Interaction of Cardiotonic Thiadiazinone Derivatives with Cardiac Troponin C*

Bo-Sheng Pant and Robert G. Johnson, Jr.

From the Department of Pharmacology, Merck Research Laboratories, West Point, Pennsylvania 19486

The cardiotonic effects of thia diazinone derivative EMD 57033 are mediated by direct actions on myofilaments (Lues, I., Beier, N., Jonas, R., Klockow, M., and Haeusler, G. J. (1993) Cardiovasc. Pharmacol. 21, 883-892). Cardiac troponin C has been postulated to be a potential target of the drug (White, J., Lee, J. A., Shah, N., and Orchard, C. H. (1993) Circ. Res. 73, 61-70). This study tested whether EMD 57033 interacts directly with recombinant human cardiac TnC (hcTnC). EMD 57033 caused concentration-dependent quenching of tyrosine (Tyr) fluorescence of hcTnC in the presence of Ca$^{2+}$ (100 μM) and little change of the fluorescence in the presence of Mg$^{2+}$ (2 mM). $K_d$ for the drug-hcTnC interaction in the presence of Ca$^{2+}$, determined by Tyr fluorescence titrations, was approximately 40 μM. The binding of EMD 57033 was stereo-selective: the optical isomer of EMD 57033 bound hcTnC much more weakly. The Ca$^{2+}$ dependence and stereo-selectivity of EMD 57033 binding were substantiated by a dialysis-based direct binding assay. EMD 57033 was found to interfere with Ca$^{2+}$-dependent binding of hydrophobic probe 1,1'-bi-(4-anilinopheno)-naphthalene-5,5'-disulfonate (bis-ANS) to hcTnC. The relationships between [Ca$^{2+}$] and Tyr fluorescence of hcTnC and between [Ca$^{2+}$] and bis-ANS fluorescence in the presence of hcTnC were substantially altered by EMD 57033 in the range of [Ca$^{2+}$] where Ca$^{2+}$/Mg$^{2+}$ sites of hcTnC were titrated by Ca$^{2+}$. EMD 57033 was found to bind as tightly to 2 Ca$^{2+}$-hcTnC as to 3 Ca$^{2+}$-hcTnC. These observations were interpreted as indicating that a EMD 57033-binding site is induced by Ca$^{2+}$ binding, but not Mg$^{2+}$ binding, to the Ca$^{2+}$/Mg$^{2+}$ sites of hcTnC. The drug-binding site most likely resides in the carboxyl domain of hcTnC.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: EMD 57033, (100)-5-[1-(3,4-dimethoxybenzoyl)-1,2,3,4-tetrahydro-6-quinyl]-6-methyl-3,6-dihydro-2H-1,3,4-thiadiazino-2-one; EMD 57439, (−)-5-[1-(3,4-dimethoxybenzoyl)-1,2,3,4-tetrahydro-6-quinyl]-6-methyl-3,6-dihydro-2H-1,3,4-thiadiazino-2-one; bis-ANS, 1,1'-bi-(4-anilinophenyl)naphthalene-5,5'-disulfonate; TnC, troponin C; TnI, inhibitory subunit of troponin; hcTnC, recombinant human cardiac TnC; cTnC, cardiac TnC; sTnC, skeletal TnC; DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid.

* The molecular mechanism(s) underlying the Ca$^{2+}$-sensitizing effects of EMD 57033 is not well understood. The compound appears to have a direct effect on actin/myosin interactions (6). It has also been hypothesized that EMD 57033 may bind cardiac troponin C and increase its affinity for Ca$^{2+}$ (4, 7).

Troponin C (TnC) is the Ca$^{2+}$-binding subunit of troponin, a hetero-trimeric protein containing, in addition, inhibitory subunit (TnI) and tropomyosin binding subunit. Troponin, together with tropomyosin, constitutes a thin filament-based regulatory protein complex. The two known isoforms of TnC, i.e. TnC found in cardiac and slow twitch skeletal muscles (cTnC) and that found in fast skeletal muscles (sTnC) share extensive sequence homology (8–10) and both belong to the superfamilies of helix-loop-helix or EF-hand Ca$^{2+}$-binding proteins (11). Crystallographic structure of avian sTnC revealed a dumbbell-shaped molecule consisting of two globular domains connected by a long central helix (12, 13). cTnC is thought to have a similar three dimensional structure (14). Both sTnC and cTnC contain four helix-loop-helix motifs (I–IV), two (II/I) in the amino-terminal domain, and two (III/IV) in the carboxy-terminal domain. While all four are functional Ca$^{2+}$-binding sites in sTnC, only three (II, III, and IV) are functional in cTnC. Site I in sTnC and II in both sTnC and cTnC bind Ca$^{2+}$ specifically ($K_d \sim 5 \times 10^3$ M$^{-1}$) and are directly involved in Ca$^{2+}$-regulation of muscle contraction (15–19). Sites III and IV bind Ca$^{2+}$ ($K_d \sim 2 \times 10^5$ M$^{-1}$) and Mg$^{2+}$ ($K_d \sim 5 \times 10^3$ M$^{-1}$) competitively, and appear to play primarily a structural role (15, 16, 20, 21). Thus, the NH$_2$- and COOH-terminal domains of TnC are often described as the regulatory and structural domains, respectively. In addition to its well documented structural role in anchoring TnC to the thin filament, the COOH-terminal domain appears to contribute directly to Ca$^{2+}$-regulation by way of interacting with the inhibitory region of TnI and residues of TnI adjacent to the inhibitory region (see, e.g., Refs. 22–24).

Both domains of TnC contains a core of hydrophobic residues, which become more exposed to solvent upon Ca$^{2+}$-binding to the domains (25, 26). The interactions of the hydrophobic patches with TnI are believed to be important for transmission of the Ca$^{2+}$ signal and the stability of troponin complex. These hydrophobic residues appear to also participate in interaction of TnC with small organic ligands, such as trifluoperazine and bepridil, which bind TnC and modulate its Ca$^{2+}$-binding properties (14, 27–30).

In the present report, we demonstrate that EMD 57033 interacts directly with cTnC in a Ca$^{2+}$-dependent and stereo-selective manner. We show that cTnC contains a EMD 57033-
binding site, which is induced by Ca\(^{2+}\) binding, but not by Mg\(^{2+}\) binding, to the Ca\(^{2+}/Mg\(^{2+}\) sites of cTnC. The drug-binding site most likely resides in the COOH-terminal domain of cTnC. In addition, the report offers new evidence that the COOH-terminal domain of cTnC, when saturated by Mg\(^{2+}\), is in a conformational state substantially different from the state it assumes when occupied by Ca\(^{2+}\).

MATERIALS AND METHODS

Expression and Purification of Recombinant hcTnC—Adult human cardiac cDNA (a gift from Dr. M. Jacobson and C. Salvatore) was subjected to polymerase chain reaction using Taq DNA polymerase (Perkin Elmer) and primers TnC5 (5'-CTCGGCCATGGATGACATC- TACAAGGC-3') and TnC3 (3'-AGTGGGGTCTTCAGGGATACGGC- CTAGGC-5') TnC5 contained the first 20 bases (underlined) of the coding sequence of human cTnC cDNA (31) and 7 additional 5' nucleotides. The bold bases in TnC5 constitute a recognition site for Ncol. TnC3 contained a 21-base sequence (underlined) complementary to a stretch of the 3'-untranslated region of hcTnC DNA and 8 additional 5' nucleotides with a BamHI recognition site (in bold). The polymerase chain reaction products were purified using low melting gel and Gene Clean II (Bio 101), digested with Ncol and BamHI, and ligated into pET3d vector (Novagen) linearized with Ncol and BamHI. The ligation reaction was used to transform competent cells of E. coli strain BL21(DE3) (Novagen). Recombinant hcTnC was purified using a modified method of Putkey et al (19). Bacteria from 1 liter of culture were harvested and washed once in distilled water, and then resuspended in 200 ml of 6 M urea, 1 M EDTA, 50 M Tris (pH 8.0), 1 M dithiothreitol (DTT). The suspension was sonicated on ice for 5 min and then centrifuged at 60,000 rpm in a Beckman ultracentrifuge with a Ti-45 rotor. The supernatant was loaded directly onto an anion exchange column (DE-52, Whatman) pre-equilibrated with the same buffer. The column was eluted with a KC1 gradient of 0–0.5 M made in the above buffer. A crude cTnC peak was eluted at a conductivity of 9–10 millimmoles/cm. Fractions enriched in cTnC were pooled, dialyzed against 1 M NaCl, 5 M CaCl\(_2\), 0.5 M DTT, 50 M Tris-HCl (pH 7.5), and then loaded on a phenyl-Sepharose (Pharmacia Biotech Inc.) column equilibrated with 1 M NaCl, 0.1 M CaCl\(_2\), 0.5 M DTT, 50 M Tris-HCl (pH 7.5). Subsequent to exhaustive wash of the column bed with the above buffer less CaCl\(_2\), pure cTnC was eluted with a buffer containing 5 M EDTA, 1 M NaCl, 0.5 M DTT, and 50 M Tris-HCl (pH 7.0). About 100 mg of purified hcTnC was obtained from each liter of bacterial culture. The purified recombinant hcTnC was indistinguishable from native bovine cTnC in terms of Ca\(^{2+}\)-binding properties and the ability to regulate bovine cardiac actomyosin ATPase.

Fluorescence Measurements—Steady state fluorescence intensities and spectra were acquired using a Fluoromax spectrofluorometer (Spex Industries Inc., Edison, NJ) equipped with a water-jacketed cuvette holder and a magnetic stirrer. Excitation and emission wave lengths were 280 and 305 nm for tyrosine, and 400 and 512 nm for bis-ANS. Free Ca\(^{2+}\) in the dialysis buffer and dialyzed sample were determined from UV absorption as described in the following paragraph. The concentrations of hcTnC in the dialyzed samples were determined by the method of Bradford (33) using hcTnC as standard. The stoichiometry of the binding was calculated as ([drug]sample - [drug]buffer)/[TnC]sample.

Drug—EMD 57033 and EMD 57439 were provided kindly by E. Merck. Concentrated stock solutions of the compounds were prepared by dissolving the compounds in 90% propylene glycol and 10% dimethyl sulfoxide. The concentration of the drugs was determined by UV absorption using a molar extinction coefficient \(\varepsilon_{280nm} = 20,000\) for both EMD 57033 and EMD 57439.

RESULTS

Binding of EMD 57033 and EMD 57439 to hcTnC—Drugs—EMD 57033 and EMD 57439 to hcTnC were detected from their effects on the intrinsic fluorescence of hcTnC. The chemical structures of EMD 57033, the (+)-enantiomer, and EMD 57439, the (-)-enantiomer, and Fig. 1. When excited at 280 nm, the fluorescence of hcTnC, which does not contain tryptophan, is attributable to tyrosine residues. Fig. 2A shows the effect of EMD 57033 and 57439 on the Tyr fluorescence of hcTnC. In the presence of 2 mM Mg\(^{2+}\) and 100 \(\mu\)M free Ca\(^{2+}\), both compounds caused concentration-dependent quenching of the tyrosine fluorescence. However, at the same concentration, EMD 57033 consistently caused significantly more quenching than did EMD 57439. For example, 50 \(\mu\)M EMD 57033 induced approximately 40% quenching of the fluorescence, while 50 \(\mu\)M EMD 57439 caused roughly 20% quenching. The double reciprocal plots (1/F versus 1/[EMD 57033] or 1/[EMD 57439]) of the data in the presence of Ca\(^{2+}\) were linear (Fig. 2B), suggesting that the binding of EMD 57033 as well as 57439 to hcTnC was non-cooperative. The \(K_{d}\) for the interaction of hcTnC with EMD 57033 and 57439, estimated from the reciprocal plots in Fig. 2B, are approximately 40 and 160 \(\mu\)M, respectively. In other words, EMD 57033, the (+)-enantiomer, bound hcTnC 4 times more strongly than the (-)-enantiomer, EMD 57439. It is also evident from Fig. 2 that the interaction of the drugs with hcTnC was Ca\(^{2+}\)-sensitive. In the presence of 2 mM Mg\(^{2+}\) and no Ca\(^{2+}\), EMD 57033 and 57439 had little or no effect on hcTnC fluorescence, while at the highest [EMD 57033] tested (50 \(\mu\)M), only a very small effect (a 5% quenching) was observed. Thus, it is clear that EMD 57033 binds to cTnC in a Ca\(^{2+}\)-dependent and stereo-selective manner.

Direct Measurement of the Binding of EMD 57033 and EMD 57439 to hcTnC—Fig. 3 shows the result of an experiment in which the binding of EMD 57033 and EMD 57439 to cTnC was measured directly by equilibrium dialysis. A small number of determinations were made because the drugs were available in limited quantities. In the presence of 100 \(\mu\)M free Ca\(^{2+}\), the EMD 57033 bound considerably more EMD 57033 than EMD 57439, given the same free [EMD 57033] and [EMD 57439]. For example, EMD 57033 bound 0.52 mol of EMD 57033/mol of cTnC at 46 \(\mu\)M EMD 57033, but only 0.29 mol of EMD 57439/mol of cTnC at 48 \(\mu\)M.
**Fig. 2. Titration of tyrosine fluorescence of cTnC with EMD 57033 and 57439.** A, Tyr fluorescence intensity of cTnC as a function of [EMD 57033] (squares) and [EMD 57439] (circles). The conditions were 2.9 μM cTnC, 100 mM KCl, 2 mM Mg²⁺, 60 mM MOPS (pH 7.0), 1 mM EGTA, pCa 9 (open symbols) or 4 (closed symbols). Excitation wavelength, 280 nm; emission wavelength, 305 nm. The data are expressed as fractions of the fluorescence intensity in the absence of the drugs. B, the data at pCa 4 replotted as double-reciprocal plots. The symbols are the same as in A. Also shown are the linear regression lines of the data.

**Fig. 3. Direct measurement of binding of EMD 57033 and EMD 57439 to hTnC by equilibrium dialysis.** The conditions were 100 mM KCl, 60 mM MOPS (pH 7.0), 1 mM EGTA, pCa 9 or 4. Each bar represents average of two or three measurements.

μM EMD 57439. This finding was consistent with the notion that EMD 57033 has higher affinity for hTnC than does EMD 57439. Based on the dissociation constants of EMD 57033 (40 μM) and EMD 57439 (160 μM) estimated from fluorescence titrations (Fig. 2), hTnC would be expected to bind 0.53 mol of EMD 57033/mol of cTnC at 46 μM EMD 57033 and 0.23 mol of EMD 57439/mol of hTnC at 48 μM EMD 57439, assuming that the protein contains a single drug-binding site. Clearly, the equilibrium dialysis data (Fig. 3) are in good agreement with these predictions. Furthermore, the data in Fig. 3 confirm that the binding of EMD 57033 and EMD 57439 to hTnC is Ca²⁺-sensitive. In the presence of 39 μM EMD 57439, cTnC bound 0.06 mol of EMD 57439/mol of cTnC at pCa 9, compared with 0.23 mol of EMD 57439/mol of cTnC at pCa 4. In the presence of 37 μM EMD 57033, the amount of EMD 57033 bound to hTnT at pCa 9 (0.09 mol of EMD 57033/mol of cTnC) was much less than the level (approximately 0.4 mol of EMD 57033/mol of cTnC) to be expected of Ca²⁺-saturated cTnC, based on extrapolation from the data obtained at somewhat higher [EMD 57033] (Fig. 3).

Ca²⁺ Titration of Tyr Fluorescence of hTnC in the Presence of EMD 57033—To determine which of the two classes of Ca²⁺-binding sites of hTnC was directly linked to the apparent Ca²⁺-dependence of the EMD 57033-cTnC interaction, we studied the relationship between [Ca²⁺] and Tyr fluorescence of cTnC in the presence and absence of EMD 57033 (Fig. 4). In the absence of EMD 57033, the Tyr fluorescence intensity increased when [Ca²⁺] was raised gradually from pCa 7.5 to 6, a range where sites III and IV (Ca²⁺/Mg²⁺ sites) were titrated. The fluorescence changed little in the range of pCa 6 to 4, where the Ca²⁺-specific site II was titrated. The Ca²⁺-induced enhancement of the Tyr fluorescence appeared to result primarily from Ca²⁺ binding to the Ca²⁺/Mg²⁺ sites of hTnC. The pCa fluorescence relation in the absence of EMD 57033 was fitted well with a single-term Hill equation, in which pCa_H = 6.87 and n = 1.8. In the presence of 42 μM EMD 57033, the pCa-Tyr fluorescence relation was strikingly different. The intensity of the fluorescence decreased with increasing [Ca²⁺] between pCa 7.5 and 6, and was essentially unchanged between pCa 6 and 4. The relation was fitted well with a Hill equation in which pCa_H = 6.82 and n = 2.5. The following scenario appears to offer the most straightforward interpretation for the observation. Ca²⁺ binding to the Ca²⁺/Mg²⁺ sites induced a EMD 57033-binding site in the COOH-terminal domain of hTnC; the subsequent binding of EMD 57033 to hTnC induced significant fluorescence quenching, which more than canceled out the fluorescence enhancement caused by Ca²⁺ binding to the Ca²⁺/Mg²⁺ sites. If instead, Ca²⁺-binding to the Ca²⁺-specific site II were necessary for EMD 57033 binding, the presence of EMD 57033 should not have significantly altered the rising phase (from pCa 7.5 to 6) of the pCa-Tyr fluorescence relation, but should have caused Ca²⁺-dependent quenching of the Tyr fluorescence between pCa 6 and 4. Such predications were not borne out in Fig. 4.

Effect of EMD 57033 on the Interaction between hTnC, Bis-ANS—Bis-ANS, a noncovalent hydrophobic fluorescence probe, has been used widely to study protein conformation. Fig. 5 shows the effect of hTnC and divalent ions (Ca²⁺ and Mg²⁺) on bis-ANS fluorescence. Addition of hTnC to a bis-ANS solution free of Ca²⁺ and Mg²⁺ induced a 100% increase of the fluorescence suggesting the presence of divalent ion-independent bis-ANS-binding site(s) in hTnC. Subsequent addition of 2
Fig. 4. Effect of EMD 57033 on the relationship between [Ca\(^{2+}\)] and Tyr fluorescence of hcTnC. Tyr fluorescence of hcTnC (5.25 μM) was titrated with Ca\(^{2+}\) in the presence of 100 mM KCl, 2 mM EGTA, 100 mM MOPS, 0.5 mM NaN\(_3\) with (circles) or without (triangles) 42 μM EMD 57033. F\(_p\) is defined as the fluorescence (F) at pCa 9.0. The lines represent the least square fits of the data with the following equation: 
\[
\frac{F}{F_0} = 1 + \Delta F_{\text{max}} \cdot [\text{Ca}^{2+}] / (K^n + [\text{Ca}^{2+}])
\]
where n and K are Hill coefficient and [Ca\(^{2+}\)] at 0.5ΔF\(_{\text{max}}\). The [Ca\(^{2+}\)]-fluorescence relation in the absence of EMD 57033 was fitted well with a Hill equation with K\(_m\) 5 mM MOPS, 0.5 mM NaN\(_3\) with (triangles) and without (circles) 42 μM EMD 57033. The lines are the best fit of the data with the following equation, which describes two classes of binding sites: 
\[
\frac{F}{F_0} = 1 + A_1 [\text{Ca}^{2+}] / (K_1^n + [\text{Ca}^{2+}]) + A_2 [\text{Ca}^{2+}] / (K_2^n + [\text{Ca}^{2+}])
\]
where K\(_m\) and A\(_m\) are the magnitudes of the transitions, n is the Hill coefficient, and K\(_1\) and K\(_2\) are [Ca\(^{2+}\)] at the midpoints of the two transitions. In the absence of EMD 57033, K\(_1\) = 0.11 μM, A\(_1\) = 0.43, n\(_1\) = 3.1, K\(_2\) = 3.9 μM, A\(_2\) = 0.205; in the presence of 42 μM EMD 57033, K\(_1\) = 0.09 μM, A\(_1\) = 0.28, n\(_1\) = 1.9, K\(_2\) = 5.8 μM, A\(_2\) = 0.22.

Fig. 5. Fluorescence spectra of bis-ANS. The spectra a, b, c and d were obtained sequentially: a: bis-ANS (5 μM) in 100 mM KCl, 100 mM MOPS (pH 7.0), 2 mM EGTA, 0.5 mM NaN\(_3\) with (circles) or without (triangles) 42 μM EMD 57033; b: after addition of 2 mM Mg\(^{2+}\)Cl\(_2\); c: after addition of 2 mM Mg\(^{2+}\)Cl\(_2\) to EMD 57033 apo-hcTnC; d: after addition of CaCl\(_2\) to pCa 4.3.

ANS-binding site(s), presumably in the COOH-terminal domain of hcTnC, and that additional bis-ANS-binding site(s), presumably in the NH\(_2\)-terminal domain, are induced by Ca\(^{2+}\) binding to the Ca\(^{2+}\)-specific site. The presence of 42 μM EMD 57033 in the titration (Fig. 6) caused a significant decrease of the magnitude of the steep rising phase of the pCa-bis-ANS fluorescence relation, with little effect on the second phase. An obvious interpretation of the observation is that EMD 57033 inhibited the binding of bis-ANS to the COOH-terminal domain of hcTnC. In the presence of EMD 57033, the Ca\(^{2+}\) affinities of the Ca\(^{2+}\)/Mg\(^{2+}\)- and Ca\(^{2+}\)-specific sites obtained from fitting the data with a two-term Hill equation were not significantly different from those in the absence of the drug (see legend for Fig. 6).

Fig. 7 shows the results of experiments in which the fluorescence of bis-ANS was titrated with EMD 57033 in the absence of hcTnC. In the presence of 2 mM Mg\(^{2+}\) and absence of Ca\(^{2+}\), EMD 57033 (up to 50 μM) had little or no effect on the fluorescence. This was expected since there was little binding of EMD 57033 to cTnC under such conditions (see Figs. 2 and 3). At pCa 6.7, EMD 57033 caused a concentration-dependent decrease of bis-ANS fluorescence. At pCa 6.7, a majority of cTnC molecules should be in the form of 2 Ca\(^{2+}\)/cTnC since the Ca\(^{2+}\)/Mg\(^{2+}\)-sites should be largely saturated by Ca\(^{2+}\), while the Ca\(^{2+}\)-specific site should be predominantly free of bound Ca\(^{2+}\). The effect of EMD 57033 at pCa 6.7 probably resulted from interference by EMD 57033 of bis-ANS binding to the COOH-terminal hydrophobic patch of cTnC. At pCa 4.3, where all the three Ca\(^{2+}\)-binding sites of cTnC were saturated by Ca\(^{2+}\), the relationship between [EMD 57033] and the fluorescence change was very similar to that observed at pCa 6.7. The double-reciprocal plots of the titration data (1/ΔF versus 1/[EMD 57033]) at pCa 6.7 and 4.3 were linear. In principle, the EMD 57033-induced bis-ANS fluorescence changes at pCa 6.7 or 4.3 (Fig. 7, A and B) could result either from 1) displacement of bis-ANS from
Fig. 7. Titration of bis-ANS fluorescence by EMD 57033 in the presence of hCTnC. A, bis-ANS fluorescence as a function of [EMD 57033]. The common conditions for the three titrations were 5 μM bis-ANS, 5 μM hCTnC, 100 mM KCl, 100 mM MOPS (pH 7.0), 2 mM EGTA, 0.5 mM azide. The unique conditions for the titrations were, respectively, 2 mM Mg²⁺ (circles), pCa 6.7 (triangles), and pCa 4.3 (squares). B, the data at pCa 4.3 and 6.7 (from Fig. 6A) replotted as double-reciprocal plots. The symbols are the same as in A. The lines represent the least square fits.

The effects of EMD 57033 on Tyr fluorescence of hCTnC and on the interaction of bis-ANS with cTnC (Figs. 2, 4, 6, and 7) revealed a close link between Ca²⁺ binding to the Ca²⁺/Mg²⁺ sites of hCTnC and EMD 57033 binding. The data indicate that the EMD 57033-binding site is induced by Ca²⁺ binding to the Ca²⁺/Mg²⁺ sites and is thus likely to reside in the COOH-terminal domain of hCTnC. The drug probably binds directly to the COOH-terminal hydrophobic pocket, which is exposed upon Ca²⁺ binding to the Ca²⁺/Mg²⁺ sites. In view of the substantial quenching of Tyr fluorescence of hCTnC induced by EMD 57033, one may further speculate that the drug-binding site is in the vicinity of Tyr-111 and Tyr-150. hCTnC contains 3 Tyr residues: 1 (Tyr-5) in the NH₂-terminal domain and 2 (Tyr-111 and Tyr-150) in the COOH-terminal domain (31). Tyr-111 and Tyr-150 are, respectively, in the 7th position of the Ca²⁺-binding loop III and the 10th position of the Ca²⁺-binding loop IV. In the crystallographic structure of skeletal TnC whose Ca²⁺/Mg²⁺ sites were occupied by Ca²⁺, loops III and IV form a short antiparallel β-sheet (12, 13). NMR examination of Ca²⁺-saturated cTnC in solution showed that the β-sheet is formed by Tyr-111–Ile-112–Asp-113 and Arg-147–Ile-148–Asp-149 (34). Thus Tyr-111 and Tyr-150 are closely positioned in space, and both may be in proximity to the bound EMD 57033.

Although our data strongly suggest that EMD 57033, at the concentrations studied here, primarily binds to the COOH-terminal domain of cTnC, it cannot be ruled out that EMD 57033, at higher concentrations, also binds to the NH₂-terminal domain of cTnC. To resolve the issue, it would be necessary to determine accurately the stoichiometry of EMD 57033 binding. In principle, this may be achieved by conducting the dialysis-based direct binding assay as described above (Fig. 3) over a much wider range of drug concentrations.

We found that the binding of EMD 57033 to the COOH-terminal domain of cTnC occurred only when the Ca²⁺/Mg²⁺ sites were occupied by Ca²⁺, but not when they were occupied by Mg²⁺. The observation is, to our knowledge, the first demonstration that the Ca²⁺-saturated and Mg²⁺-saturated COOH-terminal domain of cTnC exhibit substantially different affinities for a non-peptide organic ligand. The implication of the finding is that the region of the COOH-terminal domain involved in anchoring EMD 57033 assumes different structures depending on whether sites III and IV are occupied by Ca²⁺ or Mg²⁺. The finding is in line with previous studies, which revealed conformational differences between the 2 Ca²⁺ and 2 Mg²⁺ states of troponin C using diverse techniques including circular dichroism (35), proton magnetic resonance (36–38), Fourier transform infrared spectroscopy (39), and x-ray powder diffraction (40). Using a STnC mutant containing a tryptophan in position 154, Chandra et al. (24) showed that the structural difference between the 2 Ca²⁺ and 2 Mg²⁺ states may have functional significance. Although several TnI inhib-
ity peptides bound to the TnC mutant in the presence of either Ca$^{2+}$ or Mg$^{2+}$, it was only in the presence of Ca$^{2+}$ that the binding of the peptides induced significant changes of the Trp fluorescence of the TnC mutant (24). The findings suggest that the region of COOH-terminal domain of TnC involved in interaction with the inhibitory region of TnI is not the same structurally for the 2 Ca$^{2+}$ and 2 Mg$^{2+}$ states of TnC.

The structural differences between the 2 Ca$^{2+}$- and 2 Mg$^{2+}$-states of TnC were also revealed, in the present study, by the characteristics of the interaction between hTnC and bis-ANS, a hydrophobic probe (Figs. 5 and 6). While Ca$^{2+}$ binding to the Ca$^{2+}$/Mg$^{2+}$-sites induced bis-ANS-binding site in the COOH-terminal domain of TnC, Mg$^{2+}$ binding to the Ca$^{2+}$/Mg$^{2+}$-sites failed to do so. The finding implies strongly that when Ca$^{2+}$/Mg$^{2+}$-sites are filled by Mg$^{2+}$, the COOH-terminal hydrophobic pocket is in a state substantially different from the state it assumes when the Ca$^{2+}$/Mg$^{2+}$-sites are occupied by Ca$^{2+}$.

Assuming that the COOH-terminal EMD 57033-binding site of TnC remains accessible when TnC is an integrative part of the myofibril in a myocyte, the occupancy of the drug-binding site by EMD 57033 would be directly proportional to the Ca$^{2+}$ occupancy of the Ca$^{2+}$/Mg$^{2+}$-sites of TnC. Robertson et al. (41) have modeled the time courses of Ca$^{2+}$-exchange with cardiac troponin under ionic conditions mimicking that in a beating myocyte. They showed that when a quiescent cardiac myocyte, where the [Mg$^{2+}$]i is set at 2.5 mM, is subjected to a train of concentration stimuli of 0.33 s, the steady-state Ca$^{2+}$-occu

Acknowledgments—We are grateful to Drs. R. J. Solan, S. M. Krause, and N. Beir for critical reading of earlier versions of this paper.

REFERENCES

1. Jonas, R., Klockow, M., and Lues, I. (1992) Bioorg. Med. Chem. Lett. 2, 1059–1062
2. Lues, I., Beier, N., Jonas, R., Klockow, M., and Haeusler, G. J. (1993) Cardiovasc. Pharmacol. 21, 807–892
3. Gambassi, G., and Carbonin, P. (1991) Acta Med. Rom. 29, 359–369
4. Gambassi, G., Capecchi, M., Klockow, M., and Lakatta, E. G. (1993) Am. J. Physiol. 264, H728–H738
5. Lee, J. A., Shah, N., White, J., and Orchard, C. H. (1993) Circ. Res. 73, 1212–1214
6. Solano, R. J., Gambassi, G., Washaw, D. M., Keller, M. R., Spurgeon, H. A., Beier, N., and Lakatta, E. G. (1993) Circ. Res. 73, 61–70
7. White, J., Lee, J. A., Shah, N., and Orchard, C. H. (1993) Circ. Res. 73, 122–127
8. Collins, J. H., Greaser, M. L., Potter, J. D., and Horn, M. J. (1993) J. Biol. Chem. 258, 6356–6362
9. Wilkinson, J. M. (1980) Eur. J. Biochem. 103, 179–188
10. van Eerd, J. P., and Takakushi, K. (1975) Biochem. Biophys. Res. Commun. 64, 122–127
Binding of Cardiac Troponin C and Ca^{2+} Sensitizer EMD 57033

20. Zot, H. G., and Potter, J. D. (1982) J. Biol. Chem. 257, 7678–7683
21. Negele, J. C., Dotson, D. G., Liu, W., Sweeney, H. L., and Putkey, J. A. (1992) J. Biol. Chem. 267, 825–831
22. Leszyk, J., Collins, J. H., Leavis, P. C., and Tao, T. (1987) Biochemistry 26, 7042–7047
23. Ngai, S.-M., Sönninghen, F. D., and Hodges, R. S. (1994) J. Biol. Chem. 269, 2165–2172
24. Chandra, M., McCubbin, W. D., Oikawa, K., Kay, C. M., and Smillie, L. B. (1994) Biochemistry 33, 2961–2969
25. Striynadka, N. C. J., and James, M. N. G. (1990) Proteins 7, 234–248
26. Herzberg, O., Moutl, J., and James, M. N. G. (1986) J. Biol. Chem. 261, 2638–2644
27. Drabikowski, W., Dalgarno, D. C., Levine, B. A., Gergely, J., Grabarek, Z., and Leavis, P. C. (1985) Eur. J. Biochem. 151, 17–28
28. MacLachlan, L. K., Reid, D. G., Mitchell, R. C., Salter, C. J., and Smith, S. J. (1990) J. Biol. Chem. 265, 9764–9770
29. Dalgarno, D. C., Klevecz, R. E., Levine, B. A., Scott, G. M. M., Williams, R. J. P., Gergely, J., Grabarek, Z., Leavis, P. C., Grand, R. J. A., and Drabikowski, W. (1984) Biochim. Biophys. Acta 791, 164–172
30. Striynadka, N. C. J., and James, M. N. G. (1985) Proteins 3, 1–17
31. Gahlmann, R., Wade, R., Gunning, P., and Kedes, L. (1988) J. Mol. Biol. 201, 379–391
32. Birdsall, B., King, R. W., Wheeler, M. R., Lewis, C. A., Jr., Goode, S. R., Dunlap, R. B., and Roberts, G. C. (1983) Anal. Biochem. 132, 353–361
33. Bradford, M. M. (1976) Annu. Biochem. 72, 248–254
34. Krudy, G. A., Brito, R. M. M., Putkey, J. A., and Rosevear, P. R. (1992) Biochemistry 31, 1595–1602
35. Kawasaki, Y., and van Eerd, J. P. (1972) Biochem. Biophys. Res. Commun. 47, 859
36. Drabikowski, W., Dalgarno, D. C., Levine, B. A., Gergely, J., Grabarek, Z., and Leavis, P. C. (1985) Eur. J. Biochem. 151, 17–28
37. Tsuda, S., Hasegawa, Y., Yoshida, M., Yagi, K., and Hikichi, K. (1988) Biochemistry 27, 4120–4126
38. Tsuda, S., Ogura, K., Hasegawa, Y., Yagi, K., and Hikichi, K. (1990) Biochemistry 29, 4951–4958
39. Heidorn, D. B., and Trewella, J. (1988) Biochemistry 27, 909–915
40. Trewella, J., Liddell, W. K., Heidorn, D. B., and Striynadka, N. C. J. (1989) Biochemistry 28, 1294–1301
41. Robertson, S. P., Johnson, J. D., and Potter, J. D. (1981) Biophys. J. 34, 559–569
42. Marban, E., Rink, T. J., Tsien, R. W., and Tsien, R. Y. (1980) Nature 286, 845–850
43. Pan, B.-S., Palmiter, K. A., Plonczynski, M., and Solaro, R. J. (1990) J. Mol. Cell. Cardiol. 22, 1117–1124
44. Pan, B.-S., and Solaro, R. J. (1987) J. Biol. Chem. 262, 7839–7849
45. Nankervis, R., Lues, I., and Brown, L. (1994) J. Cardiovasc. Pharmacol. 24, 612–617
46. Strauss, J. D., Zeugner, C., and Ruegg, J. C. (1992) Eur. J. Pharmacol. 227, 437–441