Changes in Cardiac Contractility Related to Calcium-Mediated Changes in Phosphorylation of Myosin-Binding Protein C

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ABSTRACT Ca ions can influence the contraction of cardiac muscle by activating kinases that specifically phosphorylate the myofibrillar proteins myosin-binding protein C (MyBP-C) and the regulatory light chain of myosin (RLC). To investigate the possible role of Ca-regulated phosphorylation of MyBP-C on contraction, isolated quiescent and rhythmically contracting cardiac trabeculae were exposed to different concentrations of extracellular Ca and then chemically skinned to clamp the contractile system. Maximum Ca-activated force (F_{max}) was measured in quiescent cells soaking in 1) 2.5 mM Ca for 120 min, 2) 1.25 mM for 120 min, or 3) 1.25 mM for 120 min followed by 10 min in 7.5 mM, and 4) cells rhythmically contracting in 2.5 mM for 20 min. F_{max} was, respectively, 21.5, 10.5, 24.7, and 32.6 mN/mm^2. Changes in F_{max} were closely associated with changes in the degree of phosphorylation of MyBP-C and occurred at intracellular concentrations of Ca below levels associated with phosphorylation of RLC. Monophosphorylation of MyBP-C by a Ca-regulated kinase is necessary before β-adrenergic stimulation can produce additional phosphorylation. These results suggest that Ca-dependent phosphorylation of MyBP-C modulates contractility by changing thick filament structure.

INTRODUCTION

For the heart to supply the various parts of an organism with blood commensurate with the level of activity, the individual myocardial cells must be able to vary the work they do by a factor of at least five. Furthermore, these changes in power must occur very rapidly, on a time scale of fractions of a minute to a few minutes. Change in the isoforms of myofilament proteins is too slow to provide this type of regulation. There is a very large body of evidence showing that the cytoplasmic concentration of Ca ions reached during activation of contraction alters thin filament structure and its regulation of contraction. Ca is a major determinant of the work done during a contraction of the heart (Solaro et al., 1996; Marban et al., 1986). Because the contractile system in myocardial cells is rarely if ever maximally activated under normal conditions, increase in the concentration of activating Ca will increase force production and work. The force of contraction at suboptimal concentration of Ca can also be raised by an increase in the Ca sensitivity of the contractile system. The affinity of the binding sites on troponin C (TNC) for Ca is sensitive to the state of phosphorylation of troponin I (TNI), with phosphorylation of TNI decreasing the binding of Ca.

Two and possibly three myofilament proteins besides TNI can be phosphorylated by physiological reactions: the myosin-binding protein C (MyBP-C), the regulatory light chain of myosin (RLC), and the tropomyosin-binding subunit of troponin (TNT). In MyBP-C, the phosphorylation sites are specific for cardiac muscle, an occurrence that suggests a function unique to cardiac muscle (Gautel et al., 1995). Phosphorylation of MyBP-C and RLC can be produced by different Ca-calmodulin-regulated kinases present in the myofibrils (Silver et al., 1986; Hartzell and Glass, 1984; Schlender and Bean, 1991). In response to phosphorylation of RLC, there is an increase in the Ca sensitivity of activation of contraction at submaximal concentrations of Ca without alteration of the maximum Ca-activated force (F_{max}). Absence of contractile activity decreases phosphorylation of RLC to very low levels (High and Stull, 1980; Bassani et al., 1995). The level rises when contractile activity is resumed. RLC appears to be phosphorylated primarily when the concentration of Ca rises above the threshold for activating contraction. In cardiac muscle, because of the relatively low concentration of myosin light chain kinase (Stull et al., 1980; Silver et al., 1986), changes in the degree of phosphorylation of RLC occur slowly. The enzyme requires greater than 1 μM Ca to achieve a significant level of activation (Kardami and Gratzer, 1982). Protein kinase A (PKA) can also phosphorylate MyBP-C (Hartzell and Glass, 1984; Schlender and Bean, 1991).

The effect of phosphorylation of MyBP-C on cardiac contraction is not clear, but it does alter the structure of the thick filament and may modify the kinetics of cross-bridge cycling as a result (Weisberg and Winegrad, 1998). It may also alter the mechanical properties of the hinge region of myosin (Kunst et al., 2000). Extraction of a portion of MyBP-C from skinned cardiac myocytes changes the Ca sensitivity without altering F_{max} (Hofmann et al., 1991). In view of the specificity of Ca-activated phosphorylation of MyBP-C for cardiac muscle and the existence of a specific Ca-calmodulin-regulated kinase for the phosphorylation of MyBP-C (Hartzell and Grass,1984; Schlender and Bean, 1991), it is a reasonable assumption that this phosphorylation may influence the kinetics and even the maximum level of force development. To distinguish this effect of Ca on the

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contraction from that of direct Ca activation by binding to TNC, it is necessary to measure force development under conditions where the level of Ca activation is not limiting. This can be accomplished with skinned heart cells, in which the plasma membrane has been made permeable to Ca buffer systems. There is the danger that the skinned cells may lose cellular components essential for the regulatory process such as calmodulin. However, it is possible to avoid this potential problem by first changing the state of the contractile system in the intact cardiac cells and then clamping the force-developing state of the contractile system by skinning the cells with Triton X-100. Treatment with Triton X-100 does not alter F_{max} or, apparently, cross-bridge kinetics (Gao et al., 1994; Kentish et al., 1986; Saeki et al., 1991). Triton X-100 can change the relation between Ca concentration and degree of activation by shifting the Ca-force relation to higher concentrations of Ca and thereby alter force at submaximal Ca activation. Dissociation of phosphorylation of MyBP-C from that of RLC should be possible in the intact cell where regulatory components are present by distinguishing between phosphorylation taking place at Ca concentration above and below the threshold for activation of contraction (Silver et al., 1986; High and Stull, 1980; Kardami and Gratzer, 1982; Stull et al., 1980). The loss of calmodulin and the apparent inactivation of myosin light chain kinase and the Ca-calmodulin-regulated kinase in the thick filament during the skinning process inhibit both phosphorylations in skinned fibers (Sweeney and Stull, 1986; Levine et al., 1996).

Lin et al. (1991) used this approach to examine the effect of rhythmic contractile activity on the maximum level of force development. They maintained isolated rat ventricular trabeculae or papillary muscles in a quiescent state for 40–45 min and then skinned the cardiac cells with Triton X-100. These cells developed less force under maximum Ca activation (F_{max}) than cells from the same heart that had been stimulated to contract at 12–30 times per minute during the 40–45-min period. Three different procedures, a brief period of electrical stimulation producing contraction, exposure to isoproterenol, or brief exposure to 3X normal concentration of extracellular Ca, reversed most or all of the decline in maximum Ca-activated force that had occurred during the prolonged period of quiescence. Because the most obvious change common to all three procedures was an increase in intracellular Ca, it was assumed that Ca-regulated changes in the contractile mechanism could alter F_{max}.

To determine whether F_{max} can be modulated by Ca-regulated changes in myofilament proteins in the resting state we have undertaken a study of the effects of quiescence in reduced and in elevated extracellular Ca on F_{max} and phosphorylation of MyBP-C. Bassani et al. (1995) have shown that following the termination of rhythmic contractions, the intracellular concentration of Ca slowly declines; over 60 min it decreases from 290 to ~80 nM. Our results show that there are two different Ca-sensitive reactions that can modify the maximum Ca-activated force. One is regulated by Ca at concentrations that are below the threshold for activation of force, and the second is regulated by Ca in the same concentration range as force development. The changes in F_{max} that occur entirely within the range of Ca concentration below contraction threshold are correlated with changes in the phosphorylation of MyBP-C.

MATERIALS AND METHODS

Measurement of developed force

Trabeculae and papillary muscles were removed from the hearts of 39 male Wistar rats weighing between 150 and 250 g in accordance with American Association of Laboratory Animal Care (AALAC) guidelines and mounted for continuous measurement of force as previously described (Lin et al., 1991; McClellan et al., 1996). The cross-sectional area of the trabeculae and papillary muscle was between 0.01 and 0.10 mm^2. Radii varied from 0.06 to 0.24 mm, but most were between 0.08 and 0.15 mm. Sarcomere length was 2.3 ± 0.1 μm after skinning. It was not followed during contractions. The stability of F_{max} indicates that sarcomere length did not change significantly during an experiment. Maximum Ca-activated force (F_{max}) generally occurred in pCa of 5.0. The value for F_{max} that was used was always the maximum force. Confirming the adequacy of the supply of energy to the contractile system, the values for F_{max} and maximum velocity of unloaded shortening were not altered by increasing the concentration of ATP, creatine phosphate, or creatine kinase in the bathing solutions by 50%. The relaxing solution contained 100 mM KCl, 3 mM EGTA, 7 mM $MgCl_2$, 5 mM ATP, 15 mM creatine phosphate, and 25 mM imidazole at pH 7.0 and 23°C. Appropriate amounts of $CaCl_2$ were added to produce the desired pCa based on the program of Fabiato and Fabiato (1979). The skinning solution was relaxing solution with the addition of 1% Triton X-100. Because of the presence of creatine kinase in the myofibrils it was not necessary to routinely add this enzyme to the bathing solutions (McClellan et al., 1983; Lin et al., 1991).

Measurement of phosphorylation of MyBP-C

For separation of different phosphorylated forms of MyBP-C, three types of non-urea isoelectric focusing (IEF) polyacrylamide slab gels were used: 1) pH 3–7 IEF gels from Novex, Carlsbad, CA 2) pH 5–8 IEF gels from Bio-Rad, Hercules, CA and 3) in-house prepared IEF slab gels consisting of 5% acrylamide/Bis (25% T/4% C), 5% glycerol, 5% ampholites (Bio-Rad), and three catalysts added separately (riboflavin-5′-phosphate, ammonium persulfate, and *N,N,N,N,*-tetramethylethylenediamine (TEMED). Samples were loaded in buffer containing 15% glycerol and ampholites, and in some samples 1% glycine, 2% Triton X-100, or CHAPS detergent was used. The results were not significantly dependent on which of these additions was present. For the gel running buffers, the cathode buffers consisted of 40 mM lysine (free base) or 20 mM sodium hydroxide (pH 10.1), and the anode buffer consisted of 20 mM phosphoric acid (pH 2.4). The running conditions were either 2 W per gel constant for 2.5–3 h or a combination of 100 V for 1 h, 200 V for 1–2 h, and 500 V for 30 min. The transfer took place in 0.7% acetic acid (pH 3), placing the polyvinylidene difluoride (PVDF) membrane toward anode running at 10 V for 1 h. After the transfer, an immunodetection procedure was performed according to ECL Western blotting protocol (Amersham Pharmacia Biotechnology or Western Breeze from Novex), using a polyclonal antibody against MyBP-C (supplied by Dr. Mathias Gautel of EMBL at the University of Heidelberg, Heidelberg, Germany) and horseradish peroxidase or alkaline phosphatase for detection.

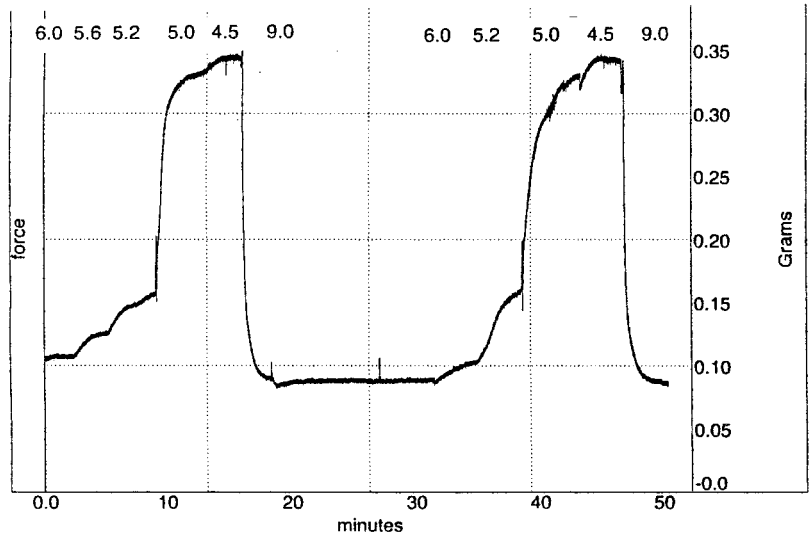

FIGURE 1 Force record showing the response of a cardiac trabecula skinned by Triton X-100 to two identical series of increasing concentrations of Ca (indicated by pCa values) separated by a 10-min period in 1 nM Ca. The response of the tissue is nearly identical in the two series.

Samples were prepared for electrophoresis by either quickly freezing the tissue, homogenizing, and then lysing or by extracting MyBP-C using the Offer-Hartzell protocol (Offer et al., 1973; Hartzell and Glass, 1984). In the first case, muscles were rinsed in relaxing solution and skinned in relaxing solution containing 1% Triton X-100 for 30 min and then rinsed again in relaxing solution. One or two bundles were taken from each dish, placed in relaxing solution containing a protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO), protein kinase inhibitor type 3 (Sigma), and okadaic acid to inhibit phosphatase and quickly frozen in dry ice/ethanol. Frozen tissue was thawed on ice for 30 min and then homogenized at a concentration of 20 mg/ml lysis buffer, which contained 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 10 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM α-glycerophosphate, protease inhibitors cocktail, 100 mM sodium fluoride, protein kinase inhibitor (type 3), and okadaic acid. The combination of NaF and the high concentration of phosphate has been shown to prevent dephosphorylation of myofibrillar protein even in the absence of more specific phosphatase inhibitors (Holyroyde et al., 1979). After homogenization, samples were spun at 13,000 $\times$ g for 20 min, and 20 μl of each supernatant was loaded on IEF gel. For extracting MyBP-C the tissue was kept in the extraction buffer (10 mM EDTA/155 mM phosphate buffer, pH 5.9, containing protease inhibitors cocktail and phosphatase and kinase inhibitors) for 1 h, 2 h, and overnight at 4°C. The extracts were collected and concentrated.

Two-dimensional gels to measure myosin regulatory light chain phosphorylation

Two-dimensional gels were prepared by fixing the first-dimension IEF mini-gel run at pH 3–10 (produced as described above) in 0.7 M trichloro acetic acid, 150 mM sulfosalicylic acid for 30 min. They were then stained with SerwaBlue for 30 min and destained in 20% ethanol. The lanes were cut out and transferred onto second-dimension gels (SDS-PAGE Tris-lycine system) for additional separation.

Statistics

Scans of diffraction patterns and gels were made with the National Institutes of Health Image program. Values for forces were expressed as the mean $\pm$ 1 SE. The Student's t-test was used to determine significance. Values were considered to be statistically significantly different when $p < 0.05$. Where multiple comparisons were made, the value for p was corrected using the Bonferroni method. The χ^2 test was used to evaluate differences in phosphorylation patterns.

RESULTS

The effect of quiescence in low extracellular Ca

Trabeculae skinned by Triton X-100 are very stable (Fig. 1). The average ratio of two successive measurements of maximum Ca-activated force (F_{max}), separated by 10 min of relaxation was 1.00 $\pm$ 0.08 (n = 8), and F_{max} does not change significantly for at least 18 h. F_{max} measured in bundles of quiescent cardiac cells that had been skinned 20–30 min after removal from the heart was 26.3 mN/mm^2

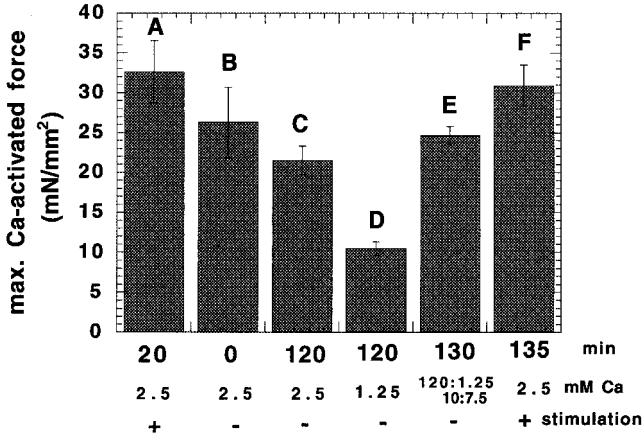

FIGURE 2 Effect of quiescence and extracellular concentration of Ca on maximum Ca-activated force of skinned cardiac tissue. The duration of the exposure of cardiac muscle to different concentrations of Ca with and without electrical stimulation was varied, and F_{max} was measured after skinning of each preparation. Each bar represents the mean $\pm$ SEM of bundles from at least six different hearts. All times are calculated from the end of the 20–30-min recovery period in 2.5 mM Ca. (see Table 1).

TABLE 1 Effects of concentration of extracellular Ca, duration of incubation, and contractions on maximum Ca-activated force in skinned cardiac muscle

	Incubation before skinning			Maximum Ca-activated force		
	Contraction	$[Ca]_e$ (mM)	Time (min)	(mN/mm^2)	n	p
A	12/min	2.5	20	32.6 ± 3.9	6	
B	no	2.5	0	26.3 ± 4.4	6	
C	no	2.5	120	21.5 ± 1.8	6	0.066*; <0.01†
D	no	1.25	120	10.5 ± 0.8	8	<0.0003‡
	no	1.25	120			
E		followed by		24.7 ± 1.1	11	<0.0005§
	no	7.5	10			
F	12/min	2.5	135	30.9 ± 2.8	6	0.045¶

Except for *A*, all times are given from the completion of the 20–30-min recovery period.
*Compared with quiescence for 10–20 min.
†Compared with quiescence for 0 min.
‡Compared with quiescence for 0 and 10–20 min.
§Compared with 90–120 min in 1.25 mM Ca.
¶Compared with quiescence for 10–20 min.

(Fig. 2; Table 1 *B*). This is ~20% lower than F_{max} in rhythmically contracting trabeculae (Fig. 2 *A*). Trabeculae that had been maintained quiescent for an additional 120 min in normal Krebs' solution containing 2.5 mM Ca produced 21.5 mN/mm^2 after skinning (Table 1 *C*). There was a greater decrease in force when the 20–30 min in 2.5 mM was followed by 120 min of quiescence in a bathing solution containing 1.25 mM Ca before skinning (Table 1 *D*). Both values were significantly different from bundles that had been quiescent and skinned after the 20-min recovery period in 2.5 mM Ca (see Table 1, compare *D* with *B*; $p < 0.01$).

When trabeculae that had been quiescent for 120 min in bathing solution containing 1.25 mM Ca were soaked in 7.5 mM Ca bathing solution for an additional 10 min, F_{max} after skinning was 24.7 mN/mm^2, a very significant increase from the value of those that had not been exposed to the high Ca for 10 min (Table 1, compare *E* and *D*; $p < 0.01$). Trabeculae that had been stimulated during the exposure to 2.5 mM Ca before skinning produced 30.9 mN/mm^2 (Table 1 *F*), a value that was significantly greater than that of the trabeculae quiescent in the 1.25 followed by 7.5 mM Ca (Table 1, compare *E* and *F*; $p < 0.05$). Resting tension in the quiescent trabeculae was very stable. There was no change when extracellular Ca was varied between 1.25 and 7.5 mM. Exposure to 7.5 mM Ca was kept to 10 min to avoid possible changes in resting tension.

Phosphorylation of MyBP-C

The degree of phosphorylation of MyBP-C was measured in bundles in each of the conditions described in Fig. 2. Before use of IEF plus Western blotting to determine the relative amounts of the different phosphorylation states of MyBP-C, it was necessary to perform certain important controls. The goal of the phosphorylation study was to compare changes in F_{max} measured in skinned fibers with the pattern of phosphorylation of MyBP-C measured in MyBP-C extracted from the skinned fiber. These values were used as indications of what actually existed in the intact cell before skinning. Therefore it was necessary that F_{max} and the phosphorylation pattern in the skinned fibers not be altered from the pattern that existed in the intact cells before skinning. Even though phosphatase and kinase inhibitors were added to all solutions, it was essential to show that they were totally effective in preventing changes in the phosphorylation of MyBP-C.

Studies reported in the literature have demonstrated that F_{max} is essentially equal to the maximum force developed during contraction of intact cardiac cells (Gao et al., 1994; Kentish et al., 1986; Saeki et al., 1991), and actomyosin ATPase activity is unchanged by Triton X-100 (Solaro et al., 1971). The Ca sensitivity is decreased by Triton treatment, but contractility was measured at the optimal concentration of Ca, eliminating the effect of a change in Ca sensitivity. The pattern of phosphorylation of MyBP-C is not altered by either the Triton solution used to skin the cells or the solution used to extract MyBP-C (Fig. 3). The patterns of phosphorylation of MyBP-C were the same in cardiac tissue quickly frozen before lysis, in cardiac tissue skinned before lysis, and in MyBP-C extracted by modified Offer-Hartzell solution (data not shown). The phosphorylation pattern of the skinned fibers in relaxing solution before any exposure to a Ca-containing contraction solution is not changed by the contraction solution used to produce maximum activation of the contractile system. Elevation of the free Ca in the contraction solution to as high as 30 μM did not produce any significant change in the relative percentage of any of the four phosphorylation forms of MyBP-C (data not shown). This is not surprising because calmodulin is lost and PKA is inactivated during the skinning (Levine et al., 1996; Sweeney and Stull, 1986).

The relative concentrations of the four different phosphorylation states in extracts and homogenates of the same tissue were the same, indicating that the efficiency of extraction was uniform among the several phosphorylation states. The amount of MyBP-C remaining in the pellet after extraction did not exceed 30% of the amount in the extract. At least 70% of the protein was extracted in the experiments included in the study. The distribution of MyBP-C among the four possible phosphorylation states was the same with extraction of MyBP-C for 1 h, 2 h, and overnight, showing that changes due either to kinases or phosphatases did not occur during the extraction procedure. IEF standards run with the extracted MyBP-C indicated that the pI for the four forms were 6.1, 5.9, 5.7, and 5.5 (Protein Bank ExPASy). These values agree quite well with the theoretical values for the unphosphorylated and phosphorylated forms of cardiac MyBP-C.

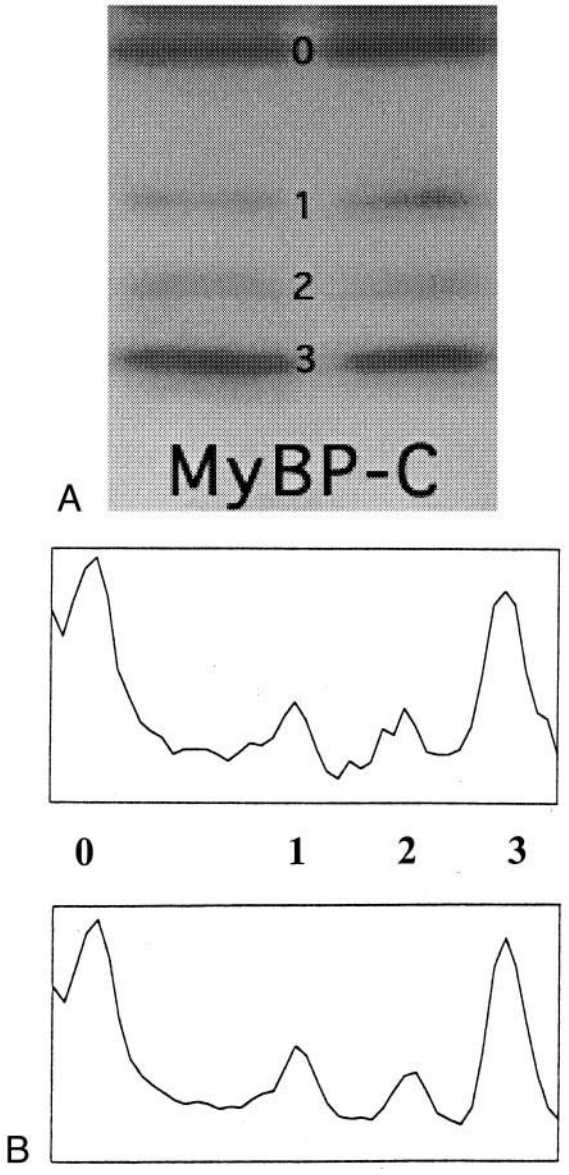

FIGURE 3 Western blot of an IEF gel stained with a MyBP-C antibody. Tissues had been soaked in 2.5 mM Ca for 120 min. (*A*) In the right lane, tissue had been skinned before MyBP-C was extracted; in the left lane, tissue was not skinned (phosphates per MyBP-C in each band is indicated by the numbers between the lanes). The right lane contains a somewhat larger amount of protein, but this is compensated for in the scans, which reveal the relative density of the bands for each form of MyBP-C. (*B*) Scan of each lane; (*top*) unskinned; (*bottom*) skinned. MyBP-C is distributed among the four forms of phosphorylation, indicated by numbers of phosphates per MyBP-C. Trabeculae with a substantial but not maximal phosphorylation are shown to demonstrate that little or no significant increase or decrease in phosphorylation was produced by skinning.

The pattern of phosphorylation of MyBP-C changes with the duration of the incubation and depends on the Ca concentration (Figs. 4 and 5). MyBP-C in cardiac muscle frozen immediately after removal of the heart from the animal (time 0 in Fig. 4) is primarily dephosphorylated. There is a large increase in phosphorylation during the first 20 min of

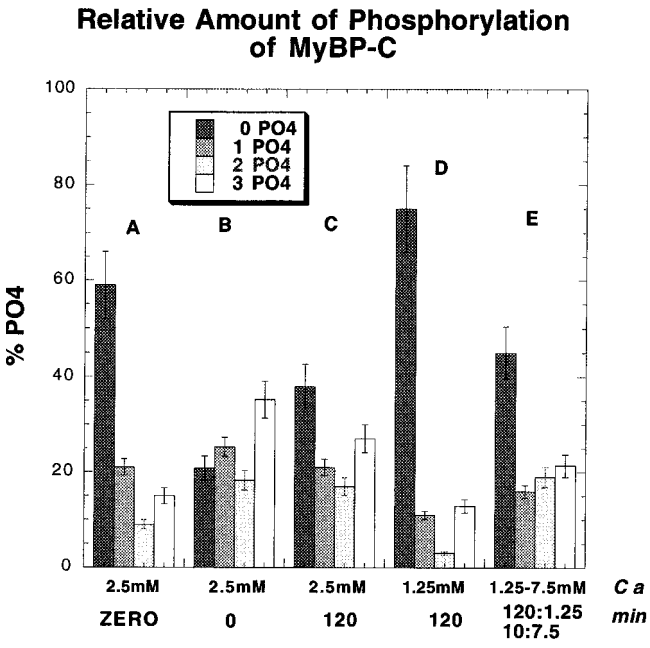

FIGURE 4 Bar graph showing the relative amounts of the four forms of MyBP-C (containing 0, 1, 2, or 3 phosphates per molecule of MyBP-C) for soaks of different duration in different concentrations of extracellular Ca as in Fig. 3. $p < 0.05$ ($n = 6$) for comparison of *A* with *B*, *C* with *D*, and *D* with *E*. All times are from the end of the 20–30-min recovery period in 2.5 mM Ca except for *A*, where zero means no recovery period (0 indicates the end of recovery and zero indicates no recovery).

recovery from the euthanasia and the dissection of the heart. Apparently the trauma of euthanasia and the period of hypoxia during the removal of the heart produce a reversible dephosphorylation of MyBP-C. After 2 h in 2.5 mM Ca solution there is a significant decrease in the level of phosphorylation from the level seen after 20–30 min of recovery in solution containing 2.5 mM Ca (Fig. 4). If the concentration of Ca in the bathing medium is reduced by 50% to

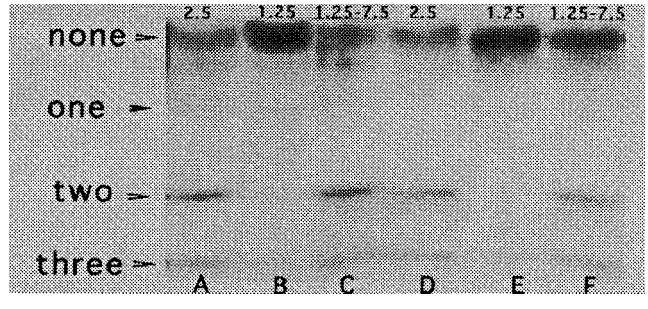

FIGURE 5 Western blot of an IEF gel stained with an antibody against MyBP-C. Cardiac trabeculae were soaked in bathing solution containing 2.5 mM Ca for 120 min (*lanes A* and *D*), 1.25 mM Ca for 120 min (*lanes B* and *E*), and 1.25 mM Ca for 120 min followed by 10 min in 7.5 mM Ca (*lanes C* and *F*). All tissues were quiescent for the entire time. Number of phosphates per MyBP-C is indicated along left of gel. Notice the changes in the relative amounts of the unphosphorylated and triphosphorylated forms with changes in Ca.

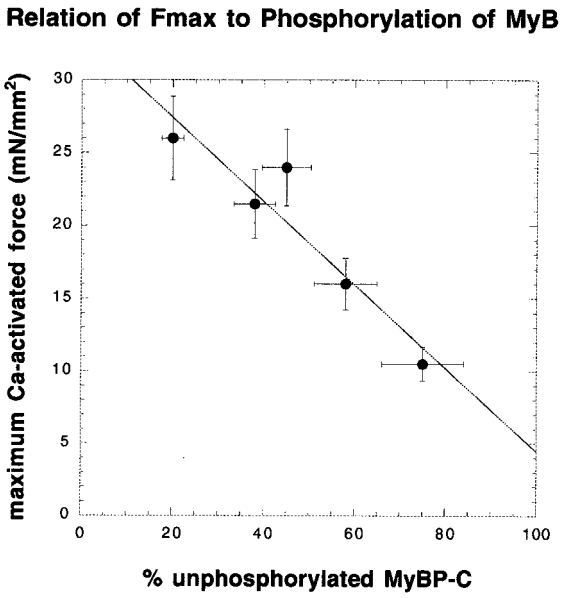

FIGURE 6 Plot of F_{max} against the percentage of unphosphorylated MyBP-C. Data have been taken from Figs. 3 and 4.

1.25 mM for 120 min there is a large decrease in the level of phosphorylation. The amount of unphosphorylated MyBP-C increases and the amount of triphosphorylated MyBP-C decreases substantially. This is the identical protocol that produced a decrease in F_{max}. Elevation of the concentration of extracellular Ca from 1.25 to 7.5 mM for 10 min with the trabeculae still quiescent caused a reversal of the phosphorylation pattern with a decrease in unphosphorylated and an increase in phosphorylated forms of MyBP-C (Figs. 4 and 5). These shifts in phosphorylation have been observed in all eight experiments in which phosphorylation patterns have been measured.

The relative concentration of each of the phosphorylated forms and the total phosphorylation were compared with F_{max} under each condition. F_{max} was inversely related to the amount of unphosphorylated MyBP-C with a high degree of significance (Fig. 6; $R = 0.94$).

Effect of Ca concentration on β-adrenergic stimulation

Paired trabeculae (unskinned) dissected from the same hearts were soaked in Krebs' solution with either 2.5 or 1.25 mM Ca for 110 min after recovery from the dissection in 2.5 mM Ca for 30 min. Isoproterenol (0.1 μM) was then added to the solutions bathing half of the trabeculae, and the incubation was continued for an additional 10 min. Although isoproterenol increases the conductance of Ca channels, the increase in intracellular Ca produced by this effect will be small in a quiescent muscle over a short period. At the conclusion of the 10 min, MyBP-C was extracted from each trabecula, and the relative amount of phosphorylation of MyBP-C was measured on IEF gels by Western blotting to separate each of the three phosphorylated forms from the unphosphorylated form (Figs. 7 and 8).

Trabeculae soaked in 1.25 mM Ca had much less phosphorylation of MyBP-C; particularly diminished were the amounts of mono- and diphosphorylated MyBP-C. Exposure to isoproterenol did not significantly alter the phosphorylation of MyBP-C from trabeculae that had been soaked in 1.25 mM Ca (Figs. 7 and 8). In contrast, MyBP-C from the trabeculae that had been soaked in 2.5 mM Ca had much more phosphorylation, including mono- and diphosphorylated forms. In these muscles the phosphorylation of MyBP-C was enhanced by exposure to isoproterenol. Triphosphorylation was significantly increased, and the unphosphorylated form was significantly decreased. Apparently, Ca-calmodulin-regulated kinase but not PKA can add the first phosphate to MyBP-C. An alternate explanation, that PKA requires a certain concentration of Ca as a co-factor, is unlikely because a co-factor is not required for other phosphorylations by PKA.

The interaction of Ca concentration and β-adrenergic stimulation on contractility and phosphorylation of MyBP-C was examined in Triton-skinned cardiac trabeculae, in which changes in excitation-contraction coupling could not obscure direct effects on myofilament proteins. No significant change in either F_{max} or phosphorylation pattern was produced by exposure to pCa 4.5 with or without added calmodulin (data not shown). Similarly, no change in F_{max} or phosphorylation of MyBP-C was produced by addition of PKA plus cAMP before or during exposure to pCa 4.5 with or without calmodulin. Absence of a change in F_{max} in skinned fibers from PKA has already been reported by Janssen and de Tombe (1997), and the need for added myosin light chain kinase (MLCK) to produce phosphorylation of RLC in skinned fibers has also been observed (Sweeney and Stull, 1986; Levine et al., 1996).

Phosphorylation of RLC

Lysed tissues were electrophoresed on two-dimensional gels to separate the phosphorylated from the unphosphorylated RLC. Western blotting and antibody staining were used to confirm that the spots measured on the gel were RLC. Fig. 9 *A* shows the results with quiescent tissue soaked in 2.5 mM Ca solution for 90 min. Three percent of the total RLC was phosphorylated. After an additional 120 min at rest in 1.25 mM Ca, the percentage of phosphorylated RLC was reduced to borderline detectable. These results are consistent with data in the literature showing that RLC is phosphorylated at Ca concentrations above the level in the resting heart (High and Stull, 1980). Electrical stimulation of an intact trabecula at 30/min for 60 min produced substantial phosphorylation of RLC (Fig. 9 *B*).

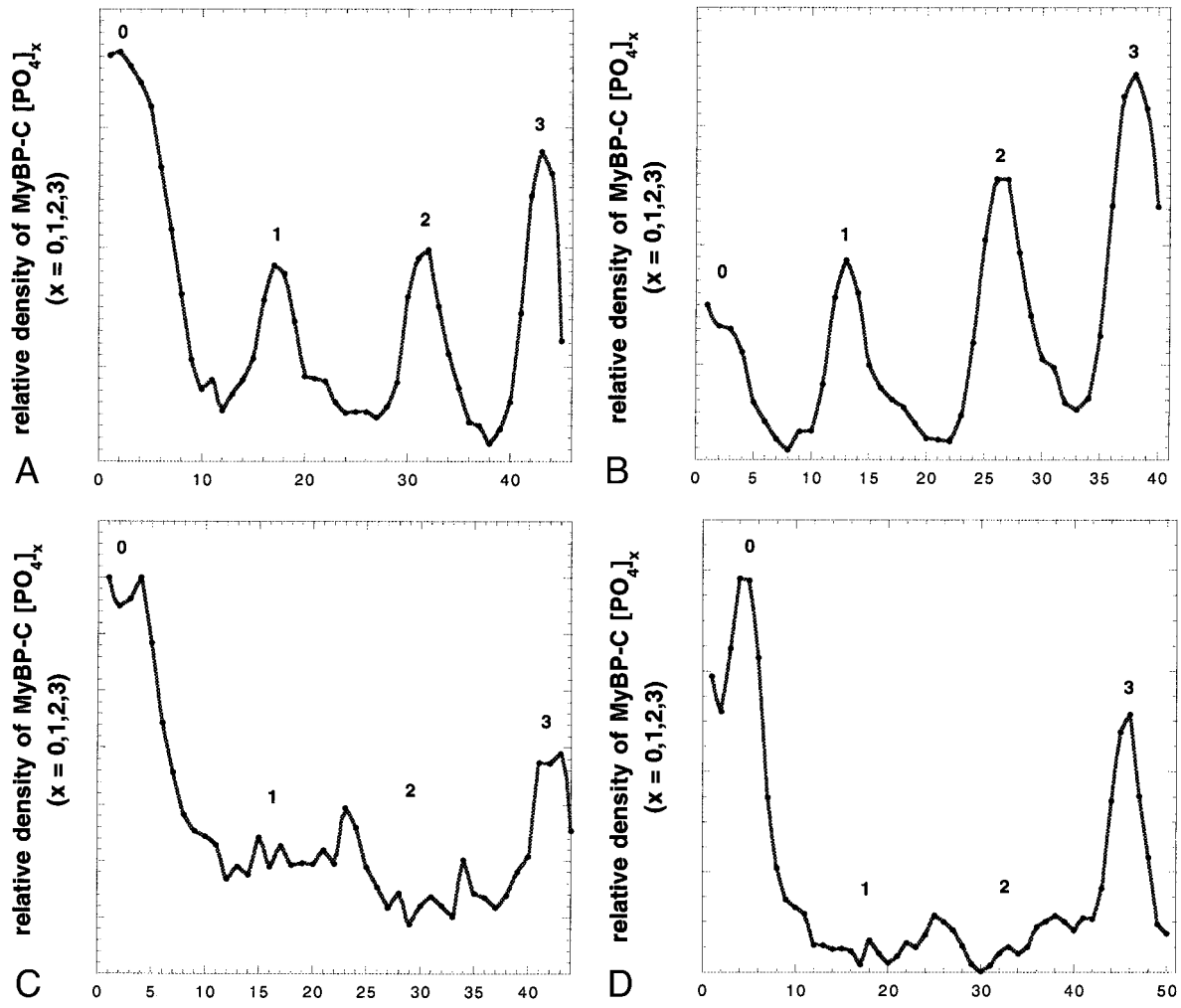

FIGURE 7 Density tracings of Western blots of IEF gels showing the relative density of the four forms of MyBP-C: from left to right containing 0, 1, 2, and 3 phosphates. Extracellular Ca concentration was 2.5 mM in *A* and *B* and 1.25 in *C* and *D*; 0.1 μM isoproterenol was present during the last 10 min of the 120-min soak in *B* and *D*.

DISCUSSION

The properties of the cardiac contractile system can be modulated by a Ca-sensitive reaction at intracellular Ca concentration below threshold for contraction. Elevation of intracellular Ca in the resting muscle alters the contractile system within a few minutes and leads to an increase in the amount of force generated by the concentration of Ca that is optimal for activation of contraction. The changes in contractility are not due to modification of the delivery of Ca during excitation-contraction coupling because they can be seen in skinned as well as intact cardiac myocytes.

MyBP-C phosphorylation and contractility

IEF gels have been used to follow the changes in the pattern of phosphorylation of MyBP-C in the resting cardiac muscle exposed to different concentrations of Ca. Closely associated with the Ca-sensitive change in contractility is a change in the pattern of phosphorylation of MyBP-C. An inverse relation exists between the percentage of unphosphorylated MyBP-C and F_{max}, suggesting that in the total absence of phosphorylation little force is generated. This effect is similar to the observations of Kunst et al. (2000). They have recently shown that the unphosphorylated fragment of cardiac MyBP-C, which contains the phosphorylation sites, reduces F_{max} in skinned skeletal muscle by 50%. Complete phosphorylation prevents the inhibition.

These changes are not due to sporadic contraction of intact individual cells in isolated bundles of rat heart. None was detected by the tension measured at the ends of the muscle bundle or by direct visualization of the magnified trabeculae. Even during the time in 7.5 mM Ca following 2 h in 1.25 mM Ca intracellular Ca did not reach a level that produced spontaneous contractions. The Ca-sensitive modulation was seen in skinned fibers, which do not have spontaneous contractions.

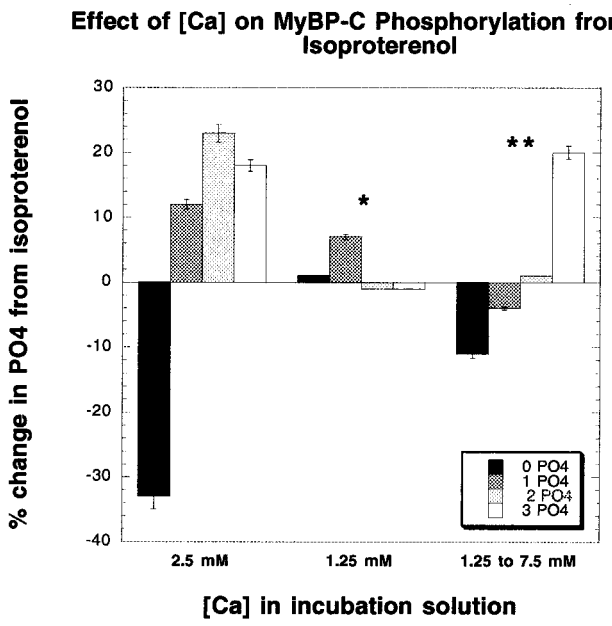

FIGURE 8 Bar graph showing the effect of extracellular Ca concentration on the change in phosphorylation of MyBP-C produced by 0.1 μM isoproterenol. Cardiac trabeculae were soaked in 2.5, 1.25, or 1.25 mM Ca followed by 7.5 mM Ca before exposure to isoproterenol. Each bar is the mean ± SEM of at least four trabeculae from four separate hearts. *$p <$ 0.01 between 2.5 and 1.25 mM Ca; **$p < 0.05$ between 1.25 and 1.25 followed by 7.5 mM Ca.

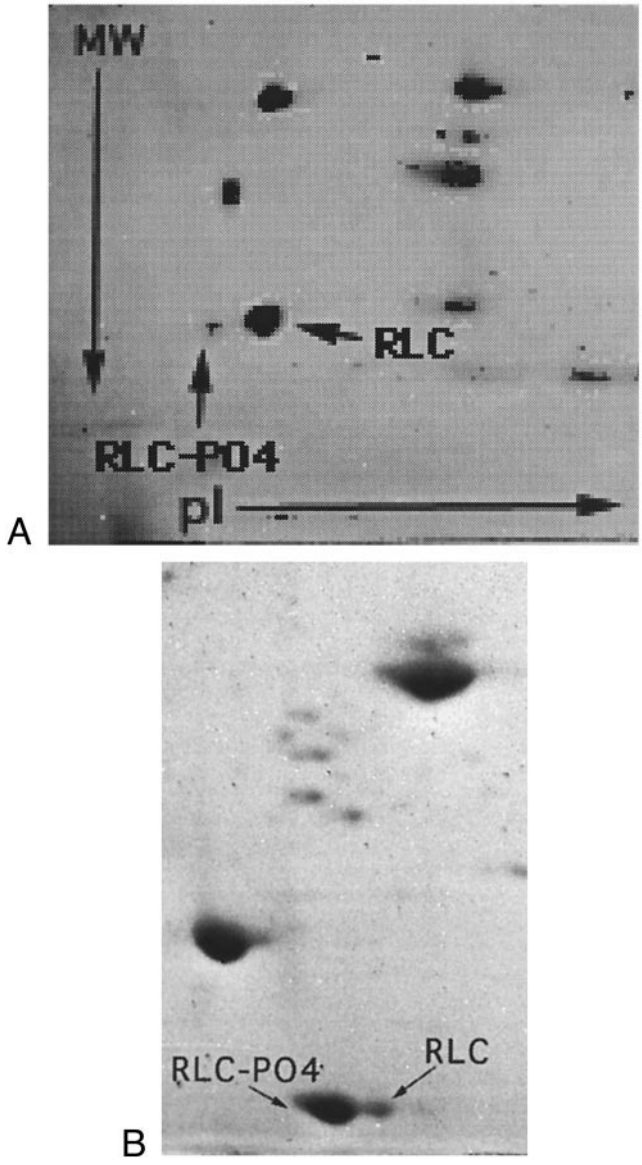

FIGURE 9 (*A*) Two-dimensional gel of trabeculae soaked for 90 min at rest in 2.5 mM Ca Krebs solution. Phosphorylated and unphosphorylated RLC are shown. The phosphorylated form is 3% of the total RLC. (*B*) Two-dimensional gel of trabecula that had been stimulated at 30/min for 60 min to show the large degree of phosphorylation of RLC that occurs.

It is very unlikely that phosphorylation of RLC contributed significantly to the change in contractility with variation in intracellular Ca concentration at subthreshold levels for three reasons: 1) the level of phosphorylation is very low in quiescent hearts soaked in 2.5 mM Ca, and the changes that occur with the different Ca concentrations used in this study are very small; 2) RLC phosphorylation in mammalian hearts occurs slowly because the concentration of the enzyme MLCK is not high; and 3) phosphorylation of RLC occurs primarily if not exclusively in contracting hearts (Stull et al., 1980; Silver et al., 1986). In association with higher concentrations of intracellular Ca produced by rhythmic contractions (Ca above the threshold for the activation of force) there was a smaller change in F_{max} that is consistent with phosphorylation of RLC.

The values for force generation and phosphorylation of MyBP-C together with results from studies of isolated thick filament structure (see Levine et al., 2001) suggest certain effects of Ca-regulated phosphorylations. The higher maximum Ca-activated force when cardiac muscle is transferred from low to high extracellular Ca is probably due to phosphorylation of MyBP-C and a structural rearrangement of myosin heads resulting in their greater order and smaller separation from the thin filament. Within the lower range of Ca concentration, phosphorylation of RLC, the other Ca-regulated phosphorylation that is associated with extension of myosin heads, is not involved.

Lin et al. (1991), in their first demonstration that quiescence of heart muscle changes its response to activator Ca, noted that there is a parallel effect on actomyosin ATPase activity but no change in the maximum velocity of unloaded shortening. This combination of effects suggests that the rate of attachment of the myosin heads to the thin filament is being regulated without change in the rate of detachment. Such a mechanism would explain why maximum force and rate of ATP hydrolysis change in the same direction without any change in velocity.

Relation between Ca and PKA-regulated phosphorylation of MyBP-C

The effect of β-adrenergic stimulation on the properties of the contraction has been controversial. Several groups have produced evidence for a direct effect on the contractile proteins that cannot be explained by phosphorylation of TNI or the regulatory light chain of myosin (McClellan et al., 1994; Hoh et al., 1988; Hasenfuss et al., 1994; Strang and Moss, 1994). Phosphorylation of TNI does not increase F_{max}, and phosphorylation of RLC is not produced by PKA. Others have found no change in contractility when the level of Ca activation in skinned fibers is controlled (Janssen and de Tombe, 1997; Hofmann and Lange, 1994). Additional evidence cited against an effect on the contractile proteins is the fact that the maximum force generated by cardiac tissue tetanized in ryanodine is not increased by catecholamines (Marban et al., 1986). None of these data, however, rules out Ca-mediated alterations of myofibrillar proteins as playing a significant role in the modulation of contractility.

In the studies reported here, we show that the intracellular Ca level can influence the response of the contractile system to β-adrenergic activity. By applying IEF and Western blotting it has been possible to determine not only the total phosphorylation of MyBP-C produced by changing intracellular Ca but also for the first time the relative concentrations of the mono-, di-, and triphosphorylated states. Sufficient reduction of intracellular Ca blocks the ability of PKA to phosphorylate MyBP-C in intact cells. The β-adrenergic agonist isoproterenol increased the relative amount of phosphorylation only when the concentration of intracellular Ca had not been reduced by soaking in low Ca.

Of the three phosphorylatable sites in the cardiac isoform of MyBP-C, a specific one of these must be phosphorylated first (Gautel et al., 1995), apparently by a Ca-regulated kinase, before phosphate can be added to the other two sites. In the intact cell when the first site is unphosphorylated, PKA appears to be unable to phosphorylate MyBP-C. The Ca-regulated kinase appears specifically to regulate the addition of the first phosphate. If monophosphorylation was present, PKA produced substantial increases in the di- and triphosphorylated forms of MyBP-C. The Ca-regulated kinase may be able to add the second and third phosphates as well, or their addition in the absence of β-adrenergic agonist may be due to adrenergic tone in the cells.

As a result of these relatively specific effects of the two kinases, it is possible that thick filaments may have as many as three (or even four) stable structures resulting from the addition of 0, 1, 2, or 3 phosphates. Three different structural states associated with changes in the phosphorylation of MyBP-C have been demonstrated (Levine et al., 2001). In vivo, the relative amount of each of these three structural states should depend on the level of rhythmic activation of contraction and presumably the time-averaged intracellular Ca concentration.

Partial phosphorylation of MyBP-C by Ca-calmodulin-regulated kinase may have a permissive effect that then allows other mechanisms to alter contractility. The existence of two different attached states, one non-force-generating, in the regulation of contraction was proposed by Matsubara et al. (1989) on the basis of their x-ray diffraction results with skinned heart. Regulated phosphorylation of MyBP-C could divide the cardiac force generators into two or three groups, each with a different probability of attachment to actin and entry into the force-generating cycle (see Levine et al., 2001). Kinetic studies have shown the existence of two separate groups of force generators with different rates of ATP hydrolysis in cardiac muscle and the conversion of the two into a single group with the higher ATPase activity by PKA (McClellan et al., 1994). The relative distribution of force generators between these two groups may be sensitive to the time-averaged concentration of intracellular Ca and the level of PKA activation. Kunst et al. (2000) have shown that F_{max} can be modulated between two values by the level of phosphorylation of a fragment of MyBP-C added to skinned fibers.

Ca-regulated changes in thick filament structure that modulate the maximum level of force production and Ca sensitivity of contraction fit well with changes in contractile activity that occur from alterations in excitation-contraction coupling. Increase in cytoplasmic Ca not only leads to greater Ca binding of the ion by TNC and greater thin filament activation but also increases the probability of myosin heads forming bonds with actin. Thus, the regulation of force through modulation of the Ca transient and binding to TNC would be complemented by another Ca-sensitive modulation that influences the distribution of force generators between forms that differ in their likelihood of entering the force-generating cycle.

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