A Comparative Study of the Interactions of Synthetic Peptides of the Skeletal and Cardiac Troponin I Inhibitory Region with Skeletal and Cardiac Troponin C†

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ABSTRACT: The cardiac and skeletal TnI inhibitory regions have identical sequences except at position 110 which contains Pro in the skeletal sequence and Thr in the cardiac sequence. The effect of the synthetic TnI inhibitory peptides [skeletal TnI peptide (104–115), cardiac TnI peptide (137–148), and a single Gly-substituted analogue at position 110] on the secondary structure of skeletal and cardiac TnC was investigated. The biphasic increases in ellipticity and tyrosine fluorescence were analyzed to determine the Ca^{2+} binding constants for the high- and low-affinity Ca^{2+} binding sites of TnC. Importantly, the skeletal and cardiac TnI peptides altered Ca^{2+} binding at the low-affinity sites of TnC, but the magnitude and direction of the pCa shifts depended on whether the peptides were bound to skeletal or cardiac TnC. For example, binding of skeletal TnI peptide to skeletal TnC (monitored by CD) caused a pCa shift of +0.30 unit such that a lower Ca^{2+} concentration was required to fill sites I and II, while binding of this peptide to cardiac TnC caused a pCa shift of −0.35 unit such that a higher Ca^{2+} concentration was required to fill site II. This is the first report of the alteration at the low-affinity regulatory sites (located in the N-terminal domain) by the skeletal TnI inhibitory peptide, even though the primary peptide binding site is located in the C-terminal domain of TnC, a finding which strongly indicates that there is communication between the two halves of the TnC molecule. As well, the amino acid residue at position 110 of the skeletal TnI sequence is critical, since the Gly-substituted analogue mainly affects the high-affinity Ca^{2+} binding sites. It is clear that the single amino acid difference in the TnI inhibitory sequence is partly responsible for the biological differences between skeletal and cardiac muscle.

Calcium activation of the skeletal and cardiac contractile systems involves Ca^{2+}-induced conformational changes in TnC. These conformational changes include the induction of α-helices, resulting in the exposure of the binding interface(s) for troponin I (TnI).[1] The physiological differences in the action of skeletal and cardiac muscle may be explained by differences in the amino acid sequences of skeleal and cardiac TnI and TnC, in particular, at the binding interfaces between these highly conserved proteins. These differences could alter the TnC–TnI interaction and hence affect muscle regulation. Skeletal and cardiac TnC have 65% amino acid homology with the majority of substitutions occurring in the first 40 residues. Cardiac TnC has eight amino acid differences located in Ca^{2+} binding site I which results in this binding site being nonfunctional. Hence, skeletal TnC binds four Ca^{2+} ions, two at the high-affinity sites (III and IV) and two at the low-affinity sites (I and II), while cardiac TnC binds three Ca^{2+} ions, two at the high-affinity sites (III and IV) and one at the single low-affinity site (site II). The high-affinity sites III and IV are located in the carboxy-terminal half of TnC and bind Ca^{2+} with an association constant of 2×10^7 and 3×10^8 M^{-1} for skeletal TnC and cardiac TnC, respectively (Potter & Gergely 1975; Johnson & Potter, 1978; Holroyde et al., 1980). Sites I and II in the amino-terminal half of the molecule bind Ca^{2+} with an association constant of 3×10^5 and 2×10^6 M^{-1} for skeletal and cardiac TnC, respectively. Although the low-affinity sites of skeletal and cardiac TnC are the regulatory sites which trigger muscle contraction, the high-affinity sites may also be involved in this process (Van Eyk et al., 1986). As well, skeletal TnI is a more effective inhibitor than cardiac TnI in both cardiac and skeletal ATPase assays (Talbot & Hodges, 1981a; Hincke et al., 1977). In addition, cardiac TnC is less effective than skeletal TnC at releasing the inhibition induced by either cardiac or skeletal TnI.

The minimum sequence of skeletal TnI required to inhibit the S1 ATPase activity is residues 104–115 (Talbot & Hodges, 1979, 1981b). The cardiac and skeletal TnI inhibitory sequences are identical except for a single-residue substitution of a Pro in the skeletal sequence at position 110 to a Thr in the cardiac sequence. The skeletal TnI peptide was a better inhibitor of the skeletal or cardiac actomyosin ATPase activity than the cardiac TnI analogue (Talbot & Hodges, 1981a). The skeletal and cardiac TnI inhibitory peptides equally inhibit force development in skeletal and cardiac muscle fibers, but the cardiac peptide is less efficient, requiring a 2–3-fold higher concentration to reach maximum inhibition (Rüegg et al., 1989, 1991). Previous work showed that the skeletal TnI inhibitory peptide can compete with TnI for TnC (Ruegg et al., 1989). In saturating Ca^{2+}, sIp dissociation constants for TnC range from 5×10^{-5} to 1×10^{-6} M (Cachia et al., 1983a; Campbell & Sykes, 1991; Malencik & Anderson, 1984). The binding of skeletal TnI inhibitory peptide 104–115 altered the secondary structure of skeletal TnC by increasing the α-helical

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[1] Abbreviations: sIp, skeletal TnI peptide (residues 104–115); cIp, cardiac TnI peptide (residues 137–148); Gly 110, Gly-substituted analogue at position 110 of the skeletal TnI peptide (104–115) which is equivalent to Gly 143 in the cardiac TnI peptide sequence 137–148; Boc, *tert*-butyloxycarbonyl; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; CD, circular dichroism; F, fluorescence; Tyr, tyrosine; sTnC, skeletal troponin C; cTnC, cardiac troponin C; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; HR, number of helical residues.

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Table I: Amino Acid Sequence of TnI Inhibitory Peptides

peptide name[a]	abbreviated name	sequence[b]
skeletal TnI Ac(104–115) amide [cardiac TnI Pro-143 Ac(137–148) amide]	sIp	Ac-G-K-F-K-R-P-P*-L-R-R-V-R-amide
cardiac TnI Ac(137–148) amide [skeletal TnI Thr-110 Ac(104–115) amide]	cIp	Ac-G-K-F-K-R-P-T*-L-R-R-V-R-amide
skeletal TnI Tly-110 Ac(104–115) amide [cardiac TnI Gly-145 Ac(139–148) amide]	Gly 110	Ac-G-K-F-K-R-P-G*-L-R-R-V-R-amide

[a,b] Since the native sequences of skeletal and cardiac TnI in this region differ only at one position as denoted by the asterisk, the nomenclature for the peptides can be given in terms of the skeletal sequence or cardiac sequence. The alternate nomenclature is given in brackets.

content and producing a small pCa^{2+} shift. In this study, the effects of the TnI inhibitory peptides (skeletal, cardiac, and a Gly analogue, Gly 110) on the secondary structure of skeletal and cardiac TnC were investigated. Circular dichroism and Tyr fluorescence were used to determine differences in the response of TnC to the three inhibitory peptides and their effect on Ca^{2+} binding.

Materials and Methods

Peptide Synthesis and Purification. The three TnI inhibitory peptides (Table I) were synthesized by using the general procedures for solid-phase peptide synthesis on either a Beckman 990 synthesizer (Berkeley, CA) or an Applied Biosystems 430A peptide synthesizer (Foster City, CA). The co-poly(styrene 1% divinylbenzene)benzhydrylamine hydrochloride resin (Institut Armand Frappier, Laval, Quebec) was substituted to 0.8 mmol of Boc-amino acid/g of resin. All α-amino groups of the amino acids used were protected with the Boc group. The following side-chain blocking groups were used: Arg(Tosyl) and Lys(2-CIZ). All amino-terminal residues of the TnI peptides were acetylated by treatment for 30 min with acetic anhydride/toluene/pyridine (1:3:3 v/v). The programs used for synthesis and HF cleavage were previously described by Hodges et al. (1988).

The crude peptides were purified on an HPLC system comprised of a Spectra Physics SP8700 solvent delivery system and Kratos SF7697 detector. Reversed-phase chromatography was carried out on an analytical Aquapore RP-300 column (4.6 mm i.d. × 220 mm, Pierce, Rockford, IL), by employing a linear AB gradient (0.1% B/min) at 1 mL/min, where solvent A was 0.05% aqueous TFA and solvent B was 0.05% TFA in acetonitrile. The sample loads varied between 20 and 50 mg per run (Mant et al., 1987; Parker et al., 1987). One-minute fractions were collected. Analytical runs were carried out by using the reversed-phase column with a gradient rate of 1% B/min, in order to identify the fractions containing the desired peptide. Fractions containing the pure peptide were pooled and lyophilized.

Preparation of Circular Dichroism and Fluorescence Samples. Rabbit skeletal TnC and bovine cardiac TnC were prepared by the method of Chong and Hodges (1982) and Strapans et al. (1972), respectively. TnC was further purified by using reversed-phase chromatography as described by Ingraham and Hodges (1988). The purity of the proteins was checked by SDS–urea–polyacrylamide gel electrophoresis (Chong et al., 1983). It is critical that the TnC be Ca^{2+}-free to avoid erroneously high ellipticity values for the minus-Ca^{2+} state which would affect interruption of the Ca^{2+}-dependent induced helical structure. In the present study, extreme care was taken to remove Ca^{2+} by initially denaturing TnC in 6 M guanidine hydrochloride in the presence of excess EDTA and DTT, and subsequent dialysis of the protein against a buffer consisting of 20 mM MOPS, 1 mM EGTA, and 50 mM KCl, pH 7.11. During the final dialysis, 1 mM DTT was added to the dialysis buffer. The protein was centrifuged for 10 min at 15000 rpm and then filtered through a low protein binding Ca^{2+}-free 0.45-μm Millipore filter. The purified peptides were dissolved in water, and the pH of the sample was adjusted to that of protein solution. The peptide and protein concentrations were determined by amino acid analysis using a Dionex 502 amino acid analyzer.

Circular Dichroism Spectra Determination. The CD experiments were conducted at 25 °C on a JASCO J-500c spectropolarimeter fitted with a thermostated cell holder and interfaced with a JASCO DP-500N data processor. Experiments were carried out in 1000-μL volumes with Ca^{2+} aliquots added to a total volume of 1030 μL. The concentrations of the cardiac and skeletal TnC varied between 22 and 30 μM in the absence and presence of 1 molar equiv of peptide.

Data were expressed as the observed ellipticity, θ (millidegrees, mdeg), and the error in the observed value was ±0.2 mdeg. The ellipticity values at 222 nm were converted to mean residue ellipticity values by using the following equation: $[\theta]_{222} = \theta M_r/100LC$ where θ = the observed ellipticity (degrees), L = the cell path length (decimeters), C the concentration in milligrams per milliliter, and M_r = the mean residue molecular weight calculated from the appropriate amino acid sequences. The theoretical curves were calculated by the summation of $[\theta]^{222}_{TnC} + [\theta]^{222}_{peptide}$ at each Ca^{2+} concentration. The fraction helix, f_H, was estimated by using the empirical equation obtained by Chang et al. (1978): $f_H = [\theta]_{222}/[[\theta]^{\infty}_{H}(1 - k/n)]$ where $[\theta]^{\infty}_{H}$ = the mean molar residue ellipticity for an infinite helix (−37400°), k = the chain length dependence factor (2.5), and n = the number of residues in a typical helix (9). The number of helical residues was then calculated by multiplying f_H by the total number of amino acid residues in the protein or protein–peptide complex. The error in the number of helical residues in TnC or the TnC–peptide complex, based on an error of ±0.2 mdeg of the observed ellipticity, is between 0 and 2 residues. Therefore, any change in the number of helical residues (HR) greater than 2 residues is significant.

Fluorescence Determination. The intrinsic Tyr fluorescence was determined by using a Perkin-Elmer MPF-44B fluorescence spectrophotometer equipped with the DCSU-2-corrected spectra accessory which allows for automatic subtraction of fluorescence due to solvent. The instrument was operated in the ratio mode, and thermostated cells were maintained at 20 °C. Detection of fluorescence was effected at 90° to the excitation beam. The emission and excitation wavelengths were 301 and 277 nm, respectively (Lakowicz, 1983). The initial volume of sample was 1000 μL, and Ca^{2+} aliquots were added to the final volume of 1030 μL. The concentrations of cardiac and skeletal TnC varied between 11 and 15 μM, to which 1 mol equiv of peptide was added.

Determination of Ca^{2+} Binding Constants and Hill Coefficients. The experimental data were analyzed by using a computer software program designed to determine biphasic binding curves (program kindly provided by Dr. B. D. Sykes, University of Alberta). The fitting program analyzed data in the form of two Hill coefficients:

$$\frac{[Ca^{2+}]^{n1}}{[Ca^{2+}]^{n1} + K_{d_1}{}^{n1}} \text{ and } \frac{[Ca^{2+}]^{n2}}{[Ca^{2+}]^{n2} + K_{d_2}{}^{n2}}$$

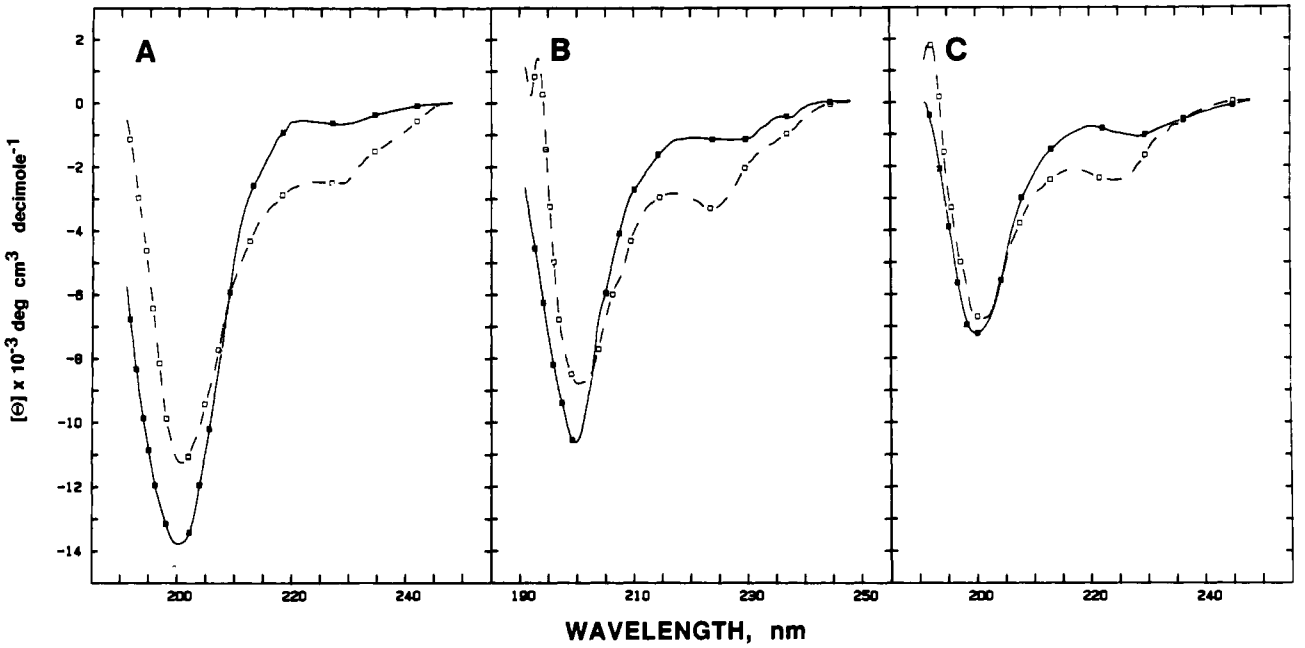

FIGURE 1: Circular dichroism spectra of TnI peptides in the absence and presence of 50% TFE. Spectra in the absence (--) and presence (—) of 50% TFE of sIp (panel A), cIp (panel B), and Gly 110 (panel C). Assay buffer: 20 mM MOPS, 50 mM KCl, and 0.1 mM EGTA, pH 7.2. Concentrations of peptide were 1 mM to which TFE was added to 50% v/v.

where K_{d_1} and $n1$ are the apparent binding constant and Hill coefficient for the low-affinity binding sites, respectively, and K_{d_2} and $n2$ are the apparent binding constant and Hill coefficient for the high-affinity Ca^{2+} binding sites, respectively. The χ value, which measures the deviation of the experimental values from the values of the best-fit curve, ranged from 0.92 to 2.05, with an average value of 1.22. Therefore, the accuracy of the experimental data is such that any difference in the calculated binding constants for the low- and high-affinity sites in the absence versus the presence of a peptide greater than 0.10 represents the effect of peptide binding on the Ca^{2+} affinity.

Results

Secondary Structure of the TnI Peptides in Solution. The CD spectra (190–250 nm) of the three TnI peptides in the absence and presence of a 50% solution of the helix-inducing solvent trifluoroethanol (TFE) were carried out in order to determine whether the peptides possess secondary structure in benign solution and whether secondary structure could be induced (Figure 1). In the absence of 50% TFE, the three peptides produced CD spectra that were typical of random coils. In the presence of 50% TFE, the CD spectra of the peptides showed a minor increase in negative ellipticity at 222 nm of approximately −2000°. A completely α-helical peptide of 12 residues would have a negative ellipticity of approximately −27 000°. Interestingly, in the absence and presence of TFE, the CD spectra of the three peptides cannot be superimposed on one another, indicating they do not have the same conformation in solution. In addition, the ellipticity ratio (200/222 nm) in the presence of TFE varied for the three peptides sIp, cIp, and Gly-110 with values of 4.3, 2.8, and 2.8, respectively.

Effect of the TnI Peptides on the Secondary Structure of Skeletal and Cardiac TnC. In the absence of Ca^{2+}, skeletal TnC has less helical residues (68) than cardiac TnC (74), while both have approximately the same number of helical residues in the presence of Ca^{2+} (108 vs 106 residues, respectively). Hence, Ca^{2+} binding to skeletal TnC induces an additional 40 helical residues compared with an additional 32 residues in cardiac TnC. This difference would suggest that in the absence of Ca^{2+} cardiac TnC contains more structure than skeletal TnC and, as well, undergoes a smaller Ca^{2+}-induced structural change. When Ca^{2+} binds to skeletal TnC, some of the α-helical residues which are induced are probably equivalent to helical residues which are already preformed in cardiac TnC in the absence of Ca^{2+}.

Since the three TnI peptides did not contain significant secondary structure even in the presence of a helix-inducing solvent, one can assume that the interaction of the peptides with TnC does not induce helix in any of the peptides. Therefore, any change in ellipticity must be due to a change in the number of helical residues in TnC. The number of additional helical residues induced by Ca^{2+} and the peptides in both skeletal and cardiac TnC is listed in Table II. If the number of additional helical residues induced by Ca^{2+} binding is the same in the presence or absence of peptide, then any additional helix that is induced upon peptide binding is unique

Table II: Number of Additional α-Helical Residues Induced in Skeletal and Cardiac TnC by the TnI Peptides and Ca^{2+}

presence or absence of metal ion	peptide[a] added	ΔHR[b] sTnC	ΔHR[b] cTnC
$-Ca^{2+}$	sIp	+15	−4
$+Ca^{2+}$		+15	−3
$-Ca^{2+}$	cIp	+3	+5
$+Ca^{2+}$		+2	+3
$-Ca^{2+}$	Gly 110	0	+7
$+Ca^{2+}$		0	+1
presence or absence of peptide	**metal ion added**	**ΔHR[b] sTnC**	**ΔHR[b] cTnC**
−sIp	Ca^{2+}	40	32
+sIp		40	33
−cIp	Ca^{2+}	41	33
+cIp		40	30
−Gly 110	Ca^{2+}	39	32
+Gly 110		39	27

[a] A 1:1 mole ratio of TnI peptide was added to TnC. [b] ΔHR is the difference in the number of helical residues indiced by addition of peptide (upper section) or Ca^{2+} (lower section) in either skeletal or cardiac troponin C, sTnC or cTnC, respectively. Any change in the number of additional helical residues (ΔHR) induced greater than two residues is significant (see Materials and Methods).

and different from that which is induced by Ca^{2+}. On the other hand, if there is a difference in the number of additional helical residues induced by Ca^{2+} binding in the absence and presence of peptide, then some of the peptide-induced helix includes at least some residues that would normally become helical when Ca^{2+} binds.

Skeletal TnI peptide (sIp) had the largest effect on the helical content in skeletal TnC. Binding of sIp resulted in the formation of an additional 15 helical residues in skeletal TnC in both the absence and presence of Ca^{2+} (Table II). The 15 helical residues induced by sIp binding are in addition or over and above that structure induced by Ca^{2+} (40 helical residues were induced by Ca^{2+} in the absence and presence of peptide, Table II). In other words, sIp causes unique structural changes in TnC. The binding of cardiac TnI peptide (cIp) to skeletal TnC had no effect on the number of helical residues induced upon Ca^{2+} binding (41 and 40 residues were induced in the absence and presence of cIp). As well, binding of cIp had only a minor effect on the helical content of TnC (three and two residues in the absence and presence of Ca^{2+}, respectively). Therefore, sIp induces considerably more α-helix than cIp (compare 15 vs 2–3 residues). Gly 110 did not alter the helical content of skeletal TnC (Table II), implying either Gly 110 does not bind TnC or the interaction does not cause any change in the helical content of TnC. Since Gly 110 binds to a skeletal TnC affinity column in the presence of Mg^{2+} or Ca^{2+}, almost as tightly as sIp (Van Eyk & Hodges, 1988), apparently the Gly 110 peptide is able to bind TnC but is unable to alter the secondary structure of skeletal TnC. As well, it is shown in a later section that Gly 110 alters the Ca^{2+} affinity of TnC.

The effects of the three TnI peptides on the helical content of cardiac TnC are different from those observed with skeletal TnC. For example, Gly 110 which caused no change in the helical content of skeletal TnC induced considerable α-helical structure in cardiac TnC in the absence of Ca^{2+}. The difference of 6 additional helical residues induced by Gly 110 in the absence of Ca^{2+} versus the presence of Ca^{2+} (7 residues minus 1 residue, Table II) is similar to the difference of 5 helical residues induced when Ca^{2+} binds in the absence versus the presence of Gly 110 (32 minus 27 residues). This indicates that the majority of structure formed upon Gly 110 binding is the same structure formed when Ca^{2+} binds. Cardiac TnI peptide induced an additional 5 and 3 helical residues in the absence and presence of Ca^{2+}, respectively, while Ca^{2+} binding induced 33 helical residues compared to 30 in the absence and presence of the peptide, respectively. The difference in the number of helical residues induced by cIp is similar to the difference in the number of helical residues induced by Ca^{2+}. This indicates that part of the structure formed upon cIp binding is the same structure formed when Ca^{2+} binds. However, the effect of cIp is minor (3–5 residues) compared to the effect of Ca^{2+} binding (30–33 residues). Surprisingly, skeletal TnI peptide, which caused a large increase in helical structure in skeletal TnC (+15 residues), actually decreased the α-helical content of cardiac TnC (−4 and −3 residues in the absence and presence of Ca^{2+}). Since sIp did not alter the number of helical residues induced upon Ca^{2+} binding, the majority of the structural changes induced by binding of sIp are not residues which are involved in the Ca^{2+}-dependent helix. The majority of the structural changes induced by sIp in skeletal TnC (+15 helical residues) and cardiac TnC (−3 helical residues) do not involve residues which are involved in Ca^{2+} binding.

Ca^{2+} Titrations of the TnI Peptide–TnC Complexes. In order to determine whether binding of the inhibitory peptides affected the Ca^{2+} affinity of skeletal and cardiac TnC, pCa curves were obtained from Ca^{2+} titration of TnC and TnC–peptide complexes. Two methods, CD and Tyr fluorescence, were used to monitor the Ca^{2+}-dependent changes. Since there is no Tyr in the amino acid sequences of the TnI inhibitory peptides, any change in the Tyr fluorescence on peptide or Ca^{2+} binding is due to changes in the environment of one or more Tyr residues located in TnC. In skeletal TnC, there are two Tyr residues at positions 10 and 109, and in cardiac TnC, there are three Tyr at positions 5, 111, and 150. Residue 109 of skeletal TnC and residue 111 of cardiac TnC are in the Ca^{2+} binding loop of site III. Previously, Johnson and Potter (1978) observed a biphasic increase in negative ellipticity (measured by CD) and Tyr fluorescence of skeletal TnC with increasing Ca^{2+} concentrations. The biphasic response was interpreted in terms of distinct changes in secondary structure in TnC upon Ca^{2+} binding to the high- and then low-affinity Ca^{2+} binding sites. The approximate pCa values obtained in this study for skeletal TnC were 7.3 and 5.6 for the high- and low-affinity sites, respectively, and are in excellent agreement with the pCa values reported previously. In the present study, the effect of various TnI inhibitory peptides on the Ca^{2+}-dependent conformational changes and the effect on the pCa values of the high- and low-affinity sites of skeletal and cardiac TnC were examined (Figures 2 and 3).

Table III: Effect of TnI Peptides on the pCa Values for the Low- and High-Affinity Ca^{2+} Binding Sites of Skeletal and Cardiac TnC

		ΔpCa[b]			
		sTnC		cTnC	
method of determination[a]	peptide	low-affinity site(s)	high-affinity site(s)	low-affinity site(s)	high-affinity site(s)
CD	sIp	+0.30	NE[c]	−0.35	NE
	cIp	+0.30	NE	NE	NE
	Gly 110	Ne	+0.21	+0.14	+0.14
F	sIp	+0.26	NE	−0.52	NE
	cIp	−0.42	NE	+0.58	NE
	Gly 110	NE	+0.16	NE	+0.30

[a] CD stands for circular dichroism and *F* for Tyr fluorescence. [b] The change in pCa (ΔpCa) was determined from the difference between the pCa value of skeletal or cardiac TnC in the presence versus the absence of the peptide. A positive ΔpCa value indicates a leftward pCa shift while a negative ΔpCa value indicates a rightward shift. The average pCa values from multiple determinations for the low- and high-affinity sites are 5.6 and 7.3 for skeletal TnC, respectively, and for the low-affinity site and high-affinity sites of cTnC, 5.7 and 7.1, respectively. [c] NE denotes no effect. If the peptide altered the pCa value of sTnC or cTnC by 0.10 or less, it was considered to have no effect.

TnI Peptide Effects on Skeletal TnC. Binding of sIp to skeletal TnC resulted in a leftward pCa shift of +0.30 unit (Figure 2, panel A, and Table III) which can be specifically attributed to a change in Ca^{2+} affinity at the low-affinity Ca^{2+} binding sites I and II. Thus, a lower Ca^{2+} concentration is required to fill the low-affinity regulatory sites of TnC in the presence of sIp. Similarly, the change in Ca^{2+} affinity was also monitored by fluorescence. sIp caused a leftward pCa shift (+0.26 unit) at the low-affinity sites, indicating the environment of at least one Tyr residue in skeletal TnC was altered in the presence of sIp. As well, cIp caused a pCa shift at the low-affinity Ca^{2+} binding sites of skeletal TnC when monitored by either CD or fluoresence (Table III). However, the direction and magnitude of the changes at the low-affinity sites are different depending on which method is used. The pCa shift was leftward (+0.30 unit) when monitored by CD (indicating less Ca^{2+} was required to induce α-helix changes at the low-affinity sites), while the pCa shift was to the right

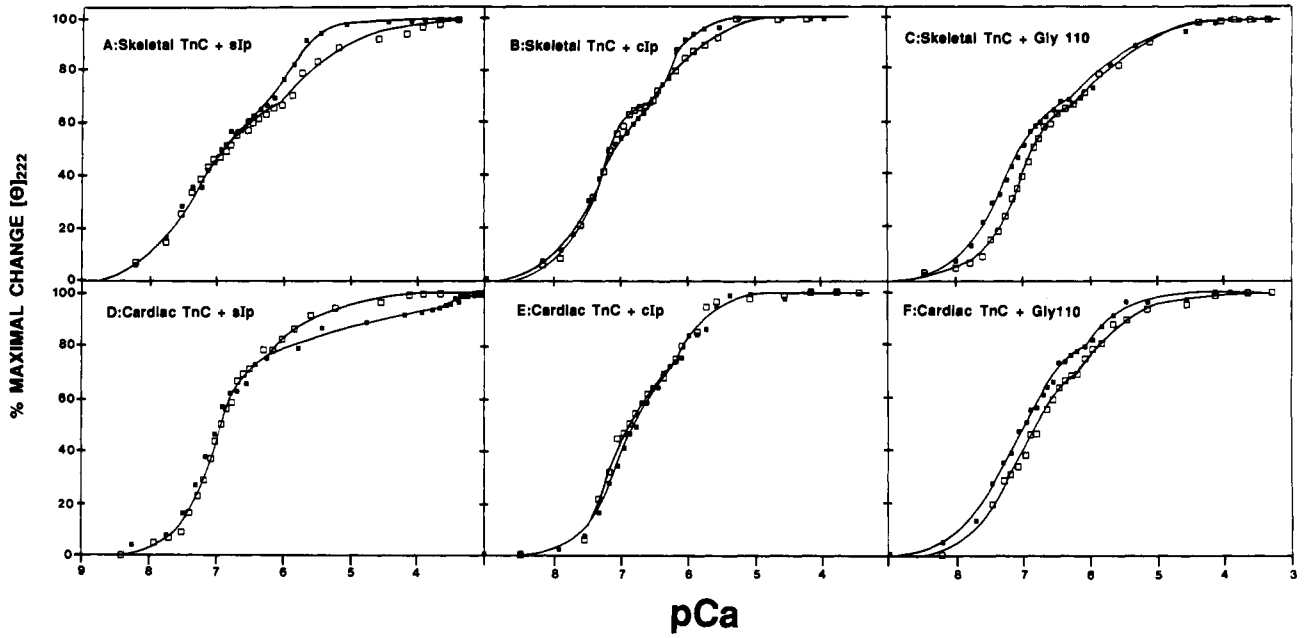

FIGURE 2: Effect of inhibitory peptides on Ca^{2+} binding to skeletal TnC and cardiac TnC monitored by circular dichroism. The percent maximal change in ellipticity of TnC in the absence (□) and presence (■) of sIp (panels A and D), cIp (panels B and E), and Gly 110 (panels C and F) is plotted versus pCa value. The top panels (A–C) show the effect of peptides on skeletal TnC while the bottom panels (D–F) show the effect of peptides on cardiac TnC. A 1:1 molar equivalent of various peptides and protein were used. The curves were calculated by a computer program that best fits the experimental data to a curve composed of two binding constants. Assays were performed as described under Materials and Methods.

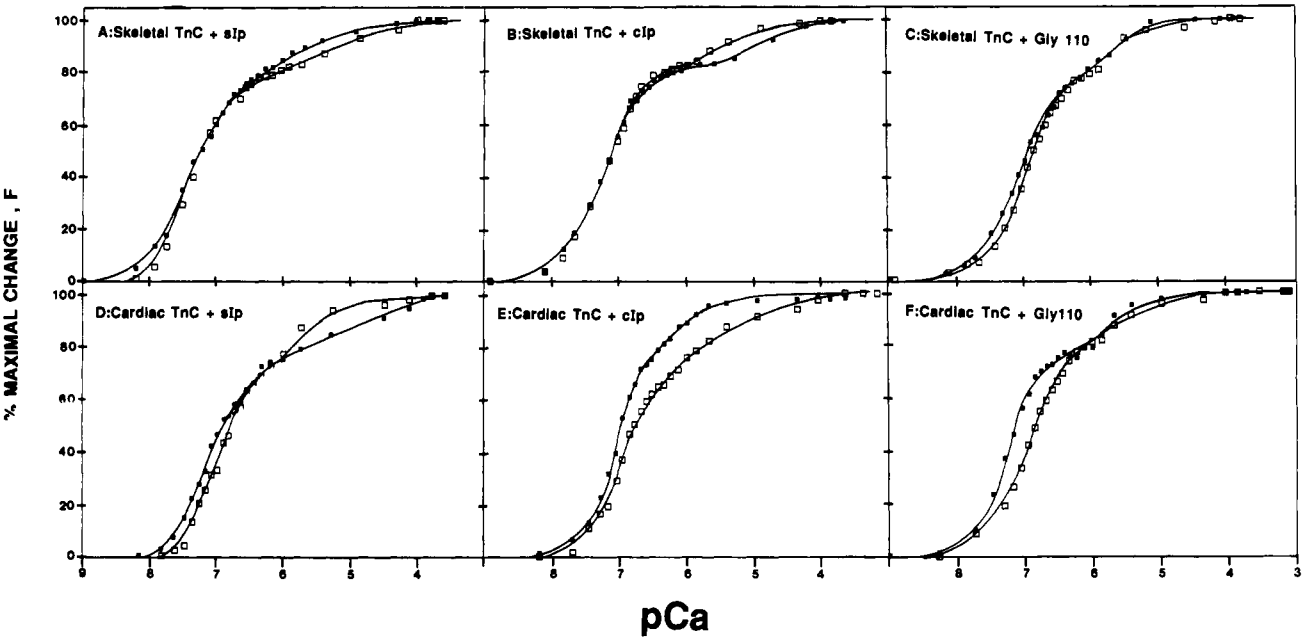

FIGURE 3: Effect of inhibitory peptides on Ca^{2+} binding to skeletal TnC and cardiac TnC monitored by Tyr fluorescence. The percent maximal change in fluorescence of TnC in the absence (□) and presence (■) of sIp (panels A and D), cIp (panels B and E), and Gly 110 (panels C and F) is plotted versus pCa values. The top panels (A–C) show the effect of peptides on skeletal TnC while the bottom panels (D–F) show the effect of peptides on cardiac TnC. A 1:1 molar equivalent of peptide to protein was used. The curves were calculated by a computer program that best fits the experimental data to a curve composed of two binding constants. Assays were performed as described under Materials and Methods.

by −0.42 unit when Tyr fluorescence was used (indicating a greater Ca^{2+} concentration was required, Table III). It would thus appear that the CD and fluorescence changes are independent of each other such that they monitor Ca^{2+} affinity at different low-affinity Ca^{2+} binding sites. Interestingly, Gly 110 caused a pCa shift at the high-affinity sites with no effect on the low-affinity sites of TnC as indicated by CD and fluorescent monitoring.

TnI Peptide Effects on Cardiac TnC. The binding of sIp to cardiac TnC resulted in a major pCa shift at the low-affinity Ca^{2+} binding sites (Figure 2, panel B). When monitored by CD or Tyr fluorescence, the pCa shift was rightward by −0.35 and −0.52 units, respectively (Table III). The interaction between cIp and cardiac TnC caused a large leftward shift (+0.58 unit) at the low-affinity site when detected by Tyr fluorescence, but when monitored by CD, there was no effect at either the high- or the low-affinity site. As discussed previously, the two methods can detect different structural changes in TnC (Figure 3, panels A and B). The effect of cIp binding on the Tyr environment is Ca^{2+}-dependent while the

effect of the peptide on α-helix structure is not sensitive to Ca^{2+}. Unlike the two native TnI peptides, Gly 110 caused pCa shifts at the high-affinity sites in cardiac TnC when the pCa curves were determined by a change in fluorescence (Figure 3, panel C). The leftward shift (+0.30 unit) indicates that a lower Ca^{2+} concentration is required to change the environment of the Tyr which is sensitive to Ca^{2+} binding at sites III and IV (Table III). On the other hand, CD detected only minor changes at the low- and high-affinity sites. Changes in the environment of Tyr residues in cardiac TnC are more sensitive to the structural changes in TnC upon peptide binding (especially cIp and Gly 110) than is the change in α-helical content as measured by CD.

Discussion

It is not surprising that the TnI peptides affect Ca^{2+} binding, since the native protein, skeletal TnI, increases the Ca^{2+} binding affinity of TnC (Wang & Cheung, 1985; Potter et al., 1976). It has been shown that the skeletal TnI peptide and other TnC binding molecules, like mastoparan and TFP, increase the Ca^{2+} binding affinity of skeletal TnC (Van Eyk & Hodges, 1987; Rüegg et al., 1989; Cachia et al., 1983b). However, in these previous studies, it was not possible to differentiate whether the TnC binding molecules were affecting the low- or high-affinity sites. The present work demonstrates for the first time that the binding of skeletal TnI peptide or cardiac TnI peptide affects Ca^{2+} affinity at the low-affinity Ca^{2+} binding sites of skeletal or cardiac TnC while the Gly 110 analogue primarily affects the high-affinity sites.

The changes in secondary structure which occur due to the binding of skeletal or cardiac TnI peptide to skeletal TnC affect the binding of Ca^{2+} at the low-affinity regulatory sites (located in the N-terminal domain). This is of interest since a vast amount of experimental evidence indicates that the major site of interaction between skeletal TnI peptide (104–115) and skeletal TnC is the N-terminal helix of Ca^{2+} binding site III (helix E) which is located in the C-domain of TnC [for a review, see Cachia et al. (1983b)]. The interaction between TnI and residues 89–100 (helix E) in the C-terminal domain of TnC is well documented (Chong & Hodges, 1981; Grabarek et al., 1981; Dalgarno et al., 1982; Leavis & Gergely, 1984; Tao et al., 1986; Lesysk et al., 1987). In fact, the CNBr fragment of TnC (residues 83–134) forms a Ca^{2+}-dependent complex with TnI and mimics the properties of the intact TnC protein in its ability to release TnI-mediated inhibition of actomyosin ATPase activity and in vitro phosphorylation of TnI at Ser-117 (Weeks & Perry, 1978). Proteolytic fragments and synthetic peptides of the inhibitory region of TnI (minimum sequence residues 104–115), which is adjacent to Ser 117, mimic the activity of TnI in their ability to inhibit actomyosin ATPase activity and bind TnC, resulting in the release of inhibition (Talbot & Hodges, 1979, 1981b; Katayama & Nozaki, 1982; Cachia et al., 1983a, 1986; Van Eyk & Hodges, 1987, 1988). Importantly, Cys-98 (helix E) of TnC is cross-linked specifically to residues within the inhibitory region of TnI whether in the IC or whole Tn complex (Lesysk et al., 1987, 1988). In addition, cross-linking of a photoaffinity-labeled TnI inhibitory peptide (benzophenone moiety attached to the α-amino group of the inhibitory peptide) to TnC results in the labeling of the C-terminal domain of TnC (sites III and IV) (Ngai and Hodges, unpublished data). Only a single cross-linking study (using TnC with its carboxyl groups activated with a carbodiimide and cross-linked to TnI) has shown that the TnI inhibitory region could be cross-linked to the N-terminal domain of TnC (Lesysk et al., 1990). The very selective nature of the cross-linking reagent (requiring amino groups within cross-linking distance to the activated carboxyl groups) could have prevented cross-linking from occurring with the C-terminal domain, either because of distance constraints or because of intramolecular cross-linking. The authors (Lesysk et al., 1990) stated that their results do not negate the importance of the interaction in the C-terminal domain. Lan et al. (1989) indicated that the preferred binding site of synthetic TnI inhibitory peptide is on the C-terminal domain of calmodulin or TnC. In fact, the TnI inhibitory peptide (104–115) did not alter the fluorescence emanating from a probe on Met-25 of TnC but did alter the environment around Cys-98. Taken together, all of the above studies strongly indicate that the primary interaction site for TnI peptide is the C-terminal domain of TnC, around Cys-98 in helix E.

It is not clear how skeletal TnI peptide and, by analogy, the cardiac TnI peptide, when bound near helix E in the C-terminal domain, can alter the binding at the low-affinity sites located in the N-terminal domain of skeletal TnC. There must be communication between the two halves of the TnC molecule possibly via the central α-helix (D/E linker) which connects the N- and C-terminal domains of TnC (Herzberg & James, 1985; Sundaralingam et al., 1985). It has been well documented that Ca^{2+} and TnI binding to TnC induces α-helix, in particular, the E-helix of Ca^{2+} binding site III (Nagy et al., 1978; Nagy & Gergely, 1979; Reid et al., 1981). The increase in helical content is seen upon binding of mastoparan (Cachia et al., 1986) to TnC or TFP binding to a proteolytic fragment of the Ca^{2+} binding site III of TnC or a synthetic peptide comprised of helix E (Gariépy & Hodges, 1983). It is conceivable that helix E, when altered by TnI (and possibly TnI peptide), could affect the N-terminal domain of TnC via the D/E helix. In fact, Wang et al. (1990), using a TnC mutant, demonstrated that binding of a cation to high-affinity sites altered the environment around the amino acid at position 57 in the N-terminal domain. In addition, binding of Ca^{2+} to the low-affinity sites alters the environment around Cys-98 which is located in helix E (Grabarek et al., 1986; Rosenfeld & Taylor, 1985). In the crystal structure, the central helix is extended, producing an elongated structure. There is some evidence derived from low-angle X-ray scattering (Heidorn & Trewhella, 1988; Hubbard et al., 1988) and fluorescence energy transfer (Wang et al., 1987) suggesting a more compact structure of TnC in solution. If the D/E linker is bent, bringing the N- and C-terminal domains in closer contact, this allows the possibility of direct interaction between the two domains rather than communication via the D/E linker.

SIp also altered the low-affinity Ca^{2+} binding site of cardiac TnC, similarly to skeletal TnC. However, the pCa shift was toward higher Ca^{2+} concentrations. The proposed binding site on skeletal TnC (residues 88–102) is highly conserved in cardiac muscle (residues 90–104) with 11 out of 15 residues identical. The effect of sIp, cIp, and Gly 110 on the secondary structure (when monitored by either CD or fluorescence) differed depending on whether the peptide was bound to skeletal or cardiac TnC. However, cIp and sIp primarily altered the Ca^{2+} affinity of the low-affinity sites while Gly 110 mainly altered the high-affinity sites of both TnC species. This indicates that although the conformational change differed depending on the species of TnC, which Ca^{2+} binding site was affected was not dependent on the TnC species, but rather on which peptide was used. Therefore, the difference in primary sequence of skeletal and cardiac TnC is not as important as the sequence difference in the TnI inhibitory region for causing the difference in the effect on the high-affinity versus the low-affinity Ca^{2+} binding sites.

The binding of cIp or Gly 110 produced more subtle changes in the secondary structure than those displayed by sIp, and these were more efficiently detected by the changes in the environment of Tyr. The single amino acid substitution between skeletal and cardiac TnI peptide of a Pro to Thr altered the effect of the TnI peptides on the secondary structure of skeletal and cardiac TnC but not the the ability of both peptides to affect Ca^{2+} binding at the low-affinity sites. It is conceivable that the secondary structure adopted by the two TnI peptides is different and the peptides may affect different amino acid residues on TnC, which in turn would alter the secondary structure of TnC differently. The $\theta_{200nm}/\theta_{222nm}$ ratio was not similar for cIp and sIp, indicating strongly that the conformations in solution and in the presence of TFE are vastly different. The structure of the skeletal TnI peptide bound to skeletal TnC in the presence of Ca^{2+}, determined by NMR, has the Pro residues forming a "turn-like" structure so that the hydrophobic residues F 106, L 111, and V 114 are in close proximity (Campbell & Sykes, 1991). It seems likely that the conformation adopted by cIp when bound to TnC is probably different from sIp. This suggestion is supported by TnC affinity chromatography of the cardiac TnI analogues in which single amino acids were individually substituted with Gly. These analogues bound to skeletal and cardiac TnC affinity columns with different strengths than the corresponding skeletal Gly analogues (Van Eyk and Hodges, unpublished data), suggesting that residues which are important for TnC binding are different for skeletal TnI 104–115 than those which are involved in the binding of cardiac TnI 137–148. Gly 110 should be more flexible than the other two TnI peptides, since Gly does not have a side chain and so can adopt a wider range of ψ and ϕ dihedral angles than Pro or Thr. Surprisingly, the $\theta_{200nm}/\theta_{222nm}$ ratios for Gly 110 and cIp were similar, but different from sIp. Since the peptide's main effect was to alter the pCa of the high-affinity sites, it is clear that the Gly 110 analogue alters the secondary structure of skeletal and cardiac TnC differently than the two native TnI peptides which affect mainly the low-affinity sites. Importantly, it is the low-affinity sites which are the regulatory sites for muscle contraction. This may be reflected in the fact that skeletal and cardiac TnI peptides induce equivalent inhibition of force development of skeletal and cardiac skinned muscle fibers while Gly 110 does not significantly alter force development (Rüegg et al., 1991).

In conclusion, the TnI inhibitory peptides upon binding caused differences, sometimes subtle, in the structure of skeletal and cardiac TnC which, in turn, altered the Ca^{2+} affinities. This indicates that even though the two TnC molecules are highly conserved, they behave differently to TnI binding. As well, the three TnI peptides acted differently from one another, indicating that the single amino acid change in inhibitory sequence is partly responsible for the differences in the biological activity of skeletal and cardiac muscle.

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Registry No. Pro, 147-85-3; Thr, 72-19-5; Gly, 56-40-6; Ca, 7440-70-2.

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6,7-Di*epi*castanospermine, a Tetrahydroxyindolizidine Alkaloid Inhibitor of Amyloglucosidase†

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ABSTRACT: A tetrahydroxyindolizidine alkaloid, 6,7-di*epi*castanospermine, was isolated from the seeds of *Castanospermum australe* by extraction with methanol and purified to homogeneity using ion-exchange, preparative thin-layer, and radial chromatography. A very low yield of a pyrrolidine alkaloid, *N*-(hydroxyethyl)-2-(hydroxymethyl)-3-hydroxypyrrolidine, was also obtained by analogous methods. The purity of both alkaloids was established by gas chromatography of their trimethylsilyl (TMS) derivatives as better than 99%. The molecular weight of each alkaloid was established as 189 and 161, respectively, by mass spectrometry, and the structure of each was deduced from their ^1H and ^{13}C NMR spectra. The structure of the pyrrolidine alkaloid is suggestive of a possible biosynthetic route to the polyhydroxyindolizidine and polyhydroxypyrrolizidine alkaloids which co-occur in *C. australe*. 6,7-Di*epi*castanospermine was found to be a moderately good inhibitor of the fungal α-glucosidase, amyloglucosidase ($K_i = 8.4 \times 10^{-5}$ M) and a relatively weak inhibitor of β-glucosidase. It failed to inhibit α- or β-galactosidase, α- or β-mannosidase, or α-L-fucosidase. Comparison of its inhibitory activity toward amyloglucosidase with those of its isomers, castanospermine and 6-*epi*castanospermine, demonstrated that epimerization of a single hydroxyl group can produce significant alteration of such inhibitory properties.

During the past decade a number of polyhydroxyindolizidine and polyhydroxypyrrolizidine alkaloids have been isolated from certain members of the plant family Leguminosae. All of these alkaloids have been shown to be inhibitors of glycosidases, exhibiting various degrees of potency and specificity (Elbein & Molyneux, 1987; Elbein, 1987). The most fruitful source of polyhydroxyalkaloids has been the Moreton Bay chestnut, or Black Bean (*Castanospermum australe*), a large leguminous tree native to northeastern Australia, which has been introduced into other subtropical areas of the world for its ornamental qualities. However, the large chestnut-like seeds produce hemorrhagic gastroenteritis when consumed by cattle (McKenzie et al., 1988) and on occasion have proved fatal to humans (Everist, 1974). The major alkaloidal constituent of the seeds, castanospermine (**1**) (Figure 1) (Hohenschutz et

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