Stereoselective Actions of Thiadiazinones on Canine Cardiac Myocytes and Myofilaments


Thiadiazinones are cardiotoxic agents that have potent, direct, and stereoselective actions on the myofilament response to Ca\(^{2+}\) in intact myocardium. Their mechanism of action is unknown. We studied the effects of racemic thiadiazinone, EMD 53998 (5-[1-(3,4-dimethoxybenzoyl)-1,2,3,4-tetrahydro-6-quinoyl]-6-methyl-3,6-dihydro-2H-1,3,4-thiadiazin-2-one), and its enantiomers on Ca\(^{2+}\) signaling in myocytes, myofilaments, and myofilament proteins. Intact canine ventricular myocytes responded to the positive enantiomer, EMD 57033, with an increase in the extent of shortening during twitch contractions without increasing the peak amplitude of the Ca\(^{2+}\) transient. The negative enantiomer, EMD 57439, also increased the extent of shortening, but in this case there was a concentration-dependent increase in the peak amplitude of the Ca\(^{2+}\) transient. This is predicted from in vitro data showing that this enantiomer is a relatively potent inhibitor of phosphodiesterase activity. There was no effect of EMD 57439 on the relation between pCa and actomyosin Mg-ATPase activity of canine heart myofibrils. In contrast, EMD 57033 shifted the pCa–Mg-ATPase activity relation to the left. There was no effect of either enantiomer on Ca\(^{2+}\) binding to myofilament troponin C. Moreover EMD 57033, but not EMD 57439, stimulated actomyosin ATPase activity of myofilament preparations in which either troponin or troponin-tropomyosin had been extracted. EMD 57033 had no effect on Mg-ATPase activity of pure ventricular myosin. EMD 57033 also stimulated the velocity of actin filament sliding on myosin heads adhered to nitrocellulose-coated glass coverslips. We propose that the action of EMD 57033 is at the actin-myosin interface on a “receptor” that may be on actin or the crossbridge. Drug binding to this domain appears to reverse the inhibition of actin-myosin interactions by troponin-tropomyosin and also to promote transition of crossbridges from weak to strong force-generating states. *(Circ Res. 1993;73:981-990)*

**Key Words** • Ca\(^{2+}\) • contraction • thiadiazinone derivatives • myofilament Ca\(^{2+}\) sensitivity • inotropic agents

In experiments reported in the present study, our aim was to investigate the mechanism of action of the cardiotoxic thiadiazinone derivative EMD 53998 (5-[1-(3,4-dimethoxybenzoyl)-1,2,3,4-tetrahydro-6-quinoyl]-6-methyl-3,6-dihydro-2H-1,3,4-thiadiazin-2-one) and its optical isomers. These agents, developed by Pharmaceutical Research at E. Merck, Darmstadt, Germany,\(^1\) belong to a chemical class that acts as so-called “myofilament Ca\(^{2+}\) sensitizers.”\(^{1-4}\) Thiadiazinone derivatives, including EMD 53998, at concentrations in the micromolar range, increase contraction amplitude in intact myocardium with no increase in the peak amplitude of the Ca\(^{2+}\) transient in most instances.\(^{2-4}\) In vitro, they act directly on the myofilaments to increase force and actomyosin Mg-ATPase activity by increasing the myofilament response to Ca\(^{2+}\).\(^{4,5}\) As is the case with other chemical classes that have Ca\(^{2+}\)-sensitizing activity, some thiadiazinone derivatives also act as inhibitors of phosphodiesterase (PDE).\(^{4-7}\) However, the thiadiazinones have properties that set them apart from other Ca\(^{2+}\) sensitizers. Their effects are greater and occur at concentrations far below those required of other agents with actions as Ca\(^{2+}\) sensitizers, such as sulmazole\(^6\) and pimobendan.\(^9\) Moreover, in the case of the thiadiazinones, PDE inhibition can be separated from Ca\(^{2+}\) sensitization by minor structural changes in the molecule.\(^{4-7}\) In fact, in the case of EMD 53998, it is now clear from earlier reports\(^5-7\) and from results we show here that the Ca\(^{2+}\)-sensitizing and PDE inhibitory activity are properties of enantiomers derived from racemic EMD 53998. EMD 57033, the positive enantiomer is effective as a Ca\(^{2+}\) sensitizer, whereas EMD 57439, the negative enantiomer, is not. EMD 57439 is 10 times more effective than EMD 57033 in its action as a PDE inhibitor\(^5\) and thus is most likely responsible for the bulk of the PDE inhibitory action of the racemic mixture.

In view of the potency and selectivity of the Ca\(^{2+}\)-sensitizing activity of these compounds, it is important to know how they work. As yet, however, there is no clear evidence on their mechanism of action. Apart from their potential as clinically useful inotropic agents, an understanding of the mechanism of action may lead
to their use as probes of domains important in the activation of myofilament proteins. They could work at one or more of the many steps in the cascade of reactions by which Ca$^{2+}$ activates the myofilaments. These include (1) Ca$^{2+}$ binding to troponin (Tn) C, (2) steps in the process by which this signal reverses the inhibitory activity of TnI, TnT, and tropomyosin (Tm) on the actin-myosin crossbridge reaction, and (3) a direct effect on either the actin filament or the crossbridge itself. Results of experiments reported here indicate that the mechanism of the Ca$^{2+}$-sensitizing activity of these agents involves a site on the thin filament important in the transduction of the Ca$^{2+}$-binding signal rather than on the Ca$^{2+}$ receptor (TnC). In addition, in the absence of Tn-Tm, the positive enantiomer affects the kinetics of the actomyosin crossbridge cycle, whereas the negative enantiomer does not.

**Materials and Methods**

*Materials*

The racemic compound EMD 53998 and its enantiomers were synthesized and purified as previously described in the Chemical Department of the Pharmaceutical Research Division of E. Merck, Darmstadt, Germany. The purity of the enantiomers was confirmed to be >99%. Stock solutions of the drugs were made in dimethyl sulfoxide (DMSO) at a concentration of 10 mmol/L and prepared fresh on the day of the experiments. All experiments contained an equivalent amount of vehicle, although pilot experiments showed that DMSO had no effect on measured myofilament activities. Pilot experiments also showed that the thiadiazones used here had no effect on assays used to measure inorganic phosphate. Collagenase B was purchased from Boehringer Mannheim, Indianapolis, Ind. Protease XIV was purchased from Sigma Chemical Co, St Louis, Mo. Indo 1-AM was from Molecular Probes, Eugene, Ore.

*Preparations*

**Canine heart cells.** Intact canine heart cells were obtained by a technique slightly different from that previously described for rat heart cells. Beagle dogs were anesthetized with pentobarbital (25 mg/kg IV). A left thoracotomy was performed, and the heart was rapidly excised. A portion of the left ventricular free wall supplied by the left anterior descending artery was excised (approximately 4 to 5 g tissue). The artery was then cannulated, and perfusion was begun with a nominally Ca$^{2+}$-free Earle's salt solution of the following composition (mmol/L): NaCl, 116; KCl, 5.4; MgSO$_4$, 1.6; NaHCO$_3$, 26.2; NaH$_2$PO$_4$, 1.2; and d-glucose, 5.6; continuously gassed with 5% CO$_2$ and 95% O$_2$ (pH 7.4) at 37°C. A curved clamp was used to occlude much of the cut surface of the tissue, and collagenase (0.4 mg/mL) in 50 µmol/L Ca$^{2+}$ Earle's salt solution was then maintained for approximately 40 minutes. After a brief final perfusion with 200 µmol/L Ca$^{2+}$ Earle's salt solution to clear the enzymes, the tissue was minced with fine scissors, agitated by pipette, and strained through a nylon mesh. The cells were resuspended in a stepwise manner in Earle's salt solution containing 0.25, 0.5, and 1 mmol/L Ca$^{2+}$. A final resuspension was made in a HEPES buffer (pH 7.4) containing (mmol/L) NaCl, MgSO$_4$, 1.2; KCl, 5; NaH$_2$PO$_4$, 1.2; HEPES, 20; d-glucose, 16; and CaCl$_2$, 1.

**Myofilament preparations.** Myofibrils and skinned fibers were extracted from ventricular muscle obtained from canine hearts. After deep anesthesia with intravenous pentobarbital, the hearts were rapidly removed, placed in ice-cold saline, and cleaned free of atria and connective tissue. Left ventricular tissue was used for preparation of cardiac myofibrils as described by Solaro et al except for the addition of proteolytic enzyme inhibitors pepstatin (0.5 µg/mL), leupeptin (0.5 µg/mL), and phenylmethylsulfonyl fluoride (0.2 mmol/L) at all steps of the isolation. Myofibrils lacking Tn-Tm (desensitized myofibrils) were prepared as described previously by a method modified from that described by Lehman. Isolated myofibrils were washed extensively and dialyzed against a buffer containing (mmol/L) dithiothreitol, 1; Na$_2$S$_2$O$_3$, 0.1; and Tris, 5 (pH 8.0); along with proteolytic enzyme inhibitors as described above. Myofibrils lacking TnC were prepared as previously described using a method modified from that reported by Morimoto and Ohtsuki. The myofibrils were extracted at 25°C, pH 7.8, in (mmol/L) CDTA (trans-1,2-diaminocyclo-hexane-N,N,N'N'-tetraacetic acid), 5; Tris buffer, 40; β-mercapto ethanol, 15; and sodium azide, 0.6; along with proteolytic enzyme inhibitors as described above. The extraction was done at a protein concentration of approximately 0.25 mg/mL for 15 minutes, after which the myofibrillar suspension was centrifuged at 3000 g for 15 minutes. The extraction was repeated (usually three times) until the ATPase activity was independent of free Ca$^{2+}$ concentration and essentially that of relaxed myofibrils. The myofibrils lacking TnC were washed three times in standard buffer (mmol/L: KCl, 60; imidazole, 30; and MgCl$_2$, 2) by centrifugation and resuspension to remove CDTA. Reconstitution was carried out by mixing the TnC-free myofibrils with pure beef cardiac TnC in a 1:1 weight ratio for 60 minutes or longer in the standard buffer. Myosin was prepared from ventricular muscle using the method of Shiverick et al. Extraction of TnC and Tn-Tm and reconstitution was verified by determination of dependence of ATPase rate on Ca$^{2+}$ and by analyzing the preparations by polyacrylamide gel electrophoresis as previously described.

**Detergent-extracted (skinned) fibers.** Skinned fibers were prepared from trabeculae cords of canine left ventricle as described by Pan and Solan. Thin-fiber bundles were first extracted at 4°C in a relaxing solution containing 1% Triton X-100, 10 mmol/L EGTA, 5.5 mmol/L ATP, 12 mmol/L creatine phosphate, 8.2 mmol/L MgCl$_2$, 30 mmol/L KCl, 60 mmol/L imidazole (pH 7.0), and proteolytic enzyme inhibitors. The bundles were stored in this same solution made 50% in glycerol without detergent and used within 2 weeks.

**Procedures**

**Simultaneous measurements of cell length and Ca$^{2+}$ transient.** Myocytes were loaded with the fluorescent Ca$^{2+}$ probe indo 1 by incubation at room temperature with the acetoxymethyl ester of indo 1 (indo 1-AM) as previously described. The loading solution consisted of 50 µg indo 1-AM, 50 µL DMSO, 90 µL fetal calf serum, and 2.5 µL of 25% Pluronic F-127 (wt/wt in DMSO). This mixture was added to 2 mL of cells in the
HEPES-buffered solution and mixed for 8 to 10 minutes. Myocytes were then centrifuged gently for 60 seconds, resuspended in HEPES-buffered solution with 1.0 mmol/L Ca\(^{2+}\), and stored in the dark at room temperature for at least 1 hour before use.

After indo loading, the cells were placed on the stage of a modified inverted microscope (model IM-35, Zeiss) equipped for simultaneous recording of fluorescence and cell length as previously described. Cells were perfused with HEPES-buffered solution containing 1 mmol/L Ca\(^{2+}\) at 23°C. Fluorescence was excited by epi-illumination with 10-microsecond flashes of 350±5 nm light. On excitation, Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free forms of indo 1 emit light, peaking at approximately 410 and 490 nm, respectively. A second dichroic mirror (465 nm) split the fluorescence emission beam, and final wavelength selection was made by two custom-designed rectangular band-pass filters (410/25 nm bandwidth and 490/20 nm bandwidth, Andover Corp) mounted directly in front of two photomultiplier tubes (EMI 9895B/350, Thorn EMI Gencom Inc). The photocurrents from the two photomultiplier tubes were integrated after each flash by a custom-designed integrator sample-and-hold circuit under the control of a VAX 11/730 computer. The ratio of emission intensity at 410 nm to that at 490 nm was computed off-line, and the resulting waveform was used as an index of the Ca\(^{2+}\), transient. In indo 1-AM-loaded cells, the Ca\(^{2+}\) transients are not calibrated with regard to the concentration of Ca\(^{2+}\), since a substantial fraction of indo AM partitions in the mitochondria.

Two platinum electrodes placed in the bathing fluid and connected to a stimulator (model SD9, Grass Instrument Co, Quincy, Mass) were used to field-stimulate the myocyte to twitch with pulses of 2 to 4 milliseconds in duration at a rate of 0.2 Hz. Cell length was monitored simultaneously with indo 1 fluorescence using red light (650 to 750 nm) to form a bright field image of the cell, which was projected onto a photodiode array with a 5-millisecond scan rate, and changes in cell length were quantified via edge tracking. The signal was then transmitted to a chart recorder (Brush 220 Gould, Inc, Cleveland, Ohio) and to a computer (VAX 11/730) for on-line analysis. Peak extent of shortening during the twitch was expressed as a percentage of the resting cell length.

Ca\(^{2+}\) binding measurements. Ca\(^{2+}\) binding to TnC in skinned heart muscle preparations was measured as described by Pan and Solaro. At the time of the experiments, bundles less than 0.2 mm in diameter were dissected from the preparations that had been stored in glycerol. The skinned fibers were first rinsed in a buffer containing 20 mmol/L imidazole (pH 7.0), 60 mmol/L KCl, and 2 mmol/L MgCl\(_2\) to remove EGTA and glycerol. They were processed for determination of Ca\(^{2+}\) binding as described by Pan and Solaro. Incubation conditions were as follows: 5 mmol/L Mg-ATP\(^{-}\), 2 mmol/L free Mg\(^{2+}\), 60 mmol/L imidazole, pH 7.0, 12 mmol/L creatine phosphate, 10 U/ml creatine kinase, 3 µCi/ml \(^{45}\)Ca, 3 µCi/ml \(^{3}H\)d-glucose, and, as a solution space marker, 1 mol/L D-glucose. The ion strength of the solution was adjusted with KCl to 0.15 mol/L. After equilibrium binding was achieved, radioactivity in the fibers was eluted as previously described in a solution containing (mmol/L) EGTA, 10; KCl, 50; MgCl\(_2\), 2; D-glucose, 1; and imidazole, 60 (pH 7.0).

Samples were assayed for \(^{45}\)Ca and \(^{3}H\), and bound Ca\(^{2+}\) was computed from the ratio of \(^{45}\)Ca and \(^{3}H\) in the binding solution and the elution solution. Protein concentration of the myofibrillar preparations and skinned fibers was determined as previously described.

ATPase rate. Ca\(^{2+}\)-dependent Mg-ATPase activity of the various myofibrillar preparations was measured as previously described by determination of inorganic phosphate release during a 10-minute incubation at 30°C. Other conditions were as follows (mmol/L): imidazole, 60; Mg-ATP\(^{2-}\), 5; Mg\(^{2+}\), 2; and ionic strength, 120. Ca\(^{2+}\) concentration was varied over a broad pCa (negative log of the molar free Ca\(^{2+}\) concentration) range, and assays were run at pH 7.0. Total concentrations of CaCl\(_2\), EGTA, KCl, MgCl\(_2\), and ATP were calculated as previously described. The same assay conditions were used for determination of myosin Mg-ATPase rate. The myofibrillar preparations and myosin were preincubated with solvent and drugs for 5 minutes. The reactions were started with ATP and stopped with ice-cold 10% trichloroacetic acid. The precipitate was removed by centrifugation, and inorganic phosphate in the supernatant fraction was determined as described by Carter and Karl.

In vitro motility assay. Motility assays were performed as previously described. Monomeric cardiac myosin (250 µg/mL) from heart muscle was adhered to a nitrocellulose-coated glass coverslip. The coverslip was part of a microchamber that was placed on the stage of an inverted microscope, which was equipped for rhodamine epifluorescence and low light level video imaging. Unregulated actin filaments from chicken pectoralis were labeled with tetramethylrhodamine (TRITC-phal-lolidin) and allowed to interact with the myosin-coated surface in the presence of 1 mmol/L Mg-ATP and various concentrations of the thiazidiones. All experiments were performed at 20°C. The velocity of actin filament sliding was determined by computer analysis of digitized video images as previously described.

Statistics

The results are given as mean±SEM. The pCa-ATPase activity relations were fit to the A.V. Hill equation using a nonlinear regression analysis (GRAPHPAD, ISI software, version 2.0) to derive the pCa\(_{0}\}(−\log [Ca\(^{2+}\) at 50% maximal activation) and n (the Hill coefficient) as previously described. Concentration-response curves were compared by one-way or two-way analysis of variance with post hoc assessment by the Bonferroni test when appropriate. A value of P<.05 was the criterion for significance.

Results

Studies With Intact Cardiac Myocytes

In approaching the mechanism of action of the thiazidiones as Ca\(^{2+}\) sensitizers, we first tested the effects of the enantiomers of EMD 53998 on contraction amplitude and on the indo 1 fluorescence transient, as a measure of Ca\(^{2+}\). The experiments were done in single myocytes isolated from the left ventricle of canine hearts. Fig 1 shows the concentration-response relation for the negative enantiomer (Fig 1A) and for the positive enantiomer (Fig 1B). The upper portion of each panel in Fig 1 shows indo 1 fluorescence, and the lower
panel depicts the recording of cell length. Seven incremental concentrations of the enantiomers ranging from 0.1 to 10 μmol/L were tested. With the negative enantiomer (EMD 57439), there was a concentration-dependent increase in twitch amplitude associated with minor changes in resting length (especially for concentrations above 3 μmol/L); there was also a parallel, large, concentration-dependent increase in Ca\(^2+\) transient amplitude (Fig 1A). In contrast, the positive enantiomer (EMD 57033) exerted a significant positive inotropic effect, which was accompanied by a progressive reduction in the diastolic cell length (beginning at 0.5 μmol/L). At concentrations of EMD 57033 up to 3 μmol/L, there were no apparent changes in the amplitude of the Ca\(^2+\) transient as indexed using indo 1 fluorescence. Thus, in concentrations up to 3 μmol/L, EMD 57033 appears to exert its effects exclusively on the myofilament response to Ca\(^2+\).

Concentrations of EMD 57033 above 5 μmol/L also caused a small increase in the peak systolic Ca\(^2+\) and further reduction of the resting cell length, most likely due to the action of the positive enantiomer as a PDE inhibitor at these concentrations. Fig 2 shows a representative example of the time course of the effects of EMD 57439, the negative enantiomer (Fig 2A), and of EMD 57033, the positive enantiomer (Fig 2B), on canine cardiac myocytes at a concentration of 1 μmol/L. The upper recording in each panel is a continuous recording of cell length on a relatively slow time base; the lower recordings show cell length and the indo 1 fluorescence transients on a faster time base in the control period before the drug (a), at a peak and stable effect of the drug (b), and after washout (c). Fig 2A shows that superfusion with the negative enantiomer resulted in a rapid increase in twitch amplitude that was accompanied by an increase of both systolic and diastolic Ca\(^2+\), without any change in diastolic length (compare Fig 1A with 1B). These effects returned to control levels on washout (c). The twitch and Ca\(^2+\), transient durations in preparations treated with the negative enantiomer, EMD 57439, are abbreviated. Fig 2B shows that the positive enantiomer exerted a rapid positive inotropic effect associated with a large decrease in resting cell length and no clear increase of the Ca\(^2+\) transient (compare Fig 1A with 1B). In contrast, there appears to be a small decrease in the peak amplitude of...
the \( \text{Ca}^{2+} \) transient. Furthermore, a reduction, rather than an increase, in the diastolic indo 1 fluorescence ratio occurred (Fig 1B). The positive enantiomer had little effect on the duration of either the twitch or the \( \text{Ca}^{2+} \) transient. Washout of the drug almost completely reversed the effect on twitch amplitude and diastolic length.

**Studies With Myofilament Proteins**

The differential effect of the enantiomers of EMD 53998 on \( \text{Ca}^{2+} \) activation of canine cardiac myofilaments is shown at a concentration of 10 \( \mu \text{mol/L} \) in Fig 3A and at a concentration of 30 \( \mu \text{mol/L} \) in Fig 3B. Results of these studies are consistent with our data showing differences in the effects of EMD enantiomers on the myofilament response to \( \text{Ca}^{2+} \) in intact canine heart myocytes (Figs 1 and 2). The negative enantiomer, EMD 57439, had no significant effect on the ATPase activity-pCa relation at 10 or 30 \( \mu \text{mol/L} \). However, there were clear alterations in the response of the myofilaments to \( \text{Ca}^{2+} \) demonstrated by the positive enantiomer, EMD 57033. These observations in canine myofilaments are similar to those obtained in guinea pig heart myofilaments. The enhanced response to \( \text{Ca}^{2+} \) involved three effects of the positive enantiomer: (1) a loss of inhibition of activity at the higher pCa values, (2) a stimulation of maximum ATPase rate, and (3) a leftward shift of the relation between pCa and ATPase activity (Fig 3C). As indicated by the parameters listed in the legend to Fig 3C, the pCa at half-maximal activation (p\( \text{C}_{50} \)) was clearly shifted to the left by the positive enantiomer at both 10 and 30 \( \mu \text{mol/L} \) of the drug. Moreover, at the higher concentration, EMD 57033 decreased the steepness of the relation between ATPase rate and pCa.

The leftward shift of the pCa-ATPase activity relation could occur as a result of a change in affinity of TnC for \( \text{Ca}^{2+} \). Yet the effect of the active enantiomer on basal and maximum ATPase activity and on the steepness of the relation between ATPase activity and pCa indicated that there may be other effects of the drug on the \( \text{Ca}^{2+} \) activation process. To test this, we measured \( \text{Ca}^{2+} \) binding to myofilament preparations under conditions in which we had previously shown that myofilament TnC is the only site titrating \( \text{Ca}^{2+} \) over the range of activating pCa values.18

Fig 4A shows that concentrations of the active enantiomer (EMD 57033) up to 30 \( \mu \text{mol/L} \) did not significantly alter the \( \text{Ca}^{2+} \)-binding properties of TnC in skinned fiber preparations of canine ventricle. Although it is not shown, as a positive control for these experiments, we measured the effects of altered pH on myofilament TnC \( \text{Ca}^{2+} \) binding.18 TnC \( \text{Ca}^{2+} \) binding was also unaffected by EMD 57439, the negative enantiomer (Fig 4B), or by racemic EMD 53998 (data not shown).

The lack of an effect of the thiadiazinones on \( \text{Ca}^{2+} \) binding to myofilament TnC indicated that the \( \text{Ca}^{2+} \)-sensitizing effect may be due to actions “downstream” from TnC. To investigate this possibility, we examined the effect of these agents on preparations from which TnC had been extracted. Myofilibrillar preparations lacking TnC demonstrate a low ATPase activity that is independent of \( \text{Ca}^{2+} \) because of the prevailing inhibitory action of myofibrillar actomyosin ATP hydrolysis by the TnI-Tnt-Tm complex.14,15 As shown in Fig 5, at the lower concentrations (1 and 3 \( \mu \text{mol/L} \)), EMD 53998 did not overcome the inhibition of the actin-crossbridge reaction by TnI-Tnt-Tm. However, at the higher concentrations, myofibrillar actomyosin ATPase activity was significantly stimulated despite the absence of TnC. This result shows clear evidence that TnC is not required for the manifestation of the effect of EMD.
We also measured the effect of EMD on myofibrillar preparations from which the entire thin-filament regulatory complex, Tn-Tm, had been extracted. As expected, extraction of Tn-Tm produced a preparation in which the actomyosin ATPase activity was turned on at all pCa values.\textsuperscript{13,14} The results we obtained with Tn-Tm-free myofibrils, which are shown in Fig 6, demonstrate that, whereas the negative enantiomer of EMD had no effect, the positive enantiomer caused a concentration-dependent increase of actomyosin ATP hydrolysis.

Results presented in Figs 3 through 6 suggest that part of the mechanism for the effect of EMD on activation of cardiac myofilaments may be a direct action of the drug on the actin-myosin reaction. This could be due to drug binding to actin or myosin. Therefore, we tested whether EMD had an effect on myosin ATPase alone. As shown in Fig 7, myosin purified from canine heart ventricular tissue showed no change in ATPase activity when incubated with EMD 57033, the active enantiomer. Thus, the stimulation of actomyosin ATPase rate by the positive enantiomer of EMD may not involve drug binding to myosin itself. However, we cannot rule out the possibility that EMD is binding to myosin at a site that interacts with actin and thus affects the actomyosin ATPase rate but that does not affect the myosin ATPase rate. Moreover, strong crossbridge interactions could be promoted by binding of EMD to the myosin head at a site that does not affect the myosin Mg-ATPase activity.

Further evidence that the thiadiazinones affect the actomyosin reaction comes from studies using pure cardiac myosin and actin filaments in the in vitro motility assay.\textsuperscript{21,22} In these experiments, actin filament motion along the heads of myosin was significantly
increased by the action of EMD (Fig 8). The velocity of actin filament motion was significantly increased with increasing concentrations of EMD 57033, the positive enantiomer. The negative enantiomer did not significantly affect actin filament velocity at any concentration.

Discussion

Results of experiments reported here show evidence for two stereoselective effects of the thiadiazinones on the contractile machinery of heart muscle. The first, Ca\(^{2+}\) sensitization, is demonstrated by results in myocytes and fully regulated myofilaments; the second, an increase in the kinetics of the actin-myosin reaction, is demonstrated by results with actomyosin ATPase rate and movement of actin filaments on myosin. Below, we discuss these effects separately as they relate to our proposed mechanism of action of the thiadiazinones.

Ca\(^{2+}\) Sensitization

Our results with intact canine heart myocytes on the effects of EMD 53998 and its enantiomers agree with data from earlier studies using rat, guinea pig, and ferret myocardial preparations.\(^1\)\(^-\)\(^4\) These reports have indicated that the thiadiazinones are unique and poten-

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Fig 4. Graphs showing measurements of Ca\(^{2+}\) binding to troponin C in skinned fibers of canine heart in the presence of thiadiazinones. A illustrates data showing a lack of effect of 10 \(\mu\)mol/L and 30 \(\mu\)mol/L of the positive enantiomer (EMD 57033) of EMD 53998 on Ca\(^{2+}\) binding to troponin C in canine heart myofibrils. B shows similar results with the negative enantiomer, EMD 57439. The results represent mean±SEM for 3 to 10 measurements at each pCa value. See "Materials and Methods" for incubation conditions. None of the values determined in the presence of drug were significantly different from the control values.

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Fig 5. Graph showing effect of extraction of tropomin C (TnC) on concentration dependence of activation of myofibrillar ATPase activity by racemic EMD 53998. Measurements were made at pCa 6.5 as indicated on control myofibrils, on myofibrils in which TnC had been extracted, and on myofibrils in which TnC was extracted and then reconstituted with TnC. Conditions were as described in the legend to Fig 3. Data represent mean±SEM preparations with \(n=6\) for control and TnC-extracted preparations and \(n=3\) for reconstituted preparations. Values at 5 and 10 \(\mu\)mol/L EMD 53998 were not significantly different from each other, whereas the value at 3 \(\mu\)mol/L EMD 53998 for TnC-extracted myofibrils was significantly different (\(P<.05\)) from both native and reconstituted preparations.
also was six measurements Mg-ATPase activity on preparations. ATPase 7.

Fig 6. Graph showing effect of concentration of enantiomers of thiadiazine derivative EMD 53998 (EMD) on ATPase activity of canine heart myofibrils from which troponin-tropomyosin had been extracted. Measurements with the negative enantiomer (○) and positive enantiomer (●) were made at pCa 6.5. Pi indicates inorganic phosphate. Data represent mean±SEM for three measurements. Other conditions were as described in the legend to Fig 3. Values obtained with the positive enantiomer were significantly higher (P<.05) than those obtained with the negative enantiomer at all drug concentrations (P<.05).

Initially important compounds that apparently react with and stimulate activity of cardiac myofilaments in situ in preparations from intact heart and in isolated cells. Using either aequorin luminescence or indo 1 fluorescence as measures of Ca²⁺, these studies showed that, in the appropriate range of concentrations, the racemic mixture (EMD 53998) can increase the ability of myocardium to shorten and develop force with little or no change in the amplitude of the Ca²⁺ transient. However, in some cases, the increase in contractile activity was associated with an increase in the peak of the Ca²⁺ transient, an effect undoubtedly due to the PDE inhibitory activity of the racemic mixture. More recent work has shown that these mixed actions of EMD 53998 represent stereoselective effects of enantiomers with separate activities as a myofilament Ca²⁺ sensitizer and PDE III inhibitor. This is the most straightforward interpretation of the present results, which demonstrate differential actions of the enantiomers of EMD 53998 on Ca²⁺, in canine heart myocytes. The increase in the Ca²⁺ transient amplitude, the decrease in its duration, and the decrease in duration of contraction are effects that would be expected to result from an elevation of cAMP. The prolongation of the contraction duration and reduction in diastolic cell length (Fig 1A) are effects expected to result from myofilament Ca²⁺ sensitzation.

The stereoselectivity of the effect of the thiadiazinones on activation of isolated myofilaments provides strong evidence that the agents affect a specific domain important in determining the state of activation of the myofilaments. Understandably, it has been proposed that this domain is on TnC and that the mechanism of the Ca²⁺ sensitization might involve an effect on Ca²⁺ binding to TnC. Lee and Allen based this proposal on a computer model of Ca²⁺ flux in the myocyte. When the affinity of TnC was increased, calculated values showed a small fall in the peak of the Ca²⁺ transient, an effect generally attributed to enhanced Ca²⁺ buffering by the myofilaments. However, in testing this idea by direct measurements on isolated myofilament preparations, our results (Fig 5) show no effects of the racemic mixture or its enantiomers on Ca²⁺ binding to myofilament TnC at concentrations that significantly increased myofilament activity. Moreover, actin-myosin interactions could be increased by EMD 57033 in preparations without TnC. Therefore, Ca²⁺ sensitization by the positive thiadiazinone enantiomer appears to involve a myofilament domain other than TnC itself.
Before considering potential mechanisms by which EMD 57033 could stimulate myofilament response to Ca\(^{2+}\) without affecting the affinity of TnC for Ca\(^{2+}\), it is useful to briefly describe current concepts of myofilament activation.\(^{10,24}\) The actin-linked regulatory system of striated muscle is composed of Tm and Tn, which is composed of three units—TnC, TnI, and TnT. In the absence of Ca\(^{2+}\), the inhibitory activity of Tn-Tm is such that crossbridges weakly attach to actin.\(^{10,24}\) On activation, Ca\(^{2+}\) binds to TnC,\(^{19}\) relieving Tn-Tm inhibition, thus allowing crossbridges to undergo the transition to a strongly bound state that generates force and promotes fiber shortening.\(^{25}\) There is also evidence that strongly bound crossbridges can themselves disinhibit the thin filament, perhaps by moving Tm from its blocking position.\(^{24,26}\) An appealing hypothesis\(^{24,27,28}\) that serves to explain a number of experimental observations is that the movement of Tm promoted by strong crossbridges acts to spread activation along the thin filament to neighboring functional units that are not activated by Ca\(^{2+}\)-TnC. The basic idea is that, with submaximal Ca\(^{2+}\) activation, strongly binding crossbridges associated with nearest neighbor functional units activated by Ca\(^{2+}\)-TnC can move Tm and spread activation to immediately adjacent functional units of the thin filament with no Ca\(^{2+}\) bound to TnC.

Based on our observations, it is apparent that the thiazidiones are able to reverse the inhibition of the actin-myosin interaction by Tn-Tm and also to promote the reaction of myosin with actin. Our hypothesis is that these effects involve a site of binding of EMD at the actin-myosin interface. One consequence of the action of EMD could be to increase the probability of the actin-myosin interaction at a given pCa value. This seems to explain the leftward shift of the pCa-ATPase activity curves seen at a concentration of 10 \(\mu\)mol/L EMD 57033 in Fig 3C. Another consequence of the action of EMD could be to affect the disinhibition of the myofilaments by strongly bound crossbridges and, in this way, to ease the spread activation along the thin filament. The significant decrease in the steepness of the pCa-ATPase activity relation associated with EMD stimulation at a concentration of 30 \(\mu\)mol/L EMD 57033 could be explained by its action on crossbridge-dependent release of myofilaments from Tn-Tm inhibition. Activation of myofilaments by strong crossbridges is a highly cooperative process involving nearest neighbor interactions.\(^{27}\) On the other hand, activation by Ca\(^{2+}\) binding to TnC is much less cooperative, reflecting binding that can be adequately fit with a model including only independent sites.\(^{18}\) In the case of stimulation of the myofilaments by this high concentration of EMD, activation by Ca\(^{2+}\) binding to TnC becomes limiting. Thus, as would be predicted and is shown by the results depicted in Fig 3C, activation of the myofilaments is much less steeply dependent on Ca\(^{2+}\). Both of these proposed effects would be most prominent at low levels of activation by Ca\(^{2+}\), at which many functional units would still be turned off by the inhibitory activity of Tn-Tm. At the higher levels of free Ca\(^{2+}\), fewer functional units would be inhibited by Tn-Tm; thus, stimulation by a mechanism involving release of the myofilaments from inhibition would be less significant.

Another mechanism for activation of the myofilaments by EMD could be an effect on the interaction of TnC with other thin-filament components. If this were the case though, we would have expected to see an effect of the drug on Ca\(^{2+}\) binding to TnC. In previous work,\(^{29}\) for example, we showed that the affinity of pure TnC for Ca\(^{2+}\) increased approximately 10-fold in the Tn1-TnC complex and in the complex of Tn1-TnC-TnT. Thus, alterations in binding between Tn1-TnT and TnC would be expected to also alter Ca\(^{2+}\) binding to TnC.

**Effects of EMD on the Kinetics of the Actin-Myosin Reaction**

Our studies on desensitized myofibrils and on preparations of pure myosin and actin filaments show strong evidence that EMD acts to stimulate turnover of the actin-crossbridge reaction in a stereoselective manner. In addition, the effect of EMD on maximum ATPase rate of regulated myofilaments at saturating levels of free Ca\(^{2+}\) could also be due to an action of the drug on the kinetics of the actomyosin ATPase. Results of experiments using the motility assay provide a direct demonstration that EMD affects the turnover of the actin-crossbridge.

How do effects of EMD on the kinetics of the actin-myosin reaction relate to its Ca\(^{2+}\)-sensitizing activity? First of all, a relation between the two activities is indicated by the same stereoselectivity of the two effects. Moreover, it is clear that reversal of thin-filament inhibition by EMD can occur without TnC present (Fig 5). Our hypothesis is that binding of EMD to a site at the actin-myosin interface promotes the reaction of myosin with the thin filament in such a way that actin filament sliding is enhanced and ATPase rate is increased without the thin-filament regulatory complex, whereas in fully regulated myofilaments loss of inhibition by Tn-Tm and disinhibition by strong binding crossbridges is enhanced. Leijendekker and Herzig\(^{30}\) also suggested that an effect of EMD 53998 on the rate constants of the actin-myosin reaction could explain the altered myofilament response to Ca\(^{2+}\). However, this effect, which was an apparent decrease in the rate of the actin-crossbridge reaction, was present only at levels of Ca\(^{2+}\) associated with less than 10% of maximum activity and thus was present only over a narrow range of activation of their preparations. In the case of the motility assay, it is difficult to know exactly which rate constants are affected by EMD, but it does appear that there are differences between fully regulated isometrically contracting fiber bundles and unloaded preparations represented in the motility assay and the assay of myofibrillar ATPase activity.

Interestingly, our proposed mechanism of action of EMD 53998 is opposite that which occurs on elevation of inorganic phosphate surrounding the myofilaments, in which case the probability of strongly bound crossbridge attachments is decreased.\(^{25,31,32}\) Product inhibition imposed by increases in inorganic phosphate concentration decreases the population of strong crossbridges, resulting in a decrease of maximum force.\(^{31,32}\) A decrease in the response of the myofilaments to Ca\(^{2+}\), and an increase in the steepness of the force-pCa relation.\(^{31,32}\) These effects cannot be accounted for by a change in the affinity of myofilament TnC for Ca\(^{2+}\).\(^{33}\) Opposite effects of inorganic phosphate and EMD on myofilament response to Ca\(^{2+}\) may be an important aspect of the therapeutic application of agents such as the thiazidiones, inas-
much as the decrease in force associated with ischemia is strong when correlated with elevated inorganic phosphate.\textsuperscript{34} Indeed, recent evidence indicates that EMD 53998 may antagonize the effect of phosphate on force generated by skinned-fiber preparations.\textsuperscript{35}

**Acknowledgments**

This study was supported by National Institutes of Health Grants ROI HL-22231 (R.J.S.) and ROI HL-45161 (D.M.W.) and by the American Heart Association Established Investigator Award (D.M.W.) and Medical Student Fellowship (M.R.K.). The technical assistance of Mr Todd R. Rice in preparing the intact canine cardiac myocytes, of Ms Mary Johnson in doing myofilament experiments, and of Ms Janet Vose in performing the motility assay is greatly appreciated.

**References**

29. Holroyde MJ, Robertson SP, Johnson JD, Solaro RJ, Potter JD. The Ca\textsuperscript{2+} and Mg\textsuperscript{2+} binding sites on cardiac troponin and their role in the regulation of adenosine triphosphatase. J Biol Chem. 1980;255:11688-11693.
34. Marban E, Kusuoka H. Maximal Ca\textsuperscript{2+} activated force and myofilament Ca\textsuperscript{2+} sensitivity in intact mammalian hearts: differential effects of inorganic phosphate and hydrogen ions. J Gen Physiol. 1987;90:609-623.
Stereoselective actions of thiadiazinones on canine cardiac myocytes and myofilaments.
R J Solaro, G Gambassi, D M Warshaw, M R Keller, H A Spurgeon, N Beier and E G Lakatta

Circ Res. 1993;73:981-990
doi: 10.1161/01.RES.73.6.981

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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