together (Sorsa et al., 2001). On the contrary, slightly longer distances were determined.

3.3. NH chemical shift changes in Ca^{2+}-saturated cardiac troponin C/troponin I-(32–79)/troponin I-(128–180)

Previously, we have shown that in the presence of the cardiac troponin C binding regions of cardiac troponin I, levosimendan binding to the C-domain of cardiac troponin C is prevented (Sorsa et al., 2003). The interaction of dextrosimendan with the C-domain of cardiac troponin C is also blocked in the presence of cardiac troponin I peptides 32–79 and 128–180. Levosimendan and dextrosimendan induced different chemical shift changes in the nuclear magnetic resonance spectrum of this protein model. In Fig. 5, such differences in fast exchange process are plotted as a function of the sequence. No differences in the effects exerted by levosimendan and dextrosimendan can be seen in the C-domain in the presence of cardiac troponin I. In addition, it also shows several sequential patches where chemical shift differences between the enantiomers interactions with cardiac troponin C in the N-domain occurred. Specifically, backbone amide resonances belonging to residues A7, A8, E19, A22, A31, G34, G42, K43, L48, G49, T53, E55, E66, D67, V72, V79, V82, and K90 exhibit stereospecific chemical shift changes. However, the differences are spread around the domain and no clear binding site could be determined.

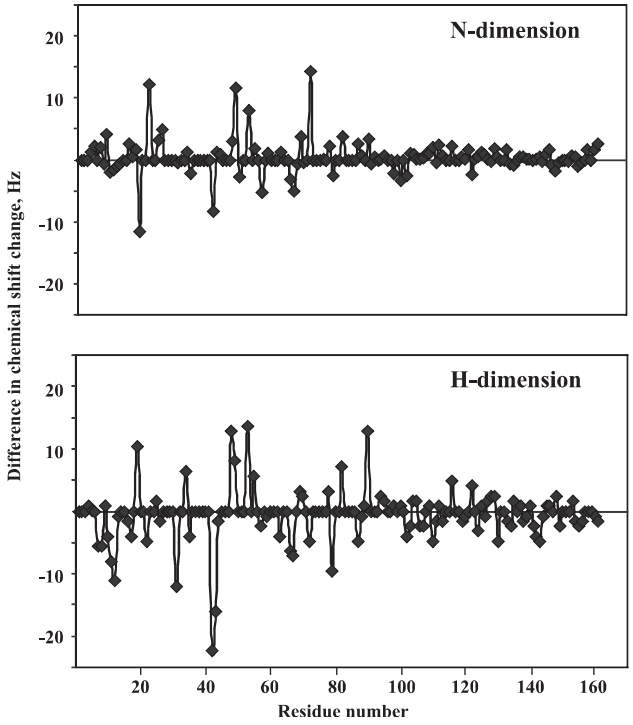

Fig. 5. Differences in the chemical shift changes resulting from fast exchange process induced by levosimendan and dextrosimendan on cardiac troponin C/troponin I-(32–79)/troponin I-(128–180) followed by $^{1}H-^{15}N$-HSQC spectra at 800 MHz. The plotted differences are calculated as $\Delta\Delta\nu = \Delta\nu_{levosimendan} - \Delta\nu_{dextrosimendan}$. Excess quantities of levosimendan and dextrosimendan was added into 0.2 mM cardiac troponin C/troponin I-(32–79)/troponin I-(128–180). The resulting chemical shift changes were compared and largest differences were found in residues A7, A8, E19, A22, A31, G34, G42, K43, L48, G49, T53, E55, E66, D67, V72, V79, V82, and K90. The upper panel shows differences in chemical shifts in nitrogen dimension and the lower panel in proton dimension.

4. Discussion

A skinned cardiac fiber in vitro experimental setup was used to study the effects of the two stereoisomers, levosimendan and dextrosimendan. In this experiment, the whole apparatus of the contractile fiber is still assembled and functional, despite of both the plasma membrane and the sarcoplasmic reticulum membrane being disrupted. The results showed that both stereoisomers had a Ca^{2+}-sensitizing effect. However, at low concentration (0.3 μM), only levosimendan showed an effect, while at higher concentrations, the concentration–response curves of the two enantiomers were parallel up to 10 μM. This behavior

suggests a specific effect of levosimendan at low concentrations and a nonspecific effect of both isomers at higher concentrations. The concentration at which the Ca^{2+}-sensitizing effect of levosimendan is half of the maximum (EC_{50}) could not be accurately determined, though we estimated it to be 0.3 μM or below.

In order to better understand the different effects observed for the two stereoisomers of simendan on cardiac muscle contraction, the binding of levo- and dextrosimendan to Ca^{2+}-saturated cardiac troponin C was studied by following changes in chemical shift of the backbone nuclei ^{1}H and ^{15}N in the presence of the respective stereoisomer.

We had previously shown that there are several interaction sites on isolated cardiac troponin C for levosimendan (Sorsa et al., 2001). The assumption we made was that the N-domain encompasses the primary binding site and that the C-domain binding sites were secondary and could possibly be blocked by cardiac troponin I. We have recently shown that this is the case (Sorsa et al., 2003). Chemical shift changes have been widely used in structure–activity relationship studies (Abusamhadneh et al., 2001; Li et al., 1999, 2000; Shuker et al., 1996). Residues, which are in or close to the binding site upon ligand binding, respond to changes in their chemical environment or changes in dynamics by shifting their cross-peaks from their original positions. In this way, a binding site for dextrosimendan in the C-domain (site 2, around residues 150–158) could be identified. However, our titration experiments revealed differences in the interaction of the two stereoisomers with the C-domain of cardiac troponin C. Dextrosimendan showed a significantly higher affinity for site 2 than levosimendan did. On the other hand, both isomers had the same low affinity for site 1 (residues 120–130).

The two stereoisomers bind to the N-domain of cardiac troponin C in a different way than to the C-domain. The addition of levosimendan or dextrosimendan resulted in the appearance of new peaks which were all assigned to the N-domain, indicative of a binding process in slow exchange on the nuclear magnetic resonance time scale ($k_{ex} < 50\ s^{-1}$). If it were a simple binding process, we might expect the intensities of the new signals to grow to the same final intensity of the original signals and then for the original signals to disappear completely. This did not happen; instead, both intensities leveled off at about 50% of the original peaks (no intensity changes between drug to troponin C ratios from 2:1 to 3:1). The "old" and "new" signals can therefore not simply be assigned to simendan-free and simendan-bound states of cardiac troponin C. This can be understood in the following way. One binding site in the N-domain of cardiac troponin C became saturated with levosimendan. However, this complex may have existed in two different protein conformations (e.g. open and closed). The exchange between these two forms is slow on the nuclear magnetic resonance time scale, whereas the simendan exchange is fast. We have previously (Pääkkönen et al., 1998, 2000) suggested that there also exists an exchange between two forms on free cardiac troponin C, though in this case, with a low concentration of the second form. We have also demonstrated two N-domain conformations corresponding to the closed and open states in the presence of the regulatory and cardiac-specific amino terminal regions of cardiac troponin I (Abbott et al., 2000). The existence of two forms in equilibrium has been demonstrated more clearly for a mutant in the C-domain of calmodulin. In this case, one of the Ca^{2+} binding sites was very weak and as such was similar to the N-domain of cardiac troponin C (Evenäs et al., 1998). In the case of calmodulin as well as for free cardiac troponin C, the exchange between the two forms was still sufficiently fast to result in nuclear magnetic resonance spectra in intermediate to fast exchange. Therefore, our present understanding of the system is that the new peaks appearing upon the addition of levo- and dextrosimendan do not directly reflect the binding, but rather they are indicative of a new conformation in the cardiac troponin C–simendan complex with a population of ca. 50%. It is possible to hypothesise that an open conformation exists, just as it was proposed in the case of calmodulin.

We found that both enantiomers interact with the N-domain of cardiac troponin C in the presence of cardiac troponin I-(32–79) and (128–180) but are blocked from the binding to the C-domain of cardiac troponin C. The similarity in chemical shifts induced by the two stereoisomers suggests similar modes of interaction with the N-domain of cardiac troponin C. Small stereospecific chemical shift differences were noted in residues belonging to A7, A8, E19, A22, A31, G34, G42, K43, L48, G49, T53, E55, E66, D67, V72, V79, V82, and K90. The number and magnitude of levosimendan- and dextrosimendan-induced chemical shift changes are consistent with both stereoisomers altering N-domain conformational exchange. It is possible that the suggested conformational isomerism of cardiac troponin C masks the chemical shift changes resulting from the change in the chemical environment adjacent to the bound drug. For the N-domain binding, we suggest that the observed chemical shift differences induced by the stereoisomers are due to the chirality of this molecule and thus a stereospecific interaction.

In this study, we have presented evidence that the Ca^{2+}-sensitizing effect of levosimendan is in part due to a stereoselective binding to the N-terminal domain of cardiac troponin C. Both the action of stereoisomers of simendan on the whole contractile apparatus and the binding to a model of a cardiac troponin C–troponin I complex show differences between the effects of levosimendan and dextrosimendan. Nuclear magnetic resonance studies of more complex protein models are called for in order to understand the function of levosimendan on a molecular level. Some work in this direction is in progress in our laboratories.

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