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THE GREEN TEA POLYPHENOL (−)-EPIGALLOCATECHIN-3-GALLATE INHIBITS MAGNESIUM BINDING TO THE C-DOMAIN OF CARDIAC TROPONIN C

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Abstract

Cardiac muscle contraction is activated via the single Ca^{2+}-binding site (site II) in the N-domain of troponin C (cTnC). The two Ca^{2+}/Mg^{2+} binding sites in the C-domain of cTnC (sites III and IV) have been considered to play a purely structural role in anchoring cTnC to the thin filament. However, several recent discoveries suggest a possible role of this domain in contractile regulation. The green tea polyphenol (–)-epigallocatechin 3-gallate (EGCg), which binds specifically to the C-domain of cTnC, reduces cardiac myofilament Ca^{2+} sensitivity along with maximum force and acto-myosin ATPase activity. We have determined the effect of EGCg on Ca^{2+} and Mg^{2+} binding to the C-domain of cTnC. In the absence of Mg^{2+} there was no significant effect of EGCg on the Ca^{2+}-cTnC affinity. Surprisingly, in the presence of Mg^{2+} EGCg caused an increase in Ca^{2+} affinity for sites III and IV of cTnC. However, in the absence of Ca^{2+} the addition of EGCg caused a significant reduction in Mg^{2+}-cTnC affinity. This reduction is presumably responsible for the increase in Ca^{2+}-cTnC affinity produced by EGCg in the presence of Mg^{2+}. We propose that the inhibitory effect of EGCg on myofilament Ca^{2+} activation may be related to an enhanced Ca^{2+}-Mg^{2+}exchange at sites III and IV of cTnC, which might reduce the myosin crossbridge dependent component of thin filament activation.

Keywords

cardiac muscle regulation; troponin; EF-hand; Ca^{2+}-binding; calcium signaling; Mg^{2+}-binding

INTRODUCTION

Cardiac troponin C (cTnC) has three functional Ca^{2+} - binding sites, namely, site II in the N-domain and sites III and IV in the C-domain. Binding of Ca^{2+} to site II (K_d~10^{-5} M) activates actomyosin ATPase and force generation. Sites III and IV can bind either Ca^{2+} (K_d~ 10^{-7} M) or Mg^{2+}(K_d~10^{-3} M) and have generally been thought to play a purely structural role in anchoring cTnC to the thin filament (Holroyde et al. 1980). At rest, when the free Ca^{2+} inside the cell is less than 10^{-7} M and the free Mg^{2+} is ~ 1.0 mM, sites III and IV would be primarily occupied by Mg^{2+}, whereas during activation the Mg^{2+} bound at sites III and IV would be displaced by Ca^{2+}. Occupation of sites III and IV by either Ca^{2+} or Mg^{2+} has been shown to be essential for the binding of cTnC to the thin filament (Holroyde et al. 1980; Negele et al. 1992).

Several investigators have raised the possibility that the C-terminal domain of cTnC may play a more complex role in cardiac muscle activation (Calvert et al. 2000; Finley et al.

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2004). Recent studies in our laboratory suggest that the ratio of Ca^{2+} to Mg^{2+} bound at sites III and IV can influence crossbridge activation of the thin filament (Fuchs and Grabarek 2011). That is, displacement of Mg^{2+} by Ca^{2+}, with site II unoccupied, weakens the interaction of strong-binding myosin crossbridges with actin. Hence Ca^{2+} and Mg^{2+} may have more complex effects on contractile regulation apart from the well-established effect of Ca^{2+} binding to site II. Also of great interest is the discovery of mutations in the C-domain which can be linked to either hypertrophic or dilated cardiomyopathy (Willott et al. 2010). Such mutations can have strong effects on C-domain Ca^{2+} binding (Swindle and Tikunova 2010).

A new approach to the study of C-domain function arises from the existence of a small number of compounds which bind to this segment of cTnC and can modify contractile activity in either a positive or negative way (Li et al. 2008). One such compound is the green tea polyphenol (–)-epigallocatechin-3-gallate (EGCg) (Robertson et al. 2009). When added in the concentration range of 25–100 μM, EGCg reduces Ca^{2+} sensitivity and Ca^{2+}-activated force in skinned cardiac fibers (Liou et al. 2008; Tadano et al. 2010).

Structural studies indicate that EGCg forms a $cTnC{\cdot}2Ca^{2+}{\cdot}EGCg$ complex and also forms a ternary complex with cTnC and the anchoring region of troponin I (Robertson et al. 2009). The goal of this study was to determine whether the effects of EGCg might be correlated with changes in C-domain cation binding. Tyrosine fluorescence titration was used to determine dissociation constants for the binding of Ca^{2+} and Mg^{2+} to sites III and IV in the absence and presence of EGCg. The data strongly suggest that EGCg has little direct effect on Ca^{2+} binding to these sites but causes a significant reduction in the affinity of Mg^{2+} for these same sites; hence, there is, paradoxically, an increase in Ca^{2+} affinity of cTnC when Mg^{2+} is present due to weaker Mg^{2+}/Ca^{2+} competition for sites III and IV. EGCg might be a useful reagent for analyzing the function of Mg^{2+} binding to cTnC, and perhaps to other EF-hand proteins.

METHODS

Protein preparations

Rat cardiac myofibrils were prepared as described previously (Fuchs and Grabarek 2011). Recombinant chicken cardiac troponin C was prepared according to (Putkey et al. 1989), using a plasmid kindly provided by Dr. J. A. Putkey. Before use the cTnC was exhaustively dialyzed against a solution of 75 mM KCl and 50 mM HEPES, pH 7.5. Protein determination of both myofibrils and cTnC was carried out using the BCA reagent (Pierce).

ATPase measurements

Myofibrillar ATPase activity was assayed as described previously (Fuchs and Grabarek 2011), using the malachite green reagent (Lanzetta et al. 1979). Assays were carried out at 30 °C in a solution containing 100 mM KCl, 20 mM MOPS, pH 7.0, 1 mM $MgCl_2$, 1 mM EGTA, 0.1–0.2 mg/ml protein, and $CaCl_2$ to set the desired pCa. The program MAXCHELATOR (Patton et al. 2004) was used to calculate free Ca^{2+} and Mg^{2+} concentrations. The effects of EGCg (Sigma) were assayed over the concentration range 0–100 μM. Before use the EGCg was dissolved in either the buffer solution (stock solution 10 mM) or dimethylsulfoxide (stock solution 20 mM). The nature of the EGCg solvent had no apparent effect on the results.

Measurement of Ca^{2+} binding

Ca^{2+} binding specifically to sites III and IV of cTnC was determined by measuring tyrosine fluorescence as a function of pCa (Leavis and Kraft 1978), using a Varian Cary Eclipse

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spectrofluorometer. Titrations were carried out at room temperature (22–23 °C) in solutions containing 75 mM KCl, 50 mM HEPES, pH 7.5, ± 2 mM $MgCl_2$, 1 mM EGTA, 1 mM NTA, ± 50 μM EGCg and either 2.5 μM or 5.0 μM cTnC. With a total [Mg^{2+}] of 2 mM the free Mg^{2+} concentration was calculated to be ~1.2 mM. The excitation wavelength was 280 nm and the emission wavelength was 305 nm. Aliquots (0.5–1.0 μl) of 100 mM $CaCl_2$ were added to 1.0 ml of cTnC solution and fluorescence was plotted as a function of calculated pCa. The fluorescence data were fitted with the Hill equation, using the Sigma Plot program.

Measurement of Mg^{2+} binding

The measurement of Mg^{2+} binding was carried out with the same instrumentation, with the temperature set at 25 °C. The cTnC solution contained 75 mM KCl, 50 mM HEPES, pH 7.5, 1 mM EGTA, and 2 μM cTnC. Aliquots (0.5–1.0 μl) of 200 mM $MgCl_2$ solution were added to 1.0 ml of cTnC solution. Using MAXCHELATOR, the free Mg^{2+} was corrected for the small amount of Mg^{2+} bound to EGTA.

RESULTS

The effect of EGCg on myofibrillar ATPase activation

In agreement with earlier data (Liou et al. 2008; Tadano et al. 2010), the myofibrillar ATPase activity at pCa 5.0 was not changed by low concentrations of EGCg but was strongly inhibited as [EGCg] was increased in the range 25–100 μM (Fig. 1). In as much as EGCg binds to the C-domain of cTnC (Robertson et al. 2009) and inhibits Ca^{2+} activation, the possibility is raised that EGCg may have a specific effect on Ca^{2+} and/or Mg^{2+} binding to sites III and IV. Given the evidence that Ca^{2+} and Mg^{2+} have different modulatory roles at the C-domain (Fuchs and Grabarek 2011), we made separate measurements of the Ca^{2+}-cTnC and Mg^{2+}-cTnC dissociation constants and the effect on each of EGCg.

Effect of EGCg on Ca^{2+} binding to the C-domain of cTnC

As shown in Fig. 2, in the absence of divalent cations there was a quenching of tyrosine fluorescence, indicating that the binding of EGCg to the C-domain does not require the presence of either Ca^{2+} or Mg^{2+} at sites III and IV. This quenching of tyrosine fluorescence by EGCg has been observed previously (Liou et al. 2008). The decrease in Tyr fluorescence is a sum of a hyperbolic curve representing an equilibrium binding isotherm and a linear quenching component due to the high extinction coefficient of EGCg. The apparent $K_{d(EGCg)}$ obtained from the computer fit (solid line in Fig. 2) is 65 μM.

The specific binding of Ca^{2+} or Mg^{2+} to the C-terminal domain of cTnC can be measured from the increase in the intrinsic tyrosine fluorescence (Leavis and Kraft 1978). Typical Ca^{2+} titration curves obtained in the absence and presence of Mg^{2+} are shown in Fig. 3. In agreement with earlier results (Leavis and Kraft 1978), in the absence of Mg^{2+} there is a maximum fluorescence increase of 30–40% with saturating concentrations of Ca^{2+}. The results of a series of titrations are summarized in Table 1. In the absence of Mg^{2+}, EGCg (50 μM) had no significant effect on the Ca^{2+}-cTnC dissociation constant, $K_{d(Ca)}$ (39.8 ± 1.6 nM without EGCg, 40.9 ± 1.4 nM in the presence of EGCg). The dissociation constant in the presence of Mg^{2+}($K_{d(Ca,Mg)}$) was 63.6 ± 3.7 nM and with addition of EGCg the $K_{d(Ca,Mg)}$ decreased to 54.8 ± 2.8 nM ($p<0.05$). Thus EGCg caused a significant increase in Ca^{2+}-cTnC affinity but only when it was present along with Mg^{2+}. Given the lack of an effect in the absence of Mg^{2+}, the possibility is raised that the increased Ca^{2+} binding caused by EGCg in the presence of Mg^{2+} is the consequence of a reduced Mg^{2+}-cTnC affinity. It should be noted that for all four titrations listed in Table 1 the mean Hill coefficients ranged in value from 1.7 to 1.9, as would be expected for the presence of two sites which bind cooperatively.

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Effect of EGCg on Mg^{2+} binding to the C-domain of cTnC

Typical normalized Mg^{2+} titration curves obtained in the absence of Ca^{2} are shown in Fig. 4. The maximum change in tyrosine fluorescence in the presence of a saturating concentration of Mg^{2+} was in the range of 10–20%. The mean values of the Mg^{2+}-cTnC dissociation constant ($K_{d(Mg)}$) obtained at 0, 50, and 100 μM EGCg are shown in Table 2. The mean increased from 522 (±35) μM in the absence of EGCg to 680 (±14) μM in the presence of 50 μM EGCg ($p<0.01$), with a further increase to 743 (±59) μM in the presence of 100 μM EGCg. Thus EGCg causes a reduction in Mg^{2+} affinity without any significant change in Ca^{2+} affinity. The increase in Ca^{2+} binding observed in the presence of both Mg^{2+} and EGCg (Table 1) presumably reflects a reduced Mg^{2+}/Ca^{2+} competition for the binding sites due to the EGCg–induced reduction in Mg^{2+} affinity. The mean Hill coefficients measured at 0, 50, and 100 μM EGCg were 1.71, 1.68 and 1.76, respectively. Cooperativity between sites III and IV evidently involves Mg^{2+} binding as well as Ca^{2+} binding.

DISCUSSION

While there is universal agreement that site II in the N-domain of cTnC is the trigger site for the initiation of cardiac contraction, there is still no consensus about the role of C-domain sites III and IV. Under physiological conditions in the resting muscle fiber, with a free Mg^{2+} concentration of ~1.0 mM and a free Ca^{2+} concentration of less than 10^{-7} M, there should be ~75% occupancy of sites III and IV by Mg^{2+}. A direct role of these sites in Ca^{2+} activation has been discounted on the grounds that Ca^{2+}-Mg^{2+} exchange is too slow relative to the kinetics of contraction and relaxation (Robertson et al. 1981). These sites clearly play a structural role inasmuch they must be occupied by either Mg^{2+} or Ca^{2+} in order for cTnC to be bound strongly to the thin filament (Holroyde et al. 1980; Negele et al. 1992).

In recent years some suggestions have been made that, in addition to their structural role, sites III and IV of cTnC may play a more complex modulating role in Ca^{2+} activation. Support for this concept comes from the recent discovery of mutations in this domain which can be linked to either dilated or hypertrophic cardiomyopathy (Willott et al. 2010). Two such mutations (E134D and D145E) have significant effects on the binding of Ca^{2+} and Mg^{2+} to sites III and IV (Swindle and Tikunova 2010). Depending upon whether Ca^{2+} or Mg^{2+} is bound at sites III and IV, there are structural differences in the C-domain, along with differences in the strength of binding of cTnC to troponin I (cTnI) (Calvert et al. 2000; Finley et al. 2004). With Ca^{2+} bound at sites III and IV the interaction between the C-domain of cTnC and cTnI is 8-fold stronger than with Mg^{2+} bound at the same sites (Calvert et al. 2000). Recent experiments in this laboratory have indicated that the strong-binding crossbridge attachments to actin (via NEM-S1 activation) may be influenced by the ratio of Ca^{2+}/Mg^{2+} bound to the C-domain (Fuchs and Grabarek 2011). With site II empty, there is a weakening of strong crossbridge interactions with actin when Ca^{2+} rather than Mg^{2+} is bound to sites III and IV. Displacement of Mg^{2+} by Ca^{2+} could have important effects on the cardiac cycle. As a consequence of the slow Ca^{2+}-Mg^{2+} exchange rate at the C-domain sites the amount of Ca^{2+} bound to this domain may reflect the rate and intensity of cardiac contraction (Calvert et al. 2000; Fuchs and Grabarek 2011). As the heart rate increases the rate of relaxation must increase as well in order to allow for adequate diastolic filling of the ventricle. This change in relaxation kinetics is accompanied by a decrease in myofilament Ca^{2+} sensitivity (Varian and Janssen 2007). One possibility is that the presence of Ca^{2+} rather than Mg^{2+} at sites III and IV might potentiate relaxation when Ca^{2+} is dissociated from site II, as would happen at the onset of relaxation. Consistent with this interpretation are data obtained with NEM-S1-activated skinned fibers showing that thin filament activation, as measured by the rate of force redevelopment following quick release (K_{tr}), is reduced as $[Ca^{2+}]$ is increased in the range where it binds primarily to the C-domain sites rather than to the N-domain (Moss et al. 2002). Also of interest are data obtained with

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skeletal TnC showing that knockout of sites III or IV (or both) not only reduces the affinity of TnC for the thin filament but also causes an increase in myofilament Ca^{2+}-sensitivity when incorporated into skinned fibers (Szczesna et al. 1996). These results are consistent with the concept that the C-domain could play a role in modulation of strong-binding crossbridge interaction.

The existence of compounds that bind to the C-domain of cTnC and alter contractile activity opens up a new approach for studying the functional role of this segment of the cTnC subunit. Our data suggest that EGCg-induced inhibition of Ca^{2+} activation in cardiac fibers might be associated, paradoxically, with an increase in Ca^{2+} affinity at sites III and IV. The results are consistent with the hypothesis that Mg^{2+} and Ca^{2+} compete for the same binding sites and that Mg^{2+} binding is the actual target of EGCg action. As indicated above, the displacement of Mg^{2+} by Ca^{2+} (in the absence of Ca^{2+} binding at site II) favors a weakening of strong-binding interactions between actin and myosin (Fuchs and Grabarek 2011). One might speculate that there is a connection between this effect and the inhibition of Mg^{2+} binding by EGCg in the sense that an increased Ca^{2+} binding at sites III and IV produced by EGCg might promote a faster rate of crossbridge detachment during the contractile cycle. Further work with reconstituted contractile systems will be needed to test this hypothesis.

It was of interest to note that, with or without EGCg, the measured Hill coefficients indicated cooperative binding at sites III and IV for both Ca^{2+} and Mg^{2+}. In the case of Ca^{2+} binding the main contribution to the cooperativity comes from the domain opening transition, i.e. the simultaneous change in the position of the helices flanking the Ca^{2+}-binding loops in both EF-hands comprising the domain (Gifford et al. 2007). In the case of Mg^{2+}, which does not induce domain opening in TnC (Grabarek 2011), it is not clear how the sites can communicate with each other. In fact, cooperativity in Mg^{2+} binding to EF-hand proteins has been noted in previous reports. In an early study of Mg^{2+} binding to the C-terminal fragment of calmodulin (Drabikowski et al. 1982) the tyrosine fluorescence data, when re-fitted with the Hill equation, yielded a Hill coefficient of 1.6. In a later study with tryptophan-substituted mutants of sTnC a Hill coefficient of 1.44 was obtained (Trigo-Gonzalez et al. 1992). More recently, a "pressure jump" technique was used to determine $K_{d(Mg)}$ values for the C-domain of sTnC (Pearson et al. 2008) and Hill coefficients of 1.9–2.0 were reported. Thus cooperative Mg^{2+} binding is a property which cTnC shares with other EF-hand proteins. In view of the fact that the Mg^{2+}-bound TnC remains in the closed-domain conformation (Grabarek 2011) cooperativity must involve a direct communication between the binding sites rather than large scale conformational transitions. The Mg^{2+} binding cooperativity may arise from decreased dynamics of the metal binding loop when one of the sites becomes occupied. Another plausible explanation based on the structure of the calmodulin-Mg^{2+} complex that we determined recently involves a transient shift of the entering helix and an expansion of the loop, both of which are required for the initial accommodation of a hydrated Mg^{2+} ion (cf. Fig. 1 in ref. (Senguen and Grabarek 2012)). It is easy to envision that such a localized destabilization of the structure in one EF-hand might facilitate a similar process in the pair-mate site, thus leading to the binding cooperativity. There is a need for further studies to establish the structural basis for communication between sites III and IV in the Mg^{2+}-bound state of cTnC.

The effects of EGCg observed with cTnC are apparently not applicable to all EF-hand proteins. EGCg had no effect on the ATPase activity of skeletal muscle myofibrils and, based on tyrosine fluorescence and CD spectra, there was no indication that it could bind to skeletal troponin C (Liou et al. 2008). It remains to be determined which amino acid sequences are responsible for the differences between cTnC and sTnC with respect to EGCg binding. On the other hand, preliminary experiments in this laboratory with the C-terminal fragment of calmodulin have yielded results similar to those seen with cTnC. There was no

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significant effect of EGCg on $K_{d(Ca)}$ in the Mg^{2+}-free state, but in the presence of Mg^{2+} the value of $K_{d(Ca,Mg)}$ was decreased 25–30 %. There are still many uncertainties about the role of Mg^{2+} in the modulation of Ca^{2+}-dependent events in the cell (Grabarek 2011). To our knowledge, this is the first case in which an organic reagent has been shown to specifically inhibit Mg^{2+} binding to a Ca^{2+}/Mg^{2+} site of an EF-hand protein. Tea polyphenols are known to interact with a long list of target sites which may influence many aspects of cardiovascular function (Stangl et al. 2007) and our data suggest that Mg^{2+} binding sites in EF-hand proteins may be added to that list. Further work along these lines may establish the basis for development of a useful approach to analyzing the functional roles of specific Mg^{2+}-protein interactions.

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Abbreviations

cTnC	Ca^{2+} binding component of troponin from cardiac muscle
sTnC	Ca^{2+} binding component from skeletal muscle
cTnI	inhibitory component of troponin from cardiac muscle
EGCg	(−)-epigallocatechin-3-gallate
K_d	apparent equilibrium dissociation constant
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
MOPS	3-morpholinopropane-1-sulfonic acid
EGTA	ethylene glycol-bis(2-aminoethylether)-*N,N,N´,N´*-tetraacetic acid
NTA	nitrilotriacetic acid

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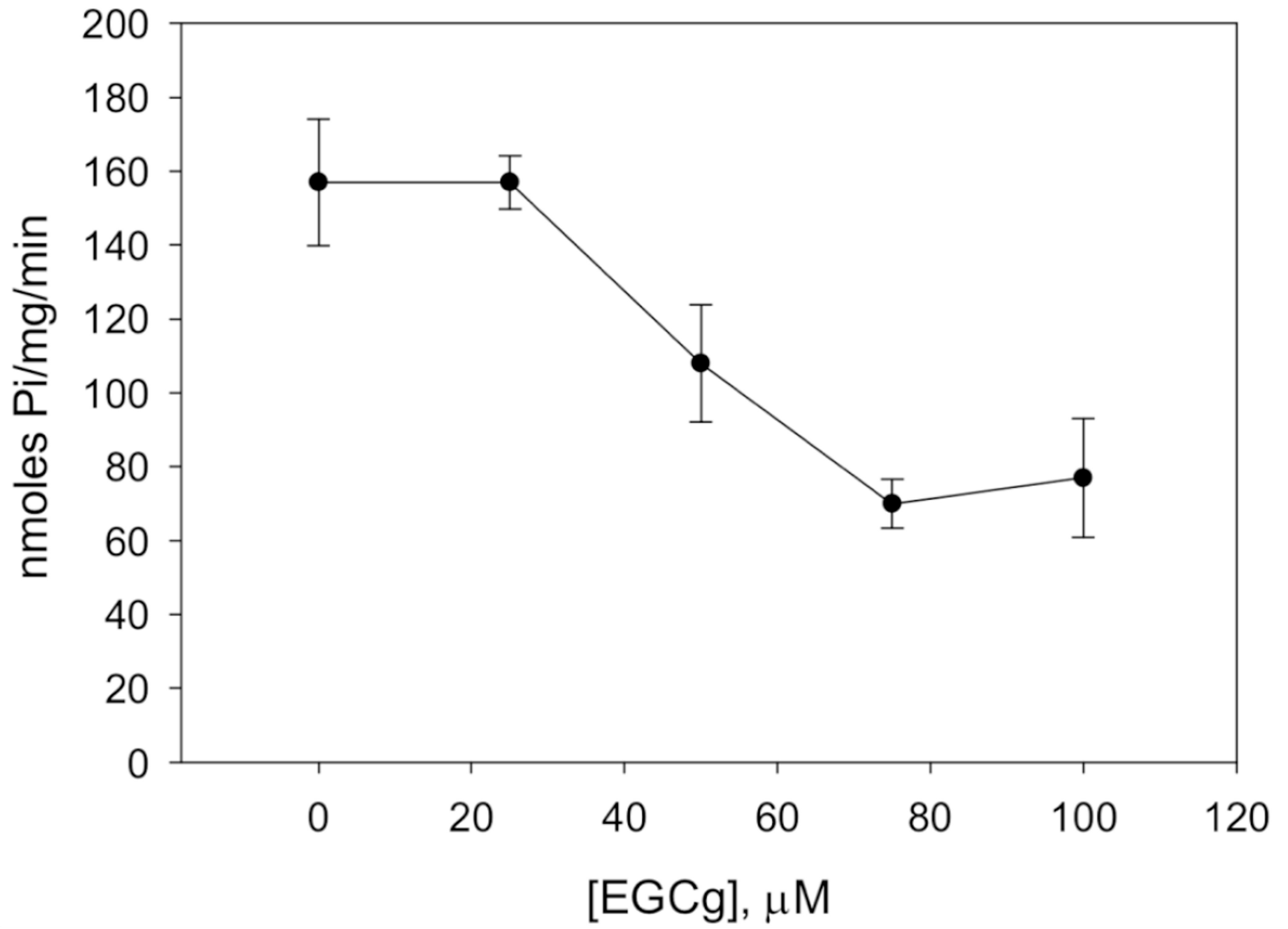

Figure 1.
The effect of EGCg on the cardiac myofibrillar ATPase activity at pCa 5.0. Each point is the mean (±SE) of 5–8 measurements.

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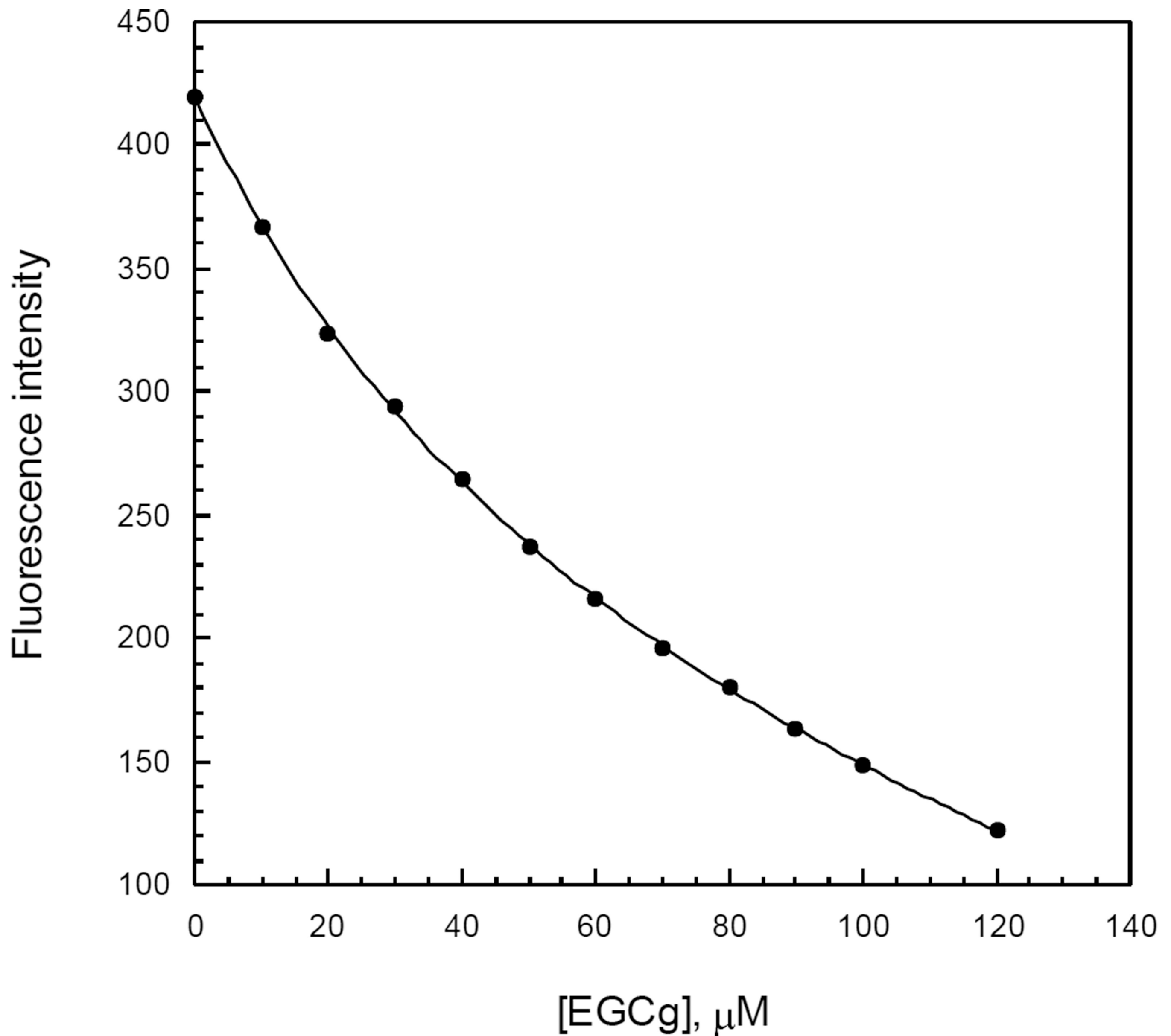

Figure 2.

Effect of EGCg on cTnC tyrosine fluorescence in the absence of divalent cations. Solution contained 75 mM KCl, 50 mM HEPES, pH 7.5, 1 mM EGTA, 1 mM NTA, and 2.5 μM cTnC. The decrease in Tyr fluorescence reflects a combined effect of quenching due to equilibrium binding of EGCg to cTnC and a filtering effect due to high extinction coefficient of EGCg. The solid line represents a computer fit to a sum of hyperbolic and linear equation as a function of EGCg concentration. The apparent $K_{d(EGCg)}$ is 65 μM.

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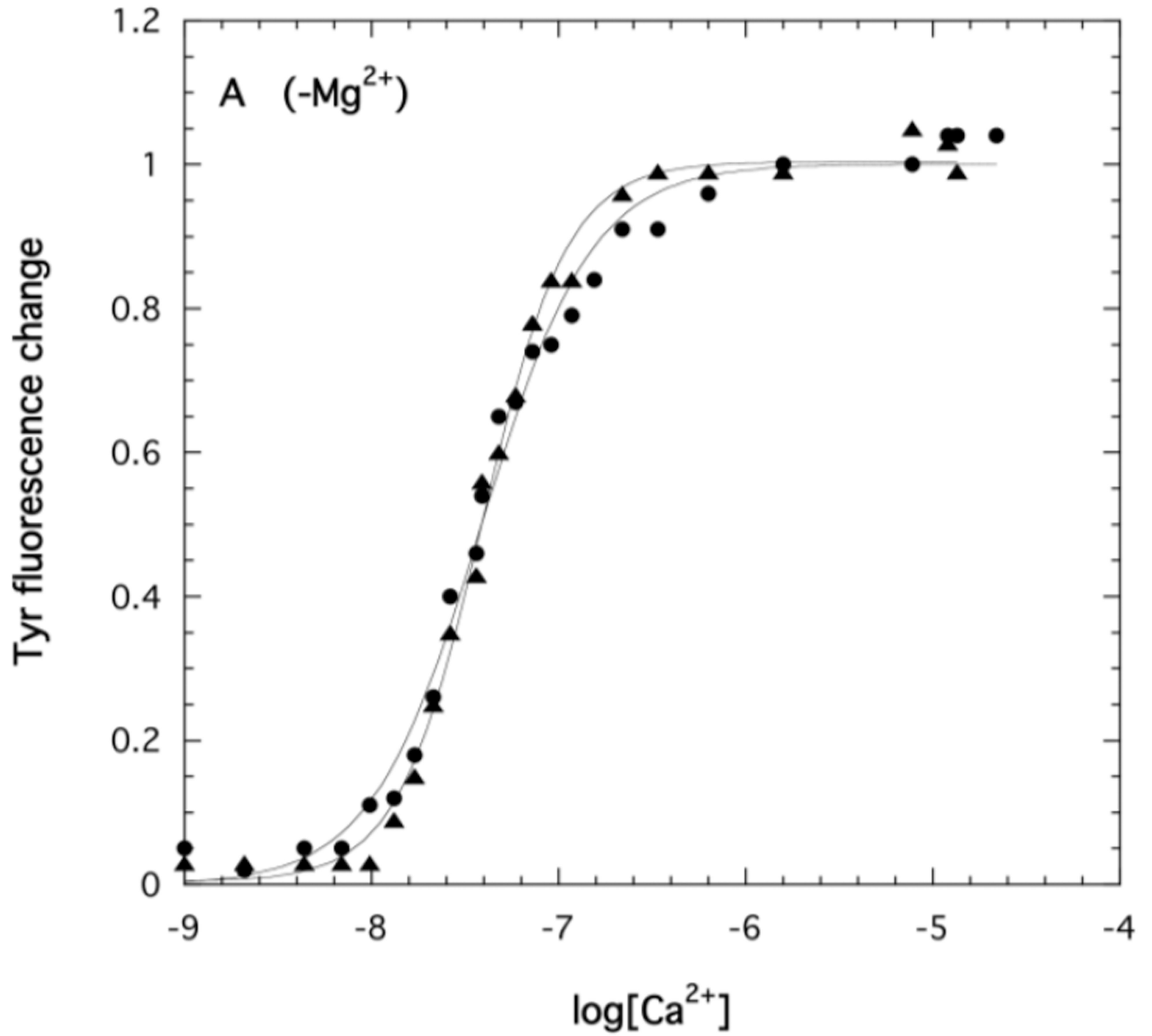

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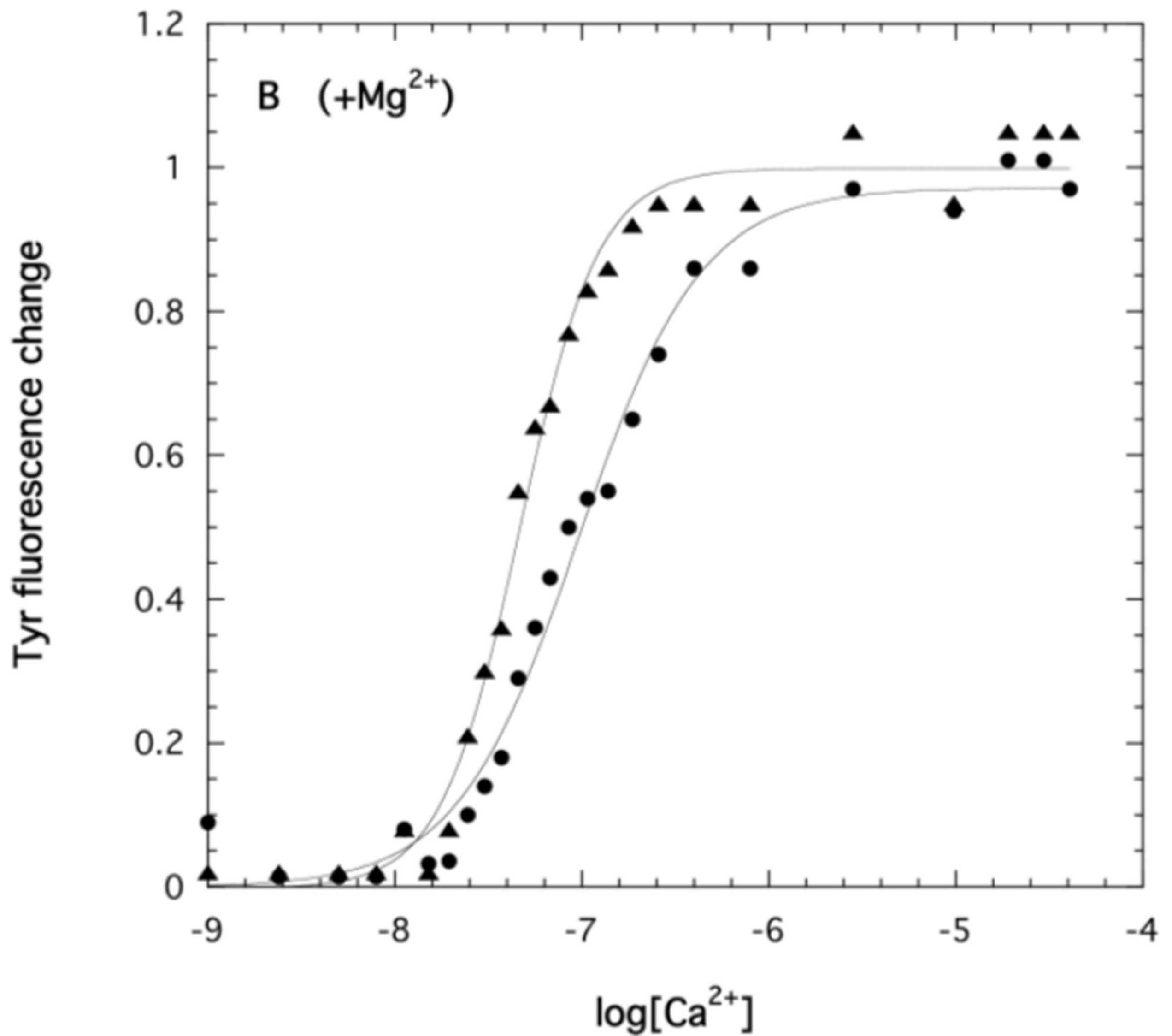

Figure 3.

The effects of Mg^{2+} and EGCg on the Ca^{2+} titration of cTnC.

Plot A, no Mg^{2+}; plot B, 2 mM $MgCl_2$ added. Circles, no EGCg; triangles, 50 μM EGCg added. Solutions contained 75 mM KCl, 50 mM HEPES, pH 7.5, 1mM EGTA, 1mM NTA 2.5 μM cTnC, and $MgCl_2$ and EGCg, as indicated. Solutions were titrated with 100 mM $CaCl_2$ added in 0.5 μl aliquots. The pCa associated with each addition was calculated with the MAXCHELATOR program. With 2 mM $MgCl_2$ added the free [Mg^{2+}] was ~1.2 mM throughout the titration.

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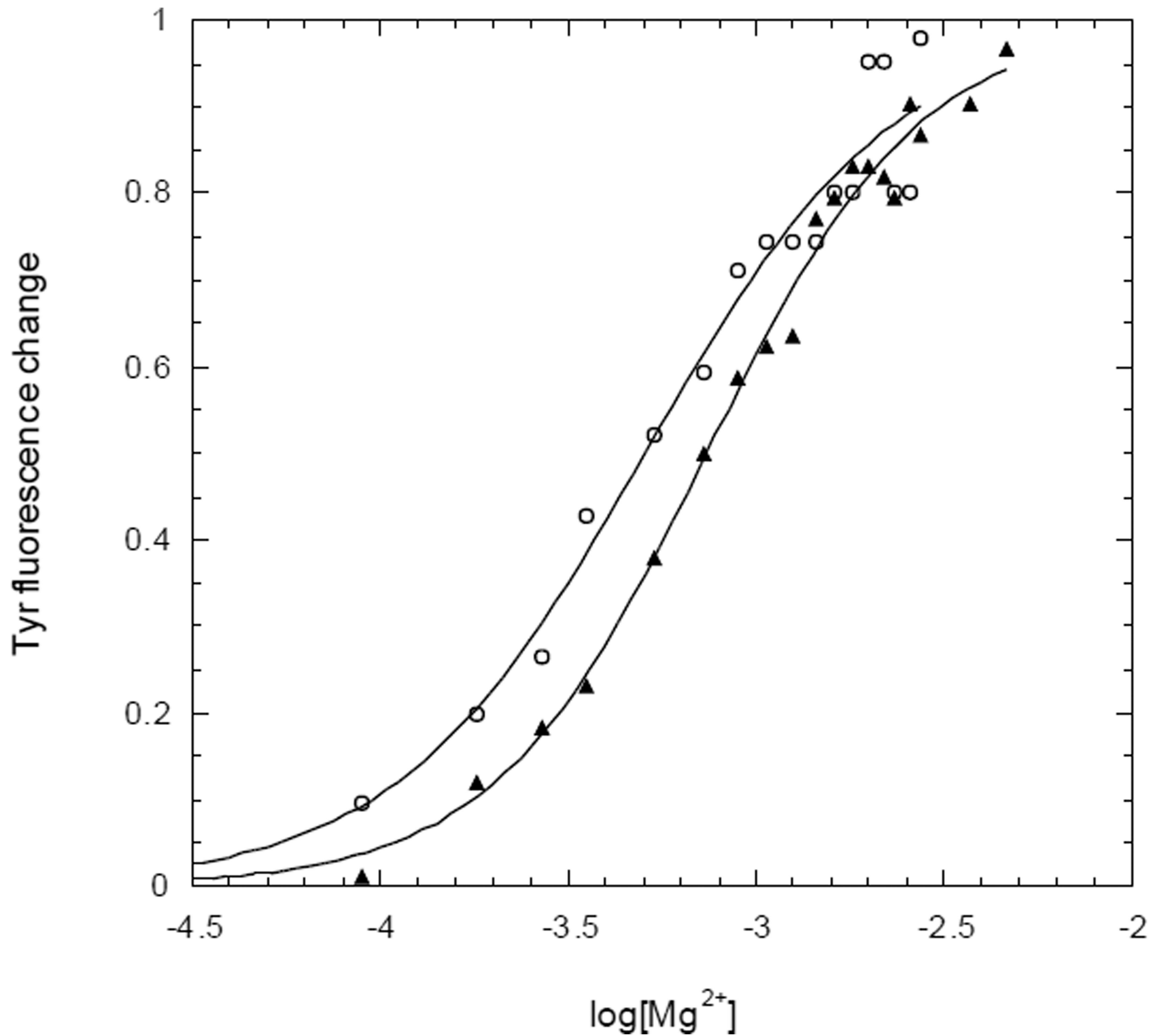

Figure 4.

Typical Mg^{2+} titration curves obtained without EGCg (circles) and in the presence of 50 μM EGCg (triangles). Solution contained 75 mM KCl, 50 mM HEPES, pH 7.5, 1 mM EGTA, and 2 μM cTnC (temperature 25°C). Solutions were titrated with 200 mM $MgCl_2$, added in 0.5–1.0 μl aliquots. In calculating the free [Mg^{2+}], correction was made for the small amount of Mg^{2+} bound to EGTA.

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Table 1

Effect of Mg^{2+} and EGCg (50μM) on Ca^{2+}-cTnC affinity ($K_{d(Ca)}$) and Hill coefficients

Conditions	n	$K_{d(Ca)}$, nM	Hill coefficient
cTnC	4	39.8 ± 1.59	1.67 ± 0.06
cTnC + EGCg	5	40.9 ± 1.44	1.92 ± 0.16
cTnC + Mg^{2+}	10	63.6 ± 3.65	1.70 ± 0.06
cTnC + Mg^{2+} + EGCg	7	54.8 ± 2.83 *	1.91 ± 0.10

Each value is mean (±S.E.).

* $p<0.05$, (cTnC+Mg^{2+}+EGCg) vs. (cTnC+Mg^{2+}).

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Table 2

Effect of EGCg on Mg^{2+}- cTnC affinity ($K_{d(Mg)}$) and Hill coefficients

[EGCg], μM	n	$K_{d(Mg)}$, μM	Hill coefficient
0	6	522 ± 35	1.71 ± 0.12
50	5	680 ± 14 *	1.68 ± 0.27
100	4	743 ± 59 *	1.76 ± 0.27

Each value is mean (±SE).

* p<0.01, 50 μM and 100 μM EGCg vs. zero EGCg..