Phosphorylation of Troponin I Controls Cardiac Twitch Dynamics

Evidence From Phosphorylation Site Mutants Expressed on a Troponin I–Null Background in Mice

YeQing Pi, Kara R. Kemnitz, Dahua Zhang, Evangelia G. Kranias, Jeffery W. Walker

Abstract—The cardiac myofilament protein troponin I (cTnI) is phosphorylated by protein kinase C (PKC), a family of serine/threonine kinases activated within heart muscle by a variety of agonists. cTnI is also a substrate for cAMP-dependent protein kinase (PKA) activated during β-adrenergic signaling. To investigate the role of cTnI phosphorylation in contractile regulation by these pathways, we generated transgenic mice harboring a mutated cTnI protein lacking phosphorylation sites for PKC (serine404 and threonine44 mutated to alanine) and for PKA (serine2324 mutated to alanine). Transgenic mice were interbred with cTnI-knockout mice to ensure the absence of endogenous phosphorylatable cTnI. Here, we report that regulation of myocyte twitch kinetics by β-stimulation and by endothelin-1 was altered in myocytes containing mutant cTnI. In wild-type myocytes, the β-agonist isoproterenol decreased twitch duration and relaxation time constant (τ) by 37% to 44%. These lusitropic effects of isoproterenol were reduced by about half in nonphosphorylatable cTnI mutant myocytes and were absent in cTnI mutants also lacking phospholamban (generated by crossing cTnI mutants with phospholamban-knockout mice). These observations are consistent with important roles for both cTnI and phospholamban phosphorylation in accelerating relaxation after β-adrenergic stimulation. In contrast, endothelin-1 increased twitch duration by 52% and increased τ by 58%. These endothelin-1 effects were substantially blunted in nonphosphorylatable cTnI myocytes, indicating that PKC phosphorylation of cTnI slows cardiac relaxation and increases twitch duration. We propose that β-agonists and endothelin-1 regulate cardiac twitch dynamics in opposite directions in part through phosphorylation of the myofilament protein cTnI on distinct sites.

(Circ Res. 2002;90:649-656.)

Key Words: transgenic mice β protein kinase C β protein kinase A β phospholamban

Catecholamines released from the sympathetic nervous system and adrenal medulla stimulate cardiac β- and α-adrenoceptors. Cardiac β-stimulation results in enhanced contractility, an increased heart rate, and an abbreviated systole due to accelerated relaxation. These changes are beneficial during the fight-or-flight response because the net result is an overall increase in power output by the myocardium. Considerable evidence has linked elevation of cAMP, activation of protein kinase A (PKA), and phosphorylation of several key substrates with the physiological outcome of β-adrenergic signaling (reviewed in Steinberg,1 Brittsan and Kranias,2 and Solaro and Van Eyk3). Among the PKA targets in the heart are L-type calcium channels, the sarcoplasmic reticulum Ca2+ pump modulator phospholamban (PLB), and 2 myofilament proteins: cardiac troponin I (cTnI) and myosin binding protein C.

Cardiac α-adrenoceptor stimulation results in mobilization of lipid second messengers including diacylglycerols and fatty acids, which in turn activate the lipid-dependent protein kinase C (PKC) family of serine/threonine kinases (reviewed in Endoh).4 Other agonists including endothelin-1 (ET-1) and angiotensin II are also potent physiological stimuli for activation of PKC in the heart.5 PKC phosphorylates a rather wide spectrum of intracellular proteins including ion channels, ion pumps, exchangers, and myofilament proteins, which has hampered efforts to understand the cellular mechanisms of PKC action in myocardium. Adding to the confusion has been the inability to mimic physiological responses to agonists that activate PKC signaling pathways via direct activation of PKC (with cell permeable diacylglycerols, phorbol esters, etc) until recently. A number of actions of ET-1 can now be mimicked by cis-unsaturated fatty acids,6

© 2002 American Heart Association, Inc.

(Circulation Research is available at http://www.circresaha.org DOI: 10.1161/01.RES.0000014080.82861.5F)
intracellular photorelease of diacylglycerol,\textsuperscript{7,8} synergism between cis-unsaturated fatty acids and diacylglycerols,\textsuperscript{9} or by picomolar doses of phorbol esters.\textsuperscript{9} The PKC-dependent effects of ET-1 on cardiac myocyte function include an increase in twitch amplitude (positive inotropic effect),\textsuperscript{7,9} an increase in systolic Ca\textsuperscript{2+},\textsuperscript{9} and upregulation of L-type channel activity.\textsuperscript{8}

In the present study, we investigated the mechanisms underlying the ability of ET-1 to regulate cardiac twitch dynamics, and in particular, the role of phosphorylation of the myofilament protein cTnI. Several studies show that ET-1 and PKC activators stimulate phosphorylation of cTnI.\textsuperscript{6,10,11} Here, we generated a transgenic mouse model in which all 5 known\textsuperscript{12} phosphorylation sites on cTnI (serines\textsuperscript{23/24}, serines\textsuperscript{43/45}, and threonine\textsuperscript{144}) were converted to nonphosphorylatable alanine. The transgene construct (designated cTnI-Ala5) was introduced into the cTnI-null background by a breeding strategy. The kinetics of twitches and Ca\textsuperscript{2+} transients in response to stimulation with ET-1 were investigated in myocytes isolated from these genetically modified mice. The well-established cardiomyocyte response to \textbeta-adrenergic stimulation was used to validate the overall approach. Some of this work was presented in preliminary form to the American Heart Association\textsuperscript{13} and Biophysical Society.\textsuperscript{14}

Materials and Methods

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

Results

Two cTnI-Ala5 constructs were examined in this study. Both contained serine/threonine to alanine mutations at the following residues: serine\textsuperscript{23/24}, serine\textsuperscript{43/45}, and threonine.\textsuperscript{144} One construct also contained a 5 amino acid FLAG tag on its amino terminus to facilitate detection and quantification. Both of these cTnI-Ala5 constructs rescued the lethal phenotype of cTnI-null mice on this specific site is not necessary to support near normal cardiac function. Also, we have detected no differences between FLAG-tagged and untagged mice in terms of cardiac pathology, myofibrillar ATPase, or force generating properties (online Figure 1 in the online data supplement available at http://www.circresaha.org). However, for unknown reasons, the FLAG-cTnI-Ala5 mice produce larger and more consistent litters, and for this reason alone, we have used these mice for all experiments described below. Figure 2 illustrates how animals were genotyped using PCR analysis. Only animals harboring the cTnI transgene and expressing residues: serine\textsuperscript{23/24}, serine\textsuperscript{43/45}, and threonine\textsuperscript{144} were included in the analysis. Native cTnI represented more than 20% of the total cTnI content (Table). However, transgenic founders (and their offspring) expressed a mixture of native and transgenic cTnI protein. The lower mobility of the FLAG-tagged cTnI (Figure 3) permitted quantification of cTnI heterogeneity in this line. By this analysis, native cTnI represented more than 20% of the total cTnI content (Table). cTnI heterogeneity was also apparent in studies of PKA catalyzed incorporation of \textsuperscript{32}P-phosphate into myofilaments. Both FLAG-tagged and non-tagged cTnI-Ala5 transgenic lines contained native phosphorylatable cTnI, representing 11% to 21% of the total cTnI content (Table). This heterogeneity was no longer observed in lines expressing mutant cTnI protein on the cTnI-null background (Figure 3). In these experiments, PKA catalyzed incorporation of \textsuperscript{32}P into cTnI was undetectable, confirming the absence of endogenous cTnI. A low level of \textsuperscript{32}P-incorporation into FLAG-cTnI-Ala5 was observed (Figure 3), suggesting the existence of a previously unidentified PKA site on cTnI. However, this \textsuperscript{32}P-incorporation represented only 3% of the phosphorylation level of WT cTnI (Figure 3, Table) and was therefore not considered further here. Using PKC-\textepsilon, \textsuperscript{32}P-incorporation into cTnI-Ala5 was less than 12% of WT (Table). Overall, these results confirm at the protein level that the primary phosphorylation sites for both PKA and PKC have been eliminated.

A widely accepted effect of cTnI phosphorylation by PKA is a desensitization of myofilament Ca\textsuperscript{2+} responsiveness,\textsuperscript{3,22-24} To assess force development and myofilament

---

**Figure 1.** Mouse breeding strategy to obtain cTnI-Ala5.\textsuperscript{6} Step 1, Transgenic founders were initially crossed with WT mice to obtain a stable transgenic line. Step 2, Transgenic mice were mated with heterozygous cTnI-knockout mice. Offspring displayed the 4 genotypes shown in roughly equal proportions. Step 3, Heterozygous knockout mice harboring the cTnI-Ala5 transgene were crossed with each other giving rise to 6 distinct genotypes. Step 4, Mice homozygous for the targeted cTnI gene and carrying the cTnI-Ala5 transgene were interbred to give nonphosphorylatable cTnI-Ala5 expressed on a null background (cTnI-Ala5,\textsuperscript{6} box). Further details can be found in Materials and Methods.
Ca\textsuperscript{2+}/H\textsubscript{11001} sensitivity, pCa-tension relationships were measured in skinned ventricular myocytes from WT and cTnI-Ala\textsubscript{5}nb myocytes (Figure 4). As expected, PKA treatment shifted the pCa-tension relationship to the right by 0.28 pCa units in WT myocytes (Figure 4A). By contrast, the pCa-tension curve was not significantly affected by PKA treatment in cTnI-Ala\textsubscript{5}nb myocytes (Figure 4B). These data support the notion that cTnI phosphorylation sites responsible for a PKA-dependent shift in myofilament Ca\textsuperscript{2+}/H\textsubscript{11001} responsiveness are absent in the transgenic myocytes. It is worth noting that basal Ca\textsuperscript{2+}/H\textsubscript{11001} sensitivity is lower in cTnI-Ala\textsubscript{5}nb than in WT myocytes (pCa\textsubscript{50}=5.30 versus 5.68, respectively). It is likely that this is related to the substitution of Ser\textsubscript{43/45} and/or Thr\textsubscript{144} with alanine, because a reduced myofilament Ca\textsuperscript{2+}/H\textsubscript{11001} sensitivity was also observed in reconstituted thin filaments containing the same mutations in cTnI.\textsuperscript{27}

Individual myocytes isolated from cTnI-Ala\textsubscript{5}nb hearts showed near normal twitches, Ca\textsuperscript{2+} transients, and responses to ET-1 and isoproterenol. In standard 1 mmol/L Ca\textsuperscript{2+}/H\textsubscript{11001} modified Ringer’s solution pH 7.4, 23°C, WT myocytes paced at 0.4 Hz shortened by about 6%, whereas cTnI-Ala\textsubscript{5}nb myocytes shortened by about 4% of resting cell length (online Table 2S can be found in the online data supplement available at http://www.circresaha.org). The kinetics of electrically stimulated twitches were also similar between WT and cTnI-Ala\textsubscript{5}nb myocytes under these conditions (online Table 2S). After exposure to 10 nmol/L ET-1, WT and cTnI-Ala\textsubscript{5}nb myocytes increased twitch amplitude by 125% and 105%, respectively (online Table 3S). The phosphatase inhibitor calyculin A (CyA) enhanced the twitch response to 10 nmol/L ET-1, but WT and cTnI-Ala\textsubscript{5}nb myocytes responses were still similar, increasing by 215% and 195%, respectively (Figure 5, online Table 3S). Intracellular Ca\textsuperscript{2+}/H\textsubscript{11001} measurements using Fluor-4 showed that ET-1 enhanced systolic Ca\textsuperscript{2+} transient amplitudes to a similar extent in WT and cTnI-Ala\textsubscript{5}nb myocytes (Figure 5). After exposure to the \beta-agonist isoproterenol at a concentration of 2.5 nmol/L, twitch amplitude increased by 250% and 280% in WT and cTnI-Ala\textsubscript{5}nb, respectively, in parallel with increases in systolic Ca\textsuperscript{2+}/H\textsubscript{11001} (online Table 5S). Thus, with only subtle differences between WT and cTnI-Ala\textsubscript{5}nb myocytes, we conclude that these 2 signaling pathways are largely intact and functional in the genetically altered myocytes.

A reduced Ca\textsuperscript{2+}/H\textsubscript{11001} sensitivity associated with PKA phosphorylation of cTnI has been suggested to play a role in accelerating relaxation during \beta-adrenergic stimulation of the heart.\textsuperscript{3} As shown in Figure 6A, WT myocytes displayed abbreviated twitches in response to 2.5 nmol/L isoproterenol. This change was quantified as a 37% decrease in time to 90% relaxation (t\textsubscript{90}) and a 44% decrease in relaxation time constant (r), both of which were statistically significant (Figure 6B). In cTnI-Ala\textsubscript{5}nb myocytes, the effects of 2.5 nmol/L isoproterenol were
not as dramatic with only an 18% decrease in $t_0$ and a 25% decrease in $\tau$. These changes were statistically significant compared with both isoproterenol-stimulated WT myocytes and unstimulated cTnI-Ala$_{5}^{ab}$ myocytes (Figure 6). Thus, twitch narrowing in cTnI-Ala$_{5}^{ab}$ myocytes was only about half the magnitude of that observed in WT myocytes.

Altered sarcoplastic reticulum (SR) function did not appear to contribute to these differences in the response of cTnI-Ala$_{5}^{ab}$ and WT myocytes to isoproterenol. Intracellular Ca$^{2+}$ transients in WT myocytes were abbreviated by 12% to 32% after treatment with 2.5 nmol/L isoproterenol (online Table 5S), consistent with findings reported in ventricular muscle from a variety of mammalian species.$^{1,2,16}$ Ca$^{2+}$ transients in cTnI-Ala$_{5}^{ab}$ myocytes were also abbreviated by 12% to 30% after isoproterenol (online Table 5S). Thus, the blunted effects of isoproterenol on cTnI-Ala$_{5}^{ab}$ twitch kinetics were not due to secondary effects on Ca$^{2+}$ handling, but most likely were a consequence of the inability of cTnI to be phosphorylated on serines 23/24.

Expression and Phosphorylation of TnI in Mouse Lines

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Tg (no FLAG)</th>
<th>Tg (FLAG)</th>
<th>cTnI-Ala$_{5}^{ab}$ (no FLAG)</th>
<th>cTnI-Ala$_{5}^{ab}$ (FLAG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total TnI content (μg/mg of myofilament protein)</td>
<td>8.4±0.7</td>
<td>8.8±0.7</td>
<td>7.8±0.3</td>
<td>7.9±0.8</td>
<td>8.5±0.9</td>
</tr>
<tr>
<td>Native cTnI content (% total TnI)</td>
<td>100</td>
<td>ND</td>
<td>23±4</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>$^{32}$P incorporation (% WT)</td>
<td>100</td>
<td>21±3</td>
<td>11±2</td>
<td>0</td>
<td>3±2*</td>
</tr>
<tr>
<td>PKA</td>
<td>100</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>12±3</td>
</tr>
</tbody>
</table>

All data are presented as mean±SEM. Values less than 1% detected on overexposed Western blots or autoradiograms are listed as zero. TnI content was determined by Western blot using a monoclonal antibody (clone 6F9, Advanced ImmunoChemical) and quantified using a standard curve generated with purified bovine cTnI. PKA treatment: 0.1 U/μL catalytic subunit of PKA (bovine brain, Sigma) for 60 minutes at 37°C; stoichiometry 1.8±0.2 mol Pi/mol WT cTnI protein. PKC treatment: 0.01 μg/μL PKC-ε (Panvera) for 60 minutes at 37°C; stoichiometry 0.9±0.3 mol Pi/mol WT cTnI protein.

*32P-incorporation into FLAG-cTnI-Ala$_{5}^{ab}$; **32P-incorporation into native cTnI; and ND, not determined.
The SERCA2 pump regulator phospholamban is also thought to play a critical role in accelerating relaxation after stimulation (reviewed in Brittsan and Kranias\(^1\)). Therefore, we crossed the cTnI-Ala\(^5\)nb mice with PLB-knockout mice to obtain mice lacking these 2 major PKA targets. Isoproterenol was essentially without effect on the twitch time course in these mice (Figure 6). These data suggest that both PLB and cTnI contribute to the enhanced relaxation rate observed with stimulation. Most importantly for this study, these results validate the cTnI-Ala\(^5\)nb mice as a useful model to investigate signaling mechanisms in the heart, in particular, those in which cell surface receptor stimulation regulates myofilament function.

The influence of ET-1 on twitch kinetics was quite different than that of isoproterenol. Figure 7A shows representative twitches in WT myocytes with a t\(_{90}\) of 375 ms in control and 480 ms after 10 nmol/L ET-1. The phosphatase inhibitor CyA was included with ET-1 in all experiments because responses were more robust and more reproducible under these conditions. However, the basic observation that myocyte twitch time course became prolonged was also observed with ET-1 alone (online Table 3S). In cTnI-Ala\(^5\)nb myocytes, t\(_{90}\) was 380 ms before and 405 ms after 10 nmol/L ET-1 (Figure 7). Thus, the large effects of ET-1 on twitch time course were blunted by \(\geq 75\%\) in myocytes that could not be phosphorylated on cTnI (Figure 7).

Intracellular Ca\(^{2+}\) measurements showed that ET-1 increased the rate of the Ca\(^{2+}\) uptake phase by 5% to 10% providing evidence that twitch broadening observed with ET-1 was not due to prolongation of the Ca\(^{2+}\) transient. ET-1 had similar effects on Ca\(^{2+}\) transient kinetics in cTnI-Ala\(^5\)nb myocytes (Figure 4, online Table 5S), arguing against differences in SR function between WT and cTnI-Ala\(^5\)nb cells. Moreover, when SR Ca\(^{2+}\) handling was deliberately altered by generating cTnI-Ala\(^5\)nb myocytes lacking PLB, twitches were larger and more abbreviated (Figure 7), consistent with the reported effects of PLB knockout alone on twitch amplitude and kinetics. This observation indicates that SR load and the rate of Ca\(^{2+}\) uptake into SR play important roles in setting the amplitude and kinetics of the twitch in both WT and cTnI-Ala\(^5\)nb myocytes. Even under these rather different starting conditions, ET-1 only broadened the twitch by 10%, similar to the modest prolongation of twitches seen in cTnI-Ala\(^5\)nb myocytes containing a normal complement of PLB. Thus, whether or not PLB is present, the cTnI phosphorylation site mutations described here result in a considerably reduced effect of ET-1 on twitch broadening. Overall, the data indicate that PKC phosphorylation on serines\(^{43/45}\) and/or threonine\(^{144}\) of cTnI during ET-1 signaling results in an increase in twitch time course, whereas PKA phosphorylation on serines\(^{23/24}\) is associated with a decrease in twitch time course.

**Figure 4.** Isometric force-generating properties of skinned myocytes. A, pCa-tension curves for WT myocytes without (pCa\(_{50}\)=5.68±0.06, n\(_{H}\)=2.3±0.2) and with PKA treatment (pCa\(_{50}\)=5.40±0.06, n\(_{H}\)=2.0±0.2, P<0.05). B, pCa-tension curves for cTnI-Ala\(^5\)nb (FLAG) myocytes without (pCa\(_{50}\)=5.30±0.05, n\(_{H}\)=2.1±0.3) and with PKA treatment (pCa\(_{50}\)=5.25±0.07, n\(_{H}\)=2.4±0.3, NS).

**Figure 5.** Twitch and Ca\(^{2+}\) transient amplitudes in response to ET-1. A, WT myocytes before (control) and 10 minutes after treatment with 10 nmol/L ET-1 (+50 nmol/L CyA). B, cTnI-Ala\(^5\)nb (FLAG) myocytes before and 10 minutes after 10 nmol/L ET-1 (+50 nmol/L CyA). Insets, Twitches (top) and Ca\(^{2+}\) transients (bottom) normalized and superimposed to reveal differences in time courses.
In order to study the role of cTnI phosphorylation in cardiac muscle function, we developed mouse lines that expressed nonphosphorylatable cTnI (cTnI-Ala5) on a cTnI-null background. These mouse hearts were relatively free of cTnI heterogeneity that could arise from the presence of both endogenous cTnI and mutant (transgenic) cTnI in the myofilaments. Indeed, considerable heterogeneity was observed in the 2 transgenic lines examined here, and similar levels of heterogeneity have been observed in other cTnI transgenic mice. It should be noted, however, that in one thoroughly investigated system, overexpression of ssTnI nearly completely replaced endogenous cTnI, giving rise to an essentially homogeneous expression of ssTnI in the adult mouse heart. The precise mechanism underlying this apparent loss of endogenous cTnI is unknown.

In the present study, native endogenous cTnI was eliminated by crossing transgenic mice with cTnI-knockout mice. This overall strategy should be generally applicable to other forms of cTnI, for example those implicated in human heart disease, as long as their function is not too impaired to rescue the lethal cTnI-knockout phenotype. Expressing cTnI proteins on a cTnI-null background works well if the mutant cTnI can support cardiac function on its own. The results of this study demonstrate that cTnI missing 5 classical phosphorylation sites can support near normal cardiac function in the complete absence of native cTnI. Therefore, phosphorylation of these sites is not essential for cTnI function.

We chose to mutate both PKA and PKC sites in the same mouse line for 2 reasons. First, with both PKA and PKC sites missing, we were able to use the β-adrenergic response as a form of internal control to provide evidence that the tissue is behaving as expected. Second, there is evidence from in vitro studies of cTnI phosphorylation that PKC can cross-phosphorylate PKA sites. Therefore, mutation of only the PKC sites would leave open the possibility of phosphorylation of PKA sites on stimulation of PKC signaling pathways that could confound the results. Importantly, the cellular signaling mechanisms appear to be intact in these mice, making this a potentially powerful system to explore the role of cTnI phosphorylation in the response of the heart to various stimuli.

The elegant work of Solaro, Potter, and coworkers has provided considerable evidence for the involvement of cTnI phosphorylation in the response of the heart to β-adrenergic stimulation. Elevation of cAMP followed by activation of PKA has been shown both in vivo and in vitro to phosphorylate serines on cTnI. The functional consequences of this reversible posttranslational modification may include a decrease in Ca²⁺ sensitivity of the thin filament regulatory system, an increase in the off rate for the dissociation of Ca²⁺ from troponin, an increase in relaxation rate, and an increase in cross-bridge cycling rate. How much cTnI phosphorylation contributes to the negative lusitropic effects (accelerated relaxation) of β-stimulation in the intact heart is still a matter of debate. Nevertheless, each of the consequences of cTnI phosphorylation described above would facilitate relaxation.
of myocardium and would complement the well-documented increase in heart rate (ie, beats per min) characteristic of the fight-or-flight response. The data presented here support the view that both cTnI phosphorylation and PLB phosphorylation play substantial roles in the negative lusitropic effects of adrenaline on the heart.

Much less is known about the effects of PKC on the function of cardiac myofilaments. A variety of agonists operate through PKC signaling in the heart, including phenylephrine, adenosine, ET-1, angiotensin II, and opiates (reviewed in Endoh 4 and Sugden and Bogoyevitch 5). Many of these agonists as well as direct activators of PKC, such as phorbol esters, diacylglycerols and fatty acids, promote phosphorylation of cardiac myofilament proteins. PKC phosphorylation of myofilament proteins, in particular, cTnI and cTnT have been linked with a reduced myofibrillar ATPase rate in a number of studies. Moreover, measurements of unloaded shortening velocity have implicated PKC signaling pathways in slowing the rate of cross-bridge cycling in ventricular myocytes. However, the precise mechanisms underlying PKC’s influence on cross-bridge function remain to be elucidated.

We investigated the hypothesis that ET-1 stimulation of ventricular myocytes causes broadening of the twitch as a direct consequence of phosphorylation of cTnI. Phenylephrine acting on α-adrenergic receptors has been shown to broaden cardiac twitches with possible contributions from intracellular alkalinization, action potential prolongation, and altered myofilament function (reviewed in Endoh 4). ET-1 was chosen as the agonist here, because in our hands, ET-1 stimulated more consistent and less complex inotropic responses than did phenylephrine, angiotensin II, or opiates. There was also less risk of activating powerful β-adrenergic signaling pathways with ET-1 than with phenylephrine, and we could avoid use of the β-blocker propranolol, which has been shown to inhibit diacylglycerol production via the signaling enzyme phospholipase D.

Conditions were optimized for ET-1 dependent twitch broadening in WT myocytes. An important factor turned out to be inclusion of the phosphatase inhibitor CYA. In the presence of 50 nmol/L CYA, 10 nmol/L ET-1 stimulated a robust and reproducible increase in twitch time course on the order of a 30% increase. A similar increase was recently reported in rat myocytes in response to a much higher dose of CYA alone. This preliminary study by Gigena et al 12 was also intriguing because, although they found the twitch time course to be dramatically broadened by CYA, they found no corresponding effects on the action potential duration or on the time course of the Ca²⁺ transient. This implied that twitch broadening was due to changes in myofilament properties.

As mentioned earlier, prior to ET-1 stimulation cTnI-Ala, myocytes behaved similarly to WT in terms of kinetics and amplitudes of twitches and Ca²⁺ transients. In response to ET-1, however, twitches were prolonged by 24% to 31% in WT but only by 5% to 8% in cTnI-Ala cells. Assuming that the blunted twitch broadening response was due to the inability of cTnI to be phosphorylated, rather than being due to altered basal twitch properties or compensatory remodeling resulting from cardiac pathology.

In summary, a nonphosphorylatable form of cTnI has been expressed on a troponin I–null background in the mouse heart. This mouse line permitted us to compare and contrast the effects of activation of PKA versus PKC signaling pathways on the kinetics of myocyte twitches. Our results are consistent with a number of studies which show that PKA phosphorylation of both cTnI and PLB play important roles in accelerating twitch relaxation during β-adrenergic signaling. In contrast, PKC phosphorylation of cTnI appears to be a major factor in prolonging the twitch time course, whereas PKC phosphorylation of PLB is not involved. The picture that emerges is that cTnI may be a critical substrate for both PKA and PKC, and that cTnI phosphorylation by these kinases may lead to opposing effects on the dynamic control of cardiac twitches. This underscores the importance of cTnI as a regulatory protein that may influence not only Ca²⁺ binding and dissociation from the thin filament, but also the kinetics of actomyosin interactions. The physiological significance of twitch broadening induced by ET-1 is not yet clear. An intriguing possibility is that twitch broadening is a reflection of a more efficient transduction of ATP hydrolysis into work production by the myocardium.
needed to determine whether PKC-mediated phosphorylation of cTnI plays a significant role in this phenomenon.

Acknowledgments
This study was supported by National Institutes of Health grants P40 RR12358 (Dr Kranias) and POI HL47053 (Dr Walker). The authors thank Dr R.J. Solaro for providing mouse cTnI cDNA and Dr Guoxiang (Simon) Chu for critical reading of an earlier version of the article.

References
34. Strang KT, Moss RL. α-Adrenergic receptor stimulation decreases maximum shortening velocity of single ventricular myocytes from rats. Circ Res. 1995;77:114–120.
Phosphorylation of Troponin I Controls Cardiac Twitch Dynamics: Evidence From Phosphorylation Site Mutants Expressed on a Troponin I-Null Background in Mice
YeQing Pi, Kara R. Kemnitz, Dahua Zhang, Evangelia G. Kranias and Jeffery W. Walker

_Circ Res._ 2002;90:649-656; originally published online February 28, 2002; doi: 10.1161/01.RES.0000014080.82861.5F

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231 Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/90/6/649

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2002/03/27/90.6.649.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the _Permissions and Rights Question and Answer_ document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org//subscriptions/
ON-LINE SUPPLEMENTARY MATERIAL

PHOSPHORYLATION OF TROPONIN I CONTROLS CARDIAC TWITCH DYNAMICS. Evidence from Phosphorylation Site Mutants Expressed on a Troponin I Null Background in Mice.

YeQing Pi, Kara R. Kemnitz, Dahua Zhang, Evangelia G. Kranias* and Jeffery W. Walker

Department of Physiology, University of Wisconsin, Madison, WI 53706
*Department of Pharmacology and Cell Biophysics, University of Cincinnati, Cincinnati,

OH 45267-0575

Running Title: Cardiac Troponin I Phosphorylation

Subject codes: cell signaling, contractile function

Corresponding Author:

Dr. Walker
Department of Physiology
1300 University Avenue
Madison, WI 53706
608-262-6941
608-265-5512 (FAX)
jwalker@physiology.wisc.edu
MATERIALS AND METHODS

Generation of genetically modified mice. The endogenous cTnI gene was replaced with a neomycin resistance cassette in S129 mice (brown coat) as described\textsuperscript{21}. cTnI cDNA (a kind gift of R.J. Solaro) was subjected to site directed mutagenesis in which codons for serine\textsuperscript{23/24}, serine\textsuperscript{43/45}, and threonine\textsuperscript{144} were converted to alanine codons. One construct contained a five residue amino terminal FLAG tag to facilitate identification, and another lacked the FLAG tag. Constructs were restriction mapped and fully sequenced prior to myoblast injection. The cardiac specific $\alpha$-myosin heavy chain promoter was used to drive expression of the transgenes in myocardium. Three to five founders were obtained for each construct, but only one of each was chosen for further experimentation based on a medium to high copy number (10 copies of cTnI-Ala\textsubscript{5} (no FLAG) transgene and 13 copies of FLAG-cTnI-Ala\textsubscript{5} transgene) and consistent breeding with large healthy litters. Transgenic mouse lines created in FVB/N strain (white coat) were crossed with cTnI knockout mice that were heterozygous for the targeted gene (see Figure 1): homozygous cTnI knockout mice could not be used because they do not survive to breeding age.\textsuperscript{1} This mating produced heterozygous cTnI knockout mice expressing the cTnI-Ala\textsubscript{5} transgene. Only brown mice were used for further breeding. Finally, these mice were crossed with each other to generate homozygous cTnI null mice that were effectively ‘rescued’ from a lethal phenotype by the presence of the transgene (see Figure 1). These mice were designated cTnI-Ala\textsubscript{5}\textsuperscript{nb} (null background). The ratio of rescued (cTnI-Ala\textsubscript{5}\textsuperscript{nb}) to knockout (cTnI\textsuperscript{/-}) pups in these litters was 2:1. This can be accounted for if the targeted and transgenic alleles segregate independently, mice are hemizygous for the transgene, and mice homozygous for the transgene die in utero.
Another transgenic mouse line was created by crossing cTnI-Ala5 with PLB knockout mice.²

**Genotyping.** DNA samples were extracted from tail tissue of 10 day old mice and polymerase chain reaction (PCR) using six pairs of primers was employed. Primer sequences are given in Table 1S. Typical PCR results for mouse genotyping are shown in the paper (see Figure 2).

**Comparison between cTnI-Ala5ₙb mice with and without the FLAG tag.** Transgene copy numbers assessed by Southern blotting were similar between these mouse lines (13 copies (FLAG); 10 copies (no FLAG)). The 5 amino acid N-terminal FLAG tag caused that protein to migrate more slowly on SDS-PAGE and on Western blots (see Figure 3A). Myocytes from both mouse lines exhibited similar steady state tension and pCa-tension relationships (Figure 1S). Myofibrillar MgATPase properties were also indistinguishable (Figure 1S). Both lines also displayed similar levels of dilated cardiac hypertrophy as measured by an increase in heart to body weight ratio (27±4 % (FLAG) versus 29±5% (no FLAG). Histological analysis (H&E and trichrome staining) revealed little or no abnormal sarcomere structure or fibrosis. Thus, the FLAG tag appears to have little influence on the physiological properties of the mouse hearts.

**Immunoblotting and phosphorylation assays.** Hearts were homogenized in relaxing buffer containing (in mM) 4 ATP, 100 KCl, 10 imidazole, 2 EGTA, 1 MgCl₂, 1 PMSF, 10 benzamidine, protease inhibitor cocktail (1 tablet Complete™/100 ml, Roche, Germany) at 4°C, and myocardial tissue was skinned in relaxing buffer containing 0.33% Triton X-100 and 0.5% BSA. Myofilaments were washed to remove Triton X-100 and BSA then solubilized by boiling for 5 min in electrophoresis loading buffer (2% SDS,
20% glycerol, 100 mM dithiothreitol, 0.1% bromphenol blue, 200 mM Tris (pH 6.8)).

Western blotting was carried out on 50 μg samples electrophoresed on 12% SDS-PAGE gels then transferred onto nitrocellulose paper. Anti-TnI monoclonal antibody (clone 6F9, Advanced ImmunoChemical Inc) was used at a 1:1000 dilution.¹

For phosphorylation, 200 μg of skinned myofilaments were incubated 60 min at 37°C in 200 μl of solution containing (in mM) 100 μCi ³²P-labeled ATP, 1 ATP, 100 KCl, 10 imidazole, 2 EGTA, 1 MgCl₂, 1 PMSF, 10 benzamidine, protease inhibitor cocktail (1 tablet Complete™/100 ml), and catalytic subunit of PKA (0.1 U/μl, Sigma Chemical Co), or purified ε-PKC (0.01 μg/μl, Panvera, Madison, WI) activated with phosphatidylycerine (200 μg/ml, Avanti Polar-Lipid), dioctanoylglycerol (20 μg/ml, Avanti Polar-Lipid), and 100 nM phorbol 12-myristate 13-acetate (PMA) (CalBiochem). Myofilaments were washed, solubilized in 200 μl electrophoresis loading buffer and electrophoresed as described above. Gels were stained with Coomassie blue, dried and subjected to autoradiography using X-OMAT film (Kodak, Rochester, NY). ³²P-labeled cTnI bands were excised from the gels and quantified by liquid scintillation counting. Signals from Western blots and autoradiograms were quantified by using a Bio-RadGS-670 imaging densitometer.

**Myocyte isolation.** Ventricular myocytes were enzymatically dissociated using a modified version of the method previously used for rat myocytes.³ Briefly, after cannulation of the aorta under a 10X dissecting microscope, the heart was perfused on a Langendorff apparatus with modified Ringer’s buffer ((in mM): 125 NaCl, 2 NaH₂PO₄, 1.2 MgSO₄, 5 KCl, 25 HEPES (pH 7.4), 5 pyruvate, and 11 D-glucose containing 1 CaCl₂ and 0.1% BSA). The heart was perfused with 20 ml Ca²⁺-free modified Ringer’s solution
containing 0.1% BSA, then with modified Ringer's buffer containing 0.25 mg/ml collagenase II and 0.1 mg/ml elastase (Worthington Biochem). CaCl₂ was added gradually over 15 min to a final concentration of 1 mM. Atria were removed and ventricles were minced and digested for another 15 min at 37°C. The suspension was filtered through Teflon mesh and resuspended in 1 mM Ca²⁺-modified Ringer's buffer containing 0.1% BSA. A typical preparation of mouse myocytes gave >70% rod shaped Ca²⁺ tolerant cells.

**Measurements of mouse myocyte twitches and Ca²⁺ transients.** Diluted cells were loaded with Ca²⁺ indicator Fluo-4/AM (5 μM, Molecular Probes, Eugene, OR) in 1 mM Ca²⁺ modified Ringer's buffer for 45 min at room temperature, then washed and suspended in indicator-free modified Ringer's. Cell twitches were initiated by electric field stimulation at 0.4 Hz using a Grass SD9 stimulator (Quincy, MA) in a modified PH1 chamber (Warner Instrument, Hamden, CT) mounted on a Nikon Diaphot inverted microscope. Twitches and Ca²⁺ transients were monitored with video edge-detector (VED 104, Crescent Electronic, Sandy, UT) and PC-based photometer system (D-140 DeltaScan, Photon Technology Inc, NJ), respectively.⁴

**Measurements of mechanical force.** The ends of skinned myocytes or small fiber bundles were glued to the arms of piezoelectric translator (model 350; Physik Instruments, Cambridge, MA) and force transducer (model 403; Cambridge Technology, Cambridge, MA), and force output was monitored as described earlier.¹ Active force was recorded at a sarcomere length of 2.1 to 2.2 μm by transferring the attached myocyte to activating solutions ranging from pCa 4.5 to 9.0 (where pCa is -log [Ca²⁺]). All tension measurements were carried out at room temperature and normalized to myocyte cross-
sectional area. To determine the Ca\textsuperscript{2+} sensitivity of isometric force, pCa-tension data was fitted with a Hill equation: \( P/P_0 = [\text{Ca}^{2+}]^n/(k^n + [\text{Ca}^{2+}]^n) \), where \( P \) is tension, \( P_0 \) is maximum tension, \( n \) is the slope (Hill coefficient) and \( k \) is the Ca\textsuperscript{2+} concentration required for half-maximal force development (pCa\textsubscript{50}). An analogous equation was used for fitting myofibrillar MgATPase versus pCa data.

**Reagents.** Stock solutions of isoproterenol and endothelin were prepared in distilled water. Calyculin A was dissolved in ethanol. PKA catalytic subunit was prepared in 2 mM dithiothreitol in water. PMA (CalBiochem) and Fluo-4/AM (Molecular Probes) were prepared in dimethylsulfoxide.

**Data analysis.** Data are expressed as mean±sem. Both paired and unpaired Student’s \( t \) tests and one-way ANOVA were used for statistical analyses. \( P<0.05 \) was taken to be a significant difference.
REFERENCES

1. Huang XP, Pi YQ, Lee KJ, Henkel AS, Gregg RG, Powers PA, Walker JW. Cardiac
troponin I gene knockout. A mouse model of myocardial troponin I deficiency.

EG. Targeted ablation of the phospholamban gene is associated with markedly
enhanced myocardial contractility and loss of β-agonist stimulation. Circ Res.
1994;75:401-409.

3. Huang XP, Pi YQ, Lokuta AJ, Greaser MG, Walker JW. Arachidonic acid stimulates
protein kinase C-epsilon redistribution in heart cells. J Cell Sci. 1997;110:1625-
1634.

4. Pi YQ, Sreekumar R, Huang XP, Walker JW. Positive inotropy mediated by
Online Figure 1. Comparison of functional properties of cTnI- Ala$_5^{nb}$ with and without the N-terminal FLAG. A. Tension versus pCa relationships. Bar graphs on the right summarize maximum force (at pCa 4.5) and pCa$_{50}$ values. B. Myofibrillar MgATPase versus pCa relationships. Bar graphs on the right summarize maximum MgATPase activity (at pCa 4.0) and pCa$_{50}$ values.
A

Relative Force

\[ \text{Relative Force} \]

\[ 9.00 \quad 6.30 \quad 5.85 \quad 5.40 \quad 4.95 \quad 4.50 \]

Maximal Force (\(\mu\)N/m²)

\[ \text{(n=18)} \quad \text{(n=7)} \]

\[ 0 \quad 5 \quad 10 \quad 15 \]

pCa

B

Relative Myofibrillar ATPase Activity

\[ \text{Relative Myofibrillar ATPase Activity} \]

\[ 9.00 \quad 6.5 \quad 6.0 \quad 5.5 \quad 5.0 \quad 4.5 \quad 4.0 \]

Maximal ATPase Activity (nmol Pi/min/mg protein)

\[ \text{(n=7)} \quad \text{(n=3)} \]

\[ 0 \quad 70 \quad 140 \quad 210 \quad 280 \]

pCa

(FLAG) (NO FLAG)

(FLAG) (NO FLAG)

(FLAG) (NO FLAG)
Table 1S. PCR primers

<table>
<thead>
<tr>
<th>Allele</th>
<th>Size (bp)</th>
<th>Name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>cTnl-KO</td>
<td>390</td>
<td>5UTF</td>
<td>5'TAGGTGTGAGGACAGAAGGCG3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PGK</td>
<td>5'GTGGAATGTGTGCGAGGCAA3'</td>
</tr>
<tr>
<td>CTnl-WT</td>
<td>630</td>
<td>5UTF</td>
<td>5'TAGGTGTGAGGACAGAAGGCG3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1IR</td>
<td>5'CGTGGAAGAGGAAATCATGATGGTGTC3'</td>
</tr>
<tr>
<td>cTnl-Ala5</td>
<td>289</td>
<td>KK2</td>
<td>5'CCTTACGCTGTCTTCTCCTAC3'</td>
</tr>
<tr>
<td></td>
<td>304*</td>
<td>KK1</td>
<td>5'GCATCAGAGTCTTCAACTGAA3'</td>
</tr>
<tr>
<td>cTnl-Ala5</td>
<td>1026</td>
<td>NP</td>
<td>5'GAGAGCCATAGGCTACGGTG3'</td>
</tr>
<tr>
<td></td>
<td>1041*</td>
<td>KK1</td>
<td>5'GCATCAGAGTCTTCAACTGAA3'</td>
</tr>
<tr>
<td>PLB-KO</td>
<td>450</td>
<td>HLT7</td>
<td>5'TCCTCGTGCTTTACGGTATC3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Neo3'</td>
<td>5'TGTGGGTGCAAAAGTTAGGC3'</td>
</tr>
<tr>
<td>PLB-WT</td>
<td>550</td>
<td>HLT7</td>
<td>5''TCCTCGTGCTTTACGGTATC3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JS940</td>
<td>5'CTTGTTCACTACTGTCAGAAC3'</td>
</tr>
</tbody>
</table>

Size refers to the number of base pairs in the predicted PCR fragment. * The same primers were used for cTnl-Ala5 with and without the FLAG. The larger PCR fragment pertains to the cTnl-Ala5 allele with the FLAG.
Table 2S. Summary of basal twitch properties.*

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>cTnl-Ala₅ⁿᵇ</th>
<th>cTnl-Ala₅ⁿᵇ/PLB-KO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Basal Twitches</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Amplitude</strong> (% resting cell length)</td>
<td>6±2</td>
<td>4±2</td>
<td>7±3</td>
</tr>
<tr>
<td><strong>Kinetics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time to 50% relax (ms)</td>
<td>285±5</td>
<td>301±5</td>
<td>256±4‡</td>
</tr>
<tr>
<td>Time to 90% relax (ms)</td>
<td>385±7</td>
<td>404±7</td>
<td>308±5‡</td>
</tr>
<tr>
<td>Time to 90% peak (ms)</td>
<td>167±4</td>
<td>187±5</td>
<td>181±4</td>
</tr>
<tr>
<td>Relaxation τ (ms)</td>
<td>99±1</td>
<td>103±1</td>
<td>43±1§</td>
</tr>
</tbody>
</table>

* All data is presented as mean ± sem. Symbols indicate statistical significance as follows: ‡ p<0.05 vs WT and cTnl-Ala₅ⁿᵇ; § p<0.005 vs WT and cTnl-Ala₅ⁿᵇ.
Table 3S. Effects of calyculin A on the twitch response to ET-1.*

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>cTnl-Ala5&lt;sup&gt;nb&lt;/sup&gt;</th>
<th>cTnl-Ala5&lt;sup&gt;nb&lt;/sup&gt;/PLB-KO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ET-1</td>
<td>ET-1+CyA</td>
<td>ET-1</td>
</tr>
<tr>
<td>Twitch Amplitude</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(%change)</td>
<td>125±9</td>
<td>215±15</td>
<td>105±20</td>
</tr>
<tr>
<td>Twitch Kinetics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time to 90% relax</td>
<td>15±5</td>
<td>32±2</td>
<td>5±2</td>
</tr>
<tr>
<td>(%change)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Effects of 50 nM calyculin A (CyA) on the myocyte response to 10 nM ET-1. All data are presented as mean ± sem.
Table 4S. Summary of ET-1 and isoproterenol (Iso) effects on twitches.*

<table>
<thead>
<tr>
<th>Agonist Effects</th>
<th>WT ET-1</th>
<th>Iso</th>
<th>cTnl-Ala5 ET-1</th>
<th>Iso</th>
<th>cTnl-Ala5/PLB KO ET-1</th>
<th>Iso</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Twitch Amplitude (% change)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>215±9</td>
<td>227±29</td>
<td>195±13</td>
<td>209±19</td>
<td>158±17</td>
<td>181±32</td>
<td></td>
</tr>
<tr>
<td><strong>Twitch Kinetics (% change)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time to 90% peak</td>
<td>5.6±1.80</td>
<td>-11.4±1.09</td>
<td>5.0±1.47</td>
<td>-8.2±1.08</td>
<td>4.7±2.59</td>
<td>-4.7±4.24</td>
</tr>
<tr>
<td>Time to 50% relax</td>
<td>24.0±0.78</td>
<td>-20.6±1.47</td>
<td>8.3±1.07</td>
<td>-4.4±1.21</td>
<td>4.8±1.80</td>
<td>-3.6±3.34</td>
</tr>
<tr>
<td>Time to 90% relax</td>
<td>27.7±1.16</td>
<td>-37.0±1.76</td>
<td>6.2±1.24</td>
<td>-17.6±1.43</td>
<td>7.4±1.38</td>
<td>-5.0±3.35</td>
</tr>
<tr>
<td>Relaxation τ</td>
<td>58.3±1.91</td>
<td>-44.2±1.39</td>
<td>10.9±1.20</td>
<td>-25.0±1.65</td>
<td>11.3±1.50</td>
<td>-2.6±3.26</td>
</tr>
</tbody>
</table>

All data is presented as mean ± sem. ET-1 indicates response to 10 nM ET-1 (+50 nM CyA). Iso indicates response to 2.5 nM isoproterenol. Data are represented as % change compared to untreated control in the parameter of interest. A positive number indicates an increase in the kinetic parameter compared to control, and a negative number indicates a decrease. Symbols indicate statistical significance as follows: ‖: p<0.05 vs WT; ‖‖: p<0.05 vs cTnl-Ala5<sup>nb</sup>. nd: not determined.
Table 5S. Summary of ET-1 and isoproterenol (Iso) effects on \( \text{Ca}^{2+} \) transient.

<table>
<thead>
<tr>
<th>Agonist Effects on ( \text{Ca}^{2+} )</th>
<th>WT</th>
<th>cTnl-Ala(_5)nb</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amplitude</strong> (%) change</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ET-1</td>
<td>36±12</td>
<td>114±74</td>
</tr>
<tr>
<td>Iso</td>
<td>38±18</td>
<td>42±9</td>
</tr>
<tr>
<td><strong>Kinetics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time to 50% decline (%) change</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ET-1</td>
<td>-5±2</td>
<td>-12±2*</td>
</tr>
<tr>
<td>Iso</td>
<td>-3±1</td>
<td>-12±1*</td>
</tr>
<tr>
<td>Time to 90% decline (%) change</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ET-1</td>
<td>-12±3*</td>
<td>-27±2*</td>
</tr>
<tr>
<td>Iso</td>
<td>-10±2*</td>
<td>-22±1*</td>
</tr>
<tr>
<td>Ca decline ( \tau ) (%) of control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ET-1</td>
<td>-11±2*</td>
<td>-32±2*</td>
</tr>
<tr>
<td>Iso</td>
<td>-12±1*</td>
<td>-30±2*</td>
</tr>
</tbody>
</table>

Data is presented as mean ± sem. Data are represented as % change compared to untreated control in the parameter of interest. A positive number for % change indicates an increase in the parameter compared to control, and a negative number indicates a decrease. * \( p<0.05 \) versus unstimulated control.