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**Research Article**

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# Differential Effect of DPI 201-106 on the Sensitivity of the Myofilaments to $Ca^{2+}$ in Intact and Skinned Trabeculae from Control and Myopathic Human Hearts

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## Abstract

**The effects of DPI, a new inotropic agent, were compared in trabeculae carneae from control and myopathic human hearts loaded with aequorin, a bioluminescent calcium indicator that emits light when it combines with calcium, and in saponin-skinned trabeculae carneae from the same hearts. The force-pCa curves in saponin-skinned fibers and the peak force-peak $Ca^{2+}$ curves in aequorin-loaded preparations were not significantly different between the control and myopathic tissues. The force-pCa curve in the skinned and aequorin-loaded preparations from the same control hearts displayed no significant shifts with the addition of DPI. In contrast, a leftward shift was present in the force-calcium relationship in the presence of DPI in aequorin-loaded and skinned muscles from myopathic hearts, indicating an increase in the sensitivity of the myofilaments to calcium. These differences in the modulation of calcium activation between myopathic and control tissues indicate that pharmacological agents may produce differential effects in normal and diseased hearts.**

## Introduction

Cardiac contraction is activated by the binding of $Ca^{2+}$ to the myofibrils (1). The delivery of $Ca^{2+}$ to the contractile proteins is complex and includes many steps: $Ca^{2+}$ entry across the sarcolemma, release from the sarcoplasmic reticulum (SR),[1] sequestration by the SR, and extrusion into the extracellular space. We were interested in comparing the sensitivity of the myofibrils to $Ca^{2+}$ in normal versus myopathic human hearts and the possible alterations of this sensitivity by the new inotropic agent, DPI, which has a positive inotropic effect and prolongs relaxation (2). To bypass excitation-contraction coupling, we used chemically skinned trabeculae in which the sarcolemma and the SR membrane were removed and the intracellular calcium concentration could be precisely controlled, to examine whether DPI exerted its positive inotropic effect by increasing the responsiveness of the contractile elements to activation by calcium ions (3–7). We also compared the peak calcium response to peak tension response in aequorin-loaded preparations to address calcium handling in intact, isometrically contracting trabeculae carneae.

Myopathic hearts have different metabolic and structural properties and function under load conditions that are different from those of normal hearts. When compared with normal hearts, preparations from myopathic hearts show differences in rates of tension development, intracellular calcium handling (2), creatine kinase activity (8), and sensitivity to inotropic interventions such as adrenergic stimulation (9). These differences in the activity of the myopathic hearts have been shown to be due to alterations in excitation-contraction coupling (2), but they may be also due to differences in activity, and regulation of the myofilaments. It is therefore not surprising that in our experiments, DPI had differential effects on calcium activation of myofilaments in normal and myopathic hearts, which were probably due to alterations in thin filament regulation. It is therefore important, when studying various inotropic agents, to account for these differential effects of the drugs on the normal and myopathic hearts.

## Methods

*Muscle preparation.* Right ventricular trabeculae were removed from control human hearts and hearts from patients with end-stage heart failure who were undergoing transplant surgery as described by Gwathmey et al. (2). They were then placed into an oxygenated solution with the following composition in millimolars: 120, NaCl; 5.9, KCl; 25, $NaHCO_3$; 1.2, $NaH_2PO_4$; 1.2, $MgCl_2$; 2.5, $CaCl_2$; and 11.5, dextrose. The solution was bubbled with 95% $O_2$ and 5% $CO_2$ to a pH = 7.4, at 20°C. Trabeculae with a width < 200 μm were selected for skinned fiber experiments. The base of each muscle was attached to a muscle holder, while the other end was tied to a force transducer in a bath containing the same physiologic solution as above. The muscles were then stimulated with a square-wave pulse of 5 ms duration at threshold voltage for 1 h, at a frequency of 0.33 Hz. Before skinning, the muscle length was adjusted to $L_{max}$.

*Skinning procedure and solutions.* The human trabeculae were chemically skinned by exposure to a solution containing: 250 μg/ml saponin, 5 mM $K_2ATP$, 7 mM $MgCl_2$, 5 mM EGTA, 60 mM KCl, 60 mM imidazole, 12 mM creatine phosphate, 15 U/ml creatine phosphokinase, pH = 7.1 at 20°C. As we were interested in removing the SR membrane, we used 250 μg/ml of saponin, a concentration that has been reported to severely damage the SR membrane (10).

The total salt concentrations necessary for obtaining the desired pCa, pMg, pMgATP, and pH at a constant ionic strength, were calculated using the program described by Fabiato and Fabiato (11). The absolute stability constants used for calculating the compositions of the solutions were as reported by Fabiato (12). 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 (*n*-tris[Hydroxymethyl]methyl-

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1. *Abbreviations used in this paper:* ANOVA, analysis of variance; SR, sarcoplasmic reticulum.

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2-amino-ethanesulfonic acid). The solutions also contained 12 mM creatine phosphate and 15 U/ml creatine phosphokinase. The skinned muscles were initially subjected to a relaxation-activation cycle using the method of Moiescu et al. (13). The relaxation solution had a pCa > 8.0, and EGTA was replaced with HDTA ($K_2$-2,6-diaminohexane-*N,N,N',N'*-tetraacetic acid) in the solution, whereas the activation solution had a pCa of 4.0. During the relaxation cycle, the muscle length was adjusted to $L_{max}$, a length at which an increase in resting tension was first observed as described by Maughan et al. (14).

DPI 201-106 was added to the buffer solutions of different pCa values at concentrations between $10^{-8}$ and $10^{-5}$ M.

*Aequorin technique.* Muscles were obtained from the same hearts as described above. The mean muscle diameters for control and myopathic hearts used for the aequorin studies were, respectively, 1.0±0.08 mm ($n$ = 14) and 1.20±0.08 mm ($n$ = 11). The muscles were placed into an oxygenated physiologic salt solution, stimulated to contract at 0.33 Hz at 30°C, and allowed to equilibrate for 1 h after being stretched to $L_{max}$. Aequorin was introduced intracellularly by a chemical loading technique as described elsewhere (15). The light emitted by the aequorin was detected with a photomultiplier tube (9635QA; Thorn EMI, Gencom, Inc., Fairfield, NJ) attached to a collecting apparatus similar to that described by Blinks et al. (16). The light signals are reported in amperes of anodal current. Isometric tension was normalized to cross-sectional area using the formula milliNewtons/cross-sectional area and is expressed as milliNewtons/squared millimeters.

*Tension-pCa analysis.* The tension versus $[Ca^{2+}]$ curves were fitted to the modified Hill relation: $T = (T_{max}\ [Ca^{2+}]^n/Q + [Ca^{2+}]^n)\ 100\%$, where $T$ is developed tension, $T_{max}$ is the maximal tension developed at pCa of 4.0, $n$ is the Hill coefficient, and Q is an affinity constant. The $[Ca^{2+}]$ for 50% activation can then be derived: $[Ca^{2+}]_{50\%} = 10^{(\log_{10}Q)/n}$.

*Chemicals.* The following drugs and chemicals were used: DPI 201-106 (Sandoz Ltd., Basal, Switzerland), $CaCl_2$ (British Drug Houses Chemicals Ltd., Poole, England), isoproterenol bitartrate, and caffeine (Sigma Chemical Co., St. Louis, MO). The aequorin used in these experiments was purchased from the laboratory of Dr. J. R. Blinks at the Mayo Foundation, Rochester, MN.

*Statistical analysis.* Experiments comparing the effects of increases in the concentrations of DPI on myofibrillar tension were done in parallel with control measurements appropriate for the particular experimental study. Results are presented as means±SEM. Statistical significance of differences was determined by $t$ test or one-way analysis of variance (ANOVA), with the level of significance set at a probability of 0.05. In the case of experiments in which we measured relations between pCa and relative tension either between two groups (myopathic versus control), or in the presence of 1 μM DPI, differences were determined after normalization of the data for a particular experiment to maximum $Ca^{2+}$-activated force and fitting of the data to the Hill equation. Differences between means of the half-maximally activating free $Ca^{2+}$ were tested for significance using ANOVA.

## Results

*Effect of DPI on intracellular calcium handling.* To investigate whether there was a difference in calcium sensitivity between the myopathic and control tissue, we compared, in aequorin-loaded muscles, the percent maximal tension (milliNewtons/squared millimeters) and percent maximal light (nanoAmperes) response in the presence of increasing concentrations of extracellular calcium. Fig. 1 illustrates that the percent maximal tension response and the percent light response at various $[Ca^{2+}]_0$ were not significantly different for control and myopathic muscles, suggesting no changes in the sensitivity of the myofilaments to $Ca^{2+}$.

To determine whether DPI exerts some part of its positive inotropic effect by increasing myofilament sensitivity to calcium in intact and actively contracting trabeculae carneae, the amplitude of the aequorin light signals and tension responses

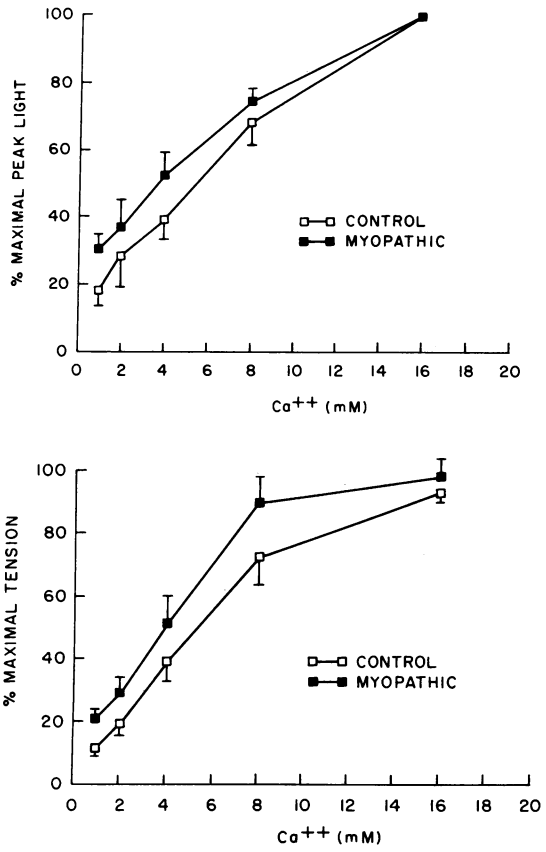


*Figure 1.* (*A*) Percent maximal tension (milliNewtons/squared millimeters), and (*B*) percent maximal light (nanoAmperes) to increasing calcium concentrations in control and myopathic tissue. Mean values±SE are shown. Number of muscles = 14 and 11 in the control and myopathic groups, respectively.

were compared under the influence of various inotropic interventions. This analysis requires the comparison of the effects of an inotropic agent to the effect of changing the extracellular calcium concentration ($[Ca^{2+}]_0$) in the same muscle preparation. Peak light (i.e., intracellular calcium) was compared with peak tension for varying extracellular calcium concentrations, isoproterenol, caffeine, and DPI concentration response curves. Fig. 2 illustrates two agents whose subcellular mechanisms have previously been characterized. Isoproterenol, whose mechanism of action involves an increase in cAMP and a decreased sensitivity of the myofilaments to calcium (17), shifted the peak calcium–peak force relationship to the right of the calcium concentration response curve. Caffeine, an agent that blocks calcium reuptake and subsequent release by the sarcoplasmic reticulum, increases cAMP, and sensitizes the myofilaments to calcium (17), shifted the relationship to the left of the calcium curve.

The peak calcium–peak force relationship for increasing concentrations of extracellular calcium and DPI concentration response curves in a control and a myopathic muscle are illustrated in Fig. 3. Peak calcium–peak force relationships were plotted before the addition of DPI and in the presence of DPI in both a control and a myopathic muscle. In the control muscle, there was no shift in the DPI concentration response curve (Fig. 3 *A*). We found that in 13 out of the 14 control

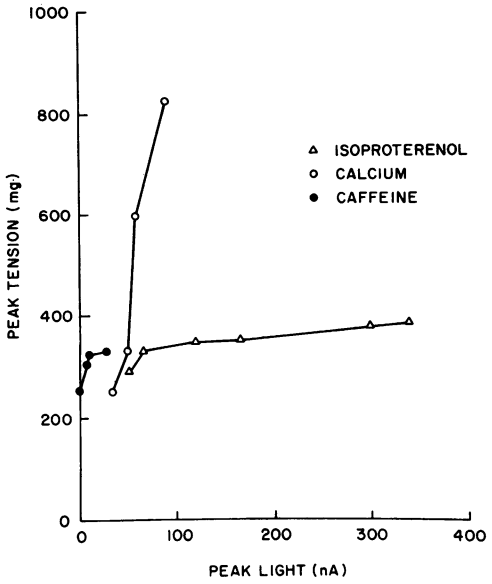


*Figure 2.* Peak tension (milligrams) and peak light (nanoAmperes) for increasing concentrations of extracellular calcium (2, 4, 8, and 16 mM), isoprotenerol ($10^{-9}$, $3 \times 10^{-9}$, $10^{-8}$, $3 \times 10^{-8}$, $10^{-7}$, and $3 \times 10^{-7}$ M, at a fixed $[Ca^{2+}]_0$ = 2.5 mM), and caffeine (0.2, 0.6, 2, and 20 mM, at a fixed $[Ca^{2+}]_0$ = 2.5 mM) in a myopathic muscle.

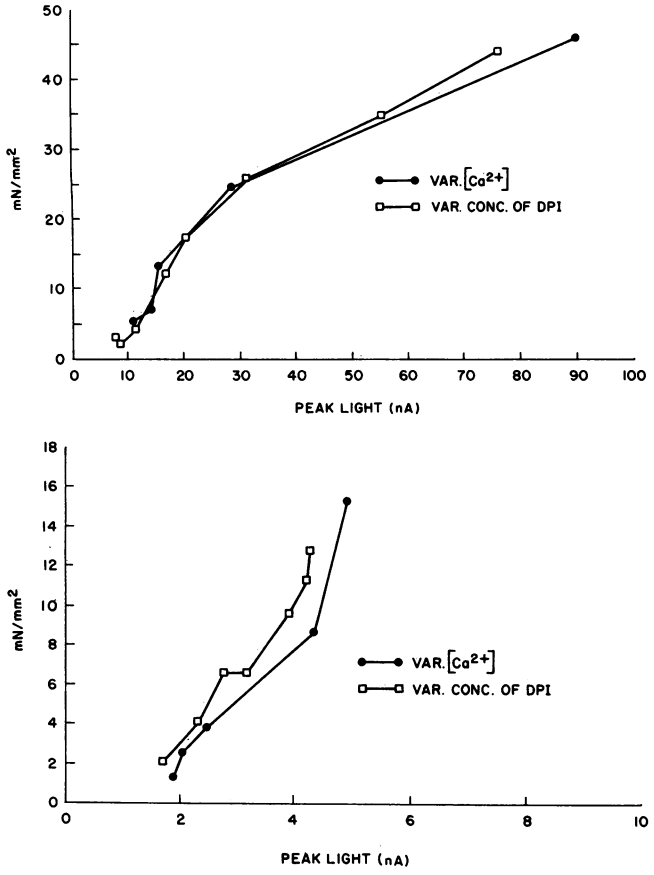


*Figure 3.* Peak tension (milliNewtons/squared millimeters) vs. peak light (nanoAmperes) for increasing extracellular calcium and increasing concentrations of DPI ($3 \times 10^{-7}$, $10^{-6}$, and $3 \times 10^{-6}$ M, at a fixed $[Ca^{2+}]_0$ = 2.5 mM) in control (*A*) and myopathic (*B*) myocardium.

muscles, the peak force–peak calcium relationships were superimposable in the absence and presence of DPI. In contrast, in the myopathic muscle, the concentration response curve to DPI was shifted to the left of the $[Ca^{2+}]_0$ concentration response curve, suggesting that there was an increase in the sensitivity of the myofilaments (Fig. 3 *B*). A similar leftward shift was present in 9 out of the 11 myopathic muscles.

*Force-$Ca^{2+}$ relationship in skinned fibers.* When skinned ventricular muscles, bathed in resting solution, were exposed to a solution with a pCa of 4.5, tension development occurred within 30–60 s in the myopathic group, and 10–40 s in the control group. We tested for hysteresis in the skinned preparations of both the myopathic and control hearts by examining the changes in isometric tension development at a fixed muscle length ($L_{max}$) and pCa value throughout an experiment. Preparations that displayed a decrease in isometric tension of > 5% during one activation-relaxation cycle were discarded. Approximately 60% of the control muscles met that criterion, whereas only 45% of the muscles from the myopathic hearts achieved the above standard. Tension oscillations were not observed in the skinned human myocardium. These observations are different from the results of Pagani et al. (18), in which phasic contractions were present in saponin-treated rabbit ventricular cells.

The activation range in both control and myopathic bundles was from $10^{-7}$ to $10^{-4}$ M free calcium ion. The results in Fig. 4 illustrate the isometric forces developed at saturating free $[Ca^{2+}]$ in the skinned preparations of both groups. Tension development (corrected for the cross-sectional area of each muscle) was not significantly ($P > 0.1$, *t* test) changed in the myopathic muscles as compared with the control muscles.

The $[Ca^{2+}]$-tension relationships (Fig. 5) of the myopathic and control muscles showed no significant differences in $Ca^{2+}$ sensitivity or cooperativity (see Table I). The $[Ca^{2+}]$ required for half-maximal activation was 1.33±0.19 μM ($n$ = 12) in the myopathic group and 1.53±0.32 μM ($n$ = 11) in the control group. The Hill coefficients were 2.05±0.19 and 2.36±0.21. The slope of the force-pCa curve in the myopathic muscles was not significantly ($P > 0.1$) steeper than the control's slope.

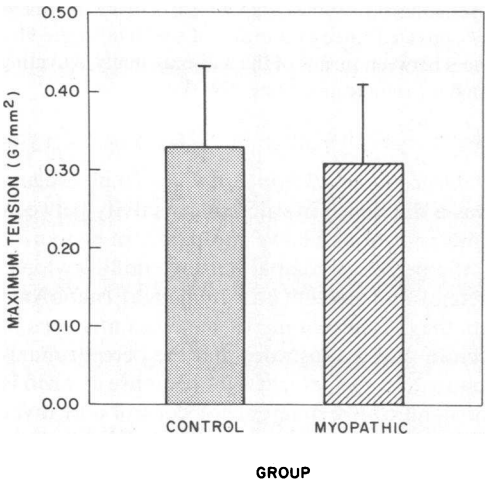


*Figure 4.* Mean maximal tension developed in control and myopathic muscles at a calcium ion concentration of $10^{-4}$ M. Number of muscles = 11 and 12 in the control and myopathic groups, respectively.

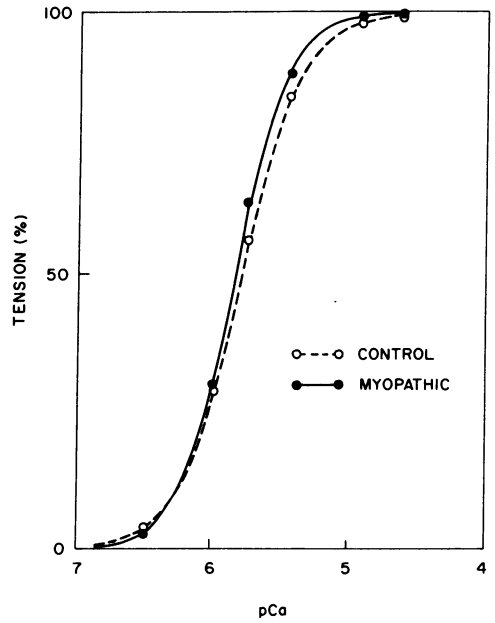


*Figure 5.* Tension-calcium relationship for control and myopathic muscles. Mean values for 11 control and 12 myopathic fibers. The solid curves are approximated with the Hill relationship.

*Effect of DPI in the skinned preparations.* Fig. 6 depicts the concentration-dependent increase in activation of the skinned fibers with DPI. At a calcium ion concentration of 1 $\mu$M, increasing concentrations of DPI increased force production by the skinned trabeculae from the control hearts by up to 7%, whereas a 23% force increase was observed in the myopathic hearts, which was significantly larger than the increase observed in the control tissue ($P < 0.01$). The DPI concentrations needed for the $Ca^{2+}$ sensitization are two to three orders of magnitude higher in our preparations than the ones reported by Herzig et al. (5). The discrepancies could be explained by the differences in species (porcine versus human) and differences in skinning procedures.

*Table I. Hill Equation: Averaged Data from Individual Experiments*

| Group | $n$ | $[Ca^{2+}]_{50\%}$ | No. |
|---|---|---|---|
| | | $\mu M$ | |
| Control | 2.05±0.19 | 1.53±0.32 | 11 |
| Myopathic | 2.32±0.21 | 1.33±0.19 | 12 |
| Control predrug | 2.10±0.21 | 1.48±0.27 | 9 |
| Control + 1 $\mu$M DPI | 2.10±0.30 | 1.36±0.25 | 9 |
| Myopathic predrug | 2.28±0.14 | 1.35±0.13 | 10 |
| Myopathic + 1 $\mu$M DPI | 2.31±0.15 | 0.85±0.29* | 10 |

All results are expressed as means±SEM calculated from individual experiments (no.).
* $P < 0.01$ as compared with the myopathic predrug.

The relationship between force and free calcium was not significantly altered (see Table I) by the addition of 1 $\mu$M of DPI in the control hearts (Fig. 7 *A*) but in myopathic muscles the half-maximal effective concentration ($EC_{50}$) was shifted significantly to the left ($P < 0.01$) by 0.20 pCa units (Fig. 7 *B*). As demonstrated in Table I, cooperativity was not significantly changed after the addition of 1 $\mu$M DPI in both groups of muscles.

To test the hypothesis that DPI binds to the myofilaments of human cardiac muscle, we subjected a muscle from a myopathic heart to a series of calcium buffers with decreasing pCa, and then added $10^{-6}$ M of DPI at a pCa of 6. After 10 min, we activated the muscle with buffers that did not contain DPI, and as depicted in Fig. 8, force development was consistently larger for each pCa value (except at pCa of 4.5), indicating that DPI was bound to the myofilaments and was responsible for the enhanced force development.

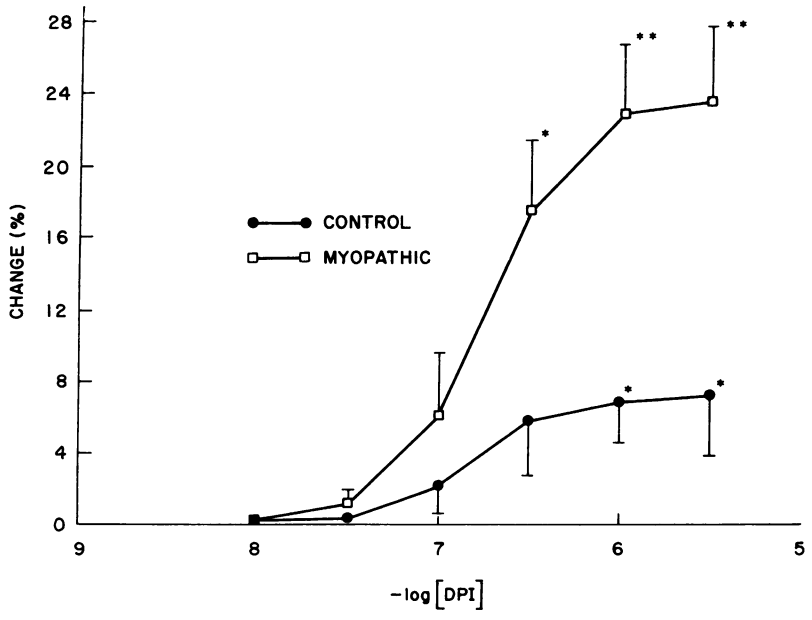


*Figure 6.* Dose dependence of the activating effect of DPI on cardiac myofibers in a solution containing 1 $\mu$M calcium (pCa 6). Mean values±SEM for 9 preparations for the control group and 10 preparations in the myopathic group. * refers to a significant difference with $P < 0.01$ between control and myopathic values, whereas ** refers to a significant difference with $P < 0.001$.

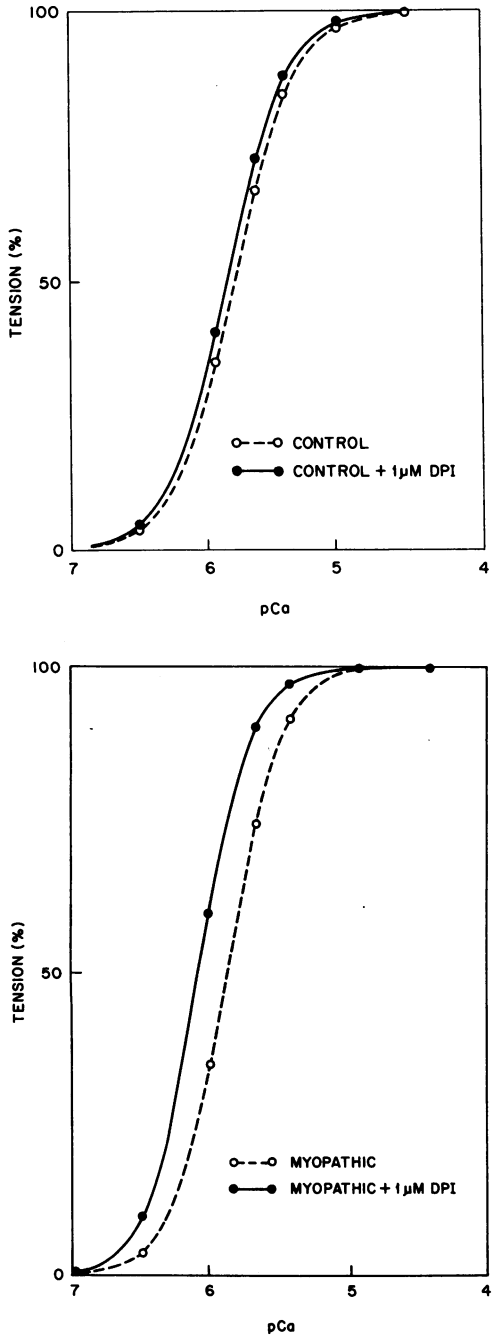


*Figure 7.* Effect of DPI on the relation between pCa and percent maximal tension in control (*A*) and myopathic (*B*) myofibers. A DPI concentration of 1 μM was added to each of the calcium buffer solutions. The solid curves are approximated with the Hill relationship.

As we were interested in examining the effect of DPI on troponin C, which binds to both $Mg^{2+}$ and $Ca^{2+}$, we varied the concentration of $Mg^{2+}$ from a pMg of 2.5 to 3.5 while keeping pMgATP fixed at 2.5 in the buffer solutions. As seen in other studies (19, 20), at the lower $Mg^{2+}$ concentrations the tension-pCa curve shifted to the left, so that a larger tension was developed for a given calcium concentration (Fig. 9). DPI (1 μM) shifted the force-calcium relationship in the myopathic tissue at the lower concentration of $Mg^{2+}$ (pMg = 3.5) by 0.19 pCa units ($n$ = 6). At the higher concentrations of $Mg^{2+}$ (pMg = 2.5), DPI shifted the force-pCa curve by 0.20 pCa units ($n$ = 9). This suggests that the enhancement of $Ca^{2+}$ binding in the presence of DPI is not due to a decrease in the $Mg^{2+}$ affinity of the regulatory sites. If this were the case, we would have expected a much larger shift to the left in the presence of DPI at the lower $Mg^{2+}$ concentrations.

## Discussion

*Myofilament sensitivity to intracellular calcium* $[Ca^{2+}]_i$ *as detected with aequorin.* The influence of various drugs upon the relationship between intracellular calcium and developed tension can be estimated by comparing the peak aequorin signal (intracellular calcium) to the peak tension response (6, 21, 22). However, limitations of this analysis have been recently reported (23, 24). We found the percent change in calcium and tension were similar between control and myopathic tissue. The upward shift seen in the myopathic tissue may reflect altered calcium handling by the sarcolemma and/or sarcoplasmic reticulum. Gwathmey et al. (2) have recently reported that in myopathic tissue, there is abnormal calcium handling by the sarcolemma and SR. The fact that the force-$[Ca^{2+}]_0$ and peak calcium-$[Ca^{2+}]_0$ relationships were similarly shifted indicates that there was no change in the sensitivity of the myofilaments to calcium in the myopathic state.

Comparing the peak aequorin signal with the peak tension responses, we found that the addition of isoproterenol resulted in a rightward shift of the relationship, indicating a decrease in the sensitivity of the myofilaments, as has been previously reported (6, 17). Caffeine, on the other hand, produced a leftward shift, indicating an increase in the sensitivity of the myofilaments (17). DPI has been reported to increase the sensitivity of the myofilaments to calcium (25). An increased sensitivity of the myofilaments to calcium could explain both the positive inotropic effect of DPI and its relaxant effect. After correcting for the increase in resting calcium, there was still a leftward shift in the peak force versus peak calcium for the myopathic tissue (i.e., for any given level of the peak intracellular calcium transient there is greater force developed), suggesting a sensitization of the myofilaments. A similar leftward

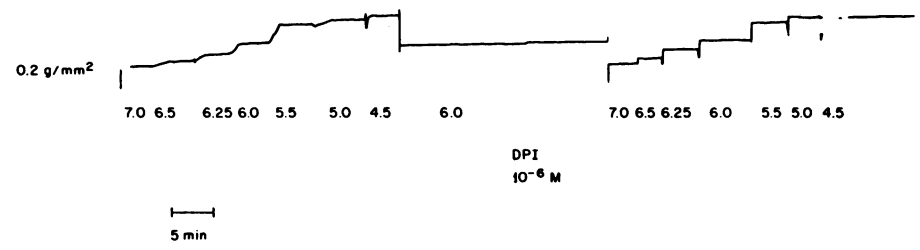


*Figure 8.* Trace record of activation cycles for a skinned human ventricular muscle from a myopathic heart. 1 μM of DPI was added when the muscle was activated at a pCa of 6.0.

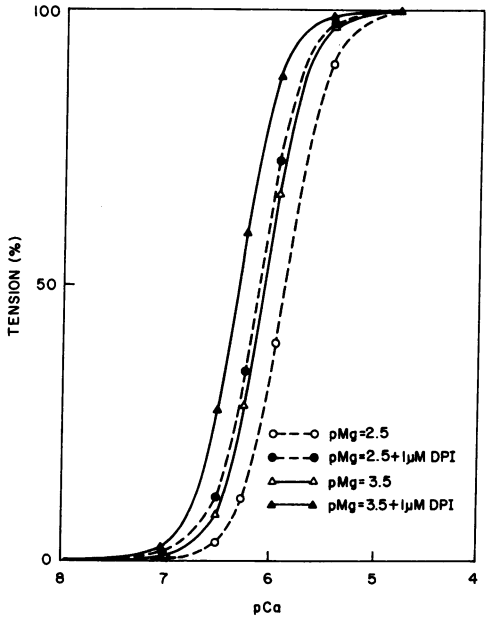


*Figure 9.* Effect of 1 μM of DPI on force-pCa relation with a normal (pMg = 2.5) and a low (pMg = 3.5) $Mg^{2+}$ concentration ($n$ = 9 for pMg = 2.5, and $n$ = 6 for pMg = 3.5). MgATP concentration was kept constant at pMg = 2.5.

shift was observed in eight myopathic muscles, in the presence of 1 μM DPI.

*DPI and myofilament sensitivity to $Ca^{2+}$ in skinned preparations.* The interaction between the myofilaments and $Ca^{2+}$ can be altered in three ways. Most currently available positive inotropic drugs act by increasing intracellular calcium, which then increases tension development. More recently, new positive inotropic drugs have emerged that alter the relationship between force development and the free calcium ion concentration. These latter agents either shift the force-pCa curve to the left, by increasing the sensitivity of the myofilaments, or upwards, by increasing the maximal force developed at high calcium concentrations.

The results of our experiments with the skinned preparations described here indicate that DPI acts directly on human cardiac myofilaments. DPI increased calcium activation in muscles from the myopathic hearts and there was strong evidence that DPI was bound to the myofilaments as illustrated in Fig. 8. Fig. 6 provides further evidence of the importance of this $Ca^{2+}$ sensitization to the overall inotropic effect of DPI. The concentration-dependent increase of the activating effect of DPI on the myofibrils correlates with the concentration-dependent increase in the twitch force (26). It is nevertheless important to note that DPI has membrane-related functions that indirectly influence force development by altering the $Ca^{2+}$ availability to the myofilaments (26). The observed changes in $Ca^{2+}$ sensitivity can be achieved through different mechanisms. The molecular mechanism by which calcium controls contraction is explained by the steric hindrance model (1). In this model, $Ca^{2+}$ allows cross-bridge interaction with the thin filament by binding to troponin C, which has one high- and two low-affinity sites for $Ca^{2+}$ (27), allowing tropomyosin to move on the thin filament away from its site, blocking actin-myosin interaction, namely troponin T. Phosphorylation of troponin I, which in the resting state is bound to actin, inhibits actin and myosin from interacting, thus affecting force production. A change in the cAMP-dependent phosphorylation of troponin I can therefore alter the sensitivity of the myofilaments to $Ca^{2+}$. DPI does not inhibit cAMP-dependent protein kinase in the concentration ranges studied in these experiments (25); therefore, the influence of DPI cannot be due to a decrease in cAMP-dependent phosphorylation of troponin I. Another site where DPI could be acting is troponin C, the cardiac myofibrillar receptor activating the actin myosin interaction. As $Mg^{2+}$ competes with $Ca^{2+}$ for the troponin C binding site, we varied the concentration of $Mg^{2+}$ while keeping pMgATP constant to test whether DPI increases the sensitivity of troponin C to calcium or decreases the sensitivity of troponin C to $Mg^{2+}$. In the presence of DPI, the same shift to the left was obtained (Fig. 9) at the lower and higher concentrations of $Mg^{2+}$, suggesting that the enhancement of myofibrillar $Ca^{2+}$ activation is probably not due to a decreased sensitization of troponin C to $Mg^{2+}$. It is possible, however, that troponin C sensitivity to $Ca^{2+}$ ions is increased. Further studies examining the amount of calcium bound to the myofibrils before and after DPI addition should be performed using $^{45}Ca^{2+}$ and [$^{3}$H]D-glucose[4] to test further the above hypothesis.

As depicted in Fig. 7, the force-pCa relation in the muscles from the myopathic hearts exhibited a larger shift to the left in the presence of DPI, than did the muscles from the control hearts. This means that at any concentration of $Ca^{2+}$, more force was developed by the addition of DPI to the myocardium from the myopathic hearts as compared with the muscles from the control hearts. The differential effect of DPI on the control and myopathic hearts may lie in the thin filament. Because the effects of DPI on cAMP-dependent phosphorylation have not been studied in myopathic cardiac tissue, we cannot rule out the possibility that troponin I may exhibit different isozymes in the myopathic hearts and be responsible for this added sensitization. Troponin C might be another myofibrillar protein that is altered in myopathic hearts; however, only one type of troponin C has been found in cardiac muscle (28). Finally, the troponin T-tropomyosin complex should be considered as another site at which functional changes in the myocardium can be correlated with changes in isozyme. It has been recently reported that two types of troponin T isozymes exist in bovine ventricular muscles (29). Histological studies on the human muscles have shown that the fibers in the myopathic tissues had different geometries (2) as compared with the fibers from control tissues, which can also explain the greater force enhancement if DPI affects in any way the actin-myosin interaction (30, 31).

## Acknowledgments

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