Protein kinase C phosphorylation of cardiac troponin T decreases Ca^{2+} -dependent actomyosin MgATPase activity and troponin T binding to tropomyosin–F-actin complex

Thomas A. NOLAND, Jr. and J. F. KUO

Department of Pharmacology, Emory University School of Medicine, Atlanta, GA 30322, U.S.A.

Effects of phosphorylation of bovine cardiac troponin T (TnT) by protein kinase C on the Ca^{2+} -stimulated MgATPase activity of reconstituted actomyosin complex and the binding of TnT to tropomyosin(Tm)–F-actin were investigated. The Ca^{2+} -stimulated MgATPase of actomyosin containing phosphorylated TnT (1.8 mol of P/mol), compared with that containing unphosphorylated TnT, was decreased by up to 48 %. Phosphorylation of TnT also decreased (up to 48 %) its maximum binding to Tm–F-actin, which was accompanied by a decrease (up to 3.5-fold) in its apparent binding affinity. The findings indicate that the effects of phosphorylated TnT in decreasing actomyosin MgATPase might be secondary to its decreased interactions with the other components of the thin filament, representing a new mechanism underlying the negative inotropic responses of various cardiac preparations to protein kinase C-activating phorbol esters.

INTRODUCTION

Actomyosin MgATPase, the basis of cardiac myofibril contractility at the molecular level, is regulated by troponin (Tn), which is composed of the Ca^{2+} -binding TnC, the MgATPase-inhibiting TnI and the tropomyosin (Tm)-binding TnT subunits. Both TnI and TnT are phosphoproteins and are phosphorylated *in vitro* by a variety of protein kinases (for reviews, see refs. [1–3]). The protein kinase C (PKC) activators, phorbol esters, have been shown to have negative inotropic effects on various cardiac preparations [4–11]. Treatment of human skinned cardiac fibres with phorbol esters has resulted in decreased contractility and Ca^{2+} sensitivity [12]. Furthermore, activation of PKC has been implicated in the actions of α -adrenergic agonists on the heart [13–15]. A precise causal relationship between the biochemical and physiological events, however, remains to be established. One suggested mechanism [8,9] that can account for this effect is that PKC phosphorylation of phospholamban [16] inhibits Ca^{2+} transport by sarcoplasmic reticulum.

We have previously reported that TnI and TnT from heart [17] and skeletal muscle [18] are excellent substrates for PKC, and have identified the sites of phosphorylation in these proteins from bovine hearts [19]. Recently, we demonstrated that phosphorylation by PKC of TnI or TnT, or both, in various Tn preparations caused a reversible decrease in Ca^{2+} -stimulated actomyosin MgATPase activity of the reconstituted contractile apparatus, suggesting that negative inotropic effects of phorbol esters can also be mediated through phosphorylation of contractile proteins by PKC [20]. Specific regions in the structure of TnT interact with Tm, TnI and TnC. The interaction of the N-terminal region (T1) of TnT with a region near the C-terminus of Tm is insensitive to Ca^{2+} , whereas the interactions of the C-terminal region (T2) of TnT with Tm (Cys-190 of skeletal muscle Tm), TnI and TnC are Ca^{2+} -sensitive [2,21]. Since the major sites of phosphorylation by PKC in TnT [19,22] are located in or near the T2 region, it is possible that phosphorylation of these sites alters the interactions of TnT with Tm, TnC and TnI, and may account for, in part, the decrease in the Ca^{2+} -stimulated actomyosin MgATPase activity [20]. Our present studies were aimed at testing this hypothesis, by investigating the effects of

phosphorylation of TnT on the Ca^{2+} stimulation of MgATPase activity of reconstituted actomyosin and on the binding of TnT to the Tm–F-actin complex. We found that phosphorylated TnT, in the presence or absence of TnC and TnI, had lower apparent affinity for binding to Tm–F-actin and that this decreased binding correlated with the decrease, caused by phosphorylated TnT, in the Ca^{2+} -stimulated MgATPase activity of actomyosin reconstituted from various molar ratios of thin-filament components.

EXPERIMENTAL

Materials

Phosphatidylserine, diolein and alkaline phosphatase were purchased from Sigma; the alkyl-lysophospholipid 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18- OCH_3) was from Calbiochem; fresh bovine hearts and pig brains were from local slaughterhouses; frozen rabbit skeletal muscle was from Pel-Freez (Rogers, AR, U.S.A.).

Preparation of enzymes and bovine heart contractile proteins

PKC, purified (about 50 % homogeneity and devoid of other contaminating protein kinases) from pig brain extracts through the phenyl-sepharose step [23], was kindly provided by Robert Raynor of this laboratory. Type I(γ), II(β) and III(α) isoforms of PKC were prepared by subsequent hydroxyapatite chromatography [24]. Protein phosphatase 1 was purified from rabbit skeletal-muscle extracts by the method of Stewart *et al.* [25]. Bovine heart ventricles were used for the purification of all of the contractile proteins. TnC, TnI and TnT were purified by the method of Potter [26] and stored at -70°C in 50 mM-Tris/HCl (pH 8.0) containing 6 M-urea, 1 mM-EDTA and 15 mM-2-mercaptoethanol. Throughout all experiments, the oxidation of TnI was kept to a minimum by including 1 mM-dithiothreitol in all solutions. Reconstituted Tn preparations containing oxidized TnI were less responsive to Ca^{2+} (results not shown). Tm was prepared by the method of Stull & Buss [27] and F-actin by the procedures of Pardee & Spudis [28] and Hartzell [29]. Throughout this paper, the term actin will be used in reference to the total concentration of actin, whereas 'F-actin' will refer to

Abbreviations used: Tn, troponin; TnI, troponin I; TnT, troponin T; TnC, troponin C; Tm, tropomyosin; PKC, protein kinase C; ET-18- OCH_3 , 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycerol-3-phosphocholine.

polymerized actin as determined by ultracentrifugation. Myosin and myosin S1 were prepared as described by Siemankowski & White [30] as modified by Hartzell [29]. In most MgATPase experiments, whole myosin was used because the MgATPase activity of actomyosin S1 was greatly decreased at the high ionic strengths used in the assay.

Phosphorylation of contractile proteins

Before the experiments, the stored TnT was dialysed against dialysis buffer (10 mM-imidazole, pH 7.0, 0.3 M-KCl, and 1 mM-dithiothreitol) by the method of Potter [26]. Dephosphorylation to remove endogenous phosphate was carried out by incubating TnT (500 μ g/ml) with protein phosphatase 1 (40 μ g/ml) in the dialysis buffer for 3 h at 30 °C, or with alkaline phosphatase (50 μ g/ml) in 0.1 M-triethanolamine (pH 9.0) containing 0.3 M-KCl and 1 mM-dithiothreitol for 8 h at 30 °C. Dephosphorylated TnT was dialysed against 6 M-urea/25 mM-citric acid (pH 6.2)/1 mM-EDTA/15 mM-2-mercaptoethanol, separated from the phosphatases by chromatography on CM-Sephadex by the method of Potter [26] and finally redialysed against the dialysis buffer as described above. The conditions for phosphorylation by PKC were essentially the same as described recently [19]. Briefly, reaction mixtures (2 ml) contained 50 mM-Tris/HCl (pH 7.5), 10 mM-MgCl₂, 100 μ M-CaCl₂, 10 μ g of phosphatidylserine, 2 μ g of diolein, 150 mM-KCl, 0.4 mM-ATP, 30 mM-2-mercaptoethanol, 0.5 mg of TnT (6.2 μ M) and 5 μ g of PKC. Reactions were initiated by addition of ATP, carried out for 3–4 h at 30 °C and terminated by the addition of the PKC inhibitor ET-18-OCH₃ (20 μ M final concentration) [31] or by dialysis overnight against 10 mM-Mops/NaOH (pH 7.0) containing 0.3 M-KCl and 15 mM-2-mercaptoethanol. To determine the stoichiometry of phosphorylation, parallel reaction mixtures, containing 0.4 mM-[γ -³²P]ATP (4×10^6 – 8×10^6 c.p.m.), were prepared for quantification of acid-stable ³²P content [19]. Control incubations were conducted in parallel experiments using heat-inactivated PKC to yield unphosphorylated TnT. Total phosphate content in TnT was determined by the method of Martensen [32]. Because PKC isoenzymes α , β and γ all similarly phosphorylated cardiac TnI and TnT (results not shown) and ET-18-OCH₃ and other PKC inhibitors similarly inhibited each isoenzyme [33], the present PKC preparation [23], presumably a mixture of all isoenzymes originally present in the pig brain extracts, was used in all studies reported herein.

Actomyosin MgATPase assay

Before assay, the thin filament was reconstituted by mixed fixed concentrations of TnI and TnC with various concentrations of TnT, Tm and actin in the dialysis buffer and incubating at 4 °C for 2 h. Ca²⁺-stimulated actomyosin MgATPase was assayed at 30 °C for 10 min in reaction mixtures (0.5 ml) containing 60 mM-KCl, 30 mM-Mops (pH 7.0), 2.3 mM-[γ -³²P]ATP (5×10^6 – 8×10^6 c.p.m.), 3.2 mM-magnesium acetate, 2.0 mM-EGTA, with or without 2.1 mM-CaCl₂ (free Ca²⁺ was calculated to be 20 μ M), 0.2 μ M-myosin and samples of the reconstituted thin-filament mixtures. The final concentrations of each thin-filament component in individual experiments are described in the Figure legends. CaCl₂, when present, was added as a mixture of EGTA and Mops/KOH to give a free Ca²⁺ concentration of 20 μ M in the assay, and appropriate volumes of 5 M-KOH were added to adjust the pH to 7.0. The reactions were initiated by the addition of [γ -³²P]ATP and terminated by addition of charcoal, and the released [³²P]P_i was determined as described [20,34]. Absolute association constant values, used to calculate total concentrations of Mg²⁺, ATP and Ca²⁺ required to give the necessary free Ca²⁺ concentrations in the presence of 1 mM free Mg²⁺ and 2.2 mM-MgATP, were those reported by Fabiato &

Fabiato [35] and Grynkiewicz *et al.* [36]. The association constants for ATP were adjusted for temperature and ionic strength as suggested by Phillips *et al.* [37]. MgATPase activity was linear as a function of incubation time or amount of actomyosin complex under the assay conditions.

F-actin-binding assay

Binding of TnT and Tm to F-actin was assessed by co-sedimentation during ultracentrifugation essentially as described by Mehegan & Tobacman [38]. Samples (500 μ l) containing various concentrations (up to 5 μ M) of F-actin, Tm (up to 0.65 μ M) and TnT (up to 0.8 μ M) were incubated for 30 min at 24 °C in a solution containing 10 mM-imidazole (pH 7.0), 3 mM-MgCl₂, 0.3 M-KCl, 0.4 mM-EGTA, 1.0 mM-dithiothreitol and, when present, 0.45 mM-CaCl₂ (free Ca²⁺ was calculated to be 55 μ M). In some experiments, TnI (0.4 μ M) and TnC (0.4 μ M) were included in the incubation mixtures. After incubation, 200 μ l portions of the mixtures were centrifuged at 250 000 g for 30 min at 24 °C in a TLA-100 rotor using a Beckman TL ultracentrifuge. The compositions of the pellets and supernatants were analysed by SDS/PAGE by the method of Laemmli [39]. The pellets and the supernatants (precipitated with 10% trichloroacetic acid) were dissolved in the Laemmli sample buffer and electrophoresed on either 8.5% or 8–15% linear-gradient SDS/polyacrylamide gels. The gels were stained with 0.15% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol/10% (v/v) acetic acid and destained in 20% (v/v) ethanol/10% (v/v) acetic acid. The relative quantities of actin, Tm, TnT, TnI and TnC on each gel were determined by densitometry using an E-C Apparatus (St. Petersburg, FL, U.S.A.) scanning densitometer with a 605 nm filter. Calibration curves for the individual proteins were constructed from samples containing known quantities of standards applied to adjoining wells during electrophoresis. When TnT was incubated in the absence of actin and Tm, under the above conditions (0.3 M-KCl and pH 7.0), 96% of the total TnT remained in the supernatant. The remainder was presumed to be aggregated and incapable of binding to Tm–F-actin. Parallel binding experiments were also conducted at 0.1 M-KCl and pH 8.0 using the conditions described by Jin & Lin [40]. Under these conditions, 10% of the total TnT was found to be insoluble and presumed to be aggregated. Under both incubation conditions, less than 4% of the Tm was determined to be insoluble or aggregated and less than 20% of the total actin failed to sediment which suggested that more than 80% of the actin was present as F-actin. Under both sets of conditions, the amount of bound TnT or bound Tm was determined as that amount of TnT or Tm appearing in the pellet with F-actin after first correcting for the insoluble or aggregated forms of TnT or Tm. Free (unbound) TnT or Tm was calculated by subtracting the amounts bound from the total amount added to the assay mixtures after correcting for the insoluble or aggregated TnT or Tm. In order to calculate the maximum binding of the various proteins and their free or total concentrations at 50% maximum binding, as well as to provide a comprehensive measure of the co-operativity of the interactions of the components of the reconstituted thin-filament complex, all of the binding data were analysed by non-linear least-squares regression (SigmaPlot Version 4.1, Jandel Scientific, Corte Madera, CA, U.S.A.) using the Hill equation [41]. All values (means $\pm$ S.D.) reported in the text were calculated using the regression analysis.

Statistical analysis

All concentration-dependence experiments were conducted as paired experiments, i.e. 'unphosphorylated' versus 'phosphorylated' and statistical comparisons were made for each specific

concentration using two-way analysis of variance and, when appropriate, followed by Dunnett's test [42].

RESULTS

In order to better assess effects of PKC-catalysed phosphorylation of bovine cardiac TnT on the Ca^{2+} -stimulated MgATPase activity of actomyosin, preparations of TnT were subjected to dephosphorylation by alkaline phosphatase or phosphoprotein phosphatase 1 before phosphorylation by PKC and reconstitution into Tn. Endogenous phosphate content in TnT as prepared was decreased from 0.6 to 0.3 mol of P/mol by the phosphatase pretreatments. Subsequent exhaustive phosphorylation by PKC resulted in the incorporation of 1.8 mol of P/mol for dephosphorylated TnT compared with 1.5 mol of P/mol for TnT not previously dephosphorylated.

Since our previous studies [20] have shown that PKC phosphorylation of TnT and/or TnI decreased the maximum Ca^{2+} stimulation of actomyosin MgATPase with little or no

Fig. 1. Concentration-dependent effects of unphosphorylated and phosphorylated bovine cardiac TnT on Ca^{2+} -stimulated MgATPase activity of reconstituted actomyosin

Purified bovine cardiac TnT was incubated with alkaline phosphatase or phosphoprotein phosphatase 1 and then either exhaustively phosphorylated by PKC or incubated with the heat-inactivated enzyme (termed 'unphosphorylated'). Actomyosin complex was reconstituted from TnI (0.2 μM), TnC (0.2 μM), various concentrations of unphosphorylated (○, △) or phosphorylated (●, ▲) TnT (up to 1 μM), actin (1 μM), Tm (0.2 μM) and whole myosin (0.2 μM) and the MgATPase activity of the complex was assayed in the presence (○, ●) or absence (△, ▲) of 20 μM - Ca^{2+} as described in the Experimental section. The MgATPase activity of each actomyosin preparation was calculated as a percentage of the maximum activity of the control preparation containing unphosphorylated TnT, which was taken as 100%. The mean maximum MgATPase activity, from this and all subsequent control samples was 60 ± 10 nmol of P/min per mg of protein ($n = 17$). The arrow in this and subsequent Figures denotes the total molar ratio of TnI/TnT/TnC/actin/myosin equal to 1:1:1:5:1 at which maximum Ca^{2+} stimulation of MgATPase activity was usually observed. The data presented are means $\pm$ S.E.M. of four paired experiments (assayed in triplicate), each using a different phosphorylated TnT preparation. In this and subsequent Figures, the asterisks denote that the values are significantly different ($P < 0.05$) from the respective controls, which contained unphosphorylated TnT.

Fig. 2. Concentration-dependent effects of bovine cardiac Tm on Ca^{2+} -stimulated MgATPase activity of actomyosin containing unphosphorylated or phosphorylated TnT

Actomyosin complex was reconstituted from unphosphorylated (○, △) or phosphorylated (●, ▲) TnT (0.2 μM), TnC (0.2 μM), TnI (0.2 μM), actin (1 μM), Tm (concentrations up to 1.0 μM) and whole myosin (0.2 μM) and the MgATPase activity of the complex was assayed in the presence (○, ●) or absence (△, ▲) of 20 μM - Ca^{2+} as described in the Experimental section and in the legend of Fig. 1. The symbols, abbreviations and presentation of enzyme activity are as defined in Fig. 1. The data presented are means $\pm$ S.E.M. of three paired experiments, each using a different phosphorylated TnT preparation per experiment and assayed in duplicate.

effect on the Ca^{2+} sensitivity of the reconstituted contractile apparatus, we suspected that these effects might be due to altered interactions among the thin-filament components. In order to explore the possibility that PKC-phosphorylated TnT might interact differently with the other components of the thin filament, the concentration-dependent effects of unphosphorylated and phosphorylated TnT on actomyosin MgATPase were first examined (Fig. 1). While the total concentrations of the other components of the thin filament were held at a constant molar ratio of 1:1:1:5 (TnC/TnI/Tm/actin), unphosphorylated TnT was found to regulate the activity of actomyosin MgATPase biphasically in the presence of Ca^{2+} ; the activity was progressively stimulated by TnT up to a concentration of 0.2 μM (a molar ratio of 1:1:1:1:5 of TnC/TnI/TnT/Tm/actin, indicated by the arrow) and decreased at higher TnT concentrations. The Ca^{2+} -stimulated activity, in the presence of increasing concentrations of phosphorylated TnT, was decreased relative to unphosphorylated TnT and this difference was maximal (a 48% decrease in the net Ca^{2+} -stimulated activity) at 0.2 μM -phosphorylated TnT (Fig. 1). In contrast with the biphasic effects seen in the presence of Ca^{2+} mentioned above, the MgATPase activity in the absence of Ca^{2+} progressively decreased with increasing concentrations of TnT and, moreover, attenuation of this effect was noted for phosphorylated TnT only at 0.1 μM (Fig. 1).

Since the decreased Ca^{2+} -stimulated MgATPase activity of the reconstituted actomyosin containing Tn with phosphorylated TnT might be explained by altered interactions of Tn with Tm and actin, the concentration-dependent effects of Tm (Fig. 2) and actin (Fig. 3) on the Ca^{2+} -stimulated MgATPase activity was examined next. In the presence of constant molar ratios of

Fig. 3. Concentration-dependent effects of bovine cardiac actin on Ca^{2+} -stimulated MgATPase activity of actomyosin containing unphosphorylated or phosphorylated TnT

Actomyosin complex was reconstituted from phosphorylated (●) or unphosphorylated (○) TnT (0.2 μM), TnC (0.2 μM), TnI (0.2 μM), actin (concentrations up to 5 μM), Tm (0.2 μM) and whole myosin (0.2 μM), and the MgATPase activity of the complex was assayed in the presence or absence of 20 μM - Ca^{2+} as described in the Experimental section and in the legend of Fig. 1. The symbols, abbreviations and presentation of enzyme activity are as defined in Fig. 1. The data presented (the net Ca^{2+} -stimulated activity) are means $\pm$ s.e.m. of three paired experiments each using a different phosphorylated TnT preparation and assayed in triplicate.

TnC/TnI/TnT/actin/myosin equal to 1:1:1:5:1, we found that Tm in the presence of Ca^{2+} progressively stimulated the actomyosin MgATPase activity and slightly inhibited the activity at higher concentrations (Fig. 2). When the reconstituted actomyosin complex contained phosphorylated TnT, the Ca^{2+} -stimulated activity was decreased by 20–30%. Tm progressively inhibited the Ca^{2+} -independent (basal) MgATPase activity of actomyosin containing unphosphorylated or phosphorylated TnT (Fig. 2). Similarly, at the constant total molar ratio of TnC/TnI/TnT/Tm/myosin equal to 1:1:1:1:1, phosphorylated TnT decreased (up to 50%) the actin-dependent Ca^{2+} -stimulated actomyosin MgATPase activity (Fig. 3).

The above results indicated that PKC-phosphorylated TnT decreased MgATPase activity presumably due, in part, to altered interactions with other components of the actomyosin complex. To examine these interactions more closely, we investigated the binding of TnT with Tm-F-actin. Since TnT is insoluble at low ionic strengths (concentrations of KCl less than 0.3 M) [38], we performed the majority of these studies using the conditions (0.3 M-KCl, pH 7.0) established by Mehegan & Tobacman [38] for the binding of TnT-TnI to Tm-F-actin. The concentration-dependent binding of unphosphorylated TnT to Tm-F-actin (at a total molar ratio of 1:5) compared with that of phosphorylated TnT is shown (Fig. 4). Maximum binding of unphosphorylated TnT to Tm-F-actin, calculated from least-squares regression analysis of the data using the Hill equation, was not significantly different from that of phosphorylated TnT (0.17 ± 0.01 compared

Fig. 4. Concentration-dependent binding of unphosphorylated and phosphorylated bovine cardiac TnT to Tm-F-actin

Tm (0.4 μM) and actin (2.0 μM) were mixed with various concentrations of unphosphorylated (○) or phosphorylated (●) TnT (up to 0.6 μM) in the presence of 0.3 M-KCl. The binding of TnT to Tm-F-actin was determined by cosedimentation with F-actin (polymerized actin, determined to be at least 1.6 μM) followed by SDS/PAGE of the pellets and supernatants as described in the Experimental section. The symbols and abbreviations are as defined in Fig. 1. The data represent means $\pm$ s.e.m. of four paired experiments using two different phosphorylated TnT preparations.

Fig. 5. Concentration-dependent effects of Tm on the binding of unphosphorylated and phosphorylated TnT to Tm-F-actin

Unphosphorylated or phosphorylated TnT (0.4 μM) and actin (2.0 μM) were mixed with various concentrations of Tm (up to 0.65 μM) and the binding of unphosphorylated (○) and phosphorylated (●) TnT was analysed by cosedimentation with F-actin as described in the legend of Fig. 4 and in the Experimental section. The symbols and abbreviations are as defined in Fig. 1. The data represent means (ranges being $< \pm 10\%$) from two paired experiments using two phosphorylated TnT preparations.

Fig. 6. Concentration-dependent effects of actin on the binding of phosphorylated and unphosphorylated TnT to Tm-F-actin

Phosphorylated (●) or unphosphorylated (○) TnT ($0.4 \mu\text{M}$) and Tm ($0.4 \mu\text{M}$) were mixed with various concentrations of actin (up to $5 \mu\text{M}$) and the binding of TnT to Tm-F-actin was analysed by cosedimentation with F-actin as described in the legend to Fig. 4 and in the Experimental section. The symbols and abbreviations are as defined in Fig. 1. The data represent means $\pm$ S.E.M. from three paired experiments using one phosphorylated TnT preparation.

with $0.16 \pm 0.01 \mu\text{M}$). In contrast, phosphorylation of TnT by PKC resulted in a 1.7-fold increase (from 0.06 ± 0.01 to $0.10 \pm 0.02 \mu\text{M}$) in the concentration of free TnT at 50% maximum binding (Fig. 4), suggesting a decreased affinity of phosphorylated TnT for Tm-F-actin. A Hill coefficient of 1.5 ± 0.2 was determined for the binding of both phosphorylated and unphosphorylated TnT, indicating that phosphorylation of TnT did not affect the co-operativity of its binding. In the same experiments, the concentration-dependent effects of unphosphorylated and phosphorylated TnT on the binding of Tm to F-actin were also assessed (results not shown). We observed that

the binding of Tm to F-actin was dependent on the TnT concentration, that the total concentration of phosphorylated TnT that caused 50% maximum Tm binding was 1.5-fold higher than that of unphosphorylated TnT (0.25 ± 0.02 compared with $0.17 \pm 0.02 \mu\text{M}$), and that maximum binding of Tm was the same in the presence of unphosphorylated or phosphorylated TnT ($0.2 \pm 0.03 \mu\text{M}$). The free TnT concentrations at 50% maximal Tm binding were $0.10 \pm 0.01 \mu\text{M}$ and $0.15 \pm 0.02 \mu\text{M}$ for unphosphorylated and phosphorylated TnT respectively. In contrast, in similar experiments using the conditions of Jin & Lin [40] (0.1 M-KCl , pH 8.0), we found that Tm binding to $1.8 \mu\text{M}$ -F-actin was already 50% of maximal in the absence of TnT, and further binding (maximum of $0.26 \mu\text{M}$) was not appreciably different in the presence of unphosphorylated or phosphorylated TnT (results not shown). However, the maximum bound unphosphorylated TnT ($0.26 \mu\text{M}$) was 1.4-fold greater than that of phosphorylated TnT ($0.18 \mu\text{M}$) and the concentration of free unphosphorylated TnT at 50% maximal binding was 30% less than that of phosphorylated TnT (0.12 compared with $0.17 \mu\text{M}$).

At a constant molar ratio of total TnT/actin of 1:5, the concentration-dependent effects of Tm on the binding of unphosphorylated and phosphorylated TnT to Tm-F-actin were next investigated (Fig. 5). Under these conditions, the maximum binding of phosphorylated TnT was 20% less than that of unphosphorylated TnT (0.15 compared with $0.19 \mu\text{M}$), but the concentrations of Tm that caused 50% maximum binding of unphosphorylated or phosphorylated TnT were identical ($0.15 \mu\text{M}$ total Tm or $0.1 \mu\text{M}$ free Tm). The same Hill coefficient (1.7) was found for the binding of either unphosphorylated or phosphorylated TnT to Tm-F-actin. In the same experiments, the concentration-dependent binding of Tm to F-actin, in the presence of unphosphorylated and phosphorylated TnT, was also assessed (results not shown). The findings indicated that the maximal binding ($0.21 \mu\text{M}$), the apparent binding affinity (free Tm concentration of $0.14 \mu\text{M}$ at 50% maximum binding) and the binding co-operativity (Hill coefficient of 2.5) of Tm for F-actin were essentially unaltered by phosphorylated TnT.

The binding of TnT to Tm-F-actin as a function of the concentration of F-actin was assessed as a constant molar ratio of total TnT/Tm equal to 1:1 (Fig. 6). We found that maximal binding of TnT to Tm-F-actin was lower for phosphorylated TnT ($0.14 \pm 0.01 \mu\text{M}$) than that for unphosphorylated TnT

Fig. 7. Concentration-dependent binding of unphosphorylated and phosphorylated TnT to Tm-F-actin in the presence of TnI and TnC

TnI ($0.4 \mu\text{M}$), TnC ($0.4 \mu\text{M}$), Tm ($0.4 \mu\text{M}$) and actin ($2.0 \mu\text{M}$) were mixed with various concentrations of unphosphorylated or phosphorylated TnT (up to $0.8 \mu\text{M}$). The binding of TnT to Tm and F-actin was analysed by cosedimentation with F-actin as described in the legend of Fig. 4 and in the Experimental section. Binding of unphosphorylated (○) and phosphorylated (●) TnT to Tm and F-actin was carried out in the absence (a) or presence (b) of $50 \mu\text{M-Ca}^{2+}$. The symbols and abbreviations are as defined in Fig. 1. The data are means (ranges being $< \pm 5\%$) from two paired experiments using one phosphorylated TnT preparation.

($0.20 \pm 0.01 \mu\text{M}$), whereas the concentration of F-actin that caused 50% maximal TnT binding was higher for phosphorylated TnT ($0.50 \pm 0.04 \mu\text{M}$) than that for unphosphorylated TnT ($0.39 \pm 0.02 \mu\text{M}$). Binding of Tm to F-actin under the same experimental conditions, in contrast, was unaffected by phosphorylation of TnT by PKC (results not shown).

Finally, we determined the concentration-dependent binding of unphosphorylated and phosphorylated TnT to Tm-F-actin in the presence of TnC and TnI (Fig. 7). In the absence of Ca^{2+} , the maximum binding of phosphorylated TnT ($0.15 \pm 0.01 \mu\text{M}$) was lower than that of unphosphorylated TnT ($0.19 \pm 0.01 \mu\text{M}$) (Fig. 7a). In addition, the concentration of free (unbound) phosphorylated TnT ($0.21 \pm 0.02 \mu\text{M}$) at 50% maximum binding was much higher than that for unphosphorylated TnT ($0.06 \pm 0.01 \mu\text{M}$). Others have shown that Tn binding to Tm-F-actin in the presence of Ca^{2+} is decreased [2,38,43,44]. We found that Ca^{2+} ($50 \mu\text{M}$) also increased the concentration of free unphosphorylated TnT at 50% maximum binding ($0.19 \pm 0.03 \mu\text{M}$) with little or no effect on its maximum binding ($0.17 \pm 0.02 \mu\text{M}$) (Fig. 7b). In contrast, Ca^{2+} did not affect the concentration of free phosphorylated TnT ($0.20 \pm 0.02 \mu\text{M}$) at 50% maximum binding to Tm-F-actin, but substantially decreased its maximum binding ($0.10 \pm 0.01 \mu\text{M}$). In addition, Ca^{2+} significantly increased the co-operativity (Hill coefficients) in binding for both phosphorylated TnT (from 1.4 to 2.2) and unphosphorylated TnT (from 1.1 to 1.6). We also observed, in the same experiment, that unphosphorylated TnT caused a greater maximum binding of Tm to F-actin than that caused by phosphorylated TnT (0.2 compared with $0.15 \mu\text{M}$ in the absence of Ca^{2+} ; 0.24 compared with $0.17 \mu\text{M}$ in the presence of Ca^{2+}). Furthermore, Ca^{2+} increased the concentration of unphosphorylated TnT required to cause 50% maximal binding of Tm (from $0.14 \mu\text{M}$ in the absence of Ca^{2+} to $0.22 \mu\text{M}$ in its presence) and these values were unaffected by phosphorylated TnT (results not shown).

DISCUSSION

In striated muscle, Tn (composed of TnC, TnI and TnT subunits) and Tm are regulatory components of the contractile apparatus required for Ca^{2+} -dependent contraction [2,21]. In the relaxed state (in the absence of Ca^{2+}), TnI and Tm inhibit actomyosin MgATPase by blocking the interaction of myosin

heads with actin and hence prevent actin-myosin cross-bridge cycling. In the presence of Ca^{2+} , the contractile apparatus becomes 'activated', beginning with a conformational change in the structure of TnC on the binding of Ca^{2+} [45], which results in an increased affinity of TnC for the MgATPase-inhibitory region of TnI [46-48] and functional detachment of TnI from actin [49]. This allows Tm to roll toward the centre of the actin helical groove, thus uncovering myosin-binding sites on actin and ultimately leading to actomyosin MgATPase activation and muscle contraction [2,21]. The interactions among the thin-filament components in the relaxed (Scheme 1a) and fully activated (Scheme 1b) states, as suggested by Leavis & Gergely [2] and Solaro & Pan [50], are illustrated. We [19] and others [22] have shown that PKC phosphorylates bovine cardiac TnT at residues 190, 194, 199 and 280, all of which are located in the C-terminal half of TnT where binding to Tm, TnI and TnC occurs [2,21]. Phosphorylation of TnT would conceivably result in alteration (possibly weakening) of the interactions of TnT with Tm, TnI and possibly TnC (Scheme 1c), while still allowing full inhibition by TnI of the MgATPase activity in the absence of Ca^{2+} (Fig. 1). Since TnT bound to Tn-F-actin is also required for full activation of actomyosin MgATPase activity in the presence of Ca^{2+} (Fig. 1 and refs. [2,21]), weakened interactions of phosphorylated TnT with TnI, Tm and TnC would not affect the binding of TnC- Ca^{2+} to TnI, yet hinder the detachment of TnI from actin. Thus full movement of Tm into the helical groove of actin would be prevented, leading to attenuation of the Ca^{2+} -stimulated MgATPase activity or partial activation. The above hypothesis is consistent with our data showing that phosphorylated TnT, under a variety of conditions, decreased Ca^{2+} -stimulated actomyosin MgATPase activity (Figs. 1-3, and ref. [20]) and that, in the presence or absence of TnC and TnI, phosphorylated TnT had decreased maximal binding to and affinity for Tm-F-actin (Figs. 4-6).

The inhibitory effects of both PKC-phosphorylated cardiac TnT and TnI on the Ca^{2+} -stimulated MgATPase activity of actomyosin [20] may have important consequences in the development and pathophysiology of the heart. Both TnI and TnT undergo isoform switching in the developing heart, where fetal isoforms of the two subunits are replaced by adult isoforms shortly after birth, and TnT in the adult heart is present as two isoforms differing at their N-termini [51-60]. Altered Ca^{2+} sensitivity of reconstituted Tn, containing different adult TnT isoforms, has been reported [58]. Furthermore, changes in Ca^{2+} -activation and Ca^{2+} -binding properties of TnC have been shown to occur in developing cardiac myofibrils [61] and these changes may be associated with isoform switching in TnI and TnT. In addition, human myopathic cardiac tissue has two distinct isoforms of TnT as opposed to the single major species in normal human myocardial tissue [62]. It is clear that additional studies are required to determine if PKC phosphorylation of the different isoforms of TnT or TnI would result in changes in the interaction of the components of the thin filament, and hence the Ca^{2+} sensitivity and/or activity of actomyosin MgATPase.

Finally, the PKC-activating phorbol esters have been shown to have negative inotropic effects on various cardiac preparations [4-11]. Recent evidence [8-9] suggests that one possible mechanism for these effects is through the phosphorylation of phospholamban by PKC [16] and the resulting inhibition of Ca^{2+} transport by the sarcoplasmic reticulum. We have previously hypothesized that PKC phosphorylation of TnI and TnT could also exert a negative inotropic effect at the level of the contractile apparatus [20]. This notion is supported by our recent findings that phorbol ester stimulated the phosphorylation of TnI and TnT in cardiac myocytes from neonatal [63] and adult rats (R. Venema & J. F. Kuo, unpublished work). Furthermore,

Scheme 1. Postulated effects of PKC phosphorylation of TnT on interactions among the thin-filament components

See the text for explanation. Symbols are: C, TnC; I, TnI; T, unphosphorylated TnT; T(P), phosphorylated TnT; Tm, tropomyosin; A, actin; M, myosin; (+) and (-), stimulatory and inhibitory effects on actin to activate myosin MgATPase, respectively; =, — and ———, strong, moderate and weak interactions at functional sites respectively.

Gwathmey & Hajjar [12] have reported that phorbol ester treatment of human intact and skinned myocardial muscle fibres resulted in decreased peak twitch force, Ca^{2+} release from the sarcoplasmic reticulum and Ca^{2+} sensitivity of myofibrillar force development. It remains to be determined if PKC phosphorylation of TnT and TnI is involved here and which phosphorylation sites are responsible for these functional changes. We have also observed that adult rat cardiac myofibrillar preparations subjected to phosphorylation by PKC had decreased Ca^{2+} -stimulated MgATPase activity (T. Noland & J. F. Kuo, unpublished work), in line with our earlier reports that phosphorylation of TnI and/or TnT in various troponin preparations decreased the enzyme activity in reconstituted actomyosin complex [20].

This work was supported by U.S. Public Health Institutes Research Grants HL-15696, CA-36777 and NS 17608. We thank Marlise Casteel and Stephanie Sanders for their expert preparation of the manuscript.

REFERENCES

- Stull, J. T. (1980) *Adv. Cyclic Nucleotide Res.* **13**, 39–93
- Leavis, P. C. & Gergely, J. (1984) *CRC Crit. Rev. Biochem.* **16**, 233–305
- Solaro, R. J. (1986) in *Protein Phosphorylation in Heart Muscle* (Solaro, R. J., ed.), pp. 129–156, CRC Press, Boca Raton, FL
- Leatherman, G. F., Kim, D. & Smith, T. W. (1987) *Am. J. Physiol.* **253**, H205–H209
- Yuan, S., Sunahara, F. A. & Sen, A. K. (1987) *Circ. Res.* **61**, 372–378
- Dosemeci, A., Dhallan, R. S., Cohen, N. M., Lederer, W. J. & Rogers, T. B. (1988) *Circ. Res.* **62**, 347–357
- Karmazyn, M., Watson, J. E. & Moffat, M. P. (1990) *B. J. Pharmacol.* **100**, 826–830
- Capogrossi, M. C., Kaku, T., Filburn, C. R., Pelto, D. J., Hansford, R. G., Spurgeon, H. A., & Lakatta, E. G. (1990) *Circ. Res.* **66**, 1143–1155
- Rogers, T. B., Gaa, S. T., Massey, C. & Dosemeci, A. (1990) *J. Biol. Chem.* **265**, 4302–4308
- Edes, I. & Kranias, E. G. (1990) *Circ. Res.* **67**, 394–400
- Watson, J. E. & Karmazyn, M. (1991) *Circ. Res.* **69**, 1114–1131
- Gwathmey, J. K. & Hajjar, R. J. (1990) *Circ. Res.* **67**, 744–752
- Kaku, T., Lakatta, E. & Filburn, C. (1991) *Am. J. Physiol. (Cell Physiol.)* **260**, C635–C642
- Talosi, L. & Kranias, E. G. (1992) *Circ. Res.* **70**, 670–678
- Fuller, S. J., Gaitanki, C. J., Hatchett, R. J. & Sugden, P. H. (1991) *Biochem. J.* **273**, 347–353
- Movsesian, M. A., Nishikawa, M. & Adelstein, R. S. (1984) *J. Biol. Chem.* **259**, 8029–8032
- Kato, N., Wise, B. C. & Kuo, J. F. (1983) *Biochem. J.* **209**, 189–195
- Mazzei, G. J. & Kuo, J. F. (1984) *Biochem. J.* **218**, 361–369
- Noland, T. A., Jr., Raynor, R. L. & Kuo, J. F. (1989) *J. Biol. Chem.* **264**, 20778–20785
- Noland, T. A., Jr. & Kuo, J. F. (1991) *J. Biol. Chem.* **266**, 4974–4978
- Zot, A. & Potter, J. D. (1987) *Annu. Rev. Biophys. Chem.* **16**, 535–559
- Swiderek, K., Jaquet, K., Meyer, H. E., Schachte, C., Hofmann, F. & Heilmeyer, L. M. G., Jr. (1990) *Eur. J. Biochem.* **190**, 575–582
- Girard, P. R., Mazzei, G. J. & Kuo, J. F. (1986) *J. Biol. Chem.* **261**, 370–376
- Huang, K.-P., Nakabayashi, H. & Huang, F. L. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8535–8539
- Stewart, A. A., Hemmings, B. A., Choen, P., Goris, J. & Merlevede, W. (1981) *Eur. J. Biochem.* **115**, 197–205
- Potter, J. D. (1982) *Methods Enzymol.* **85**, 234–241
- Stull, J. T. & Buss, J. E. (1977) *J. Biol. Chem.* **252**, 851–857
- Pardee, J. D. & Spudich, J. A. (1982) *Methods Enzymol.* **85**, 164–181
- Hartzell, H. C. (1985) *J. Mol. Biology* **186**, 185–195
- Siemankowski, R. F. & White, H. D. (1984) *J. Biol. Chem.* **259**, 5045–5053
- Helfman, D. M., Barnes, K. C., Kinkade, J. M., Jr., Vogler, W. R., Shoji, M. & Kuo, J. F. (1983) *Cancer Res.* **43**, 2955–2961
- Martensen, T. M. (1984) *Methods Enzymol.* **107**, 3–23
- Raynor, R. L., Zheng, B. & Kuo, J. F. (1991) *J. Biol. Chem.* **266**, 2753–2758
- Oishi, K., Zheng, B. & Kuo, J. F. (1990) *J. Biol. Chem.* **265**, 70–75
- Fabiato, A. & Fabiato, F. (1979) *J. Physiol. (Paris)* **75**, 463–505
- Gryniewicz, G., Poenie, M. & Tsein, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- Phillips, R. C., George, P. & Rutman, R. J. (1966) *J. Am. Chem. Soc.* **88**, 2631–2640
- Mehegan, J. P. & Tobacman, L. S. (1991) *J. Biol. Chem.* **266**, 966–972
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Jin, J.-P. & Lin, J. J.-C. (1988) *J. Biol. Chem.* **263**, 7309–7315
- Hill, A. V. (1910) *J. Physiol. (London)* **40**, iv–vii
- Dunnett, C. W. (1964) *Biometrics* **20**, 482–491
- Wegner, A. & Walsh, T. P. (1981) *Biochemistry* **20**, 5633–5642
- Heeley, D. H., Golosinska, K. & Smillie, L. B. (1987) *J. Biol. Chem.* **262**, 9971–9978
- Grabarek, A., Leavis, P. C. & Gergely, J. (1986) *J. Biol. Chem.* **261**, 608–613
- Van Eyk, J. E. & Hodges, R. S. (1988) *J. Biol. Chem.* **263**, 1726–1732
- Talbot, J. S. & Hodges, R. S. (1979) *J. Biol. Chem.* **254**, 3720–3723
- Talbot, J. A. & Hodges, R. S. (1981) *J. Biol. Chem.* **256**, 2798–2802
- Tao, T., Gong, B.-J. & Leavis, P. C. (1990) *Science* **247**, 1339–1341
- Solaro, R. J. & Pan, B.-S. (1989) in *Physiology and pathophysiology of the heart*, 2nd edn., pp 291–303, Kluwer Academic, Boston
- Saggini, L., Gorza, L., Ausoni, S. & Schiaffino, S. (1989) *J. Biol. Chem.* **264**, 16299–16302
- Sabry, M. A. & Dhoot, G. K. (1989) *J. Muscle Res. Cell Motil.* **10**, 85–91
- Murphy, A. M., Jones, L., II, Sims, H. F. & Strauss, A. W. (1991) *Biochemistry* **30**, 707–712
- Leszyk, J., Dumaswala, R., Potter, J. D. & Collins, J. H. (1988) *Biochemistry* **27**, 2821–2827
- Leszyk, J., Dumaswala, R., Potter, J. D., Gusev, N. B., Verin, A. D., Tobacman, L. S. & Collins, J. H. (1987) *Biochemistry* **26**, 7035–7042
- Gusev, N. B., Barskaya, N. V., Verin, A. D., Duzhenkova, I. V., Khuchua, Z. A. & Zheltova, A. O. (1983) *Biochem. J.* **213**, 123–129
- Risnik, V. V., Verin, A. D. & Gusev, N. B. (1985) *Biochem. J.* **225**, 549–552
- Tobacman, L. S. & Lee, R. (1987) *J. Biol. Chem.* **262**, 4059–4064
- Saggini, L., Ausoni, S., Gorza, L., Sartore, S. & Schiaffino, S. (1988) *J. Biol. Chem.* **263**, 18488–18492
- Cooper, T. A. & Ordahl, C. P. (1985) *J. Biol. Chem.* **260**, 11140–11148
- McAuliffe, J. J., Gao, L. & Solaro, R. J. (1990) *Circ. Res.* **66**, 1204–1216
- Anderson, P. A. W., Oakley, A. & Allen, P. D. (1989) *Circulation* **80** (suppl. II), II-503 (abstr.)
- Liu, J. D., Wood, J. G., Raynor, R. L., Wang, Y.-C., Noland, T. A., Jr., Ansari, A. A. & Kuo, J. F. (1989) *Biochem. Biophys. Res. Commun.* **162**, 1105–1110

Received 16 December 1991/27 April 1992; accepted 19 May 1992