Effect of Protein Kinase C Activation on Sarcoplasmic Reticulum Function and Apparent Myofibrillar Ca$^{2+}$ Sensitivity in Intact and Skinned Muscles From Normal and Diseased Human Myocardium

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Protein kinase C regulates the activity of a diverse group of cellular proteins including membrane ion channel proteins. Although protein kinase C and its substrate protein have been identified in both membrane and cytosolic fractions in the heart, the physiological role of this kinase in the regulation of cardiac function remains unknown. We examined the physiological role of protein kinase C by stimulating its activity with 12-deoxyphorbol 13 isobutyrate 20 acetate (DPBA) in human trabeculae carnea. This resulted in decreased peak isometric twitch force and peak intracellular sarcoplasmic reticulum calcium release as detected with aequorin. Furthermore, in the presence of DPBA, steady-state force–[Ca$^{2+}$] relations were shifted to higher intracellular calcium concentrations, and the Hill coefficient was reduced, indicating a decrease in responsiveness of the myofilaments to calcium and a change in cooperativity among thin filament proteins, respectively. Thus, DPBA affects not only intracellular calcium concentration, but myofilament calcium interactions as well. The effect of DPBA on Ca$^{2+}$ activation probably reflects phosphorylation of thin-filament regulatory proteins by protein kinase C. (Circulation Research 1990;67:744–752)

Protein kinases are known to modulate the electrical activity of various cells through their actions on membrane channels. In cardiac muscle and neuronal cells, protein kinase C, activated by diacylglycerol or synthetic phorbol ester compounds and intracellular calcium ions (Ca$^{2+}$), regulates many calcium and potassium currents. In cardiac tissue, calcium current has been reported to be variably unchanged, increased, or decreased in the presence of protein kinase C stimulation. Because a number of neurotransmitters and hormones as well as related second messengers appear to cause an increase in intracellular calcium concentration ([Ca$^{2+}$]), the inhibitory effect of protein kinase C on cardiac function is of considerable interest. A clear understanding of second messenger mechanisms is important not only in physiological myocardial functions, but also in pathophysiological states of the heart such as cardiac failure and hypertrophy. β-Adrenergic stimulation is associated with changes in phosphatidylinositol turnover. It has been suggested that there is a substantial loss of the capacity of isoproterenol to increase cyclic AMP generation in myopathic tissue, suggesting desensitization of the receptor–adenylate cyclase system. Recent reports that protein kinase C activation can result in a decline in the affinity of β-receptors and a decrease in their total number may therefore have direct pathophysiological implications in heart failure. Further, through signal pathways, protein kinase C activation and calcium mobilization may also play a role in long-term responses such as gene expression and cell hypertrophy.

There is relatively little information on the functional significance of this regulatory system in the heart. It has become clear that thin-myofilament regulation can affect force–[Ca$^{2+}$] relations. Protein

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kinase C has been shown to phosphorylate both the inhibitory subunit of troponin I (TnI) and the tropomyosin-binding subunit troponin T (TnT).\textsuperscript{11} Recent studies suggest a functional role of TnT in modulating Ca\textsuperscript{2+} sensitivity of force production in cardiac muscle.\textsuperscript{12,13} Multiple isoforms of troponin T have been identified in bovine and rabbit myocardium as well as in developing myocardium.\textsuperscript{12-15} In normal human myocardium, one isoform of TnT is found, whereas two TnT isoforms are expressed in myopathic human ventricles.\textsuperscript{16} Therefore, it became of interest to study the effect of protein kinase C activation on myofilament regulation in normal and myopathic human myocardium. Differences detected in the force-[Ca\textsuperscript{2+}], relation in response to protein kinase C activation might then prove insightful to the mechanism of modulation of myofibrillar contractile activity by troponin.

The effect of 12-deoxyphorbol 13 iso-butylate 20 acetate (DPBA) on Ca\textsuperscript{2+} uptake and release from the sarcoplasmic reticulum (SR) was measured using the bioluminescent Ca\textsuperscript{2+} indicator aequorin. Furthermore, steady-state force-[Ca\textsuperscript{2+}] relations were derived in the presence and absence of DPBA. To directly investigate the relation between force and Ca\textsuperscript{2+} at the level of the myofilaments, we examined the effects of DPBA in skinned fiber preparations.

**Subjects and Methods**

**Muscle Preparation**

Cardiac tissue was obtained from the hearts of patients undergoing cardiac transplantation because of end-stage heart failure and hearts not suitable for transplantation from brain-dead organ donors without known cardiac disease. Informed consent was obtained from all heart transplant recipients and from the families of all prospective heart donors. The tissue was quickly transported to the laboratory in an oxygenated physiological solution (see composition below) at room temperature, and a thin right ventricular trabecular carnea was excised. Ventricular trabeculae carnea were removed from 13 human hearts (five control and eight myopathic). Five of the eight from the end-stage heart failure group were from patients with idiopathic dilated cardiomyopathy, two were from patients with ischemic cardiomyopathy caused by coronary artery disease, and one was from a patient with viral cardiomyopathy (age range, 24–59 years). Muscles were placed into an oxygenated solution with the following composition (mM): NaCl 120, KCl 5.9, NaHCO\textsubscript{3} 25, Na\textsubscript{2}HPO\textsubscript{4} 1.2, MgCl\textsubscript{2} 1.2, CaCl\textsubscript{2} 2.5, and dextrose 11.5 bubbled with 95% O\textsubscript{2} and 5% CO\textsubscript{2}, pH 7.4, at 30\textdegree C. The base of each muscle was attached to a muscle holder, while the other end was tied to a force transducer/ergometer (Cambridge Technology, Inc., Watertown, Mass.). Square wave pulses of 5-msec duration were delivered at threshold voltage through a punctate electrode located at the base of the muscle. Muscles were stimulated at 0.33 Hz and allowed to equilibrate at a length that produced maximal peak isometric twitch force for 1 hour (L\textsubscript{0}).

**Measurement of [Ca\textsuperscript{2+}].**

Muscles were loaded with aequorin by a modified chemical loading procedure. Muscles were first exposed to an oxygenated solution composed of (mM) TES 20, MgCl\textsubscript{2} 2, KCl 120, Na\textsubscript{2}ATP 5, and EGTA 10 for 2 minutes at 2\textdegree C. Aequorin in a solution containing (mM) EGTA 0.1, Na\textsubscript{2}ATP 5, KCl 120, MgCl\textsubscript{2} 2, TES 20, and aequorin 0.5 mg/ml was then introduced into the muscle via macrinjection through a glass micropipette. The muscle was then placed in a Ca\textsuperscript{2+}-free Krebs’ solution at 20\textdegree C, and Ca\textsuperscript{2+} was added at 20-minute intervals in increasing concentrations (mM): 0.025, 0.25, 1.25, and 2.5. The temperature of the bath was then slowly returned to 30\textdegree C. Light emitted by the aequorin was detected with a photomultiplier tube (Thorn EMI 9635QA, Gencom Inc., Fairfield, N.J.) attached to a light-collecting apparatus. To estimate [Ca\textsuperscript{2+}]\textsubscript{i}, we converted normalized light signals obtained from the preparations to calcium concentrations by using an in vitro calibration curve, which was modeled using the following equation:\textsuperscript{17}

\[
\frac{L}{L_{\text{max}}} = \frac{1+K_R[Ca^{2+}]^3}{1+K_{TR}+K_R[Ca^{2+}]^3}
\]

where L is the luminescence signal, L\textsubscript{max} is the maximum light emitted after the lysis of the muscle preparation exposed to saturating [Ca\textsuperscript{2+}] with 2% Triton-X 100. K\textsubscript{R} and K\textsubscript{TR} are constants. The conditions of the in vitro medium were 150 mM KCl, 2 mM MgCl\textsubscript{2}, and 5 mM PIPES, pH 7.1, at 30\textdegree C. The rate constant for aequorin consumption measured in vitro in 10 mM [Ca\textsuperscript{2+}], 150 mM KCl, and 2 mM MgCl\textsubscript{2}, pH 7.1, at 30\textdegree C, was 1.98 seconds\textsuperscript{-1}. The rate constant was taken into account when measuring L\textsubscript{max} and converting light signals to fractional luminescence. To generate the calibration curve, specific pCas were obtained as described for skinned fiber preparations (see below). Ca\textsuperscript{2+} was added in the form of CaEGTA prepared with CaCO\textsubscript{3}, EGTA, and KOH.

**Tetanization of Muscles**

Tetani were induced by stimulating the muscles at 15–20 Hz with a stimulus duration of 50 msec for periods of 4–6 seconds without pharmacological intervention. An interval of at least 3 minutes, during which the muscles were stimulated at 0.33 Hz, separated tetani at each extracellular calcium concentration ([Ca\textsuperscript{2+}]). [Ca\textsuperscript{2+}]\textsubscript{i}, was increased from 0.5 to 16 mM in the absence and presence of DPBA, and tetani were elicited. Phosphate was removed from the bathing medium to avoid calcium precipitation. To assure maximal Ca\textsuperscript{2+} availability, 0.1 \textmu M Bay K 8644 was added to the bathing medium, and the calcium concentration response relation was derived in the presence of DPBA. Addition of atropine, a cholinergic receptor antagonist, and propranolol, a
nonselective β-receptor antagonist, to the bathing medium did not alter the reported results.

Skinning Procedure

Trabeculae carnea were selected (diameter, <250 μm) from the human hearts for skinning preparations. They were chemically skinned by exposure to a solution containing saponin 250 μg/ml, Na2ATP 5 mM, MgCl2 7 mM, EGTA 5 mM, KCl 60 mM, imidazole 60 mM, phosphocreatine 12 mM, creatine phosphokinase 15 units/ml, pH (−log10 [H⁺]) 7.1 at 20°C. The muscles were exposed to this solution for 30 minutes. Because we were interested in removing the SR membrane, we used 250 μg/ml saponin, a concentration that has been reported to destroy the SR membrane.18

The total salt concentrations necessary for obtaining the desired pCa (−log10 [free Ca2+], Mg (−log10 [Mg2+]), pMgATP (−log10 [MgATP]), and pH at a constant ionic strength were calculated using the program described by Fabiato and Fabiato.19 The absolute stability constants used for calculating the compositions of the solutions were as reported by Fabiato.20 The solutions were prepared at a temperature of 20°C, with a pMg of 2.5, a pMgATP of 2.5, an EGTA concentration of 10 mM, an ionic strength of 0.16 M, and a pH of 7.1 adjusted using 30 mM TES. The solutions also contained 12 mM phosphocreatine and 15 units/ml creatine phosphokinase. All stock solutions were prepared in plasticware: 5 mM K2EGTA was prepared with CaCO3, EGTA, and KOH at 80°C; 5 mM K2EGTA was prepared with EGTA and KOH. KOH and KCl (1 M each), 50 mM Na2ATP, and TES were prepared the day of the experiment. Creatine phosphate and creatine phosphokinase were added immediately before the experiment. The solutions of different pCAs were stored frozen at −20°C for no more than 2 weeks in plastic bottles (because glass exchanges calcium) and were thawed before the experiment. Calcium was added as CaEGTA. The relaxation solution had a pCa>8.0, and EGTA was replaced with HDTA (K2·2·diaminohexane-Ν,Ν,N',N'-tetraacetic acid) in the solution, whereas the activation solution had a pCa of 4.0. In the relaxed state the muscle length was adjusted to L0, a length at which an increase in resting tension was first observed.

Force-pCa Analysis

The force versus [Ca2+] curves were fitted to a modified Hill relation:

$$F = F_{\text{max}} \frac{[Ca^{2+}]_{\text{m}}}{Q + [Ca^{2+}]_{\text{m}}} \times 100\%$$

where F is developed force, $F_{\text{max}}$ is the maximal force developed at pCa 4.0, $n_h$ is the Hill coefficient, and Q is an affinity constant. The [Ca2+] for 50% activation can then be derived:

$$[Ca^{2+}]_{50\%} = 10^{(lo\text{g}_{10} Q)/n_h}$$

Chemicals

DPBA was purchased from LC Services, Woburn, Mass. All other chemicals were purchased from Sigma Chemical Co., St. Louis. The aequorin used in these experiments was purchased from the laboratory of Dr. J.R. Blinks in Rochester, Minn.

Statistical Analysis

Data derived from myopathic hearts were pooled because we have previously demonstrated that there were no differences in myofilament Ca2+ responsiveness among muscles obtained from myopathic hearts of varying etiologies.21 Results are presented as mean±SEM. Statistical significance was determined by one-way analysis of variance. Statistical significance was set at $p=0.05$. When comparing the various force-[Ca2+] relations that were fitted to the Hill equation, differences between means of the half maximally activating [Ca2+] were tested for significance.

Results

Figure 1 shows the effect of DPBA on twitch force and [Ca2+]. A time-dependent decrease in peak [Ca2+], and isometric force was observed. The second slower component of the calcium transient seen in myopathic human myocardium was reduced in amplitude in the presence of DPBA. The effect of DPBA on contractility, steady-state force−[Ca2+], relations,22 and peak force−peak [Ca2+] curves were derived. Figure 2 shows the effect of DPBA on twitch peak force−peak [Ca2+], relations and steady-state force−[Ca2+] relations. In the presence of DPBA, $F_{\text{max}}$ for peak twitch force decreased 66±3.0% and 55.4±9% in control and myopathic muscles, respectively. Steady-state $F_{\text{max}}$ also decreased 45.5±6.1% and 30±7.1% in control and myopathic muscles, respectively. The aforementioned findings for steady-state force−[Ca2+] relations and peak force−peak [Ca2+] relations are summarized in Table 1. Figure 2 demonstrates a rightward shift in the force−[Ca2+] relations in the presence of DPBA for both control and myopathic tissue. This shift to higher [Ca2+] indicates a decrease in sensitivity of the myofilaments to Ca2+. The first posttetanic twitch was larger than the pretetanic twitch force in the absence and presence of DPBA, which suggests that SR calcium handling was not impaired (Figure 2).

The ability of DPBA to affect sensitivity of the myofilaments to Ca2+ directly was investigated using chemically skinned fiber preparations. In these preparations the sarcolemma and SR are disrupted. Therefore, specific receptors located in the sarcolemmal membrane are removed, and SR calcium release is eliminated. DPBA (1 μM) was added at each pCa. Figure 3 demonstrates a trace record of activation cycles for a skinned trabeculae. Figure 4 shows a shift to higher [Ca2+]$,L$, a reduction in maximal force development, and a Hill coefficient similar to what was found in intact preparations.
To investigate whether DPBA might be diminishing the release of calcium from the SR without impairing sequestration or affecting myofilament sensitivity to Ca\(^{2+}\), we investigated the effect of DPBA in the presence of caffeine, an agent known to induce release of SR Ca\(^{2+}\) and to increase the sensitivity of the myofilaments to Ca\(^{2+}\).\(^{23,24}\) The addition of 10 mM caffeine did not result in an enhanced release of intracellular Ca\(^{2+}\) in the presence of DPBA. This result suggests that diminished SR Ca\(^{2+}\) release is due to reduced Ca\(^{2+}\) storage. Further, the negative inotropic effect seen with DPBA was partially reversed, supporting the idea that myofilament response is affected (Figure 5).

Protein kinase C has been reported to affect sarcolemmal calcium channels.\(^{2}\) The addition of 0.1 \(\mu\)M Bay K 8644 in the presence of increased [Ca\(^{2+}\)], resulted in maximal myofilament Ca\(^{2+}\) activation. In the presence of DPBA, Bay K 8644, a calcium channel agonist that primarily affects open time of L-type channels, increased [Ca\(^{2+}\)], and peak steady-state force (Figure 6). However, the [Ca\(^{2+}\)] relation remained shifted to the right, and \(F_{\text{max}}\) was not returned to pre-DPBA levels. These data indicate that diminished intracellular Ca\(^{2+}\) cannot fully explain the diminished force seen with protein kinase C stimulation.

**Discussion**

It has been suggested that phorbol esters can affect sarcolemmal calcium channels in cardiac muscle.\(^{6,7}\) The results reported here are consistent with the possibility that protein kinase C stimulation reduces transsarcolemmal calcium flux resulting in diminished SR calcium loading and subsequent release. Addition of 1 \(\mu\)M DPBA diminished SR calcium release in a time-dependent manner and was associated with a decrease in peak isometric twitch force. The fast component of the calcium transient (\(L_1\)), which has been shown in normal and diseased human myocardium to reflect SR calcium release and reuptake, was diminished in amplitude after exposure of the muscle to DPBA. The second, slower component (\(L_2\)) seen only in myopathic human myocardium, which has been reported to reflect SR calcium rerelease and enhanced slow, inward calcium current through voltage-dependent calcium channels,\(^{25}\) was similarly reduced in amplitude. The effect of DPBA on \(L_2\) in myopathic tissue could result from
either a reduced transsarcolemmal calcium flux in the presence of DPBA or improved SR calcium handling.

If, as previously demonstrated, the time course of the calcium transient as detected with aequorin predominantly reflects SR calcium release and reuptake, our data suggest that SR function is not impaired in the presence of DPBA. No change in the time course of the calcium transients was detected with aequorin. Further SR calcium handling did not appear to be altered in the presence of DPBA since posttetanic potentiation of force was still present (Figure 2). It has been reported that in cardiac SR, protein kinase C phosphorylates phospholamban, resulting in activation of Ca\(^{2+}\),Mg\(^{2+}\)-ATPase and can increase Ca\(^{2+}\) uptake from the cytosol.\(^{26}\) Our data do not refute this finding but do suggest that SR calcium mobilization is not impaired in the presence of protein kinase C stimulation.

SR calcium release was not impaired, as indicated by a lack of increased Ca\(^{2+}\) release by the SR in the presence of caffeine and DPBA. Caffeine has been shown to induce SR calcium release. These data indicate that Ca\(^{2+}\) release from the SR was probably diminished secondary to reduced transsarcolemmal calcium flux with a resultant reduction of SR Ca\(^{2+}\) loading. Nevertheless, diminished [Ca\(^{2+}\)]\(_i\) can only explain in part the effect of protein kinase C stimulation on force development. As demonstrated by the addition of Bay K 8644, a calcium channel agonist, enhanced availability of intracellular calcium did not restore the pre-DPBA force-[Ca\(^{2+}\)], relation or maximal Ca\(^{2+}\)-activated force. However, addition of caffeine, an agent known to increase the sensitivity of the myofilaments to calcium,\(^{24}\) reversed in part the altered force-[Ca\(^{2+}\)] relation seen with DPBA at a given [Ca\(^{2+}\)]. These data suggest a direct effect of protein kinase C activation on the Ca\(^{2+}\) responsiveness of the myofilaments.

The force-[Ca\(^{2+}\)] relation can be described by the modified Hill equation. The Hill coefficient has been
**TABLE 1.** Hill Coefficients and $[Ca^{2+}]_{50\%}$ From Relations Between Force and $[Ca^{2+}]_{i}$

<table>
<thead>
<tr>
<th>Group</th>
<th>$[Ca^{2+}]_{50%}$ (μM)</th>
<th>$n_h$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steady-state force $-[Ca^{2+}]_{i}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.58±0.01 5.00±0.02 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-DPBA</td>
<td>0.70±0.02* 2.60±0.10* 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myopathic</td>
<td>0.60±0.04 5.60±0.23 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+DPBA</td>
<td>0.97±0.06* 6.00±0.15* 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak twitch force $-peak$ $[Ca^{2+}]_{i}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.00±0.02 5.20±0.08 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-DPBA</td>
<td>1.30±0.05* 3.20±0.10* 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myopathic</td>
<td>0.72±0.13 4.57±0.21 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+DPBA</td>
<td>0.98±0.11* 3.76±0.33* 3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All results are expressed as mean±SEM. The mean values of the Hill parameters were derived from curves fitted individually to the data of each experiment. Differences between means of $[Ca^{2+}]_{50\%}$ of two groups were tested for significance by analysis of variance. $n_h$, Hill coefficient; $n$, number of samples.

*$p<0.01$ vs. pre-DPBA (analysis of variance).

Table 1 indicates that the Hill coefficients for control and myopathic fibers are different, with the myopathic fibers having a higher Hill coefficient. This suggests a change in the cooperativity of calcium binding at the troponin complex.

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used as an index of cooperativity between thick and thin myofilaents. However, the mechanism of cooperativity is not clear. The Hill coefficient does not reflect the number of $Ca^{2+}$ binding sites on the troponin complex. Troponin C in cardiac tissue has been shown to have two low-affinity $Ca^{2+}$ binding sites and one high-affinity $Ca^{2+}$ binding site. Hill coefficients higher than three have been reported in skinned cardiac muscles. Therefore, the steepness of the force-$[Ca^{2+}]$ relation in skinned fiber preparations and intact tetanized preparations must be attributed to mechanisms other than individual troponin C molecules. Interactions of neighboring troponin molecules mediated through actin monomers bound to neighboring tropomyosin-troponin complexes probably affect the steepness of the force-$[Ca^{2+}]$ relation. Alternatively, the attachment of cross bridges during force production may increase the apparent affinity of neighboring myosin cross bridges to actin. This might in turn facilitate calcium binding. There is also evidence that cross bridges communicate with calcium binding sites on troponin C by actin-tropomyosin and TnI, thereby changing the calcium affinity of the binding sites. Despite these discrepancies in the interrelation of the Hill coefficient to troponin C $Ca^{2+}$ binding sites, the Hill coefficient still remains a useful tool for parameterization of activation curves and cooperativity studies.

Protein kinase C is the only kinase known to phosphorylate both cardiac TnI and TnT in vitro. The time-dependent negative inotropic effect seen with DPBA may reflect the reported kinetics of phosphorylation of TnI and TnT. Phosphorylation of TnI has an apparent $K_m=3.4$ μM and $V_{max}=2.6$ μM.

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**FIGURE 3.** Trace record of activation cycles for a skinned human ventricular muscle. 12-Deoxyphorbol 13 isobutyrate 20 acetate (DPBA; 1 μM) was added at each activation cycle.

**FIGURE 4.** Force-pCa relations in skinned preparations. In the presence of 12-deoxyphorbol 13 isobutyrate 20 acetate (DPBA), the force-$[Ca^{2+}]$ relation is shifted toward higher $[Ca^{2+}]$ with a substantial decrease in $F_{max}$. The Hill coefficient and $[Ca^{2+}]_{50\%}$ were 2.15 and 1.45 μM pre-DPBA (●), respectively, and 1.55 and 2.09 μM post-DPBA (○), respectively.
μmol/min/mg enzyme, and TnT $K_m=0.3 \ \mu\text{M}$ and $V_{max}=0.5 \ \mu\text{mol/min/mg enzyme}$. Therefore, the reduction in peak force that occurs over a relatively slow period might reflect the low $V_{max}$ of TnT phosphorylation.

Phosphorylation of TnI is known to decrease the affinity of the troponin complex for Ca$^{2+}$ with no effect on maximal Ca$^{2+}$-activated force. The result of TnI phosphorylation by protein kinase C would be expected to result in a rightward shift of the force-

\[\text{[Ca}^{2+}\text{]}_0\] relation in the presence of 1 μM 12-deoxyphorbol 13 isobutyrate 20 acetate (DPBA). After addition of DPBA the parameters were 0.89 μM and 4.44, respectively, with an $F_{max}$ of 77%.

\[\text{DPBA}\]

\[\text{[Ca}^{2+}\text{]}_0\]

\[\text{16 mM}\]

\[\text{0.1 mM BAY K 8644}\]

\[\text{F}_{\text{max}} \%\]

\[\text{50}\]

\[\text{100}\]

\[\text{PRE-DRUG}\]

\[1 \ \mu\text{M DPBA} + 0.1 \ \mu\text{M BAY K 8644}\]

\[\text{[Ca}^{2+}\text{]}_0\]

\[\text{0.01}\]

\[\text{10.0}\]
[Ca$^{2+}$], relation. TnI phosphorylation by cyclic AMP has a lower $K_m$ than its phosphorylation by protein kinase C.\textsuperscript{11} Katoh et al\textsuperscript{3} have suggested that protein kinase C phosphorylates at least two sites in both TnI and TnT compared with one site on TnI for the cyclic AMP–dependent kinase. This may explain the relatively large increase in the [Ca$^{2+}$]$_{100}$ in the presence of DPBA. The greater effect of DPBA on peak twitch force compared with steady-state force is probably due to the fact that twitch force resides on the steep portion of the force-[Ca$^{2+}$] relation, and small changes in sensitivity of the myofilaments to Ca$^{2+}$ are likely to result in relatively large alterations in peak isometric twitch force. It has been shown that phosphorylation of TnT or TnI by protein kinase C decreases the $V_{\text{max}}$ of Ca$^{2+}$-dependent activation of actomyosin ATPase in reconstituted troponin–tropomyosin–actin–myosin complexes (J.F. Kuo and T.A. Noland, personal communication, 1990).

The role of TnT in Ca$^{2+}$ activation of the myofilaments has been poorly defined. Recently, investigators have proposed that shifts in TnT isoform populations can affect myofilament Ca$^{2+}$ responsiveness. Moreover, a specific interaction between TnT and tropomyosin has been demonstrated in skeletal muscle. These findings suggest a possible role for TnT in the interaction between tropomyosin or between tropomyosin and the actin filament. In this study the lower Hill coefficient for steady-state force–[Ca$^{2+}$]$^n$ relations seen in normal myocardium in the presence of DPBA most likely reflects phosphorylation of TnT, whereas the shift to higher [Ca$^{2+}$], most likely reflects phosphorylation of TnI. Because no new isoforms of TnI have been reported in myopathic hearts, it is unlikely that the differential effect of DPBA on the Hill coefficient seen between control and myopathic myocardium is due to phosphorylation of TnI. Similarly, no isoforms of troponin C have been reported in diseased myocardium. However, different protein kinase C isoforms may be activated under physiological and pathological conditions. Therefore, the reduction in the Hill coefficient seen in the control myocardium could result from phosphorylation of TnT.

In myopathic tissue there are two TnT isoforms, as opposed to one isoform seen in normal myocardium.\textsuperscript{16} Changes in the proportion of TnT isoforms found in developing rabbit heart have been related to a postnatal increase in Ca$^{2+}$ sensitivity.\textsuperscript{13} In rat neonatal myocardium, which expresses two TnT isoforms, the force-pCa relations are shifted to higher pCa values by 0.4 pCa units as compared with force-pCa relations from adult rats, which express one TnT isoform.\textsuperscript{12} Bovine myocardium expresses two isoforms of TnT.\textsuperscript{30} In the presence of the smaller isoform, investigators have found that the Ca$^{2+}$-activation curve was shifted by 0.1–0.15 pCa units to the left.\textsuperscript{30} This observation may explain why in myopathic tissue, which has been shown to express two TnT isoforms, steady-state force and twitch force are less affected by protein kinase C activation. Steady-state force–[Ca$^{2+}$]$^n$ relations in myopathic tissue in the presence of DPBA have an increased Hill coefficient, which might be the result of a predominant effect of phosphorylation of TnT isoforms in the presence of saturating troponin C binding sites, resulting in altered calcium responsiveness. This would also explain the decreased Hill coefficient for twitch force in myopathic tissue. Another potential explanation for the increase in the Hill coefficient in myopathic tissue is the effect of relative states of phosphorylation of the two TnT isoforms. As previously mentioned, TnT and TnI both have at least two phosphorylation sites.\textsuperscript{11}

Hypertrophied myocardium has been reported to have lower actomyosin ATPase activity.\textsuperscript{31} This results in a slower cross-bridge cycling rate and greater force economy. As human ventricular myocardium contains predominantly the myosin heavy chain isoform V$_s$, shifts in the myosin isoform cannot explain the differential effect seen with DPBA. A lower actomyosin ATPase activity should result in a leftward shift of the force–[Ca$^{2+}$]$_{100}$, contrary to what was found in this study. If actomyosin ATPase inhibition was solely responsible for the effects seen with DPBA, the effect should have been greater in the hypertrophied hearts. Although Mg$^{2+}$-ATPase activity of myofibrils from myopathic hearts has been reported to be lower, the Ca$^{2+}$-ATPase activity of myosin from myopathic hearts has been reported to be the same as that of myosin from normal hearts.\textsuperscript{16} Because cyclic AMP–dependent phosphorylation of cardiac TnI is known to decrease the calcium sensitivity of actomyosin ATPase activity by altering the interaction between troponin C and TnI,\textsuperscript{32} one would expect a similar effect with protein kinase C phosphorylation. The $V_{\text{max}}$ for actomyosin ATPase activity is reduced with protein kinase C stimulation (J.F. Kuo and T.A. Noland, personal communication, 1990).

Interestingly, in skinned fiber preparations the shift of the force-pCa curve toward higher Ca$^{2+}$ was more pronounced, and the decrease in maximal force was larger. Protein kinase C also phosphorylates the Na$^+$–H$^+$ antiporter, leading to alkalization that increases the responsiveness of the myofilaments to Ca$^{2+}$. In the intact muscle, the effect of phosphorylating TnI probably outweighs the alkalinizing effect, resulting in a net decrease in the sensitivity of the myofilaments. In skinned fibers in which the sarclemma is removed, phosphorylation of TnI is unopposed because of destruction of the Na$^+$–H$^+$ antiporter, and the desensitization of the myofibrillar proteins is much more pronounced.

Our data demonstrate for the first time the effect of protein kinase C activation on Ca$^{2+}$ sensitivity of the myofilaments and contractile activation. This effect is most likely due to phosphorylation of TnI and TnT. These data suggest that protein kinase C stimulation may play a physiological role in cardiac muscle activation.
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Effect of protein kinase C activation on sarcoplasmic reticulum function and apparent myofibrillar Ca2+ sensitivity in intact and skinned muscles from normal and diseased human myocardium.

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