Sensitization of Dog and Guinea Pig Heart Myofilaments to Ca\(^{2+}\) Activation and the Inotropic Effect of Pimobendan: Comparison With Milrinone

Kazuya Fujino, Nicholas Sperelakis, and R. John Solaro

We compared the effects of the newer inotropic drugs, pimobendan (UD-CG 115 BS) and milrinone (Win 47203), on the electrical, mechanical and biochemical activity of intact and detergent-skinned preparations of cardiac muscle. Both of these agents increased contractile force of guinea pig papillary muscle preparations bathed under physiological conditions or depolarized with 25 mM K\(^{+}\). The positive inotropic action was associated with potentiation of the Ca\(^{2+}\)-dependent slow action potentials (APs). Contractile force developed in the presence of 25 mM [K], and 1 \(\mu\)M isoproterenol was increased further by addition of 50 \(\mu\)M pimobendan with no effect on the slow action potential. Milrinone (50 \(\mu\)M) did not produce a further increase in the force or potentiate the slow APs. Pimobendan, in a dose-dependent manner, increased active tension developed by chemically-skinned dog heart muscle fibers at submaximally activating concentrations of Ca\(^{2+}\), whereas milrinone did not. At pCa 6.25, the half-maximal concentration of pimobendan for stimulation of force development was about 40 \(\mu\)M. At maximally activating levels of Ca\(^{2+}\) (pCa 4.5), pimobendan had little or no effect on force development. The effect of pimobendan on force was paralleled by changes in the Ca\(^{2+}\)-activated Mg-ATPase activity of the isometric skinned fiber preparations. Moreover, the tension-cost (unit increase in ATPase rate/unit increase in force) was unchanged in the presence of pimobendan. Milrinone did not affect ATP hydrolysis by the skinned fiber preparations. Force-pCa and ATPase-pCa relations of skinned fiber preparations contracting isometrically were shifted to the left by 0.15–0.20 pCa units in the presence of 50 \(\mu\)M pimobendan. In contrast, there was no effect of pimobendan on the ATPase activity of unloaded myofibrillar preparations. The stimulation of force and ATPase activity of the skinned heart muscle fibers could be accounted for by an effect of pimobendan on the affinity of the regulatory (low affinity, Ca\(^{2+}\)-specific) binding sites of cardiac troponin C. Ca\(^{2+}\) binding to the “structural” high affinity sites of troponin C was slightly inhibited. The results indicate that the positive inotropic actions of pimobendan, but not milrinone, may involve activation of the cardiac myofilaments by a direct effect involving an increased affinity of the regulatory site on troponin C for Ca\(^{2+}\).

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Pimobendan (UD-CG 115 BS; 4,5-dihydro-6-2-(4-methoxyphenyl)-1H-benzimidazole-5-yl-5-methyl-3(2H)-pyridazinone) and milrinone (Win 47023; 1,6-dehydro-2-methyl-6-oxo(3,4'-bipyridine)-5-carbonitrile) are two drugs belonging to a new class of nondigitalis inotropic agents.

Both pimobendan\(^{1-3}\) and milrinone\(^{4,5}\) have been shown to have positive inotropic effects, and both act as peripheral vasodilators.

Although the inotropic action of these drugs is not clearly understood, there is evidence that they might affect contractility by inhibition of the breakdown of cyclic AMP (cAMP) by phosphodiesterase.\(^{4,6,7}\) Thus, the mechanism of action of these agents could be similar and involve protein phosphorylations associated with elevation of cAMP, as occurs with sympathomimetic agents. On the other hand, there are also data indicating that the positive inotropic actions of these two agents may differ with respect to direct effects on Ca\(^{2+}\) activation of...
the myofilaments. Milrinone\textsuperscript{8} appears to be without direct effect on the activation of the myofilaments by Ca\textsuperscript{2+}, whereas pimobendan\textsuperscript{9} has been shown to alter the sensitivity of the cardiac myofilaments to Ca\textsuperscript{2+}.

In the present experiments, we have compared the inotropic actions of milrinone and pimobendan in the same preparations for the purpose of 1) establishing whether they directly affect Ca\textsuperscript{2+} sensitivity of the myofilaments, 2) determining the mechanism by which myofilament Ca\textsuperscript{2+} sensitivity is enhanced, and 3) determining whether the energy cost of force development is altered. Our results indicate that the positive inotropic effects of pimobendan, but not milrinone, may involve an alteration in the relations among Ca\textsuperscript{2+}, myofilament force, and ATPase activity independent of alterations in Ca\textsuperscript{2+} delivery to the myofilaments. A preliminary report of part of this work has been published in abstract form.\textsuperscript{10}

Materials and Methods

Electrophysiological Experiments

Measurements of force and electrical activity of papillary muscle preparations were done essentially as described previously.\textsuperscript{11} Papillary muscles (less than 1 mm in diameter) were dissected from right ventricles of male guinea pigs (250–350 g) and placed in a tissue chamber (1.0 ml). The solution was circulated at a rate of 3.0 ml/min. The tendon end of the muscle was tied to a force transducer and the other end was fixed. The muscles were stimulated through two platinum electrodes about 10% above threshold with rectangular voltage pulses, 2 msec in duration and at a frequency of 0.5 Hz. The preparations were first equilibrated for 60 minutes in the following order: relaxing solution, activating solution with drug, activating solution without drug, relaxing solution without drug, activating solution with drug, and then stored at –20°C in a solution consisting of 50% glycerol and 50% skinning solution without detergent. The prolonged treatment of the muscle fibers with Triton X-100 was to solubilize cellular membranes including sarcolemma, sarcoplasmic reticulum, and mitochondria as well as to render the sarcolemma permeable to solutes in the bathing solutions.\textsuperscript{12} At the time of experiments, preparations less than 0.2 mm in diameter were dissected from the fiber bundles and used for measurement of force, ATPase activity, and Ca\textsuperscript{2+} binding. Thin papillary muscles from the right ventricle of guinea pig hearts were chemically skinned by a 30-minute incubation in the skinning solution.

Cardiac myofibrils were purified from dog heart ventricles by extraction in Triton X-100 according to the method of Solaro et al.\textsuperscript{13} The purified myofibrillar fraction was washed by resuspension and centrifugation first with 10 volumes of 60 mM KC1, 2 mM MgCl\textsubscript{2}, 30 mM imidazole, pH 7.0 (standard buffer) with 2 mM EGTA and finally in standard buffer alone. The protein was suspended in the standard buffer to a protein concentration of 10–15 mg/ml as determined by the method of Lowry et al.\textsuperscript{14}

Force and ATPase Measurements

Force developed by the skinned fiber preparations was measured by mounting the fibers (5–7 mm in length and approximately 0.1 mm in diameter) between an AME801 force transducer (Aksjeselskapet, Horten, Norway) and a glass rod extending from a micromanipulator. After the fibers were mounted, glycerol was washed out by immersing the fiber in a 1 ml chamber containing a relaxing solution consisting of 10 mM EGTA, 8.2 mM MgCl\textsubscript{2}, 14 mM KC1, 60 mM imidazole (pH 7.0), 5.5 mM ATP, 12 mM creatine phosphate, and 10 IU/ml creatine phosphokinase. The fibers were then stretched in the relaxing solution to the sarcomere length of 2.0 μm, as determined by laser diffraction. The fibers were activated at various free Ca\textsuperscript{2+} concentrations achieved by varying the ratio of total CaCl\textsubscript{2} to EGTA while maintaining 5 mM MgATP\textsuperscript{2−}, 2 mM free Mg\textsuperscript{2+}, and ionic strength 0.15 M as computed for pH 7.0 with binding constants compiled by Fabiato.\textsuperscript{15} The isometric force developed by the skinned fibers with and without added drugs was measured between pCa 8.0 and 4.5. Generally, each solution was applied to the muscle in the following order: relaxing solution, activating solution without drug, activating solution with drug, activating solution without drug, relaxing solution. This procedure was repeated from activating solution of high pCa to activating solution of low pCa.
When the order of activation was changed, the responses were the same. The pCa 4.5 activating solution was applied frequently as a check for any deterioration in maximum force generating capabilities.

The hydrolysis of ATP by the skinned fiber preparations was measured with two to four fibers mounted in the chamber. Pimobendan interfered with our usual assay of ATPase, which follows the oxidation of reduced nicotinamide adenine dinucleotide in a coupled assay. Moreover, in our hands, measurements of creatine production in incubation solutions in which the ATP was buffered with creatine phosphate/creatine phosphokinase were not sensitive enough to pick up possible differences. Therefore, we determined the ATPase activity by measurement of the release of inorganic phosphate with a relatively sensitive assay described by Carter and Karl. The incubation conditions for these experiments were similar to those used in the force measurements, except that no creatine phosphate was added and the concentration of MgATP was increased to 15 mM to mimic the ATP gradient across the fiber in the presence of 5 mM ATP and 10 mM creatine phosphate. Sodium azide (10 mM) was added to inhibit mitochondrial ATPase activity. Drug effects on isometric force were the same whether the ATP was buffered with the 10 mM creatine phosphate or by the addition of 15 mM MgATP. Measurements of inorganic phosphate were made in a linear phase of the reaction (the last 10 minutes of a 15-minute incubation) in the following order: relaxing solution, activating solution without drug, activating solution with drug, relaxing solution. The protein content of the skinned muscle fibers was determined by the method of Lowry et al as previously described by Pan and Solaro. The ATPase activity of unloaded myofibrillar preparations was also determined by measurement of the release of inorganic phosphate as previously described. Reactions were initiated by the addition of ATP and were quenched by the addition of ice-cold trichloroacetic acid. The inorganic phosphate was assayed by the method of Carter and Karl. The ATPase activity of unloaded myofibrillar preparations was measured as described by Pan and Solaro. The skinned fibers were first rinsed in a buffer containing 20 mM imidazole (pH 7.0), 60 mM KCl, and 2 mM MgCl₂ to remove EGTA and glycerol. Then, the fibers were transferred to the "binding" solution, which was similar to that for force measurement except for the addition of 4 μCi/ml of ⁴⁰Ca and 4 μCi/ml of (³H) D-glucose as a solution space marker and 1 mM D-glucose. When calcium binding was studied in a rigor condition, the binding solution did not contain MgATP or creatine phosphate. After incubation in the binding solution, radioactivity in the fibers was eluted as previously described in a solution containing 10 mM EGTA, 50 mM KCl, 2 mM MgCl₂, 1 mM D-glucose, and 60 mM imidazole (pH 7.0). Samples were assayed for ⁴⁰Ca and ³H and bound Ca²⁺ was computed from the ratio of ⁴⁰Ca and ³H in the binding solution and the elution solution. Protein concentration of the skinned fibers was determined as previously described. Ca²⁺ binding data were fit by means of a computerized nonlinear least-square curve fitting procedure as previously described.

Solutions

The concentration of free Ca²⁺ in incubation solutions was calculated using absolute stability constants compiled by Fabiato. Pimobendan was obtained from Dr. Karl Thomae GmbH and dissolved in DMF (N,N-dimethyl formamide) or DMSO (dimethyl sulfoxide). Milrinone was obtained from Sterling-Winthrop Pharmaceuticals (New York), dissolved in equimolar HCl, and adjusted to pH 7.0 with 50 mM imidazole before use. Equal amounts of solvents were added to solutions in the control experiments. Neither of the drugs affected the stability constant of EGTA as determined in experiments in which partition of ⁴⁰Ca between a soluble phase containing EGTA and Chelex resin was measured. All experiments were done at room temperature (23 ± 1°C).

Statistical Analysis

All results were expressed as mean ± SEM. The Student’s t test for paired data and analysis of variance were used for statistical analysis. Values of p<0.05 were considered significant.

<table>
<thead>
<tr>
<th>Table 1. Effect of Pimobendan and Milrinone on Normal Fast Action Potentials of Guinea Pig Papillary Muscles</th>
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<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>RMP (mV)</td>
</tr>
<tr>
<td>APA (mV)</td>
</tr>
<tr>
<td>Vₘₐₓ (Volts)</td>
</tr>
<tr>
<td>APD₉₀ (msec)</td>
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<tr>
<td>Force (% control)</td>
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</table>

Data are shown as mean±SEM from five experiments for pimobendan and from four experiments for milrinone. RMP, resting membrane potential; APA, action potential amplitude; Vₘₐₓ, maximum rate of voltage rise; APD₉₀, action potential duration at 90% repolarization. Dimethyl sulfoxide (0.5%) was used throughout in the experiments with pimobendan. The β-adrenergic blocker, propranolol (1 μM), was used throughout with the two drugs being tested to insure that any positive inotropic effect was not mediated by the β-receptor. *p<0.05 compared with control.
Results

We first compared the effects of pimobendan and milrinone on the normal fast action potentials (APs) and contractile force of guinea pig papillary muscles. The results, which are summarized in Table 1, showed that pimobendan significantly prolonged the AP duration, whereas milrinone significantly shortened the AP duration. Both compounds significantly increased the developed force even in the presence of 1 μM propranolol.

To assess possible effects on the slow inward current, we measured slow APs in guinea pig papillary muscles depolarized by an extracellular potassium concentration of 25 mM. Typical effects of pimobendan on the slow APs and contraction are shown in Figure 1A, and Table 2 summarizes effects of pimobendan on parameters of the slow AP. Superfusion of the preparations with 50 μM pimobendan, in the presence of 1 μM propranolol to prevent any adrenergic effects, resulted in an increase in developed force as well as an increase in the slow AP duration, amplitude, and maximum rate of rise (Vmax). In the presence of isoproterenol there was a potentiation of the slow APs and contractions. Addition of pimobendan under these conditions resulted in a further increase in the force of contraction (Figure 1B; Table 2). As also shown in Figure 1B, this increase in force occurred without significant effect on the contours of the slow APs. Milrinone (50 μM) also significantly increased the AP amplitude, Vmax, AP duration, and force in the presence of 1 μM propranolol. In contrast to the results with pimobendan, milrinone did not further potentiate the slow APs or contractile force above that produced by 1 μM isoproterenol (Table 3). These results provide indirect evidence that pimobendan, but not milrinone, may have as part of its mechanism of action a direct effect on the response of the myofilaments to Ca2+.

To investigate this possibility more directly, we measured the effects of pimobendan and milrinone on Ca2+-activated activity of “chemically skinned”

<table>
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<tr>
<th>Table 2. Effect of Pimobendan on the Ca2+-Dependent Slow Action Potentials of Guinea Pig Papillary Muscles</th>
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<tr>
<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td>RMP (mV)</td>
</tr>
<tr>
<td>APA (mV)</td>
</tr>
<tr>
<td>Vmax (V/s)</td>
</tr>
<tr>
<td>APD90 (msec)</td>
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<tr>
<td>Force (% control)</td>
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</table>

Slow action potentials (APs) were induced in 25 mM (K)o. Data are shown as mean±SEM for four experiments. RMP, resting membrane potential; APA, action potential amplitude; Vmax, maximum rate of rise of voltage potential; APD90, action potential duration at 90% depolarization. Dimethyl sulfoxide 0.5% was used throughout in the pimobendan experiments. The slow APs were induced in two ways: (a) basal conditions (no added stimulant) plus high-voltage stimulation; (b) isoproterenol (1 μM) added throughout to potentiate the slow APs. In the basal condition, propranolol (1 μM) was present throughout to insure that any effects of the drug were not mediated by the β-receptor. *p<0.05 compared to control.
TABLE 3. Effect of Milrinone on the Ca²⁺-Dependent Slow Action Potentials of Guinea Pig Papillary Muscles

<table>
<thead>
<tr>
<th></th>
<th>Propranolol (1 μM)</th>
<th>Isoproterenol (1 μM)</th>
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<tr>
<td></td>
<td>Control</td>
<td>Milrinone (50 μM)</td>
</tr>
<tr>
<td>RMP (mV)</td>
<td>-44.6±0.5</td>
<td>-45.0±0.5</td>
</tr>
<tr>
<td>APA (mV)</td>
<td>63.2±0.9</td>
<td>73.2±2.3*</td>
</tr>
<tr>
<td>V₉₀ (V/s)</td>
<td>5.7±1.2</td>
<td>11.3±2.5*</td>
</tr>
<tr>
<td>APD₅₀ (msec)</td>
<td>73.3±7.3</td>
<td>95.5±10.8*</td>
</tr>
<tr>
<td>Force (% control)</td>
<td>100</td>
<td>164.6±5.2*</td>
</tr>
</tbody>
</table>

Slow action potentials were induced in the same ways as in Table 2. Data are shown as mean±SEM for four experiments. RMP, resting membrane potential; APA, action potential amplitude; V₉₀, maximum rate of voltage rise; APD₅₀, action potential duration at 50% depolarization. *p<0.05 compared with control.

Preparations of dog and guinea pig heart muscle. Records illustrating the effect of pimobendan on force of skinned heart muscle preparations at pCa 6.00, 6.25, and 8.00 are shown in Figure 2. Force was reversibly increased in all these conditions and at this concentration of pimobendan, it is apparent that the threshold pCa for activation of force is reduced to lower levels of free Ca²⁺. The dose dependence of the effect of pimobendan and milrinone on skinned fiber force at pCa 6.25 is shown in Figure 3A. The half-maximally effective concentration (EC₅₀) value obtained at pCa 6.25 was about 40 μM, and this is similar to the value reported by Ruegg et al. at pCa 6.1. Isometric force developed by skinned dog heart muscle fibers was approximately doubled at 50 μM pimobendan, and there were significant effects of pimobendan at concentrations above 25 μM. As shown by results depicted in Figure 3B, maximal Ca²⁺ activated force at pCa 4.5 was only slightly affected by pimobendan. Isometric force developed by the skinned fibers was unaffected by milrinone at all pCa values studied.

Similar results were obtained with skinned fibers of guinea pig hearts. At pCa 6.25, force developed by the fibers (n = 4) was significantly increased to 158 ± 17% of control by 50 μM pimobendan and was unaffected by 50 μM milrinone (102.9 ± 6.3% of control).

Results presented above suggest that the sensitivity of myofilament contractile activity to the level of free Ca²⁺ in the bathing media is enhanced in the presence of pimobendan. To investigate this directly, we did experiments in which the pCa-force relation was determined in control conditions and in presence of 50 μM pimobendan. As shown in Figure 4, there was a marked effect of pimobendan at relatively high pCa values and a small effect at relatively low pCa values. The half-maximally activating pCa (pCa₅₀) was shifted about 0.15 pCa units to the left by 50 μM pimobendan. On the other hand, milrinone had no effect on the pCa-force relation (Figure 4B).

An important question with regard to the mechanism of action of Ca²⁺-sensitizing agents, such as...
pimobendan, is whether the increase in force at submaximally activating levels of pCa involves alterations in the energy cost or economy of tension development, that is, the ratio ATP hydrolyzed to force developed. In a first line of experiments, we tested whether pimobendan affected the ATPase activity of cardiac myofibrillar preparations. As shown in Figure 5, there was no effect of pimobendan on the ATPase activity of these unloaded myofibrillar preparations. Pimobendan had no significant effect on the dog cardiac myofibrillar ATPase activity, even when dissolved in either DMSO or polyethyleneglycol. As we have previously reported,\textsuperscript{8} milrinone was without effect on cardiac myofibrillar ATPase activity.

Results with myofibrillar preparations showing no effect of pimobendan on ATPase activity were difficult to reconcile with results such as those in Figures 2 and 3 showing that the same drug levels were associated with increases in the submaximal force of the skinned fiber preparations. Myofibrils shorten and supercontract during activation. We therefore tested the effect of pimobendan on the ATPase activity of the skinned fiber preparations held isometric and developing force. Figure 6A shows the time course of ATP hydrolysis by the skinned fiber preparations in the control condition and in the presence of 50 and 200 \( \mu M \) pimobendan. Liberation of inorganic phosphate was linear with time in each case, and as shown in Figure 6B, the increase in the ATPase rate associated with pimobendan was in proportion to the increase in force. This was true both at pCa 6 and at pCa 6.25. Data shown in Figure 7A illustrate the effect of pimobendan on the ATPase activity of skinned muscle fibers over a range of free Ca\(^{2+}\) concentrations. There was no effect of pimobendan on the maximum ATPase activity, and pimobendan induced about a 0.2 pCa unit leftward shift of the pCa\(_{50}\) for activation of ATPase activity. This degree of increase in the Ca\(^{2+}\) sensitivity of ATPase activity of the skinned muscle fibers to Ca\(^{2+}\) is close to that for force development (Figure 4A). On the other hand, milrinone did not affect the ATPase activity of skinned fibers over a range of free Ca\(^{2+}\) concentration (Figure 7B).

The most straightforward mechanism for Ca\(^{2+}\) sensitization of the cardiac myofilaments by pimobendan is an increase in the Ca\(^{2+}\) affinity of troponin C (TNC), the thin filament Ca\(^{2+}\) receptor responsible for activation of the myofilaments. To examine this mechanism directly, we measured the effect of pimobendan on the Ca\(^{2+}\) binding properties of TNC in skinned muscle fibers. Data shown in Figure 8A illustrate the effect of pimobendan on Ca\(^{2+}\) binding to the skinned muscle fibers over a broad range of pCa values in the presence of MgATP. We have previously shown that above pCa 5 (i.e., at free Ca\(^{2+}\) concentrations lower than 10 \( \mu M \)) Ca\(^{2+}\) bound by the skinned fibers is on myofilament TNC.\textsuperscript{12,18} The data obtained in the control condition agree with our previous work\textsuperscript{12,18} in which we have shown that there are 3 mol Ca\(^{2+}\) bound/mol TNC, that is, about 2 nmol Ca/mg protein at full activation of the myofilaments. Ca\(^{2+}\) binds to two high affinity sites, the so-called "structural sites" or Ca\(^{2+}\)-Mg\(^{2+}\) sites, and to a single low affinity regulatory site. There is clear evidence that activation is associated with the titration of the single low affinity class of Ca\(^{2+}\)-binding site.\textsuperscript{12,19} The data in Figure 8A show that the curve relating free Ca\(^{2+}\) to TNC-bound Ca\(^{2+}\) was shifted up and to the left in the presence of 50 \( \mu M \) pimobendan. Importantly, it is apparent, even
from visual inspection of the data shown in Figure 8A, that Ca$^{2+}$ binding is more greatly affected by pimobendan at the relatively low pCa values. There was no effect of pimobendan above pCa 6.5, but Ca$^{2+}$ binding to myofilament TNC was significantly increased at pCa values less than pCa 6.5, as determined by analysis of variance. It is in this range of free Ca$^{2+}$ that Ca$^{2+}$ binds to the single regulatory site on TNC. Values of the binding parameters for myofilament TNC, obtained from nonlinear curve fitting, are summarized in Table 4. These data also show that the main effect of pimobendan was to increase the binding affinity constants of the regulatory sites ($K_0$) by twofold to threefold. We also measured the effect of pimobendan on Ca$^{2+}$ binding to myofilament TNC in the absence of MgATP (i.e., under rigor conditions) in which all cross-bridges are connected to the thin filament (Figure 8B; Table 4). Under these conditions, Ca$^{2+}$ binding to the regulatory sites is potentiated by cooperative interactions among myofilament proteins. Even with TNC in this high affinity "potentiated state," there was a small, but significant, increase in Ca$^{2+}$ binding to the regulatory class of binding sites when the skinned fibers were incubated with 50 µM pimobendan (Figure 8B).

Discussion

Our results provide evidence in support of the hypothesis that pimobendan has a direct action on cardiac myofilament calcium activation. Concentrations of pimobendan similar to those producing the inotropic effect in intact preparations increased developed force of skinned fibers at submaximally activating levels of free Ca$^{2+}$. This result confirms an earlier study by Ruegg et al. Our results indicate that the mechanism for this increase in submaximal force is a direct effect of pimobendan on the Ca$^{2+}$-
binding affinity of myofilament TNC. Analysis of the Ca\(^{2+}\)-binding data shows that the single low affinity site for Ca\(^{2+}\) on TNC is stimulated by pimobendan. On the other hand analysis of the Ca\(^{2+}\) binding to the higher affinity sites on myofilament TNC indicates that these sites are slightly inhibited by pimobendan in the concentrations used here. (We think that this is the reason for the lack of a difference in the total myofilament bound Ca\(^{2+}\) at pCa 6.5, where the Ca\(^{2+}\) saturation of the regulatory site 2 is relatively low and the Ca\(^{2+}\) bound to site 1 is inhibited by pimobendan to a small extent.) The specificity of action of pimobendan on site 2 is important, in view of evidence\(^{12}\) that this low affinity site is responsible for regulation of the contraction-relaxation cycle of the heart. To our knowledge this is the first inotropic agent showing specificity for the regulatory Ca\(^{2+}\)-binding domain of cardiac TNC.

The effect of pimobendan on Ca\(^{2+}\) affinity of TNC could occur indirectly through alterations in troponin I (TNI), the inhibitory protein in the TN complex that binds to TNC. Changes in the level of TNI phosphorylation have been shown to affect the regulatory sites of cardiac TNC,\(^{20}\) but our results indicate that the increase in Ca\(^{2+}\) sensitivity and Ca\(^{2+}\) binding associated with treatment of myofilaments with pimobendan is not likely to be due to alterations in level of the TNI phosphorylation. Ca\(^{2+}\) affinity of the regulatory sites of TNC was increased by pimobendan in the absence of MgATP, that is, without a substrate for phosphorylation reactions. It is also possible that the effect of pimobendan could occur indirectly through binding to TNI and an alteration of the interaction between TNI and TNC. It is known that the affinity of Ca binding to TNC, when complexed with TNI, is about 10-fold greater than the Ca\(^{2+}\)-binding affinity in the case of free TNC.\(^{19}\) Yet there is evidence that pimobendan may bind directly to cardiac TNC. Recently, Jaquet and Heilmeyer\(^{21}\) reported that pimobendan affected Ca\(^{2+}\) binding by isolated cardiac TNC. Although this demonstrates indirectly that pimobendan binds to TNC, the interpretation of their result is difficult in view of evidence that pimobendan increased self-association of the solubilized TNC. Obviously, self-association of TNC cannot occur in our skinned fiber preparations.

Although there was no measurable increase in ATPase activity of unloaded myofilbrillar preparations, pimobendan increased the ATPase activity and Ca\(^{2+}\) bound by skinned muscle fibers contracting isometrically. This difference might be related to the difference in the procedures for preparations of myofilbrils and skinned fibers although we have found no evidence from gel electrophoresis that the two preparations are different in terms of their content of myofilbrillar proteins. We think it more likely that this difference may be related to the fact that the cross-bridge cycle of isometric muscle, in which the rate limitation is imposed by an external load on the transformation of attached force-generating states, is different from that of freely shortening muscle.\(^{22}\) Whether this is the basis for the difference or not, our results emphasize the importance of measuring ATPase activity in skinned muscle fibers held isometric. Moreover, our results also indicate that in some cases, effects of Ca\(^{2+}\)-sensitizing actions of agents such as pimobendan might be missed if they are evaluated solely by the measurement of myofilbrillar ATPase activity.

Additional evidence that pimobendan may have activity as a Ca\(^{2+}\) sensitizer is provided by comparison of results of the effects of pimobendan on slow action potentials and force in preparations superfused with propranolol and those maximally activated with isoproterenol. In the presence of pro-

### Table 4. Effect of Pimobendan on Ca\(^{2+}\)-Binding Parameters of Skinned Dog Cardiac Muscle Fibers

<table>
<thead>
<tr>
<th>MgATP (mM)</th>
<th>(n_1) (nmol/mg)</th>
<th>(K_1) (M(^{-1}))</th>
<th>(n_2) (nmol/mg)</th>
<th>(K_2) (M(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>1.13 ± 0.06</td>
<td>8.34 ± 1.10 × 10^4</td>
<td>0.57 ± 0.03</td>
</tr>
<tr>
<td>Pimobendan</td>
<td>5</td>
<td>1.21 ± 0.01</td>
<td>6.96 ± 0.66 × 10^4</td>
<td>0.61 ± 0.00</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>1.07 ± 0.02</td>
<td>1.69 ± 0.19 × 10^4</td>
<td>0.54 ± 0.01</td>
</tr>
<tr>
<td>Pimobendan</td>
<td>5</td>
<td>1.11 ± 0.02</td>
<td>1.39 ± 0.08 × 10^4</td>
<td>0.56 ± 0.01</td>
</tr>
</tbody>
</table>

Values of \(n\) and \(K\) are mean ± SEM of the individual values of \(n\) and \(K\) obtained by fitting data from several experiments separately. The number of experiments is three, except in the case of pimobendan plus ATP, where the number of experiments is four. Data from same experiments depicted in Figures 9 and 10.
pranolol, slow APs induced by K⁺ depolarization were potentiated by pimobendan. In the presence of 1 μM isoproterenol, there was a significant increase in force and the parameters of the slow action potential and contraction. Under these conditions, addition of 50 μM pimobendan did not effect the slow action potential, but force was significantly increased. These results confirm and extend an earlier report by Honerjager et al⁶ and indicate that pimobendan may have effects not only on Ca²⁺ entry via increases in cAMP but also have effects independent of Ca²⁺ entry, that is, a sensitization of myofilament force development to Ca²⁺.

In contrast to the case with pimobendan, we could find no evidence that milrinone has myofilament Ca²⁺-sensitizing activity. Yet, both milrinone and pimobendan have activity as phosphodiesterase inhibitors in vitro,⁴,⁶,⁷ and, thus, there is a question as to whether direct Ca²⁺ sensitization of the myofilaments by pimobendan forms part of its profile of inotropic agent. An important point here is that it is apparent that milrinone is more effective than pimobendan as a phosphodiesterase inhibitor. For example, in preparations from guinea pig hearts, the EC₅₀ of milrinone for phosphodiesterase inhibition is about 100 μM, whereas a maximal inhibition of only 27% was obtained with 300 μM pimobendan.⁶ Whether this difference in effectiveness is sufficient to argue against a strong role for phosphodiesterase inhibition in the mechanism of action of pimobendan is not clear. However, the relatively weak action of pimobendan as an inhibitor of phosphodiesterase, together with its clear direct effects on myofilament Ca²⁺ sensitivity, points to the possibility that pimobendan may have both mechanisms of action. In support of this idea are data reported by Honerjager et al,⁶ who showed that isometric force developed by guinea pig papillary muscles was

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**Figure 6.** Effect of pimobendan on the ATPase activity by the dog heart skinned muscle fibers and on the relation between force and ATPase activity of skinned fibers. A: Time course of inorganic phosphate (Pi) liberation in 50 μM and 200 μM and without pimobendan at pCa 6.0. Mean±SEM for three preparations. B: Effect of pimobendan on the relation between force and ATPase activity of skinned cardiac muscle. At pCa 6.0, ATPase data are the same as in (A) at 50 and 200 μM pimobendan. Force (mean±SEM, n=6) was measured separately in control conditions and at 50 and 200 μM pimobendan. At pCa 6.25, force and ATPase activity were measured simultaneously, and the skinned fibers were incubated in the order, 0 μM (control), 10 μM, 25 μM, 50 μM, 100 μM, 200 μM, and 0 μM pimobendan again. Before and between each contraction, the fiber was incubated in the relaxing solution. ATPase activity in the relaxing solution (pCa>8.0) was 7.6±0.9 nmol Pi/mg/min, n=9.
increased by pimobendan in a dose-dependent manner, while levels of cAMP were not. At 10 μM pimobendan, cAMP levels were raised 1.64-fold, but with increases in pimobendan concentration up to 300 μM, force increased but the levels of cAMP did not. On the other hand, there is good evidence that inotropic effects of milrinone are more closely correlated with levels of cAMP. Endoh et al. showed that milrinone elevated the tissue cAMP level essentially in parallel with the increase in the force of contraction of dog heart ventricular trabeculae. In these experiments, it was also shown that the increases in force paralleled a dose-dependent increase in the amplitude of the Ca²⁺ transient, measured with the aequorin technique. This result is compatible with our data showing that milrinone does not directly enhance the sensitivity of the myofilaments to Ca²⁺. We have also shown that the elevation of cAMP levels by milrinone is not associated with an increase in sarcoplasmic reticulum phosphorylation or myofilament protein phosphorylation, which could also affect myofilament Ca²⁺ sensitivity. Thus, the positive inotropic effect of milrinone could be explained by effects on Ca²⁺ currents, which presumably increase in association with cAMP-dependent sarcolemma protein phosphorylation. A cAMP-dependent potentiation of Ca²⁺ entry via calcium channels is indicated by earlier results and by data reported here showing enhancement of the parameters describing slow Ca²⁺-channel activity.

In summary, our results indicate that the positive inotropic effects of pimobendan, but not milrinone, may involve increases in the Ca²⁺ sensitivity of myofilaments. The increase in force of the skinned fibers was associated with a proportional increase in
ATPase activity, indicating that tension-cost is not altered by pimobendan. The mechanism of the Ca\(^{2+}\) sensitization involves an interaction of pimobendan with a domain of cardiac TNC that produces specific enhancement of Ca\(^{2+}\) binding to the single regulatory site.

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K Fujino, N Sperelakis and R J Solaro

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