Phosphorylation of Troponin I by Protein Kinase A Accelerates Relaxation and Crossbridge Cycle Kinetics in Mouse Ventricular Muscle

Jonathan C. Kentish, Diana T. McCloskey, Joanne Layland, Sue Palmer, Jeffrey M. Leiden, Anne F. Martin, R. John Solaro

Abstract—Phosphorylation of cardiac myofibrils by cAMP-dependent protein kinase (PKA) can increase the intrinsic rate of myofibrillar relaxation, which may contribute to the shortening of the cardiac twitch during β-adrenoceptor stimulation. However, it is not known whether the acceleration of myofibrillar relaxation is due to phosphorylation of troponin I (TnI) or of myosin binding protein-C (MyBP-C). To distinguish between these possibilities, we used transgenic mice that overexpress the nonphosphorylatable, slow skeletal isoform of TnI in the myocardium and do not express the normal, phosphorylatable cardiac TnI. The intrinsic rate of relaxation of myofibrils from wild-type and transgenic mice was measured using flash photolysis of diazo-2 to rapidly decrease the [Ca\(^{2+}\)] within skinned muscles from the mouse ventricles. Incubation with PKA nearly doubled the intrinsic rate of myofibrillar relaxation in muscles from wild-type mice (relaxation half-time fell from ~150 to ~90 ms at 22°C) but had no effect on the relaxation rate of muscles from the transgenic mice. In parallel studies with intact muscles, we assessed crossbridge kinetics indirectly by determining \(f_{\text{min}}\) (the frequency for minimum dynamic stiffness) during tetanic contractions. Stimulation of β-adrenoceptors with isoproterenol increased \(f_{\text{min}}\) from 1.9 to 3.1 Hz in muscles from wild-type mice but had no effect on \(f_{\text{min}}\) in muscles from transgenic mice. We conclude that the acceleration of myofibrillar relaxation rate by PKA is due to phosphorylation of TnI, rather than MyBP-C, and that this may be due, at least in part, to faster crossbridge cycle kinetics. (Circ Res. 2001;88:1059-1065.)

Key Words: protein kinase A ■ phosphorylation ■ relaxation ■ troponin I ■ myosin binding protein-C

Stimulation of β\(_1\)-adrenoceptors in the myocardium during sympathetic stimulation not only increases contractile force but also accelerates myocardial relaxation (positive lusitropy). This increase of relaxation rate is important for proper pump function, because it allows adequate time for diastolic filling of the ventricles despite the raised heart rate during sympathetic stimulation. The activation of β\(_1\)-adrenoceptors stimulates the cAMP/protein kinase A (PKA) pathway, and the faster relaxation of the myocardial cells is partly due to an enhanced reuptake of Ca\(^{2+}\) into the sarcoplasmic reticulum (SR) as a result of phosphorylation of phospholamban by PKA.\(^1\) In addition, PKA phosphorylates the cardiac myofibrils during β-stimulation.\(^2\)–\(^4\) This may lead to an acceleration of the intrinsic rate of myofibrillar relaxation, thereby contributing to the abbreviation of the twitch. Using flash photolysis of the caged chelator of Ca\(^{2+}\), diazo-2, to rapidly decrease Ca\(^{2+}\) concentration inside skinned fibers, Zhang et al.\(^8\) reported that PKA accelerated relaxation in pig skinned muscles. However, a later study by Johns et al.\(^9\) found no effect in similar experiments using guinea pig skinned muscles. Recent work with intact mouse muscles has suggested that β-stimulation can produce an SR-independent, presumably myofibril-mediated, acceleration of relaxation that is seen in isometric but not isotonic contractions.\(^4\)

Assuming that phosphorylation does increase the relaxation rate of cardiac myofibrils, how might this be produced? It is known that phosphorylation of troponin I (TnI) by PKA decreases myofibrillar Ca\(^{2+}\) sensitivity\(^2,3,5–8\) and increases the rate at which Ca\(^{2+}\) dissociates from TnC,\(^9\) which could lead to faster relaxation by increasing the rate of thin filament deactivation. Alternatively, there is some evidence that phosphorylation by PKA can directly accelerate some steps in the crossbridge cycle. For example, the maximum velocity of shortening in skinned cells or muscles was increased by PKA in some studies,\(^3,10,11\) although not in others.\(^12\) Under isometric conditions, myofibrillar ATPase activity was either increased\(^11\) or unchanged\(^13\) by PKA. More consistent results under near-isometric conditions have been obtained with

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perturbation analysis, which showed that the frequency of minimum dynamic stiffness of muscles \( f_{\text{min}} \) during sinusoidal length perturbations was increased during \( \beta \)-stimulation.14–17 This increase in \( f_{\text{min}} \) is likely to reflect an acceleration of strain-dependent transitions in the crossbridge cycle. If the increase in crossbridge kinetics were related to a faster rate of crossbridge detachment, it would produce a faster relaxation of the myofibrils. However, it is still not clear whether the positive lusitropic effect of phosphorylation is due to a direct action on the rate of \( \text{Ca}^{2+} \) loss from TnC or on the rate of crossbridge detachment during relaxation.

Another uncertainty is that we do not know which myofilament protein mediates the effect of PKA on relaxation rate. As stated above, PKA phosphorylates TnI and increases the rate of \( \text{Ca}^{2+} \) loss from TnC. In addition, PKA phosphorylates the N-terminal motif of the thick-filament protein myosin binding protein-C (MyBP-C).2–4,8,18–21 This may abolish a restraining influence of MyBP-C on the flexibility of the myosin head.21 Consistent with this, electron microscopy of isolated thick filaments has shown that phosphorylation of MyBP-C causes the crossbridges to move away from the thick-filament backbone.18 This action may increase the rates of crossbridge attachment and detachment from actin, thereby accelerating relaxation.19 Because both TnI and MyBP-C are phosphorylated concurrently by PKA, it has so far proved impossible to establish the relative roles of phosphorylation of TnI and MyBP-C in the acceleration of myofibrillar relaxation. In the present study, we resolved this difficulty by using transgenic mice in which the normal cardiac form of TnI and MyBP-C in the acceleration of myofibrillar relaxation. In the present study, we resolved this difficulty by using transgenic mice in which the normal cardiac form of TnI (cTnI) is replaced by the slow skeletal form of TnI using transgenic mice in which the normal cardiac form of TnI and MyBP-C in the acceleration of myofibrillar relaxation. In the present study, we resolved this difficulty by using transgenic mice in which the normal cardiac form of TnI (cTnI) is replaced by the slow skeletal form of TnI.3 ssTnI, which is normally expressed in the heart only during fetal and early postnatal life, lacks an N-terminal sequence of 32 to 33 amino acids compared with cTnI. In cTnI, this N-terminal sequence contains the two serine residues (23 and 24 in the mouse) that can be phosphorylated by PKA. Thus, MyBP-C is the only phosphorylatable target for PKA in ssTnI transgenic mice. The aims of this study were (1) to establish whether phosphorylation accelerates the intrinsic relaxation rate of wild-type mouse myofibrils; (2) if so, to use the transgenic mice to determine whether the effect is due to phosphorylation of TnI or of MyBP-C; and (3) to see whether differential effects of phosphorylation on relaxation of wild-type and transgenic mouse myofibrils are associated with differences in crossbridge cycle kinetics under near-isometric conditions, as assessed by measurement of \( f_{\text{min}} \).

**Materials and Methods**

Briefly, heterozygous ssTnI transgenic (TG) mice were produced from CD-1 mice by inserting a vector containing the mouse ssTnI transgene under the control of the cardiac-specific \( \alpha \)-myosin heavy chain promoter.3 Genotyping (using specific PCR primers) and phenotyping (using SDS-PAGE of isolated myofibrils) were done only after the experiments so that the experimenter did not know which type of mouse was being used. The results were the same using either wild-type (WT) CD-1 mice or nontransgenic littermates of transgenic mice, so we pooled the results from these two groups.

For skinned-muscle experiments, mice (30 to 40 g) were killed by cervical dislocation in accordance with UK Home Office guidelines (section 1). Suitable papillary muscles or trabeculae (diameters: WT, 136±12 \( \mu \)m, n=6; TG, 129±10 \( \mu \)m, n=5) from the right ventricles were skinned with 1% Triton X-100 and attached to a force transducer.22 For rapid-relaxation experiments, the photolysis solution contained 0.25 mol/L diazo-2 and varied [\( \text{Ca}^{2+} \)] to produce forces of 40% to 80% of maximum force. When force had stabilized, a xenon flashlamp focused on the muscle was triggered to photolyze diazo-2. Results shown are those in which muscles relaxed to 0% to 15% of maximum force. Initially, relaxation trajectories were assessed by calculation of the \( R_{\text{rel}} \) (time taken for force to fall by 50% over the 10-second recording period). Subsequently, further analysis was carried out by fitting the relaxation to a double-exponential decay: force at time \( t \) = \( a \times e^{-k_1 \times t} + b \times e^{-k_2 \times t} + c \). This was done to assess which of the two exponential components of the relaxation trajectory contributed to the observed changes in \( R_{\text{rel}} \). Phosphorylation was carried out by incubating the muscles with 500 \( \mu \)mol/L PKA (porcine catalytic subunit, Sigma) in relaxing solution for 30 minutes at 22°C.

For the intact-muscle experiments, muscles were mounted between a servomotor and a force transducer in a flow-through bath and superfused with Krebs solution (1 mmol/L \( \text{Ca}^{2+} \), 24°C). Fused tetani were produced by stimulating the muscles at 10 Hz in 1 \( \mu \)mol/L ryanodine and 30 \( \mu \)mol/L cyclopiazonic acid (to inhibit the SR) plus 12 mmol/L \( \text{Ca}^{2+} \) and 1 \( \mu \)mol/L BAY K 8644 (to increase \( \text{Ca}^{2+} \) influx). During the tetanus, muscle-length oscillations of 0.6% peak-peak were applied, and the dynamic stiffness was calculated from the resulting force excursions. All data are expressed as mean±SEM. Differences were analyzed using Student’s paired or unpaired t tests, as appropriate, with \( P<0.05 \) being regarded as statistically significant.

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

**Results**

**Effects of Phosphorylation on Myofibrillar \( \text{Ca}^{2+} \) Sensitivity**

Western blot analysis has shown that overexpression of ssTnI in the adult mouse heart leads to a lack of expression of the cTnI gene.3 This was verified in the present study by the absence of cTnI in SDS gels from the TG mice (Figure 1A). The ssTnI band is not visible in the gel of TG myofibrils because ssTnI co-migrates with myosin light chain-I. The lack of phosphorylatable TnI in myofibrils from TG mice was further demonstrated by autoradiograms of myofibrils after incubation with PKA and \( ^{32} \text{P} \)-ATP (Figure 1B): both TnI and MyBP-C were phosphorylated in myofibrils from WT mice, whereas only MyBP-C was phosphorylated in myofibrils from TG mice.
from TG mice. Western blot analysis has shown that ssTnI completely replaces cTnI. This was confirmed in the present study by the fact that the maximum Ca\textsuperscript{2+} activated force of skinned muscles from TG mice (16.0±0.6 mN · mm\textsuperscript{-2}) was the same as in muscles from WT mice (15.8±0.8 mN · mm\textsuperscript{-2}). The only difference in the steady-state properties was that skinned muscles from TG mice had a higher Ca\textsuperscript{2+} sensitivity than WT muscles under control conditions (Figure 2). The [Ca\textsuperscript{2+}] required for 50% activation ([Ca\textsuperscript{2+}]\textsubscript{50}) was 0.74±0.11 μmol/L (n=5) in TG myofibrils compared with 1.52±0.12 μmol/L (n=6) for WT myofibrils, corresponding to a difference of 0.31 pCa units (P<0.05).

As shown in Figure 2A, phosphorylation of the WT Triton-skinned muscles with PKA increased the [Ca\textsuperscript{2+}]\textsubscript{50} to 2.15±0.19 μmol/L (n=6), which corresponds to a decrease in Ca\textsuperscript{2+} sensitivity of 0.15±0.02 pCa units (P<0.001, paired t test). In contrast, incubation of the TG muscles in PKA did not alter the Ca\textsuperscript{2+} sensitivity (Figure 2B): the [Ca\textsuperscript{2+}]\textsubscript{50} rose only to 0.86±0.07 μmol/L (n=5), a change in pCa\textsubscript{50} of 0.05±0.03 pCa units. This shift was not significantly different from zero and was exactly the same as in time-matched controls incubated without PKA (0.05±0.03, n=4). The lack of change in the Ca\textsuperscript{2+} sensitivity of myofibrils in TG muscles, despite the phosphorylation of MyBP-C under these conditions (Figure 1B), confirms previous work\cite{1} and demonstrates that the fall in Ca\textsuperscript{2+} sensitivity in WT mice results from phosphorylation of cTnI rather than of MyBP-C. In neither type of mouse muscles was the maximum Ca\textsuperscript{2+} activated force altered significantly by incubation with PKA (Figure 2).

**Effects of Phosphorylation on Myofibrillar Relaxation Rate**

Rapid relaxation of skinned muscles during activation by Ca\textsuperscript{2+} (Figure 3) was produced by flash photolysis of diazo-2, a "caged" chelator of Ca\textsuperscript{2+} that exhibits a near-instantaneous increase in Ca\textsuperscript{2+} affinity after photolysis with a flash of near-UV light.\cite{23} This causes the [Ca\textsuperscript{2+}] surrounding the myofilaments to be reduced rapidly (in ~1 ms) so that the rate of the ensuing relaxation is limited only by the properties of the myofibrils. In skinned muscles from WT mice, incubation with PKA had a large effect on relaxation rate (Figure 3B). After PKA, relaxation was accelerated dramatically, with the half-time for relaxation (RT\textsubscript{50}) falling by 41% from 152±29 to 90±16 ms (Figure 3C). This increase in relaxation rate was almost entirely due to a change in the slower rate constant (k\textsubscript{s}). The faster rate constant (k\textsubscript{f}) rose slightly from 35.7±3.1 s\textsuperscript{-1} to 44.4±6.3 s\textsuperscript{-1}, but this change was not statistically significant.
The amplitudes of the two exponential components or of the final force level \((a, b, c)\) in Materials and Methods) were not altered significantly (results not shown).

Under control conditions, skinned muscles from the TG mice (Figure 4) had a faster rate of relaxation than WT mice (RT\(_{50}\) of 70±6 ms, \(n=9\); \(P<0.05\) versus WT). This was due to a greater value for \(k_1\) (44±4 versus 36±3 s\(^{-1}\)) in WT mice; \(P<0.05\), because \(k_2\) (4.0±1.0 s\(^{-1}\), \(n=9\)) was the same as in the WT muscles. (However, it should be noted that the initial conditions were different for the two types of muscle, in that the higher [Ca\(^{2+}\)] sensitivity of TG muscles required that the preflash [Ca\(^{2+}\)] of the diazo-2 solution was lower than with the WT muscles, to give the same initial level of activation in the two muscle types). It is clear that, in contrast to the WT muscles, the TG muscles exhibited no change in the rate of myofibrillar relaxation after incubation in PKA, with no significant alteration in RT\(_{50}\) or the relaxation rate constants (Figure 4) or in the amplitudes of the exponential components (not shown). Thus, the lack of phosphorylation of TnI in the TG myofibrils (Figure 1B) is associated with a lack of effect of PKA on the myofibrillar relaxation rate.

**Effects of Phosphorylation on \(f_{\text{min}}\)**

There is evidence that the intrinsic rate of relaxation of cardiac myofibrils may be governed by the kinetics of the crossbridge cycle rather than by the rate at which Ca\(^{2+}\) is lost from TnC.\(^{22,24}\) We therefore examined whether the differential effects of phosphorylation on the relaxation dynamics of WT and TG mice were reflected in differential effects on crossbridge kinetics. This was done under near-isometric conditions (as in the relaxation experiments) by measuring the oscillation frequency \((f_{\text{min}})\) that produced minimum dynamic stiffness during small sinusoidal length perturbations in tetanized muscles (Figure 5A). Intact muscles were used because it was difficult to obtain clear stiffness minima in skinned muscles; an added advantage was that intact muscle contains the functional \(\beta\)-adrenoceptor pathway. In the WT muscle illustrated in Figure 5B, \(f_{\text{min}}\) was 2 Hz under control conditions. Isoproterenol was subsequently applied at a concentration (5 \(\mu\)mol/L) that gave a maximal increase of the isometric twitch in the absence of SR blockers (results not shown). This stimulation of \(\beta\)-adrenoceptors caused \(f_{\text{min}}\) to increase to 3 Hz in the muscle shown. In 17 WT muscles, isoproterenol increased \(f_{\text{min}}\) by 63% from 1.91±0.17 Hz to 3.12±0.22 Hz (Figure 5C). This suggests that \(\beta\)-adrenoceptor stimulation accelerates crossbridge kinetics in mouse heart muscle. The apparent effects of isoproterenol on the maximum amplitude of dynamic stiffness in Figure 5B were not typical of other muscles. Isoproterenol had no significant effects on the magnitudes of isometric tetanic stress (Figure 5D), as seen previously,\(^{25}\) or of dynamic stiffness (Figure 5E; stiffness measured at 10 Hz, the highest oscillation frequency used in these experiments).

The basal level of TnI phosphorylation under control conditions is undetectable in TG myocytes\(^{26}\) but is 10% to 20% of maximum in WT myocytes (see online date supplement for details). Given that phosphorylation increased \(f_{\text{min}}\) in WT mice, we might therefore expect that \(f_{\text{min}}\) under control conditions would be smaller in TG muscles than in WT muscles.
muscles. In fact, \( f_{\text{min}} \) in the TG muscles (1.75 ± 0.16 Hz, \( n = 8 \), Figure 5C) was lower than in WT mice, but the difference was not statistically significant. The isometric tetanic stress and the stiffness at 10 Hz were also similar to those in WT mice (Figures 5D and 5E). However, a major difference between the two types of mice was apparent with the effects of isoproterenol on \( f_{\text{min}} \). In the example TG muscle shown (Figure 5B), the addition of isoproterenol in fact decreased \( f_{\text{min}} \) although on average in the TG muscles, \( f_{\text{min}} \) was not altered significantly (Figure 5C). This was not due to a general lack of responsiveness to \( \beta \)-adrenoceptor stimulation, because the twitch amplitude was still increased by isoproterenol (not shown). These results indicate that activation of PKA speeds crossbridge kinetics in WT but not in TG mice. This suggests that the faster crossbridge kinetics are associated with phosphorylation of TnI rather than of MyBP-C.

Recently, it was reported that phosphorylation of the cardiac MyBP-C motif, incorporated into skinned skeletal fibers, increased maximum force production without altering muscle stiffness (a measure of the number of attached crossbridges).26 However, although we also found no effect of \( \beta \)-adrenoceptor stimulation on the dynamic stiffness of TG muscles (measured at 10 Hz, Figure 5E), we did not see any change in the tetanic force of TG muscles during \( \beta \)-stimulation (Figure 5D).

**Discussion**

**Phosphorylation Effects in WT Mice**

It is well established that PKA phosphorylates cardiac myofibrils, both in isolated myofibrils and in intact cells during \( \beta \)-adrenoceptor stimulation.2–13,18–21,26 However, it is less clear whether this action is beneficial for cardiac function. The resulting decrease in myofibrillar \( \text{Ca}^{2+} \) sensitivity, which is now known to be due to phosphorylation of TnI,2–3,8 appears counterproductive, because it would tend to offset the positive inotropic action resulting from the enhanced \([\text{Ca}^{2+}]\), transient during \( \beta \)-adrenoceptor stimulation.1 A beneficial effect would however result if phosphorylation acted to increase the intrinsic rate of myofibrillar relaxation. Previous work, using flash photolysis of pig or guinea pig skinned cardiac muscles in air at \( \approx 12^\circ \text{C} \), has produced contradictory results, that phosphorylation by PKA does5 or does not6 increase the myofibrillar relaxation rate. Our results, using skinned muscles in solution at 22°C, show that phosphorylation does increase the intrinsic relaxation rate of mouse cardiac myofibrils (Figure 3). It remains to be determined whether these divergent results from diazo-2 experiments are due to differences in species or in methodology. The faster relaxation resulting from phosphorylation of the myofibrils is likely to contribute to the beneficial effect of twitch abbreviation during \( \beta \)-adrenoceptor stimulation, although the magnitude of the contribution by the myofibrils, relative to the SR, may depend on the load placed on the muscle.4

It is difficult to say precisely how phosphorylation accelerates myofibrillar relaxation, because the factors that determine relaxation rate are poorly understood. Relaxation is a complex process, involving \( \text{Ca}^{2+} \) removal from TnC, thin-filament deactivation, crossbridge dissociation, and loss of cooperativity in the thin filament, particularly the activating effect of strongly bound crossbridges (for review, see Gordon et al27). In addition, even in muscles held isometric, there is likely to be some internal shortening of the muscle during activation and a corresponding internal relengthening (ie, sarcomere relengthening) during relaxation; this will influence the rate of decay of force. All these processes may occur simultaneously, so it is difficult to define their individual contributions to the relaxation process, let alone to state how phosphorylation accelerates relaxation. However, it is likely that the faster relaxation after phosphorylation (Figure 3) does not result directly from the faster loss of \( \text{Ca}^{2+} \) from TnC, because even before phosphorylation, the loss of \([\text{Ca}^{2+}]\) is probably too rapid to limit relaxation.22,24 Further support for this view is given by the results from the TG muscles. These muscles had an enhanced \( \text{Ca}^{2+} \) sensitivity compared with WT mice, which if anything should reflect a slower dissociation of \( \text{Ca}^{2+} \) from TnC. However, the TG mice showed a faster, not slower, relaxation compared with the WT mice.

We and others have provided evidence that the kinetics of the crossbridge cycle may be a major determinant of the intrinsic rate of myofibrillar relaxation.22,24 Consistent with this, the present results suggest that the faster relaxation caused by phosphorylation of myofibrils in the WT skinned muscles is associated with faster crossbridge kinetics, as assessed by measurements of \( f_{\text{min}} \), in intact muscles (Figure 5C). During the maintained tetanus, thin-filament activation and cooperative mechanisms should be constant, so the increase in \( f_{\text{min}} \) will reflect a true acceleration of the underlying crossbridge kinetics, unrelated to activation effects. Although in the past \( f_{\text{min}} \) has been regarded as a measure of crossbridge cycling rate, it is more likely to be a composite, indirect measure of the forward and backward transitions between zero-force (detached and weakly bound) and force-generating (strongly bound) crossbridge states.27 (The overall crossbridge cycling rate can be determined from measurements of myofibrillar ATPase activity, but this has produced divergent results, with an increase11 or no change13 after phosphorylation with PKA). The present results indicate that \( f_{\text{min}} \), and therefore crossbridge kinetics, are increased in intact mouse muscles during \( \beta \)-stimulation, as has been found in the hearts of other mammalian species.14–17 At present, we cannot be sure of the relationship (if any) between \( f_{\text{min}} \) and the biexponential relaxation trajectory typically seen in diazo-2 experiments (Figures 3 and 4).6,22 However, a commonality between \( f_{\text{min}} \) and the slower rate constant (\( k_s \)) of relaxation is suggested by the finding that both \( f_{\text{min}} \) and \( k_s \) showed no difference between WT and TG muscles and that in WT mice both \( f_{\text{min}} \) and \( k_s \) were increased (by \( \approx 60\% \) to \( 80\% \)) by phosphorylation with PKA. In contrast, \( k_s \) was greater in TG than in WT mice and was not altered by phosphorylation. One possible explanation for our results is that both \( f_{\text{min}} \) and \( k_s \) are influenced by the rate of forward detachment of crossbridges and that phosphorylation enhances this detachment rate. An alteration in the strain dependency of crossbridge transitions might cause these increases in \( f_{\text{min}} \) and \( k_s \) without necessarily altering the isometric ATPase activity. The idea that an acceleration of crossbridge kinetics may be responsible for
the increases in $f_{\text{min}}$ and relaxation rate is supported by the finding that these increases were both due to phosphorylation of TnI by PKA (see below). Whether there is an additional contribution from accelerated kinetics of thin-filament deactivation or of other cooperative mechanisms remains to be determined.

**Phosphorylation Effects in TG Mice**

A major aim of the present study was to use TG mice that overexpressed nonphosphorylatable ssTnI to elucidate the relative roles of phosphorylation of TnI and MyBP-C to the positive lusitropic effects of PKA. Fortunately for our purposes, the overexpression of ssTnI results in a lack of expression of the normal cTnI, as shown by Western blots and SDS-PAGE gels of myofibrils (Figure 1). Thus, in the TG myofibrils only MyBP-C is phosphorylated by PKA (Figure 1B). Similarly, in intact myocytes from TG mouse hearts, β-adrenoceptor stimulation leads to phosphorylation of MyBP-C but not TnI.³

Under control conditions, the muscles from TG mice were different in two respects from WT mouse muscles. First, as seen previously,³,⁹,¹⁸ TG muscles expressing ssTnI had a higher Ca²⁺ sensitivity than WT muscles (Figure 2). This illustrates that the isoform of TnI is a major determinant of Ca²⁺ sensitivity and helps to explain why neonatal myofibrils, which express ssTnI rather than cTnI, have a higher Ca²⁺ sensitivity than adult myofibrils.⁰ The second difference was that the relaxation rate of TG muscles (Figure 4) was faster than that of the WT muscles (Figure 3). This unexpected finding could indicate that the isoform of TnI also determines the dynamics of myofibrillar relaxation. However, the conditions were different for the two types of muscle, in that preflash [Ca²⁺] was lower for the TG muscles, to achieve the same initial level of activation. Further studies will be required to establish if the disparity in relaxation rates between WT and TG mice is due to true differences in myofibrillar properties or merely to differences in preflash [Ca²⁺]. One potential problem is that the elevated relaxation rate of the TG myofibrils under control conditions could have masked any potential acceleration of relaxation from the subsequent phosphorylation of MyBP-C. We think this is unlikely because the faster relaxation of TG myofibrils was in fact due to a higher value for rate constant $k_1$; the value of $k_2$, which was the rate constant increased by phosphorylation in WT muscles, was no greater in TG myofibrils than in WT myofibrils under control conditions $4 \text{s}^{-1}$, Figures 3 and 4), and so should not have been limiting.

At first sight, the faster relaxation of skinned muscles from TG mice is surprising, given that in intact cells and hearts of TG mice relaxation is slower than in WT mice, leading to diastolic dysfunction in vivo.³ One reason for this difference is that the Ca²⁺ transient decays more slowly in TG myocytes than in WT myocytes,³ for unknown reasons. This, coupled with the higher Ca²⁺ sensitivity of TG myofibrils, makes it likely that the rate of relaxation in intact TG myocytes is determined only by the slow fall of [Ca²⁺], rather than by the faster intrinsic relaxation rate of the myofibrils.

A major finding in the present work was that the PKA-induced phosphorylation of both TnI and MyBP-C in skinned muscles from WT mice increased myofibrillar relaxation rate substantially, but that phosphorylation of MyBP-C alone in the TG muscles had no effect on relaxation. Similarly, in the intact muscles β-stimulation increased $f_{\text{min}}$ in the muscles from WT mice but not in those from TG mice. Thus, the increases in both relaxation rate and $f_{\text{min}}$ are likely to be due to phosphorylation of TnI rather than of MyBP-C. As discussed above, the faster crossbridge kinetics shown by the $f_{\text{min}}$ measurements may be responsible, at least in part, for the increase in relaxation speed of the myofibrils.

Nevertheless, other studies have suggested that phosphorylation of MyBP-C may influence the contractile properties of cardiac muscle. Kunst et al²⁶ diffused the phosphorylatable motif of cardiac MyBP-C into skinned skeletal fibers and showed that force and force per attached crossbridge were higher, and myofibrillar Ca²⁺ sensitivity lower, if the motif was phosphorylated. In contrast, Calaghan et al⁹ obtained indirect evidence that phosphorylation of MyBP-C, mimicked by introduction of myosin S2 into intact cardiomyocytes (to reduce the endogenous interaction between MyBP-C and myosin S2), increased myofibrillar Ca²⁺ sensitivity. Our results indicate that phosphorylation of MyBP-C alone (in the TG muscles) does not alter the maximum force or Ca²⁺ sensitivity of skinned muscles (Figure 2B)²³,²⁸ nor the tetanic force or stiffness of intact muscles (Figure 5D). Clearly, more work is needed to resolve these discrepancies and to elucidate the functional consequences of MyBP-C phosphorylation. Because MyBP-C phosphorylation has been reported to increase the flexibility or extension of myosin crossbridges,¹⁸,¹⁹,²¹ there may be other effects of MyBP-C phosphorylation that were not uncovered by the experiments reported in the present study. Our experiments do not, for example, rule out a potential permissive effect, in which phosphorylation of MyBP-C is necessary for the effects of TnI phosphorylation to be seen.

In summary, by using transgenic mice that lack phosphorylatable TnI in the adult myocardium, we have established that it is phosphorylation of TnI, rather than of MyBP-C, that is responsible for an increase in the intrinsic relaxation rate of cardiac myofibrils. This may be due, at least in part, to an acceleration of crossbridge cycle kinetics. The acceleration of myofibrillar relaxation induced by PKA is likely to contribute to the faster relaxation of the myocardium during β-adrenoceptor stimulation.

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**References**

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