Our goal was to define the role of phosphorylated cardiac troponin-I in the adult myocyte contractile performance response to activated protein kinase C. In agreement with earlier work, endothelin enhanced both adult rat myocyte contractile performance and cardiac troponin-I phosphorylation. Protein kinase C participated in both responses. The role of cardiac troponin-I phosphorylation in the contractile function response to protein kinase C was further investigated using gene transfer into myocytes of troponin-I isoforms/mutants lacking one or more phosphorylation sites previously identified in purified cardiac troponin-I. Sarcomeric replacement with slow skeletal troponin-I-abrogated protein kinase C-mediated troponin-I phosphorylation. In functional studies, endothelin slowed relaxation in myocytes expressing slow skeletal troponin-I, while the relaxation rate increased in myocytes expressing cardiac troponin-I. Based on these results, acceleration of myocyte relaxation during protein kinase C activation largely depended on cardiac troponin-I phosphorylation. Experiments with troponin-I isoform chimerae provided evidence that phosphorylation sites in the amino portion of cardiac troponin-I mediated the protein kinase C acceleration of relaxation. The cardiac troponin-I Thr-144 phosphorylation site identified in earlier biochemical studies was not significantly phosphorylated during the acute contractile response. Thus, amino-terminal protein kinase C-dependent phosphorylation sites in cardiac troponin-I are likely responsible for the accelerated relaxation observed in adult myocytes.

Troponin I (TnI) is a key regulatory protein within the thin filament of the contractile apparatus. Different isoforms of TnI influence myofilament Ca$^{2+}$ sensitivity (1) and contribute to developmental changes in myofilament function. In particular, the transition from slow skeletal TnI (ssTnI) expression in embryonic/fetal hearts to the cardiac isoform expressed exclusively in adult hearts appears to "fine tune" myofilament regulatory function (1, 2). The Ca$^{2+}$ sensitivity properties of the myofilaments also can be modified by TnI phosphorylation (3). For example, β-adrenergic activation of protein kinase A (PKA) phosphorylates cardiac TnI (cTnI), which reduces myofilament Ca$^{2+}$ sensitivity (3) and contributes to accelerated relaxation in intact adult myocardium (4). TnI also is phosphorylated by activated protein kinase C (PKC) (5, 6), and while an association exists between PKC-mediated cTnI phosphorylation and contractile function (7), the specific role of TnI in the contractile response remains unclear.

The activation and expression pattern of multiple PKC isoforms in the heart changes under pathophysiological conditions, which may influence the role of PKC-mediated TnI phosphorylation in the contractile function response. In adult myocardium, the classical α isoform, as well as the novel δ and ε isoforms are normally expressed in both rats and humans (8–10), while expression of the classical β isoform is absent from adult myocytes from these species (8, 11). However, pathophysiological conditions, such as pressure overload-induced hypertrophy and diabetic hypertrophy are associated with increased α and δ expression in rat models (12, 13). Increased levels and activity of PKC α, βI, and βII are observed in heart failure patients (14). Moreover, the contractile response to PKC activation also is altered in myocytes from heart failure patients (15).

The phosphorylation state of cTnI also changes under situations associated with cardiac pathophysiology. Troponin I phosphorylation significantly decreases during heart failure (16–18), and a component of this decrease is attributed to alterations in cTnI phosphorylation by PKC (17, 18). In transgenic mice, levels of TnI phosphorylation increase with up-regulation of at least one PKC isoform, prior to the development of cardiac dysfunction (19). Collectively, TnI phosphorylation and studies of PKC expression/activation provide evidence that the relationship between PKC activation, cTnI phosphorylation, and contractile function may change during pathophysiological conditions associated with cardiac dysfunction. Thus, it is important to first establish the function of phosphorylated cTnI in the acute physiological contractile function response to PKC.

The goal of the present study is to define the physiological role of cTnI in the adult cardiac myocyte contractile response to endothelin (ET). ET is a potent endogenous mediator of contractile function in the heart (20–22), and the short term contractile response to ET is largely mediated via activation of PKC (23, 24). The cTnI phosphorylation sites of interest are Ser-23/24, Ser-43/45, and Thr-144 (6). Our results from myocytes expressing endogenous cTnI are comparable with myocytes expressing ssTnI, cTnI/ssTnI chime-
ras, and TnI mutants after viral-mediated gene transfer. In earlier studies, this gene transfer approach produced stoichiometric replacement of endogenous cTnI in the sarcomere (1, 25, 26). The rationale for using the selected isoform, chimeras, and mutants is that each of these TnI proteins lacks one or more of the 3 PKC-mediated phosphorylation sites. The ssTnI isoform lacks the Ser-23/24 and Thr-144 sites, but contains a consensus sequence for PKC at the residues analogous to cTnI Ser-34/45 (ssTnIThr-11/Ser-13) (27, 28). Chimera N-card/slow-C TnI (25) contains the Ser-23/24 and Ser-45/46 sites, while Thr-144 and the amino-terminal Thr/Ser of ssTnI are present in the N-slow/card-C TnI sequence (26). The cTnI Thr-144 site is of particular interest because it lies within the inhibitory peptide (IP) region of TnI. The IP region is believed to act as a molecular switch as it toggles from actin to troponin C in response to Ca2+ (29), and the ability of this region to be modified by phosphorylation would indicate an important potential target for therapeutic approaches designed to modify myofilament function. Thus, we compared the ability of Thr-144 to be phosphorylated in intact myocytes treated with ET to the two sets of phosphorylation sites located in the amino portion of cTnI. In these experiments, the Pro-112 present in the analogous position of the ssTnI sequence is substituted for Thr-144 in cTnI, and vice versa. Individual gene transfer and expression of cTnI T144P and ssTnI P112T in adult myocytes is followed by a comparison of ET-induced phosphorylation of these mutant proteins versus wild-type cTnI in the intact adult myocyte. Taken together, studies described here provide important insight into the role of cTnI in the acute ET-induced, PKC-activated enhancement of contractile performance, and the relative significance of cTnI phosphorylation sites in the intact myocyte.

EXPERIMENTAL PROCEDURES

Generation of Adenoviral Vectors—Recombinant adenoviral vectors were constructed by co-transfection of shuttle plasmids containing TnI cDNAs (cTnI, ssTnI, N-slow/card-C TnI, ssTnIT112P, cTnIT144P, and cTnIT144PFLAG) and pJM17 into HEK293 cells (1). Virus replication was carried out in the HEK293 cell line, which expresses the E1 region of the adenoviral genome in trans. Recombinant adenoviral DNA was identified by Southern blot analysis (1). Mutagenesis Strategy—Full-length wild-type ssTnI and cTnI cDNAs were kind gifts of Ann Murphy (30). In addition to preparing recombinant adenovirus from each of these constructs, the cDNAs were used to separately generate N-slow/card-C TnI and N-slow/card-C TnI in pGEM-3Z, as described earlier (25, 26). The TnI constructs, cTnIFLAG, cTnIT144P, cTnIT144PFLAG, and ssTnIP112T, were prepared using the Stratagene QuikChange mutagenesis kit. Preparation of cTnIFLAG was performed as described earlier (31). The following oligonucleotide primers were used to make cTnIT144P (primer 1: GCAAGGTTAACGCGGCGCTTCCTCCCGAAGACTGT; primer 2: CACTTCTGGAAGAACGCCCGTTAAACTGCG; underlined regions indicate mutated residues), and ssTnIP112T (primer 1: GGAGGAATGGCAAGCGGCGCTGCAGCTCGCCGGTC; primer 2: GGAGCCGCGGCAAGATGGCGCGCTTACCTG). Each cDNA was then subcloned into the pcA4 shuttle vector. Chimeric and mutant protein production were verified in HEK293 cells prior to virus making.

Primary Cultures of Adult Cardiac Myocytes—Ventricular myocytes were isolated from adult rats, as described previously (32). A aliquots of Ca2+ -tolerant myocytes (2 × 105) were plated on laminin-coated coverslips and incubated at 37 °C in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin (P/S). After 2 h, media was replaced with adenovirus diluted in serum-free Dulbecco’s modified Eagle’s medium with P/S. Serum-free media (2 ml) was added after an hour incubation with adenovirus, and media was changed the day after adding virus and then every 2–3 days. Western Blot Analysis of Protein Composition—Control and adenovirus-treated adult rat cardiac myocytes maintained in culture for 4 days were scraped from coverslips into 10 μl of sample buffer (1). Samples were separated by gel electrophoresis and transblotted onto polyvinylidene difluoride membrane for 2000 V-h with immunodetection carried out as described by Westfall et al. (33) using a 1:1000 dilution of anti-TnI monoclonal antibody (mAb; Fitzgerald), a monoclonal antibody which recognizes all isoforms of TnI. In separate experiments, blots from adult cardiac myocytes collected 1–7 days after gene transfer were probed for the major endogenous protein kinase C isoforms observed in rat hearts using anti-PKCα (1:400, Santa Cruz Biotechnology), anti-PKCβ1 (1:100, Santa Cruz Biotechnology), anti-PKCδ (1:100, Santa Cruz Biotechnology), and anti-PKCγ (1:100, Santa Cruz Biotechnology) Abs, followed by secondary goat anti-rabbit Ab (α, β) or donkey anti-rabbit Ab (γ) conjugated to peroxidase (Amersham Biosciences; 1:2000; Sigma 1:1000, respectively).

Phosphorylation of Myofilament Proteins in Intact Cardiac Myocytes—Myocytes cultured for 4–5 days were incubated with [32P]orthophosphate (100 μCi/ml) in Dulbecco’s modified Eagle’s medium with P/S at 37 °C. After a 2-h labeling interval, radioactive media was replaced with unlabelled media containing calycin A (10 μg/ml) in addition to endothelin-1 (ET, 10–250 nm), a PKC inhibitor (e.g. chelerythrine, 10 μM; bisindolylmaleimide, 500 nm to 1 μM), or ET plus a PKC inhibitor, and incubated for 5–40 min at 37 °C. Experiments with calycin A + ET added along with the inactive bisindolylmaleimide structural analog that lacks PKC-inhibiting properties, bisindolylmaleimide V (Bio-V; 1 μM), the EKI1/2 inhibitor PD98059 (50 μM), the PKA inhibitor SP203580 (12 μM), the JNK inhibitor SP600125 (0.5 μM), or the PKA inhibitor, H-89 (0.1 μM) also were carried out at 37 °C for 10 min. Phosphorylation was terminated by replacing media with ice-cold relaxing solution (RS; 7 mM EGTA, 20 mMimidazole, pH 7.0, 1 mM free Mg2+), 14.5 mM creatine phosphate, and 4 mM MgATP with sufficient KCI to yield anionic strength of 180 mM, pH 7.0. Myocytes were then rinsed twice in ice-cold RS containing 0.1% Triton X-100 followed by several rinses in RS alone. Cells were collected in sample buffer, and proteins were separated by SDS-PAGE (34). Gels were silver-stained, dried down, and then exposed to a phosphorimager to determine the extent of phosphorylation. Radioactive bands were quantified using Quantity One software (Bio-Rad), and contractile proteins were identified based on their migration relative to molecular weight markers (7).

Electrical Stimulation of Myocytes Maintained in Primary Culture and Measurement of Sarcomere Length in Cardiac Myocytes—Myocytes used for shortening assays were transferred the day after isolation to a plexiglas stimulation chamber consisting of 8 wells containing platinum electrodes mounted to the sides of each well and a glass bottom. Myocytes were electrically stimulated (2.5-ms pulse, 0.4 Hz) for 4 days in media 199 supplemented with P/S, 10 mMHEPES, 0.2 mM/1mg bovine serum albumin, and 10 mMglutathione (M199+), and media was replaced every 12 h (35). The voltage was set so that more than 50% of the myocytes were stimulated on each coverslip. Four days after gene transfer, individual coverslips are transferred to a temperature-controlled chamber mounted on a Nikon microscope stage and the chamber was filled with M199+. A video-based detection system (Ionoptix, Milton, MA, Ref. 36) was used to detect sarcomere length in intact myocytes. The cell chamber temperature was maintained at 37 °C, and myocytes were stimulated at 0.2 Hz. Experiments were recorded for up to 30 min in 5-min intervals using Sarcom software (Ionoptix). An average of 10 twitches per myocyte were collected for each condition, and for each time point after introducing media containing ET, and/or PKC inhibitors. Maximal positive and negative time derivatives (±dl/dt max) and −dl/dt max were normalized with respect to shortening amplitude.

Statistical Analysis—Values are expressed as mean ± S.E. Grouped comparisons for phosphorimage densitometry and contractile function measurements made using the Sarcomen program were analyzed using a 1-way analysis of variance and a Newman-Keuls multiple comparison test with P < 0.05 considered significantly different.

RESULTS

Basal and Agonist-mediated TnI Phosphorylation by PKC in Adult Cardiac Myocytes—Our results on myocytes cultured in serum-free media demonstrate that cTnI phosphorylation observed during the 2-h [32P]orthophosphate labeling period is primarily due to PKC (Fig. 1). Evidence supporting this conclusion is based on the reduced phosphorylation observed during the labeling period in the presence of the PKC inhibitors chelerythrine (10 μM) or bisindolylmaleimide (bis-1, 500 nm, Fig. 1). Western blot analysis of adult myocytes cultured for 1–4 days demonstrated isoform expression of endogenous PKCα, δ, and ε remained unchanged during the culture period (Fig. 2), and PKCβ1 remained below detectable levels of expression over the same time period (results not shown). Thus,
changes in PKC isoform expression were not responsible for the basal cTnI phosphorylation. Endogenous cTnI phosphorylation also was observed in previous studies on freshly isolated adult cardiac myocytes (7, 37, 38), yet the signaling pathway(s) responsible for this basal phosphorylation remained undefined until now. Basal phosphorylation was not significantly influenced by inhibition of the ERK1/2 (PD98059, Fig. 1), or PKA (H-89; results not shown) pathways. Collectively, these results provide direct evidence that PKC is responsible for a significant component of the basal cTnI phosphorylation observed in intact myocytes (7).

ET-mediated activation of PKC significantly stimulated acute cTnI phosphorylation above basal levels within 10 min (Fig. 3, A and B, Table I). Enhanced contractile function was observed within this same time interval in previous studies on rat myocytes (24), as well as in the present study (Fig. 4A, Table II). A representative time course with 10 nM ET in Fig. 3A shows an increase in cTnI phosphorylation near maximal levels by 5 min, maintenance of elevated phosphorylation for up to 20 min, and then a decline over the next 20 min. Maximal phosphorylation was observed with 100 nM ET, and 10 nM ET produced near maximal phosphorylation of cTnI (Fig. 3B). An increase in myosin light chain 2 phosphorylation was observed in addition to cTnI with ET (Fig. 3B), in agreement with earlier work (7, 39). Previously, troponin T (TnT) phosphorylation also was observed in response to other PKC agonists (40). In the present study, TnT was not significantly phosphorylated in multiple experiments with 10–250 nM ET (Fig. 3B) over the 10–15 min time interval used for subsequent contractile function studies.

The PKC pathway was primarily responsible for phosphorylation of cTnI in response to ET, as indicated by the reduction of cTnI phosphorylation to basal levels in myocytes treated with bis-1 or chelerythrine (Fig. 5A), but not by inhibitors of JNK (e.g. SP600125, Ref. 41, Fig. 5B), p38 (e.g. SB203580, Refs. 42 and 43), or ERK1/2 (e.g. PD98059, Ref. 43, results not shown).

Activation of the ERK1/2, JNK, and p38 pathways were examined because each of these MAPKs can act as downstream effectors of activated PKC, and each pathway influences cardiac contractile function (23, 41–43). An inhibitor of protein kinase A, H-89 (13) also did not significantly influence cTnI phosphorylation in response to ET (results not shown). In experiments with ssTnI, ET activation of PKC did not result in phosphorylation of the ssTnI isoform over 40 min (Fig. 3A), despite the presence of a consensus sequence analogous to cTnI Ser-34/37 for PKC phosphorylation at Ser-11/Thr-13 (27).

Influence of PKC Activation on Contractile Function—Positive inotropy is usually observed in response to ET (24, 44, 45), while the lusitropic effects on the myocardium are more controversial with both increased and diminished rates of relaxation being reported (44–46). Sarcomere shortening in isolated, intact cardiac myocytes was performed to determine the influence of PKC activation on cardiac myocyte contractile function. Representative tracings in Fig. 4 show sarcomere shortening and re-lengthening in a control myocyte under basal conditions, and the significant increase in peak shortening and relaxation rate observed in response to 10 nM ET (Table II). Addition of the PKC antagonist bis-1 largely inhibited these functional effects of ET on adult cardiac myocytes (44, inset), in addition to inhibiting ET-induced cTnI phosphorylation (Fig. 5A). The role of cTnI in the contractile response to PKC activation by ET was examined in subsequent experiments. Replacement of endogenous cTnI with ssTnI in the myofilaments of adult cardiac myocytes resulted in loss of TnI phosphorylation (Figs. 3A and 6), and prolongation of all indices of relaxation in response to ET (Fig. 7, Table II). The increased peak amplitude of shortening observed in response to ET also was significantly blunted in myocytes expressing ssTnI.
PKC-mediated cTnI phosphorylation in adult myocytes

Densitometric comparison of TnI phosphorylation in response to ET in myocytes expressing cTnI (e.g. control and AdcTnI), and N-card/slow-C TnI. Phosphorylation is expressed as a fraction of basal phosphorylation in control myocytes, which is set at 1.0. Note that TnI phosphorylation detected in myocytes expressing N-card/slow-C TnI includes phosphorylation of the chimera protein as well as residual endogenous cTnI. Phosphorylation was not detected in the region of migration identified for ssTnI or N-slow/card-C TnI on SDS-PAGE.

<table>
<thead>
<tr>
<th>Control</th>
<th>cTnI</th>
<th>N-card/slow-C TnI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>1.0 (20)</td>
<td></td>
</tr>
<tr>
<td>10 nm ET</td>
<td>1.58 + 0.25 (9)</td>
<td>1.13 + 0.11 (16)</td>
</tr>
<tr>
<td>100 nm ET</td>
<td>1.68 + 0.26 (3)</td>
<td>1.71 + 0.31 (9)</td>
</tr>
<tr>
<td>250 nm ET</td>
<td>1.47 + 0.12 (6)</td>
<td>2.09 + 0.37 (6)</td>
</tr>
<tr>
<td>10 nm ET + 500 nm Bis-1</td>
<td>0.73 + 0.09 (4)</td>
<td>1.93 + 0.64 (4)</td>
</tr>
<tr>
<td>10 nm ET + 10 μM Chelerythrine</td>
<td>0.82 + 0.08 (6)</td>
<td>0.99 + 0.23 (4)</td>
</tr>
</tbody>
</table>

Fig. 4. Representative traces of sarcomere shortening made in isolated adult myocytes. Contractile function is shown under basal conditions, and 10 min after addition of 10 nm ET. Peak shortening and the rate of re-lengthening are increased in this trace. Comparable results are observed in other cardiac myocytes, as shown in Table II. Based on these findings, the role of cTnI phosphorylation during PKC activation by ET is to accelerate relaxation and contribute to increasing the amplitude of contraction.

Phosphorylation of TnI Chimeras and Mutants by PKC and Effects on Contractile Function—Experiments with myocytes expressing mutants and isoform chimeras of TnI were performed to further investigate the role of phosphorylated TnI in the contractile response to PKC, and to begin investigating the role of individual phosphorylation sites within TnI. In previous biochemical studies, incubation of purified cTnI with PKC results in phosphorylation of Ser-23/24 located in the 32 amino acid extension, which is unique for the cTnI isoform (28, 30, 47), of Ser-43/45, and Thr-144, which is located within the inhibitory peptide (IP) region of TnI (28, 29, 47). The IP region consists of amino acids 130–149, and is the minimum sequence necessary to inhibit strong interactions between actin and myosin (29, 48). This domain presumably binds to actin under conditions, and 10 min after addition of 10 nm ET. Peak shortening and the rate of re-lengthening are increased in this trace. Comparable results are observed in other cardiac myocytes, as shown in Table II. Based on these findings, the role of cTnI phosphorylation during PKC activation by ET is to accelerate relaxation and contribute to increasing the amplitude of contraction.

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Endogenous cTnI was replaced by chimeras of the ssTnI and cTnI isoforms in adult myocytes to determine whether the remaining 4 sites were phosphorylated in response to ET. The N-card/slow-C TnI chimera contains the putative Ser-23/24 and Ser-43/45 sites, but lacks Thr-144, while only the Thr-144 site is present in the chimera N-slow/card-C TnI. Replacement of endogenous cTnI with either chimera exceeded 60% within 4 days after gene transfer (Fig. 6A). In phosphorylation studies, consistent phosphorylation of N-card/slow-C TnI was observed in response to ET (Figs. 3B and 6B), over a comparable time course and dose-response effect as cTnI (Fig. 3, A and B, Table 1). As with ssTnI, no significant phosphorylation of N-slow/card-C TnI chimera was detected in myocytes over 5–40 min of ET (Fig. 6B). The effects of these chimeras on myocyte relaxation responses to ET also were consistent with the phosphorylation results. Analysis of indices of relaxation in myocytes expressing N-slow/card-C TnI indicated relaxation was prolonged in response to ET (mean ± S.E. % change from basal of −dl/dtmax = −4.81 ± 1.20; % change from basal of TTRs50% = 0.101 + 0.002, n = 16), in agreement with the direction of measurements observed in myocytes expressing ssTnI. In contrast, myocytes expressing N-card/slow-C TnI responded similarly to control myocytes, with accelerated indices of relaxation (mean ± S.E. % change from basal of −dl/dtmax = 48.47 ± 17.94; % change from basal of TTRs50% = −0.013 ± 0.001; n = 3). These results indicate cTnI phosphorylation is an important pathway contributing to the accelerated relaxation observed in response to ET activation of PKC.

DISCUSSION

A key finding in the present study is that cTnI phosphorylation in intact adult myocytes acts to accelerate relaxation during PKC activation by ET. This conclusion is based on results from several experiments. In parallel cTnI phosphorylation and sarcomere shortening studies, there is a strong temporal correlation between increases in cTnI phosphorylation and enhanced contractile function in response to ET (Figs. 3 and 4). A similar association between ET-induced PKC-activated cTnI phosphorylation and contractile function was observed earlier (7), although the relationship between these 2 responses in the intact myocyte remained uncertain. Gene transfer of ssTnI, the subsequent absence of ssTnI phosphorylation (Figs. 3 and 6), and the accompanying prolongation of myocyte relaxation in response to ET (Fig. 7) provides evidence that PKC-mediated cTnI phosphorylation works to enhance relaxation rate. Our results also show that PKC directly phosphorylates cTnI in the intact myocyte. This point is important
because some cellular responses associated with PKC activation involve activation of kinases downstream from PKC (49). However, inhibitors of downstream MAPK and PKA pathways had no effect on cTnI phosphorylation in response to ET in the present study (Fig. 5). Another new finding of the present study is that the cTnIThr-144 site identified in biochemical studies (6) and located in the IP region of TnI is not a significant target for acute PKC-dependent phosphorylation in the intact myocyte. Instead, activated PKC targets one or both sets of dual serine residues in the amino-portion of cTnI (e.g., Ser-23/24 and Ser-43/45), which is/are then responsible for the TnI-dependent acceleration of myocyte relaxation observed in response to ET.

The Contractile Response to PKC Activation by ET—In adult myocardium, PKC activation variably influences contraction, with reports of either increased or decreased peak contractile function (24, 44, 50, 51). The direction and magnitude of the contractile response depends on several variables including the PKC agonist, agonist dose, incubation interval, as well as the animal model (7, 24, 52). In our experiments, the PKC agonist and endogenous neuroendocrine ET increased peak sarcomere shortening in the isolated adult rat myocytes (Fig. 4). These results agree with previously reported ET-induced increases in peak contractile performance amplitude typically observed in rat and human myocardium (7, 24, 53–55). Analysis of relaxation rates indicated that ET accelerated the rate of re-lengthening (Table II), and results from earlier work support this finding (21, 45). This ability of ET to enhance relaxation rate is predicted to occur in human myocardium, based on similar positive inotropic effects of ET previously observed in rat and human myocytes (53, 54). Enhanced relaxation rates in response to positive inotropic agonists, such as ET, may help to preserve diastolic function and/or avoid the development of arrhythmias.

Contribution of TnI Phosphorylation to the Contractile Response to Activated PKC—Our results demonstrate that cTnI phosphorylation in response to ET contributes to the increased relaxation rate in adult cardiac myocytes. In earlier studies, investigators predicted that TnI phosphorylation played a significant role in the contractile function response to PKC activation based on the association between cTnI phosphorylation and changes in function (7, 24, 50). However, the role of TnI in this response remained unclear, in part because multiple end-targets contribute to the contractile response to PKC activation (24, 56, 57). In the present study, myocytes expressing non-phosphorylatable ssTnI (Fig. 7, Table II) or the chimera, N-slow/card-C TnI (results not shown) responded to ET with delayed relaxation, an indication that an important function of PKC-mediated cTnI phosphorylation is to maintain myocardial relaxation. In general, factors responsible for decreasing myofilament Ca\(^{2+}\) sensitivity also cause accelerated myocyte relaxation (3, 4). Indeed, earlier work showing decreases in the Ca\(^{2+}\) sensitivity of reconstituted actomyosin-S1 ATPase (6), and in isometric force studies on permeabilized myocytes (50) in response to activated PKC also provide support for our conclusion.

An end-target that could be responsible for prolonging relaxation in the absence of phosphorylatable cTnI is the Na\(^+\)/H\(^+\) exchanger. ET activation of PKC stimulated Na\(^+\)/H\(^+\) exchange in earlier studies (24), and the resulting intracellular alkalosis increased myofilament Ca\(^{2+}\) sensitivity (58) and led to prolonged relaxation. Thus, accelerated myocyte relaxation resulting from cTnI phosphorylation appears to counterbalance the slowed relaxation caused by alkalosis during PKC activation. Non-physiological ET doses reportedly decrease overall relaxation rate (54), which may result from a shift in the relative contributions of phosphorylated cTnI and Na\(^+\)/H\(^+\) exchange to relaxation.

Myocytes expressing cTnI also demonstrated a PKC-sensitive positive inotropic response to ET, which was absent in myocytes expressing ssTnI. Direct enhancement of peak myofilament function via PKC-mediated cTnI phosphorylation has not been observed in biochemical studies (6). The mechanism responsible for enhanced peak shortening during ET phosphorylation and diminished shortening in myocytes expressing non-phosphorylatable ssTnI remains unclear. One explanation may lie in the already enhanced peak shortening observed under basal conditions in myocytes expressing ssTnI compared...
FIG. 5. Representative phosphorimages of myofilament phosphorylation in myocytes expressing cTnI (e.g. control or AdcTnI-treated) or the chimera, N-card/slow-C TnI in response to ET and various signaling pathway inhibitors. A, ET-induced myofilament phosphorylation and the ability of PKC inhibitors to block phosphorylation. Phosphorylation is shown for control myocytes in lanes 1, 4, 7, and 10, for myocytes expressing cTnI (AdcTnI) in lanes 2, 5, 8, and 11, and for myocytes expressing N-card/slow-C TnI in lanes 3, 6, 9, and 12. Results shown in lanes 1–3 are under basal conditions, lanes 4–6 are in response to 10 min of 10 nM ET, lanes 7–9 indicate 10 min of 10 nM ET plus chelerythrine (10 μM), and lanes 10–12 after 10 min of 10 nM ET plus 500 nM bisindolylmaleimide-1 (bis-1). B, ET-induced myofilament phosphorylation in the presence and absence of the p38 inhibitor, SB203580 (2 μM), or the JNK inhibitor, SP600125 (0.5 μM). Neither inhibitor significantly altered the ability of ET to stimulate cTnI phosphorylation. In similar phosphorylation experiments carried out with the ERK inhibitor, PD98059 and PKA inhibitor H89, there was no significant alteration in ET-induced cTnI phosphorylation (results not shown). The diminished phosphorylation observed in response to ET in the presence of chelerythrine or bis-1 indicates that the majority of ET-mediated TnI phosphorylation is via activated PKC.

with myocytes expressing cTnI (Table II). Similar increases in basal peak shortening were observed in myocytes from transgenic mice expressing ssTnI (4). Endothelin activation of PKC may be unable to further stimulate peak shortening beyond this already enhanced level in myocytes expressing ssTnI.

Factors Influencing the Role of cTnI Phosphorylation in PKC-Mediated Relaxation Response—Recent studies by Pi et al. (46) using transgenic mice expressing a cTnI mutant lacking all 5 putative PKC-dependent phosphorylation sites (cTnI-Ala_6_9) indicated that cTnI phosphorylation decreased, rather than increased relaxation rate in response to ET. Species differences in ET-induced contractile responses (54), and PKC isoform expression (8, 9, 59, 60), as well as variations in the agonist dose-response relationship (54, 61) may account for the divergent functional outcomes observed in mouse versus rat myocardium. An alternative explanation may lie in the fact that mice expressing this mutant cTnI developed dilated hearts, and compensatory remodeling may have secondarily influenced the myocyte contractile response to ET in cTnI-Ala_6_9-expressing mice compared with wildtype controls. Previously, alterations

FIG. 6. Expression of TnI isoforms, chimeras and mutants in adult cardiac myocytes after gene transfer (A), and phosphorylation of these TnI proteins in adult myocytes in response to 10 nM ET (B). A, Western blot analysis of TnI expression in adult cardiac myocyte controls (lanes 1 and 7), and in myocytes 4 and 6 days after gene transfer of adenovirus containing one of the following constructs: cTnI (lane 2), ssTnI (lanes 3 and 9), N-slow/card-C TnI (s/c TnI, lanes 4 and 11), N-card/slow-C TnI (s/c TnI, lanes 5 and 12), cTnIT144Pro (cTnIT144P, lane 8), or ssTnIP112T (lane 10). Immunodetection was carried out using monoclonal mouse anti-TnI antibody (1:1000; Fitzgerald), and peroxidase-conjugated goat anti-mouse secondary Ab. Expression of each isoform, chimera, or mutant resulted in a coordinated decrease in endogenous cTnI without significant changes in overall TnI expression. B, representative phosphorylation of TnI phosphorylation in response to ET in myocytes expressing cTnI (lane 1), ssTnI (lane 2), N-card/slow-C TnI (c/s TnI, lane 3), N-slow/card-C TnI (s/c TnI, lane 4), or ssTnIP112T (lane 5). Phosphorylation after 10 min of ET resulted in 32P incorporation into N-card/slow-C TnI, cTnI, and cTnIT144P, but not ssTnI, ssTnIP112T, or N-slow/card-cTnI. These results are consistent with phosphorylation of one or both sets of amino-terminal serines, but not Thr-144 in cTnI by PKC.

FIG. 7. Representative sarcomere shortening traces within adult cardiac myocytes expressing ssTnI under basal conditions and 10 min after addition of 10 nM ET. In myocytes expressing ssTnI, ET slightly decreases the amplitude of shortening and prolongs relaxation rate.
provides new information on the relative contribution of site(s) phosphorylated within cTnI to cause the accelerated relaxation by PKC activation. Our results demonstrate that the primary phosphorylation sites responsible for acutely accelerating relaxation in response to activated PKC do not include Thr-144 in the IP region, and instead are located within the amino-terminal of cTnI. Biochemical studies previously demonstrated that exhaustive phosphorylation of purified cTnI by PKC resulted in phosphorylation of Ser-23/24, Ser-43/45, and Thr-144 by PKC (6). This phosphorylation of cTnI by PKC resulted in diminished Ca$^{2+}$ sensitivity of actomyosin ATPase activity in a reconstituted system containing tropomyosin, troponin C, TnI, TnT, and acto-S1. Until now, the role of Thr-144 remained uncertain because substitution with cTnIS43/45A blunted the shift in Ca$^{2+}$ sensitivity to the greatest extent, followed by cTnIS23/24A, with cTnIT144A having the least ability to blunt the response (6). The current study now provides strong evidence that Thr144 in cTnI is not significantly phosphorylated during acute ET-induced PKC activation in intact myocytes (Fig. 6), and therefore does not significantly contribute to the TnI-dependent influence on relaxation during acute PKC activation. Our results with TnI chimeras also pointed to Ser-23/24 and/or Ser-43/45 as primary cTnI phosphorylation site(s) for mediating cTnI-dependent accelerated relaxation during PKC activation in myocytes (Figs. 3 and 6). However, the relative contribution of each Ser pair (e.g. Ser-23/24, Ser-43/45) remains to be determined in the PKC-mediated contractile function response of intact myocytes. For both Ser-23/24 and Ser-43/45, phosphorylation results in the introduction of negative charges to regions containing a high proportion of basic amino acids. Further experiments are needed to address whether this neutralization of charge reduces ionic interactions with adjacent myofilament proteins, such as TnC or actin. Future studies addressing these questions in intact myocytes will not only improve our understanding of the cellular relationship between PKC activation, TnI phosphorylation, and the contractile function response, but may also lead to therapeutic strategies to treat pathophysiological conditions such as diastolic dysfunction.

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REFERENCES


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