The binding of a new calcium sensitizer, levosimendan, to human cardiac troponin C (cTnC) is described. Fluorescence studies done on cTnC and the cardiac contractile structures respond in an impaired manner to activator Ca\(^{2+}\) (2). This may be a consequence of either a reduction in intracellular pH during cardiac hypoxia or ischemia, or an increase in the Ca\(^{2+}\)-dependent phosphorylation of troponin I; both of which lead to a reduction in the Ca\(^{2+}\) affinity of troponin C (3, 4). Moreover, to better characterize the binding, an NH\(_2\)-terminal cTnC fragment was produced, and its interaction with levosimendan studied by \(^1\)H NMR spectroscopy. Finally, a binding site for levosimendan at the hydrophobic pocket of the NH\(_2\)-terminal domain of cTnC is proposed by applying molecular modelling techniques.

### MATERIALS AND METHODS

**Cloning and Expression of Recombinant Human Cardiac Troponin C and Its Amino-terminal Fragment**—The coding sequence of human cTnC was cloned by using 2.5 µg of the human heart poly(A\(^+\)) RNA (purchased from Clontech, Palo Alto, CA) as template for the cDNA synthesis with the synthetic oligonucleotide 5'-ACGTAAGTCCCTACTCACAACCTCTGATG-3' acting as the 3' primer. The following PCR reaction (20) the 5' primer was oligonucleotide 5'-ACGTAAGTCCCTACTCACAACCTCTGATG-3' and the 3' primer was the same oligonucleotide used for the cDNA synthesis. The amplified DNA fragment was digested with BamHI and EcoRI, purified, and subcloned to the pGEM3-vector (Promega) (20). The cloned cTnC DNA fragment was sequenced as described previously (21-23). For protein expression, the cTnC insert from pGEM3 was isolated and ligated to BamHI-EcoRI-digested glutathione S-transferase fusion protein vector pGEX-2T (Pharmacia Biotech Inc.) to yield the clone pOGL501.

**In Vitro Mutagenesis and Expression of Troponin C**—The 5' primer and subsequent cloning procedures were the same as described for pOGL501. Amino acid substitution Asp\(^{88}\) → Ala\(^{88}\) (pOGL511) was carried out by applying the “megaprimer” technique as described (24, 25). The mutant oligonucleotide was 5'-TGCTAGAAGAACCGCAAGGGAATCTTACACCCCTGGCAAGGAGATGGCGGCTGTGTCAGGCTGGAGTTCTG-3' and 5'-ACGTAAGTCCCTACTCACAACCTCTGATG-3'.
3'. The cloning of the PCR fragment to yield the glutathione S-transferase fusion expression construct was performed as described above. The accuracy of the mutant clones was verified by DNA sequencing. The expression construct was introduced into Escherichia coli BL21 (DE3) or DH-5 cells (Promega) which were grown and induced with isopropyl-β-D-1-galactopyranoside (0.5 mM final concentration) as described by Lundstrom et al. (26). The expression construct coding for the amino-terminal fragment of cTnC (amino acids 1–91; pOGL222) was made by PCR using as the 3' primer an oligonucleotide (5'-AGCTGAAATCTCACCCTTTGCTGTCGTCTC-3') that incorporates the translation stop-codon after the sequence coding for the amino acid 91.

**Purification of Recombinant Troponin C Proteins**—After the induction, the E. coli cells from a 5-liter flask culture medium were harvested as described by Guan and Dixon (27). The cells were lysed by sonication at 100 watts (at 4 °C) and the lysate was cleared by centrifugation (10,000 x g, 20 min at 4 °C). The recombinant protein was purified by glutathione-Sepharose 4B-affinity chromatography (Pharmacia) as described previously (27). Recombinant cTnC protein was cleaved from the fusion protein with thrombin, and eluted from the column with the cleavage buffer. Aliquots from the eluted fractions were analyzed by SDS-PAGE (15% acrylamide) (28) and Coomassie Brilliant Blue staining. The fractions containing recombinant cTnC protein were pooled, concentrated, and the buffer changed by ultrafiltration (Omega Filter NMWL 3K, Filtron). Further purification was performed on an anion exchange Mono-Q chromatography column (HR 5/5, Pharmacia). The column was equilibrated with 20 mM Tris-HCl, pH 7.0, and the protein eluted with a linear gradient of sodium chloride (0–0.7 M) in 40 min. The peak containing recombinant cTnC was concentrated and the buffer exchanged to water by ultrafiltration.

**Characterization of the Purified Proteins**—The recombinant human cTnC was analyzed by reversed phase chromatography (Spherisorb, 300Å C18, 0.3 x 15-cm column). The column was equilibrated and the protein eluted as described by Tilgmann and Kalkkinen (29). The amino-terminal fragment and the substitution mutant of the recombinant cTnC were analyzed on a RP column (TSK TMS 250, C18, 0.21 x 3 cm). The proteins were eluted with a gradient of 3–100% acetonitrile in 60 min. The collected peaks were further analyzed by SDS-PAGE and the gels were stained with Coomassie Brilliant Blue. For analysis of tryptic peptides the proteins were alkylated (30) and desalted by RP chromatography as described (29). The collected proteins were dried by vacuum centrifugation, dissolved in 0.6 μL urea, and 8.0 mM Tris-HCl, pH 8.0, and digested with trypsin (3%, w/w) for 4 h at 37 °C. The resulting peptides were analyzed by capillary electrophoresis (Applied Biosystems 270 HT) on a 50-μm x 500-mm (to detector) fused silica capillary column equilibrated with 20 mM sodium citrate, pH 2.5. Electrophoresis was performed at 20 kV at 30 °C and monitored at 200 nm. To determine the amino acid sequences, the tryptic peptides were separated by RP chromatography on a Spherisorb, 300Å C18, 3 x 150-mm column equilibrated and eluted as described (29). The peptides collected were dried and sequenced by Edman degradation using a modified amino acid analyzer (31).

**Chemicals**—Levosimendan, (R)-1-[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)-phenylhydrazono-propanenitrile, CAS registry number 141505-33-1, was synthesized at Orion-Farmos, Espoo, Finland. Trifluoperazine and dansylchloride were purchased from Sigma.

**Fluorescence Studies**—Both the wild-type and substitution mutant recombinant human cTnC were labeled with dansylchloride as described previously (32). Briefly, a solution containing 55 μM protein, 10 mM NaHCO3, and 0.1 mM KCl at pH 8.0 was gently stirred, while 150 μM dansylchloride was added. The mixture was incubated in the dark for 12 h at 4 °C, under gentle stirring. The dansylated protein was thereafter dialyzed, lyophilized, and redissolved at a final concentration of 1 μM in acetate buffer containing 10 mM EGTA, at pH 7.0. Fluorescence was measured at 22 °C with a Hitachi F-4010 fluorescence spectrophotometer using 340-nm excitation and 502-nm emission wavelength, with a measuring time of 15 s and a band pass of 5 nm. Calcium chloride was added in increments of 1 μM (total calcium), and the concentration of free Ca2+ was calculated according to Fabiato and Fabiato (33). When indicated, either 10 μM trifluoperazine or 3 μM levosimendan was added (from stock solution in Me2SO) to the protein before calcium titration. Control experiments were performed in the presence of 0.7% Me2SO, and pH was readjusted to 7.0 ± 0.02 with ROH after each Ca2+ addition. Protein concentrations were determined according to Bradford (34). Statistical significances were calculated with Dunnet's two-tailed t test, by using the program ANOVA (repeated measurements).

**1H NMR Experiments**—Samples of the recombinant amino-terminal fragment of cTnC (typically 20 mg) were run on a Chlexus column and lyophilized. The freeze-dried protein was reconstituted with 0.4 ml of a solution containing 10 mM Hepes, 100 mM KCl, and 5 mM dithiothreitol, at pH 7.4, in 99.8% 2H2O (Aldrich), and again freeze-dried. Finally, the samples were redissolved in 0.4 ml of 99.995% 2H2O (Aldrich). The calcium content of the 2H2O batch (<25 μM) was measured by atomic absorption. Typical protein concentrations in samples for NMR studies were 1.5–6 μM, at a pH meter reading of 7.4. The protein was titrated by adding aliquots of a solution of 100 μM CaCl2 in 20 mM Hepes buffer, pH 7.4, in 2H2O. The calcium-saturated protein was obtained by adding CaCl2 to produce a final molar ratio of CaCl2 to protein of 2:1. Stock solutions of levosimendan were prepared by dissolving 0.5 mM levosimendan-free acid in 1 ml of 0.5 M NaOH in 2H2O, with gentle shaking. Aliquots of this oversaturated solution of 0.5 μM levosimendan sodium salt were added to the calcium-saturated protein solution to give a levosimendan to protein ratio of 1:1.

**1H NMR one-dimensional spectra** were acquired on a Bruker ARX 400 spectrometer, summing 64 free induction decays per spectrum, using a sweep width of 4800 Hz, and a digitization of 16 k. The temperature of the sample was maintained at 24 °C throughout the experiment. Phase-sensitive NOESY spectra (35) were generated by using time-proportional phase increment procedures, in 1 x 400 w data matrices (zero-filled to 1 x 1 k prior to double Fourier transformation), using a sweep width of 4800 Hz in both domains. 16 Free induction decays were acquired per increment. The mixing time for the NOESY experiment was 500 ms. When needed, suppression of the residual 2HOD signal was achieved by presaturation with a low decoupler power.
Prior to Fourier transformation, the data matrices were multiplied by a modified sine bell window function, and phase corrections were applied. Furthermore, hetero- and homo-correlated two-dimensional NMR experiments were also performed to resolve the assignment of 1H NMR signals of levosimendan (data not shown).

RESULTS AND DISCUSSION

Production of the Troponin C Recombinant Proteins—To obtain sufficient amounts of cTnC protein for the fluorescence and NMR studies, human cTnC was expressed as a gluthathione S-transferase fusion protein in E. coli, and purified by affinity and anion exchange chromatography. The analysis of the highly purified full-length recombinant troponin C proteins, the wild-type (Fig. 1A) and the substitution mutant (not shown), by SDS-PAGE and RP chromatography revealed that both contained two species of polypeptides with slightly different sizes; the difference being about 1000 Da. Tryptic peptide and amino acid sequencing analysis showed that the two forms were otherwise identical, except that the larger polypeptides, which constituted about 25% of the total cTnC protein, contained an extension of 7 amino acids (QEFIVTN) in the carboxyl terminus. This heterogeneity is probably due to the suppression of the troponin C translation stop codon (UAG) and the incorporation of glutamine into the protein, which leads to the extension of 7 amino acids and termination of translation in the stop codon of the vector sequence. This kind of heterogeneity did not occur in the production of the 91-amino acid long NH2-terminal fragment of cTnC (Fig. 1B).

Fluorescence Experiments—The change in fluorescence of a properly labeled protein can be used to follow conformational changes which occur during Ca2+ binding. cTnC has two cysteines in its N-domain (Cys54 and Cys84) which could be labeled with the sulphydryl-specific fluorescence probe molecule 2-(4'-iodoacetamidoanilino)naphthalene-6-sulfonic acid (36). However, because the proposed binding site of hydrophobic ligands on cTnC is in the proximity of Cys84 (18), it is possible that the steric hindrance of 2-(4'-iodoacetamidoanilino)naphthalene-6-sulfonic acid bound to Cys84 would disturb the interaction between levosimendan and cTnC. Therefore, dansylchloride was selected as the labeling reagent. In fact, dansyl may bind to the NH2-terminal amino group or to the side chains of tyrosine and lysine residues, which in the case of cTnC are located far from both the predicted binding site of levosimendan and the Ca2+-binding site. The effects of trifluoperazine and levosimendan on the Ca2+ dependence of dansylchloride-modified cTnC are shown in Fig. 2, A and B. A 5% decrease in the absolute fluorescence after the addition of Ca2+, which produce a free calcium concentration of 0.4 μM (data not shown), could be interpreted as an effect of the Ca2+ binding to high-affinity binding containing 10 mM EGTA, at pH 7.0. Each curve represents the average ± S.E. of n titrations. A, wild-type recombinant cTnC in the presence (10 μM) and absence (MeSO control) of trifluoperazine. B, wild-type recombinant cTnC in the presence (3 μM) and absence (MeSO control) of levosimendan. C, comparison between the wild-type recombinant cTnC and the D88A substitution mutant. D, the D88A substitution mutant in the presence (3 μM) and absence (MeSO control) of levosimendan. Statistical significance is indicated in the figures as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
sites. The binding of calcium to the low-affinity calcium-specific binding site II is shown by an increase of total fluorescence due to the increase in the values for free calcium concentration from 0.4 μM to 1 mM. In the control experiments Ca_{20} and K_{30} were calculated to be 2.73 ± 0.03 μM and 5.0 × 10^{-5} M, respectively (Fig. 2A). 10 μM trifluoroperazine and 3 μM levsimendan decreased the value of Ca_{20} to 1.60 ± 0.05 and 1.19 ± 0.03 μM, respectively (Fig. 2, A and B). These results suggest that also levsimendan, like trifluoroperazine (37), changes the calcium binding properties of cTnC.

The effect of the point mutation, Asp^{88} to Ala, on the Ca^{2+} dependence of the total fluorescence is shown in Fig. 2C. The mutation does not significantly modify the calcium affinity of the protein, and in fact, the Ca_{20} value of the low affinity binding site of the site-directed mutant cTnC is 2.84 ± 0.06 μM, which may be slightly higher than for the wild-type. Finally, the addition of 3.0 μM levsimendan to the mutant cTnC slightly increased the value of Ca_{20} (3.09 ± 0.09 μM), as shown in Fig. 2D. The finding that levsimendan significantly affects the Ca^{2+} dependence of the total fluorescence on the daisytalk wild-type cTnC, but not on the mutant suggests that the Asp^{88} residue, near the hydrophobic pocket of the NH_{2}-terminal domain, plays an important role in the effects levsimendan has on the calcium-binding properties of cTnC. This finding also supports the hypothesis that Asp^{88} participates directly in the binding of levsimendan to the protein.

**1H NMR Experiments** —To confirm the hypothesis that the levsimendan-binding site is in a hydrophobic pocket of the NH_{2}-terminal domain of cTnC, a recombinant NH_{2}-terminal fragment of human cTnC was studied by 1H NMR. It has been previously reported, in studies using limited proteolysis, that both the NH_{2}-terminal and COOH-terminal fragments of cTnC preserve their original Ca^{2+}-binding properties (38), and their structural integrity (39, 40). Recently, Li et al. (41) have shown that the Ca^{2+}-bound transition of the recombinant N-fragment of skeletal TnC was very similar to the behavior of the whole TnC. In order to assess whether our NH_{2}-terminal fragment of cTnC preserves those same characteristics, a titration of the protein with Ca^{2+} was performed and followed by 1H NMR analysis of the complex. The results from this titration are shown in Fig. 3. The variations in chemical shift, induced by the addition of Ca^{2+}, for 10 different 1H NMR signals were measured. The data confirm that the recombinant NH_{2}-terminal fragment of human cTnC has only one binding site for calcium ion, with a K_{Ca^{2+}} value of approximately 3–5 × 10^{-5} M.

These data, compared with the NMR spectra of Ca^{2+} titration curves of the whole human cTnC already described in the literature (19, 42), support the hypothesis that the structure of the NH_{2}-terminal fragment of cTnC is not affected by the COOH-terminal domain of the protein (39).

The NMR experiments presented in this paper were not aimed to establish the molecular structure of the NH_{2}-terminal fragment of cTnC, or even to accomplish the total assignment of its 1H NMR signals. However, a partial sequence-specific assignment of the aromatic spin system (i.e. Phe\(^{57}\), Phe\(^{57}\), and Tyr\(^{5}\)) was accomplished by: (i) comparing the NOE connectivities observed in NOESY spectra (Fig. 4, A and B) with the peaks in COSY spectra (not shown) and with literature data on the whole Ca^{2+}-saturated cTnC (42, 43), and (ii) by comparing the drifts of the chemical shift of the aromatic protons during Ca^{2+} titration with the data obtained for the entire protein (42). The NOESY spectra of the NH_{2}-terminal fragment of cTnC showed the signal patterns attributable to the 6 amino acids with aromatic side chains present in the protein (Tyr\(^{5}\), Phe\(^{57}\), Phe\(^{57}\), Phe\(^{57}\), Phe\(^{57}\), and Phe\(^{57}\)). The aromatic signals of Tyr\(^{5}\), which do not change their chemical shift values with the addition of Ca^{2+} (Fig. 3, trace N, peaks 2 and 3), were assigned by comparison to the literature (43).

The aromatic zone of the two-dimensional NOESY spectra of the apo- and the Ca^{2+}-saturated fragments (Fig. 4, A and B) shows that binding of Ca^{2+} alters the chemical environment of the residues in the NH_{2}-terminal hydrophobic cluster in accordance with literature data on the whole protein (42, 43). However, the presence of a NOE between Phe\(^{57}\) and Phe\(^{57}\) is shown both in the apo- and Ca^{2+}-saturated form (Fig. 4, A and B, peak d), suggesting that the binding of Ca^{2+} does not disrupt the spatial relationship between these two aromatic residues, as shown for the whole protein (43).

Information about the spatial location of levsimendan on the protein was obtained by comparing the two-dimensional NOESY spectra obtained from Ca^{2+}-saturated cTnC before and after the addition of levsimendan (Fig. 4, B, C, D, F, and G). Cross-peaks generated by the NOE between protons belonging to the two different molecules can be individuated in the relatively uncrowded spectral region shown in Fig. 4G. A cross-peak is generated by the NOE between the proton in the position para on the phenyl ring of Phe\(^{57}\) and the methylene proton antiparallel to the methyl group on the dihydropyridazine ring of levsimendan (Fig. 4G, peak g). In the same spectral region, cross-peaks due to the NOE between protons of the phenyl ring and the dihydropyridazine ring of levsimendan are shown (Fig. 4G, peaks h, i, l, and m).

In the spectrum of the NH_{2}-terminal fragment, which contains 7 methionine residues, six different signals could be tentatively assigned to methionine terminal methyls in the region from 1.8 to 2.3 ppm (Fig. 3) and 1H-m6; Fig. 4F, signals...
m1-m6). Two of these signals show cross-connectivities both with signals assigned to phenylalanine aromatic protons (Fig. 4G, spectral region from 2.15 to 2.22 ppm in the first dimension and from 7.00 to 7.35 ppm in the second dimension), and with the meta- and ortho-protons of the phenyl ring of levosimendan (Fig. 4G, peaks n, o, p, and q). In the molecular model of cTnC proposed by Ovaska and Taskinen (18), only the two terminal methyls of Met⁴⁰ and Met⁴¹ are in the proximity of the hydrophobic cluster, near the phenyl ring of Phe²⁰. In contrast, the methyls of Met¹, Met⁴¹, Met⁴², and Met⁴³ are not oriented toward any of the phenylalanine aromatic rings. The spatial location of Met¹, in the proximity of Tyr⁴ and far from the hydrophobic cluster, is confirmed also in the NOESY spectrum (Fig. 4G, peaks r and s, corresponding to the peak m3 in Fig. 4F). Moreover, additional information on the assignment of the methionine residues comes from the Ca²⁺-induced variations of chemical shifts of the six signals m1-m6. Herzberg et al. (44) proposed that as a consequence of the Ca²⁺-induced opening of the structure of skeletal troponin C, some of the methionine side chains in the N-domain should become more accessible to the solvent. In particular, the accessibility to the solvent for Met⁶⁰ on helix D and Met⁴¹ and Met⁴² on helix 3 should dramatically change, and should remain unchanged for Met²⁰ on helix A and Met⁴¹ and Met⁴² on helix D. As a consequence some Ca²⁺-dependent changes in the chemical shifts and in the relaxation times of the methyl protons belonging to Met²⁰, Met⁴¹, and Met⁴² on cTnC (corresponding to Met¹, Met⁴¹, and Met⁴² on cTnC) could be expected, while the peaks due to the methyl protons of Met⁴¹ and Met⁴² on cTnC (corresponding to Met⁴¹ and Met⁴² on cTnC) should be less affected. In Fig. 3 (expansion from 1.7 to 2.4 ppm) it is clearly evident that four of the six signals marked as m1-m6 show a Ca²⁺-dependent variation of their chemical shifts (Fig. 3, signals m1, m4, m5, and m6), while two do not (Fig. 3, signals m2 and m3). Additionally, the signal m1 shows a change in the relaxation behavior of the relative protons. Furthermore, the one-dimensional spectra of the Ca²⁺-saturated NH₂-terminal cTnC show that the addition of levosimendan causes small further shifts of the signals m1, m4, m5, and m6 (but not of the signals m2 and m3) in the same direction as in the Ca²⁺ titration (compare Fig. 3N and Fig. 4F in the spectral region from 1.7 to 2.3 ppm).

These data, in addition to the NOE connectivities already discussed, make it reasonable to assign the two peaks at 2.15 and 2.22 ppm to the terminal methyls of Met⁴¹ and Met⁴², respectively. Our data confirm also the tentative assignment of the Met¹ signal proposed by MacLachlan et al. (42) in the ¹H NMR spectrum of the whole cTnC.

In conclusion, the NMR findings support the hypothesis that the levosimendan-binding site on human cTnC is in the proximity of Met¹⁸, Met⁴¹, and Phe⁷⁷, within the hydrophobic pocket of the NH₂-terminal domain. Furthermore, the binding of levosimendan to the NH₂-terminal fragment of Ca²⁺-saturated cTnC does not seem to induce major changes in the structure of the protein (compare the NOE connectivities in Fig. 4, B and D).

A Model of the Levosimendan-cTnC Complex—The molecular model of the cTnC presented here is based on the x-ray structures of chicken skeletal TnC (9) and calmodulin (45). The model is in the three-calcium state because it is supposed that calcium sensitizers can bind to the regulatory N-domain of the Ca²⁺-saturated protein. The calcium-bound conformation of the N-domain was determined by analogy to the Ca²⁺-saturated domains of skeletal TnC and calmodulin. The construction of this cTnC model has been described in detail earlier (18).

As a first approach, levosimendan was docked to the NH₂-terminal domain of the protein model so that the N(NH) part of
the hydrazone structure was located near Asp^{ss}, and the hydrophobic phenylidihydropyridazine part was oriented toward the hydrophobic patch of the model. After energy minimization a reasonable model for the drug-protein complex was obtained, which could be used as a starting model to guide the interpretation of the NMR spectra. The results from the fluorescence and two-dimensional NMR experiments were afterwards used to refine the levosimendan-cTnC complex molecular model. Of great importance was the NMR evidence for the spatial proximity of the trans methylene proton of the dihydropyridazine ring to the para-phenyl proton of the Phe^{77} side chain.

In the resulting molecular model of the complex, the dihydropyridazine ring of levosimendan appears buried in a hydrophobic cleft formed by Phe^{20}, Ala^{29}, Ala^{29}, Phe^{34}, Val^{28}, and Phe^{77}. The distance between the trans methylene proton of the dihydropyridazine and the para-phenyl proton of Phe^{77} is 2.3 Å. In this model, Glu^{29} does not form a good binding geometry with the carbonyl oxygen of the dihydropyridazine ring, and the binding of this carbonyl group to the backbone amide NH group of Ala^{29} seems probable. Furthermore, the phenyl ring of levosimendan is lined by Met^{61}, Cys^{64}, and Met^{68}, and the smallest distances between the protons of the two methionine methyl groups and the phenyl ortho-protons of levosimendan are between 2.5 and 3.2 Å. The dinitrile tail of levosimendan binds within a groove formed by Gln^{51}, Gln^{56}, Asp^{68}, Ser^{69}, and Lys^{72}, while Asp^{88} binds to the hydrazone NH. The drug-protein complex is shown as a stereo pair in Fig. 5, with residues within 5 Å of the ligand being shown.

**Conclusions**—Fluorescence and NMR spectroscopy experiments have given evidence showing that levosimendan binds to a specific site on recombinant human cTnC, and provided data for a molecular model for the drug-protein complex. The model shows how levosimendan can bind to calcium-saturated human cTnC, in the hydrophobic patch of the N-domain near the site where the B helix is located when the protein is in its apo-form. The proposed location of the levosimendan-binding site is consistent with a stabilization of the calcium-bound conformation of the N-domain, which was also seen as a change in the calcium titration curve by fluorescence spectroscopy. Furthermore, our model is in agreement with previously reported findings for other calcium sensitizers (18).

The binding of levosimendan to cTnC may have an important role in explaining the pharmacological properties of this drug in vivo. In fact, it has been shown that levosimendan produces positive inotropic action in paced papillary muscle in the concentration range of 1–10 μM, and augments the tension in skinned fibers to the same extent in the same concentration range (7). It has been therefore suggested that the levosimendan-induced inotropy is caused by an increase in calcium sensitivity of the contractile proteins rather than on an increase in calcium influx (7).

The fact that levosimendan can bind to cTnC does not necessarily mean that it would interact in the same way with the troponin complex. In fact, some studies have pointed out that the hydrophobic pocket on the NH2-terminal domain of cTnC may be responsible for part of its critical interaction with TnI (46). However, preliminary experiments with troponin complex high affinity chromatography have given evidence of an interaction of levosimendan with the cTnC-TnI complex (8). Moreover, skinned fiber experiments show that levosimendan interacts with the whole contractile protein system. Therefore, additional experiments have been planned to investigate the existence of a tertiary complex consisting of levosimendan-TnC-TnI, and possibly to clarify its structure in more detail.

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**REFERENCES**

Binding of Levosimendan to Recombinant Human cTnC