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# The Effect of a Thiadiazinone Derived $Ca^{2+}$ Sensitizer on the Responsiveness of $Mg^{2+}$-ATPase to $Ca^{2+}$ in Myofibrils Isolated from Stunned and Nonstunned Porcine and Human Myocardium

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**ABSTRACT.** Previously, we showed, in an *in situ* porcine model, that the thiadiazinone derivative [+]EMD 60263, a putative $Ca^{2+}$ sensitizer with minimal phosphodiesterase III inhibitory properties, increased contractility more profoundly in stunned than in nonstunned myocardium. The aim of the present investigation was to study the mechanism of action by determining the *in vitro* effects of [+]EMD 60263 on the $Ca^{2+}$ responsiveness of the $Mg^{2+}$-dependent ATPases of myofibrils and sarcoplasmic reticulum membrane vesicles, isolated from normal ventricle of swine and hypertrophic septum of cardiomyopathic patients. Contamination of the myofibrils with sarcoplasmic reticulum membranes was excluded by testing the effect of the sarcoplasmic reticulum $Ca^{2+}$-pumping ATPase inhibitor thapsigargin. The plasma concentrations at which [+]EMD 60263 exerted its inotropic effect in the *in situ* porcine model were found to be submicromolar. [+]EMD 60263 stimulated concentration-dependently (1–10 μM) the submaximally activated $Mg^{2+}$-ATPase (at pCa 6.1) of pig heart myofibrils. [+]EMD 60263 (10 μM) shifted the $pCa_{50}$ of porcine myofibrillar $Ca^{2+}$-stimulated, $Mg^{2+}$-dependent ATPase from 6.00 ± 0.05 to 6.67 ± 0.05, whereas the [−]enantiomer EMD 60264 had no significant effect. Although the effect was much less at 1 and 3 μM, [+]EMD 60263 (10 μM) also stimulated maximal myofibrillar $Mg^{2+}$-ATPase activity. The Hill coefficient, reflecting the steepness of the fitted pCa/$Mg^{2+}$-ATPase curve at half-maximal activation, was not affected by [+]EMD 60263 (10 μM). [+]EMD 60263 (10 μM) had no effect on sarcoplasmic reticulum $Ca^{2+}$-stimulated, $Mg^{2+}$-dependent ATPase from swine heart. The thiadiazinone derivative [+]EMD 57033 (10 μM), but not its [−]enantiomer EMD 57439, had similar, although less potent, effects on pig heart myofibrillar $Mg^{2+}$-ATPase activity as compared to [+]EMD 60263. [+]EMD 60263 (3 μM) produced a significantly larger leftward shift of the $pCa^{2+}$/$Mg^{2+}$-ATPase activity curve of myofibrils isolated from the stunned compared to the adjacent nonstunned myocardium ($\Delta pCa_{50}$s caused by the presence of [+]EMD 60263 amounted to +0.57 ± 0.04 and +0.42 ± 0.05, respectively) in the *in situ* porcine model. The effects of [+]EMD 60263 on myofibrillar $Mg^{2+}$-ATPase of hypertrophic human heart were identical to those observed with porcine heart myofibrils. The results indicate that the positive inotropic action of [+]EMD 60263 observed in the *in situ* porcine model of stunned myocardium may be primarily due to myofilament sensitization to $Ca^{2+}$, and that this compound may have a similar action on diseased human myocardium. BIOCHEM PHARMACOL 51;9:1211–1220, 1996.

**KEY WORDS.** cardiac myofibrils; cardiac sarcoplasmic reticulum; human; pig; $Ca^{2+}$-stimulated; $Mg^{2+}$-ATPase; thiadiazinone derivatives; myocardial stunning

Myocardial contractility can be modulated by two principal mechanisms, 1. the amplitude of the cytosolic [$Ca^{2+}$] transient, 2. the responsiveness of the myofilaments to $Ca^{2+}$ [1, 2], or 3. a combination of both mechanisms. Myofilament $Ca^{2+}$ sensitivity is, in part, related to an enhancement of $Ca^{2+}$ binding to the myofilaments, reflected by increased myofibrillar $Mg^{2+}$-ATPase activity [3, 4]. Most of the positive inotropic drugs used clinically, such as digitalis, α- and β-adrenergic agonists, and PDE III§ inhibitors, act by increasing cytosolic and SR $Ca^{2+}$ loading, which leads to an increase in magnitude of the $Ca^{2+}$ transient [5]. A number of PDE III inhibitors (e.g. sulmazole, pimobendan, and several thiadiazinone derivatives) appear to produce their positive inotropic effect not only through increased cytosolic

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§ *Abbreviations:* SR, sarcoplasmic reticulum; ATPase, adenosine-5′-triphosphatase; DTT, dithiothreitol; MOPS, 4-morpholino-propane sulfonic acid; $P_i$, inorganic phosphate; PMSF, phenylmethane-sulfonylfluoride; PDE III, phosphodiesterase III; LADCA, left anterior descending coronary artery; LCXCA, left circumflex coronary artery.

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[$Ca^{2+}$] transient but they also exert a $Ca^{2+}$-sensitizing action in both skinned and intact cardiac muscle preparations [1, 6, 7]. Either method of increasing myocardial contractile function may be damaging because the increased $Ca^{2+}$ transient can potentially result in chronic overloading of the cell with $Ca^{2+}$ [8]; increased myofilament $Ca^{2+}$ sensitization has the potential to prolong the time of relaxation and to impair diastolic filling of the heart [9].

Thiadiazinone derivatives vary widely in their ability to sensitize myofilaments to $Ca^{2+}$ and to increase cellular cyclic AMP levels *via* their PDE III inhibitory action [10]. However, *in vitro* studies with skinned fibers and soluble preparations of PDE III have indicated that the optical isomers of the racemic thiadiazinone EMD 53998 possess a remarkable and distinct separation of $Ca^{2+}$-sensitizing and PDE III-inhibitory activities [11]. Thus, compared to the racemate EMD 53998, the [−]enantiomer EMD 57439 (Fig. 1) is a "pure" PDE III inhibitor with almost no $Ca^{2+}$-sensitizing activity, and the [+]enantiomer EMD 57033 (Fig. 1) is a potent $Ca^{2+}$ sensitizer with weak PDE III-inhibitory activity.

Recently, we reported on the *in vivo* cardiovascular effect of the thiadiazinone derivative [+]EMD 60263 (for its chemical structure, see Fig. 1) in pigs with regionally stunned myocardium [12, 13]. In these studies, the effect of [+]EMD 60263 on regional myocardial function was assessed by measuring the systolic segment shortening, external work, and mechanical efficiency of stunned and nonstunned myocardium [12]. It was shown that [+]EMD 60263 increased systolic segment shortening of both stunned and nonstunned myocardium although the effect on the former was much more pronounced than on the nonstunned myocardium. Furthermore, [+]EMD 60263 restored the mechanical efficiency of stunned myocardium to baseline levels, and that of nonstunned myocardium was unaffected. The action of [+]EMD 60263 was not attenuated when experiments were repeated after α- and β-adrenergic receptor blockade, thereby excluding adrenergic stimulation or PDE III inhibition as the cause of the positive inotropic action of [+]EMD 60263 [12]. The [−] enantiomer EMD 60264 (Fig. 1) has been investigated in the same model and found to have no effect on systolic segment shortening, external work or mechanical efficiency (unpublished results). In another study, we demonstrated that, in the same porcine model, the rate of SR $Ca^{2+}$ uptake was slightly increased in stunned compared to nonstunned myocardium [14]. The data supported the hypothesis that a decreased sensitivity of the myofilaments to $Ca^{2+}$, and not a decreased SR $Ca^{2+}$ pumping activity, is involved in the mechanism of stunning [15, 16].

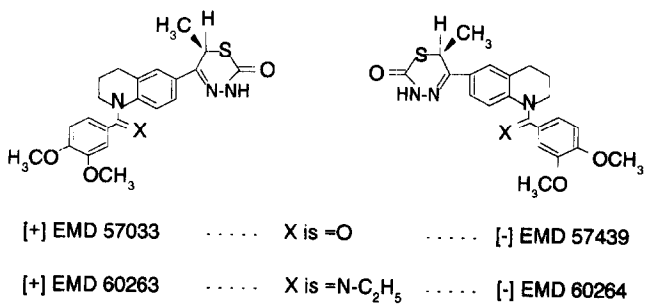


**FIG. 1. Chemical structures of the [+]enantiomers EMD 57033 and EMD 60263 (left) and [−]enantiomers EMD 57439 and EMD 60264 (right) (see also refs. 11, 12 and 17).**

The aim of the present investigation was to complement the data on the thiadiazinone derivatives of our *in vivo* studies [12, 13] with data obtained with subcellular preparations *in vitro*. We, therefore, studied the effects of [+]EMD 60263 and its [−]enantiomer EMD 60264 on the $Ca^{2+}$ responsiveness of $Mg^{2+}$-ATPases measurable in SR membrane vesicles and myofibrils isolated from normal porcine myocardium. We also studied the effect of [+]EMD 60263 on the $Ca^{2+}$ responsiveness of the $Mg^{2+}$ ATPase of myofibrils isolated from stunned and the adjacent nonstunned myocardium in the *in situ* porcine model. Moreover, the effectiveness of [+]EMD 60263 on myofibrils isolated from hypertrophic septum of patients with cardiomyopathy was tested. For comparison, we also measured the effects of the thiadiazinone derivative [+]EMD 57033, a $Ca^{2+}$ sensitizer with weak PDE III inhibitory activity, and [−]EMD 57439, a "pure" PDE III inhibitor with no $Ca^{2+}$-sensitizing action. The results show that [+]EMD 60263, at concentrations (1–3 μM) close to the plasma concentrations found to be effective in the *in situ* porcine model [12], sensitizes the $Mg^{2+}$-ATPase of isolated myofibrils to $Ca^{2+}$ and has no effect on the $Ca^{2+}$-stimulated $Mg^{2+}$-ATPase of isolated SR membrane vesicles. Consistent with the observations of our previous *in vivo* experiments [12] is the finding that, in the stunned myocardium, the $Ca^{2+}$-sensitizing effect of [+]EMD 60263 on isolated myofibrils was potentiated.

## MATERIALS AND METHODS

### *Materials*

The pure enantiomers [+]EMD 60263 and [−]EMD 60264 (5-[1-(α-ethylimino-3,4-dimethoxybenzyl)-1,2,3,4-tetrahydro-6-quinolyl]-6-methyl-3,6-dihydro-2H-1,3,4-thiadiazin-2-one) and [+]EMD 57033 and [−]EMD 57439 (5-[1-(3,4-dimethoxybenzyl)-1,2,3,4-tetrahydro-6-quinolyl]-6-methyl-3,6-dihydro-2H-1,3,4-thiadiazin-2-one) were supplied by E. Merck, Darmstadt, Germany. Fig. 1 depicts the chemical structures. The only difference between the enantiomers is the position of the methyl group ($CH_3$) bound to the C-atom beside the S-atom. Because of the asymmetrical configuration of that C-atom, the [+]-enantiomer deflects the plane of light to the right, whereas the [−]-enantiomer deflects it to the left [17]. Stock solutions (0.2 mM) of [+]EMD 60263 and [−]EMD 60264 were made in distilled water and those (0.2 mM) of [+]EMD 57033 and [−]EMD 57439 in DMSO and were prepared on the day of the experiment. All solutions contained an equivalent amount of water or DMSO that had no effect on the $Ca^{2+}$ responsiveness and activities of cardiac SR and myofibrillar $Mg^{2+}$-

ATPases. Control experiments also demonstrated that these thiadiazinone derivatives had no effect on the assay used to measure $P_i$ formation. Leupeptin, aprotinin, pepstatin, and thapsigargin were from Sigma Chemical Company (St Louis, MO, U.S.A.). All other chemicals were obtained from either E. Merck (Darmstadt, Germany), Boehringer (Mannheim, Germany), or Sigma.

### *Subcellular Preparations*

SR vesicles and myofibrils were isolated from porcine ventricular muscle and hypertrophic septum of cardiomyopathic patients undergoing open-heart surgery. Stunned and nonstunned myocardium was obtained from 4 anesthetized open-chest pigs, in which the distribution territory of the left anterior descending coronary artery (LADCA) was stunned by 2 sequences of 10-min coronary artery occlusions and 30-min reperfusion. The nonstunned myocardium was obtained from the distribution territory of the left circumflex coronary artery (LCXCA), which was not occluded. For further details of this *in situ* porcine model, see Soei *et al.* [12]. The cardiac muscle specimen (from pigs about 3 g and from humans not more than 1 g) were minced and mixed with 4 volumes 10 mM $NaHCO_3$ and 1 mM dithiothreitol (DTT) and homogenized with a Polytron PTX 10 (Kinematica, GmbH, Lucerne, Switzerland). The homogenate was centrifuged at 9000 $g_{av}$ for 20 min at 4°C and the supernatant centrifuged again at 9000 $g_{av}$ for 20 min. The final supernatant was further subfractionated for isolation of enriched SR vesicles as described [18, 19] (see below). The combined pellets were used for the isolation of the purified myofibrils according to the method described by Murphy and Solaro [20]. The pellets were resuspended in 4 volumes of solution containing 10 mM EGTA, 8.2 mM $MgCl_2$, 14.4 mM KCl, 60 mM imidazole, 5.5 mM ATP, 22 mM creatinephosphate, 10 $U \cdot mL^{-1}$ creatine kinase, 1% Triton X100, 5 $\mu g \cdot mL^{-1}$ leupeptin, 10 $\mu g \cdot mL^{-1}$ pepstatin, 10 $\mu g \cdot mL^{-1}$ aprotinin, 1.7 $mg \cdot mL^{-1}$ PMSF in a glass-Teflon homogenizer, and left on ice for 30 min and, thereafter, centrifuged for 15 min at 1100 $g_{av}$. The supernatant was discarded and the myofibrillar pellet washed twice with 2 volumes 30 mM KCl, 30 mM imidazole, and 2 mM $MgCl_2$, pH 7.0 and, finally, resuspended in this buffer containing 50% glycerol up to a protein concentration of 10 $mg \cdot mL^{-1}$. The myofibrillar suspension was stored in aliquots at −80°C.

For the isolation of SR vesicles, the final 9000 $g_{av}$ supernatant was centrifuged at 35000 $g_{av}$ for 30 min at 4°C. The supernatant was discarded and the pellet resuspended in 3 mL 0.6 M KCl, 20 mM MOPS, 1 mM DTT, pH 6.8, and again centrifuged at 35000 $g_{av}$. The purified SR vesicles were resuspended in 10 mM Tris, 0.3 M sucrose, 0.5 M KCl, and 1 mM DTT, pH 7.0 up to a protein concentration of 5–10 $mg \cdot mL^{-1}$. The SR vesicle suspension was stored in aliquots at −80°C. SR and myofibrillar protein (yields were approximately 1 and 20 mg protein/g myocardium, respectively) was determined with the method of Bradford [21].

### *Assay of $Mg^{2+}$-ATPase*

ATPase activities were determined by measuring the formation of $P_i$ according to the method of Lanzetta *et al.* [22]. Briefly, aliquots of the myofibrillar suspension were thawed and the glycerol-containing storage buffer removed by centrifugation for 15 min at 2000 $g_{av}$ (4°C). The myofibrillar pellet was washed twice with 60 mM KCl, 30 mM imidazole, and 2 mM $MgCl_2$, pH 7.0 and, finally, resuspended in a solution containing 60 mM KCl, 30 mM imidazole, 2 mM $MgCl_2$, 1 mM DTT, and 1.7 $mg \cdot mL^{-1}$ PMSF. SR vesicles (5 μg protein) and myofibrils (40 μg protein) were incubated at 30°C in a total volume of 200 μL solution containing 60 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, 25 mM MOPS, pH 7.0, 2 mM EGTA, 2 mM ATP, 5 mM $NaN_3$, 0.5 μM A23187, and various amounts of $Ca^{2+}$. Different levels of free $Ca^{2+}$ were achieved by varying the $Ca^{2+}$/EGTA ratio, thereby keeping the total EGTA concentration constant. Free $Ca^{2+}$ in the buffer was calculated using Fabiato's SPECS computer program [23] as described earlier [24].

### *Determination of Plasma Concentrations of [+]EMD 60263 in the* In Situ *Pig Model*

To 600 μL of plasma obtained from blood samples taken at various time points during the course of the previous *in vivo* experiments with [+]EMD 60263 in pigs [12], 500 μL of water-saturated ethylether were added and mixed well. The organic and aqueous phases were separated in an Eppendorf table centrifuge. The organic top layer was removed and collected in an Eppendorf vial. This extraction procedure was repeated 5 times. The ether phases were collected separately. Thereafter, the ether was evaporated in a speed-vac centrifuge. The residuals were resuspended and dissolved in 300 μL acetonitril. The amount of [+]EMD 60263 in a given plasma sample was determined on an HPLC system: 30 μL of the acetonitril solutions were injected on a LiChrosorb RP 8 (5 μm) RT 125-4 column (E. Merck, Darmstadt), which was equipped with a Hiber LiChroCart 4-4 precolumn (E. Merck, Darmstadt). The column was equilibrated and developed in a buffer composed of 35% acetonitril and 65% 0.1 M sodium phosphate, pH 6.0 at a flow rate of 1 mL/min. The elution was monitored at a wavelength of 320 nm. The concentration of [+]EMD 60263 was deduced from the area of the peaks eluting at the appropriate time from the column, by comparison with the values determined for identically treated standard samples. The plasma concentration of a given blood sample was determined by adding the peak areas of the various ether extraction samples.

### *Statistics*

The results are given as mean ± SEM. The pCa-$Mg^{2+}$ ATPase data were fitted to a sigmoid function by nonlinear regression analysis. The data, normalized to maximum activity after subtracting basal ATPase activity, were fitted to

the Hill equation ($P = P_o/(1 + Q/[Ca^{2+}]^n)$), in which $P_o$ is the maximal $Ca^{2+}$-stimulated $Mg^{2+}$-ATPase, $P$ the level of $Ca^{2+}$-stimulated $Mg^{2+}$-ATPase less than maximum, $Q$ a constant, and $n$ the Hill value as described [25, 26]. For the Hill equation, only data points were used that fulfilled the condition $0.1\ P_o \leq P \leq 0.9\ P_o$. The equation was solved for $P$, $Q$ and $n$. The $pCa_{50}$ (at 50% of the maximal $Ca^{2+}$-stimulated $Mg^{2+}$-ATPase activity) was determined by using the $n$ and $Q$ calculated from the Hill equation [25]. The pCa (i.e. $-\log[Ca^{2+}]$) corresponding to 50% activation of $Ca^{2+}$-stimulated $Mg^{2+}$-ATPase was $-(1/n)\log Q$. Data were evaluated for statistical significance by the Student's $t$-test and significance was accepted at $P < 0.05$. The $pCa_{50}$ shifts caused by the presence of [+]EMD 60263 in myofibrils isolated from the stunned and the adjacent nonstunned myocardium were assessed by two-way ANOVA with repeated measuring and Bonferroni's adjustment (BMDF, Statistical Software Inc).

## RESULTS

### *Characterization of the $Ca^{2+}$-Stimulated $Mg^{2+}$-ATPases in the Subcellular Fractions*

Before testing the effect of the EMD enantiomers on the $Ca^{2+}$ responsiveness of $Mg^{2+}$-ATPases of isolated SR and myofibrils, we characterized a specific property of the SR $Ca^{2+}$-pumping ATPase to exclude possible cross-contamination of the isolated myofibrils and SR fraction. Contamination of the myofibrils by SR membrane vesicles was unlikely because, before the last precipitation step during isolation, the myofibrils were always treated with 1% Triton X100. Thapsigargin, a specific inhibitor of the SR $Ca^{2+}$ pump, was tested on $Ca^{2+}$-stimulated $Mg^{2+}$-dependent ATPases of both subcellular fractions (Table 1). Thapsigargin (1 μM) completely blocked the $Ca^{2+}$-stimulated part of the ATPase activity in the SR fraction, whereas there was no effect on the $Ca^{2+}$-stimulated portion of the $Mg^{2+}$-ATPase activity of the myofibrils. The same results were obtained using the myofibrils isolated from human myocardium (unpublished observations). The results demonstrate that SR membrane impurities associated with myofibrils are efficiently eliminated by pretreatment with Triton X100 and that contamination of SR membrane by myofibrillar protein is also negligible.

### *Plasma Concentrations of [+]EMD 60263 at its Maximal Inotropic Effect in the* In Situ *Porcine Model*

Prior to studying the *in vitro* effects of [+]EMD 60263 on the myofibrillar and SR $Ca^{2+}$-stimulated, $Mg^{2+}$-dependent ATPases, we needed to know the plasma concentrations at which the compound was effective in our previous *in vivo* experiments [12]. When the effects of 2 consecutive doses of [+]EMD 60263 (0.75 and 1.5 $mg \cdot kg^{-1}$ intravenously, $n = 7$), administered at 15-min intervals, on segment shortening, external work, and mechanical efficiency in anaesthetized pigs were measured, plasma samples were taken for HPLC analysis of [+]EMD 60263 concentration. Each dose was infused over a 3-min period, and a second higher dose was infused 15 min later. The mean concentrations of [+]EMD 60263 at the end of the infusion period were 4.5 ± 0.4 μM (mean ± SEM, $n = 7$; lower dose) and 8.1 ± 2.0 μM (higher dose); they declined within 15 min to 0.14 ± 0.06 μM and 0.45 ± 0.12 μM, respectively. Thus, submicromolar plasma concentrations of [+]EMD 60263 were measured at times when myocardial contractility was determined and found to be elevated, which indicates that the drug may already be intracellularly effective at these low concentrations [12, 13].

**TABLE 1. Characteristics of $Ca^{2+}$-stimulated, $Mg^{2+}$-dependent ATPases of porcine ventricular sarcoplasmic reticulum (SR) membrane vesicles and myofibrils**

| | | $Mg^{2+}$-ATPase (nmol $P_i \cdot min^{-1} \cdot mg^{-1}$) | |
|---|---|---|---|
| | pCa | Control | + Thapsigargin |
| SR | 7 | 62 ± 17 | 55 ± 10 |
| | 5 | 430 ± 52 | 48 ± 3 |
| Myofibrils | 7 | 21 ± 2 | 24 ± 2 |
| | 5 | 42 ± 4 | 44 ± 4 |

$Mg^{2+}$-ATPase activities at pCa 7 of SR membrane vesicles and myofibrils are always close to the basal activity measured in the absence of $Ca^{2+}$ (compare Figs. 2 and 4) and can, therefore, be subtracted from the activity at pCa 5 to obtain the activities of $Ca^{2+}$-stimulated, $Mg^{2+}$-dependent ATPases. Thapsigargin concentration was 1 μM. Results are presented as mean ± SEM for 3 experiments with different preparations of SR membrane vesicles and myofibrils.

### In Vitro *Effects of EMD Enantiomers on Myofibrillar $Mg^{2+}$-ATPase Isolated from Normal Ventricle of Swine*

Figure 2 shows that [+]EMD 60263 increased the submaximally activated (at pCa 6.1) porcine cardiac myofibrillar $Mg^{2+}$-ATPase in a concentration range of 1–10 μM. The maximal effect was reached at drug concentrations ≥20 μM. The differential effects of [+]EMD 60263 on the $Ca^{2+}$ activation of porcine cardiac myofibrillar $Mg^{2+}$-ATPase are shown at 3 drug concentrations (1, 3, and 10 μM) in Fig. 3. The enhanced response to $Ca^{2+}$ involved 2 effects. First, a leftward shift of the relation between pCa and myofibrillar ATPase activity, indicated by the parameters listed in the legend to Fig. 3. The pCa at half-maximal activation ($pCa_{50}$) was shifted to the left by [+]EMD 60263, depending on its concentration (1, 3, and 10 μM), from 6.00 ± 0.05 under control conditions to 6.10 ± 0.08, 6.30 ± 0.03, and 6.67 ± 0.05, respectively ($P < 0.05$ vs control $pCa_{50}$ at 3 and 10 μM [+]EMD 60263. Second, an increase in maximal myofibrillar $Mg^{2+}$-ATPase activity was observed. This increasing effect on maximal $Mg^{2+}$-ATPase appears to be independent of the effect on increased $Ca^{2+}$ responsiveness because it was measured at saturating $Ca^{2+}$ concentrations; a change in $Ca^{2+}$ responsiveness of the myofilaments should be without effect. This increase in maximal $Mg^{2+}$ ATPase activity, however, seems to level off between 3 and 10 μM [+]EMD 60263, and the increase in $Ca^{2+}$ respon-

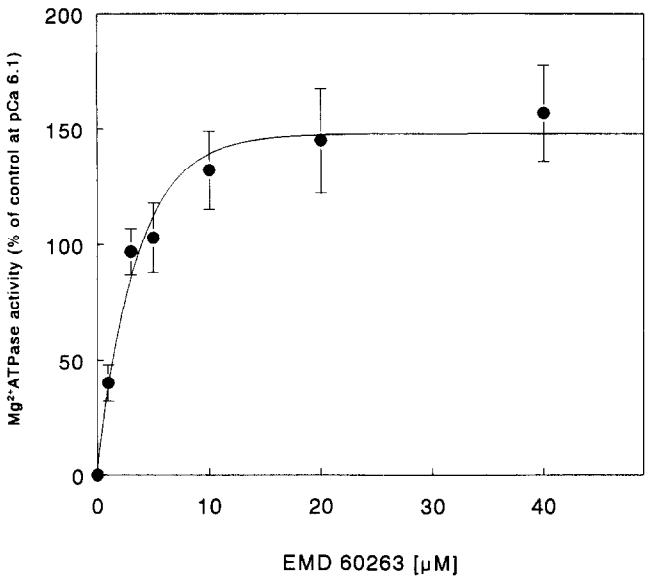


**FIG. 2. Effect of varying concentrations of [+]EMD 60263 on submaximally (pCa 6.1) activated $Mg^{2+}$-ATPase of porcine ventricular myofibrils. Stimulated ATPase activities in the presence of [+]EMD 60263 are expressed as % increase, taking the activity in the absence of the drug as 100%. See Materials and Methods for further details. Results are presented as mean ± SEM for 3 experiments with different preparations of myofibrils.**

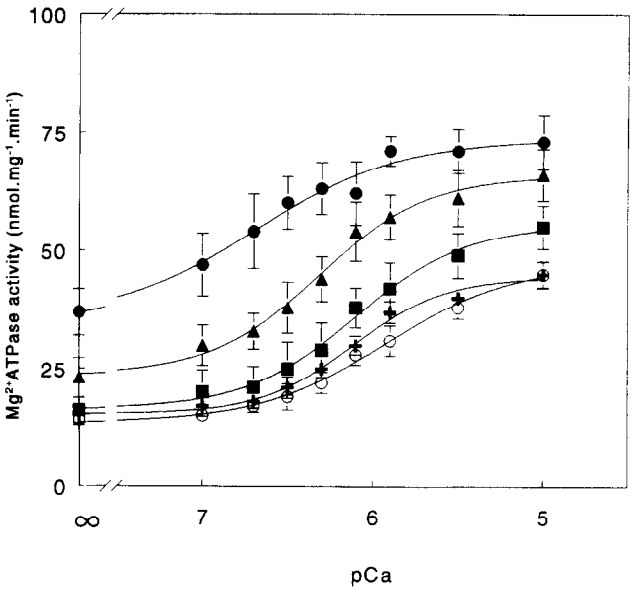


**FIG. 3. Graphs showing the relation between $pCa^{2+}$ (-log M) and $Mg^{2+}$-ATPase activity (in nmol $P_i$ • mg protein$^{-1}$ · min$^{-1}$) in porcine ventricle myofibrils under control conditions (○, $n$ = 6), in the presence of 1 μM (■, $n$ = 3), 3 μM (▲, $n$ = 3) and 10 μM [+]EMD 60263 (●, $n$ = 3) and in the presence of 10 μM [-]EMD 60264 (+, $n$ = 3). For further details, see Materials and Methods. Results are presented as mean ± SEM for 3 experiments with separate preparations of myofibrils. Data normalized to maximum activity after subtracting basal $Mg^{2+}$-ATPase activity were fitted to the Hill equation, giving the following parameters: under control conditions: $pCa_{50}$ = 6.00 ± 0.05 ($n$ = 1.46 ± 0.12); in the presence of 1, 3 and 10 μM [+]EMD 60263: 6.10 ± 0.08 ($n$ = 1.32 ± 0.09), 6.30 ± 0.03 ($n$ = 1.23 ± 0.12) and 6.67 ± 0.05 ($P < 0.05$ vs control $pCa_{50}$ at 3 and 10 μM [+]EMD concentration) ($n$ = 1.19 ± 0.18), respectively; in the presence of 10 μM [-]EMD 60264: $pCa_{50}$ = 6.10 ± 0.04 ($n$ = 1.44 ± 0.25).**

siveness continued to rise, as can be seen from the leftward and upward displacement of the $Mg^{2+}$ ATPase/$pCa^{2+}$-curve in comparison to the control curve. The average Hill coefficients, reflecting the steepness of the fitted curves at $pCa_{50}$, remained the same at each concentration of [+]EMD 60263 tested (legend to Fig. 3). The negative enantiomer EMD 60264 (10 μM) had no significant effect on the $Ca^{2+}$-activation pattern of the porcine cardiac myofibrillar $Mg^{2+}$-ATPase (Fig. 3).

In comparison, Fig. 4 shows the effects of the known optical isomers [+]EMD 57033 and [-]EMD 57439 on porcine heart myofibrillar $Ca^{2+}$-stimulated $Mg^{2+}$-dependent ATPase. Similar to [+]EMD 60263 (10 μM), but to a somewhat lesser extent, [+]EMD 57033 (10 μM) produced a leftward shift ($pCa_{50}$ changed from 6.04 ± 0.07 under control conditions to 6.55 ± 0.04; $P < 0.05$ vs control $pCa_{50}$, $n$ = 3) of the $Ca^{2+}$ activation curve of myofibrillar $Mg^{2+}$-ATPase without changing the Hill coefficient. [+]EMD 57033 (10 μM) also stimulated, although less potently than [+]EMD 60263, maximal $Mg^{2+}$-ATPase activity of the myofibrils. As expected, the negative enantiomer EMD 57439 had no effect on the $Ca^{2+}$ activation pattern of myofibrillar $Mg^{2+}$-ATPase (Fig. 4).

### In Vitro *Effect of* [+]*EMD 60263 on SR* $Mg^{2+}$-*ATPase From Normal Ventricle of Swine*

$Ca^{2+}$-stimulated $Mg^{2+}$-dependent ATPase was measured in SR vesicles isolated from porcine ventricle. In the assay of the ATPase $Ca^{2+}$ ionophore, A23187 (0.5 μM) was always present to uncouple the vesicular $Ca^{2+}$ uptake process from ATP hydrolysis, thereby preventing possible inhibitory effects of the built-up electrochemical $Ca^{2+}$ gradient on the $Ca^{2+}$-stimulated, $Mg^{2+}$-dependent ATPase of the SR, as we observed previously [18, 27]. In contrast to its dramatic effects on myofibrillar ATPase, [+]EMD 60263 (10 μM) neither affected $pCa_{50}$ nor the maximum $Ca^{2+}$-stimulated $Mg^{2+}$-ATPase of the SR (Fig. 5).

### In Vitro *Effect of* [+]*EMD 60263 on the* $pCa/Mg^{2+}$-*ATPase Activity Relationships of Myofibrils Isolated from Stunned and Nonstunned Myocardium*

Previously, we showed that [+]EMD 60263 increased systolic segment shortening of both stunned and nonstunned porcine myocardium, although the effect on the stunned was more pronounced than on the nonstunned myocardium [12]. Therefore, 4 experiments were carried out with anesthetized open-chest pigs, in which the distribution territory of the LADCA was stunned by 2 sequences of 10-min coronary artery occlusion and 30-min reperfusion. The results of the individual experiments, including the mean data, are

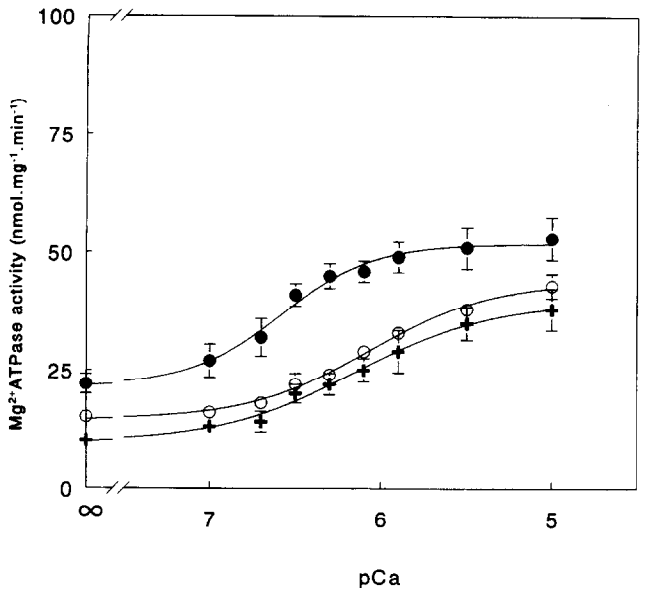


**FIG. 4. Graphs showing the relation between $pCa^{2+}$ (−log M) and $Mg^{2+}$-ATPase activity (in nmol $P_i \cdot mg\ protein^{-1} \cdot min^{-1}$) in porcine ventricle myofibrils under control conditions (○) and in the presence of 10 μM EMD 57033 (●) and 10 μM [−]EMD 57439 (+). See Materials and Methods for further details. Results are presented as mean ± SEM for 3 experiments with different preparations of myofibrils. Data normalized to maximum activity after subtracting basal $Mg^{2+}$-ATPase activity were fitted to the Hill equation, giving the following parameters: under control conditions: $pCa_{50}$ = 6.04 ± 0.06, $n$ = 1.35 ± 0.08; in the presence of 10 μM [+]EMD 57033: $pCa_{50}$ = 6.55 ± 0.04 ($P < 0.05$ vs control $pCa_{50}$), $n$ = 1.35 ± 0.09; in the presence of 10 μM [−]EMD 57439: $pCa_{50}$ = 6.11 ± 0.03; $n$ = 1.23 ± 0.25.**

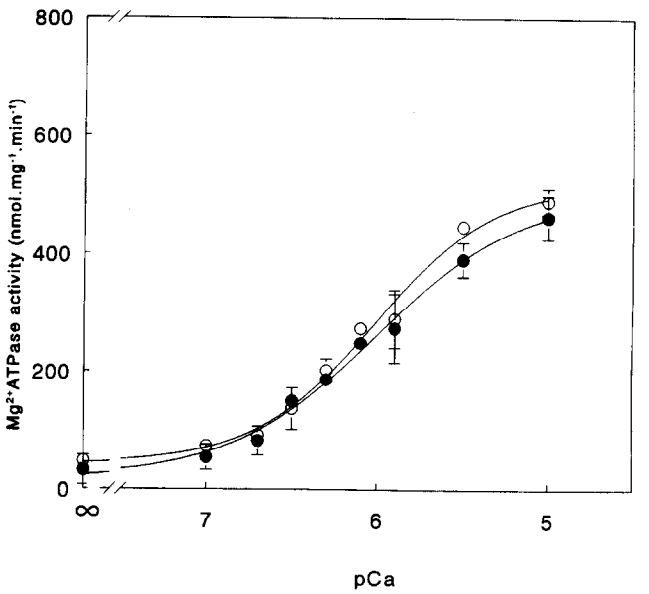


**FIG. 5. Graphs showing the relation between $pCa^{2+}$ (−log M) and $Mg^{2+}$-ATPase activity (in nmol $P_i \cdot mg\ protein^{-1} \cdot min^{-1}$) in porcine ventricular SR under control conditions (○) and in the presence of 10 μM [+]EMD 60263 (●). See Materials and Methods for further details. Results are presented as mean ± SEM for 3 experiments with different preparations of SR membrane vesicles.**

shown in Table 2. The normalized pCa/$Mg^{2+}$-ATPase activity curves, obtained from the myofibrils isolated from the stunned and nonstunned myocardium, were fitted to the Hill equation ($P = P_o/(1 + Q/[Ca^{2+}]^n)$), and the pCa corresponding to 50% activation of $Ca^{2+}$-stimulated $Mg^{2+}$-ATPase was calculated from $(-1/n)\log Q$. No differences in the maximal activity of $Ca^{2+}$-stimulated $Mg^{2+}$-ATPase were observed between nonstunned and stunned myocardium. (Table 2). In 3 of the 4 pigs, a rightward shift of the pCa/$Mg^{2+}$-ATPase activity curve of stunned myocardium was observed, which followed from the decrease in $pCa_{50}$ (Table 2). More experiments have to be carried out to establish whether or not the tendency of myofibrils from stunned myocardium to desensitize to $Ca^{2+}$ is a reproducible finding. No differences were seen between stunned and nonstunned myocardium in the increasing effect of [+]EMD 60263 on maximal $Ca^{2+}$-stimulated $Mg^{2+}$-dependent ATPase. However, the compound induced a leftward shift of the pCa/$Mg^{2+}$-ATPase activity curve in each of the pigs ($\Delta pCa_{50}$ = + 0.57 ± 0.04 in stunned versus $\Delta pCa_{50}$ = +0.42 ± 0.05 in nonstunned myocardium). The latter finding is consistent with our previous observations on the effects of [+]EMD 60263 on systolic segment shortening in the *in situ* porcine model.

### In Vitro *Effects of* [+]*EMD 60263 on the Myofibrillar* $Mg^{2+}$-*ATPase of Human Hypertrophied Septum*

Because no data were available on the effects of [+]EMD 60263 on human myocardium, we tested the drug on myofibrils isolated from the hypertrophic septum of cardiomyopathic patients (Fig. 6). Similar to its effect on porcine cardiac myofibrils, the $pCa_{50}$ shifted markedly to the left by 10 μM [+]EMD 60263, from 6.06 ± 0.09 under control conditions to 6.53 ± 0.10 ($P < 0.05$ vs control $pCa_{50}$). [+]EMD 60263 (10 μM) also increased the maximally activated $Ca^{2+}$-stimulated $Mg^{2+}$-ATPase of isolated human myofibrils.

## DISCUSSION

The thiadiazinone [+]EMD 60263 has been described as the first $Ca^{2+}$-sensitizing agent devoid of PDE III inhibitory activity [28]. In chemically skinned ventricular fibers of pig heart, [+]EMD 60263 (3 μM) shifted the $EC_{50}$ of $Ca^{2+}$ for the contractile activation from 2.41 mM to 0.73 mM, whereas the optical isomer [−]EMD 60264 was ineffective [28]. The concentrations ($IC_{50}$) of [+]EMD 60263 and [−]EMD 60264 required to reach half-maximal inhibition of PDE III activity of guinea-pig ventricles are >30 and 2 μM, respectively (N. Beier, personal communication). The results of the present experiments also provide evidence for a direct stereoselective activating effect of [+]EMD 60263 on $Ca^{2+}$-stimulated myofibrillar actomyosin $Mg^{2+}$-ATPase of pig and human myocardium. There were two effects of [+]EMD 60263: (1) the relation between pCa and actomyo-

**TABLE 2. *In vitro* effects of 3 μM [+]EMD 60263 on the $pCa^{2+}/Mg^{2+}$ ATPase activity relationship measured in myofibrils isolated from stunned and nonstunned myocardium of open-chest anesthetized pigs**

| | Maximal $Ca^{2+}$-stimulated $Mg^{2+}$ ATPase activity (nmol $P_i \cdot mg^{-1} \cdot min^{-1}$) | | | | | | $pCa_{50}$ (-log M) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Stunned | | | Nonstunned | | | Stunned | | | Nonstunned | | |
| Pig no. | -EMD | +EMD | ΔATPase | -EMD | +EMD | ΔATPase | -EMD | +EMD | $\Delta pCa_{50}$ | -EMD | +EMD | $\Delta pCa_{50}$ |
| 1 | 35 | 56 | +21 | 23 | 49 | +26 | 5.84 | 6.35 | +0.49 | 5.94 | 6.30 | +0.36 |
| 2 | 29 | 47 | +18 | 37 | 49 | +12 | 5.73 | 6.41 | +0.68 | 5.83 | 6.34 | +0.51 |
| 3 | 41 | 45 | +4 | 40 | 44 | +4 | 6.10 | 6.67 | +0.57 | 6.09 | 6.56 | +0.49 |
| 4 | 43 | 50 | +7 | 38 | 47 | +9 | 6.09 | 6.64 | +0.55 | 6.18 | 6.51 | +0.33 |
| mean | 37 | 50 | +13 | 35 | 47 | +13 | 5.94 | 6.52 | +0.57* | 6.01 | 6.43 | +0.42 |
| SEM | 3 | 2 | 4 | 4 | 1 | 5 | 0.09 | 0.08 | 0.04 | 0.08 | 0.06 | 0.05 |

Stunned and nonstunned myocardium was obtained from 4 anesthetized open-chest pigs in which the distribution territory of the LADCA was stunned by 2 sequences of 10-min coronary artery occlusion and 30-min reperfusion. The nonstunned myocardium was obtained from the distribution territory of the LCXCA, which was not occluded. The normalized $pCa/Mg^{2+}$ ATPase activity curves in the absence and presence of 3 μM [+]EMD 60263, measured at the pCa values as these were also chosen in Figs. 3, 4 and 6, were fitted to a sigmoid function by nonlinear regression analysis. The ATPase activities normalized to maximum activity were fitted to the Hill equation as described in Methods, and when analyzed gave the calculated $pCa_{50}$ values. *Significantly different ($P < 0.05$) from the [+]EMD 60263-induced $\Delta pCa_{50}$ in the nonstunned myocardium.

sin $Mg^{2+}$-ATPase activity was shifted leftward, indicative of the increased responsiveness of $Mg^{2+}$-ATPase to $Ca^{2+}$; (2) maximal $Ca^{2+}$-stimulated $Mg^{2+}$-dependent ATPase was increased, pointing towards a drug action on the kinetics of ATPase. Analogous results were obtained using the [+] and [-] enantiomers of EMD 53998. The present observations with [+]EMD 57033 and [-]EMD 57439 on pig and human

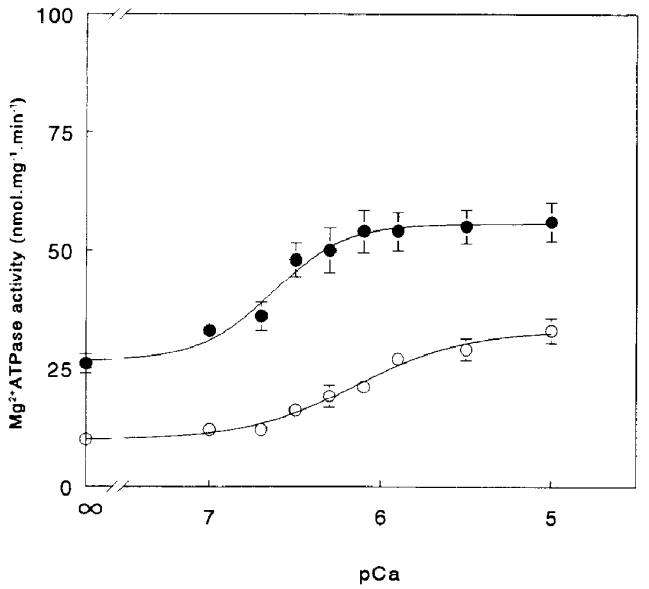


**FIG. 6. Graphs showing the relation between $pCa^{2+}$ (-log M) and $Mg^{2+}$-ATPase activity (in nmol $P_i \cdot mg$ $protein^{-1} \cdot min^{-1}$) in myofibrils isolated from human hypertrophic septum specimen measured under control conditions (○) and in the presence of 10 μM [+]EMD 60263 (●). See Materials and Methods for further details. Results are presented as mean ± SEM for 5 experiments with different preparations of myofibrils. Data normalized to maximum activity after subtracting basal $Mg^{2+}$-ATPase activity were fitted to the Hill equation, giving the following parameters: under control conditions: $pCa_{50}$ = 6.06 ± 0.09, $n$ = 1.55 ± 0.22; in the presence of [+]EMD 60263: $pCa_{50}$ = 6.53 ± 0.10 ($P < 0.05$ vs control $pCa_{50}$), $n$ = 1.61 ± 0.31.**

cardiac myofilaments are in agreement with those obtained in myofibrillar preparations from guinea pig heart [11] and canine ventricle [29]. In the former reports, changes in steepness of the $pCa/Mg^{2+}$-ATPase curves were not observed at 10 μM [+]EMD 57033, but at 30 μM [11, 29]. Likewise, in the present study, Hill coefficients reflecting the slopes of the fitted activation curves at $pCa_{50}$ were neither changed by 10 μM [+]EMD 57033 nor by 10 μM [+]EMD 60263. The results provide evidence for two stereoselective effects of the thiadiazinones on the contractile apparatus of heart muscle: First, $Ca^{2+}$ sensitization of myofilaments and, second, an increase in kinetics of actomyosin $Mg^{2+}$-ATPase. The stereoselectivity of the effects of the thiadiazinones provides strong evidence that these agents affect a specific domain important in determining the state of activation of the myofilaments. Initially, it was believed that the mechanism of $Ca^{2+}$ activation of cardiac actomyosin $Mg^{2+}$-ATPase by racemic EMD 53998 might involve an effect on $Ca^{2+}$ binding to troponin C [10, 30–32]. In subsequent studies, however, Solaro *et al.* demonstrated that $Ca^{2+}$ sensitization by [+]EMD 57033 does not appear to involve the binding of $Ca^{2+}$ to troponin C but, rather a myofilament domain other than troponin C itself [29]. The studies of Solaro *et al.* [29] on desensitized myofibrils and on preparations of pure myosin and actin filaments provide evidence that [+]EMD 57033 acts by stimulating the turnover of the actin-crossbridge reaction. In *in vitro* motility assays performed by Solaro *et al.* [29], monomeric cardiac myosin were adhered to nitrocellulose-coated glass coverslips. Unregulated actin filaments were allowed to interact with the myosin-coated surface in the presence of $Mg^{2+}$-ATP and various concentrations of [+]EMD 57033. The velocity of the actin motion significantly increased with increasing concentrations of [+]EMD 57033, but [-]EMD 57439 had no effect. This proved the effect to involve the turnover of the cyclic cross-bridge. There have been questions as to how the effects of [+]EMD 57033 on the kinetics of actomyosin interaction relate to the drug's $Ca^{2+}$-sensi-

tizing activity [29]. The relation between the two activities is indicated by the same stereoselectivity of the two effects. Leijendekker and Herzig [33] estimated turnover rates of cross-bridges by biochemical ($Mg^{2+}$-ATPase activity) and mechanical (tension development) characteristics of skinned porcine right ventricle. They concluded that the turnover rate of the myocardial cross-bridges was reduced in the presence of the racemate EMD 53998 at low (pCa $\geqslant$ 6.25), but not at high, $Ca^{2+}$ (pCa $\leqslant$ 5.85).

The relatively long-lasting myocardial contractile dysfunction after brief periods of ischemia has been termed stunning. The molecular mechanism of this phenomenon is still poorly understood, but possible candidates are a decreased $Ca^{2+}$ delivery to and a decreased $Ca^{2+}$ responsiveness of the myofilaments [15, 34–36]. Recent data from our laboratory in a model of stunned porcine myocardium have shown that the rate of SR calcium uptake increases slightly [16, 36]. Therefore, it is unlikely that a change in active $Ca^{2+}$ transport by the SR is the principal cause of contractile dysfunction of stunned myocardium. Other reports have demonstrated that the intracellular $Ca^{2+}$ transient remains the same in stunned myocardium [34, 35]. Korbmacher *et al.* [36] performed experiments investigating the effects of [+]EMD 57033 on isolated stunned rabbit hearts and showed that the thiadiazinone derivative acted as a potent positive inotropic agent. Recently, we reported on the *in vivo* cardiovascular effect of [+]EMD 60263 in pigs with or without stunned myocardium [12]. In these studies, the effect of [+]EMD 60263 on regional myocardial function was assessed by studying the systolic segment shortening of stunned and nonstunned myocardial segments. It was shown that [+]EMD 60263 increased systolic segment shortening of both the stunned and nonstunned myocardium, and that the effect on stunned was much more pronounced than on nonstunned myocardium. No effects were observed with [−]EMD 60264 (unpublished observations). Consistent with the observations of our previous *in vivo* experiments [12], is the present finding that in stunned compared to nonstunned myocardium, the $Ca^{2+}$ sensitizing effect of [+]EMD 60263 on isolated myofibrils was potentiated. Moreover, in 3 of 4 pigs we found a slight desensitization to $Ca^{2+}$ in the stunned myocardium. These slight decreases in $pCa_{50}$ observed, apparently caused by stunning, might be related to the more pronounced positive shifts of $pCa_{50}$ produced by [+]EMD 60263 in stunned compared to nonstunned myocardium. A recent study determined the $Ca^{2+}$ sensitivity of isometric tension of skinned myocytes obtained from endomyocardial biopsies taken from the LADCA-perfused bed (preischemic versus stunned myocardium) of anesthetized open-chest pigs [37]. After more severe ischemia, a reduction in myofilament $Ca^{2+}$ sensitivity of the isolated skinned myocytes was found, in agreement with our *in vitro* observations comparing the $Ca^{2+}$ sensitivities of the $Mg^{2+}$-ATPase of myofibrils from stunned and nonstunned myocardium. Moreover, it was concluded in this report that not only the decreased $Ca^{2+}$ sensitivity of the myofilaments, but also the decreased cycling rates of the cross-bridges likely form the basis of stunning [37]. Similar results were recently obtained with ventricular trabeculae from control and stunned rat myocardium [38].

At present, we report the plasma concentrations at which [+]EMD 60263 was effective in one of our previous *in vivo* studies [12]. The effects of intravenous [+]EMD 60263 infusions (0.75 and 1.50 $mg \cdot kg^{-1}$, $n = 7$) on myocardial functions (systolic segment shortening, external and mechanical efficiency) were determined 15 min after drug infusion when the plasma concentrations of [+]EMD 60263 were 0.2 and 0.5 μM, respectively. The stereoselective *in vitro* effect of [+]EMD 60263 on the $Ca^{2+}$ responsiveness of the $Mg^{2+}$-ATPase of myofibrils isolated from either normal, nonstunned, or stunned porcine myocardium became evident at concentrations (1–3 μM) that appeared to be close to the plasma concentrations required for the maximum increase in systolic segment shortening of stunned and nonstunned myocardial segments [12]. It is also noteworthy that we observed no effects of [+]EMD 60263 on the $Ca^{2+}$ responsiveness of the $Mg^{2+}$-ATPase of isolated porcine cardiac SR membrane vesicles, implying that the SR is not involved in the positive inotropic action of [+]EMD 60263 on swine myocardium. Moreover, by the present data, additional support [12, 13] is provided for the hypothesis that a decreased sensitivity of the myofilaments to $Ca^{2+}$ and not a decreased SR $Ca^{2+}$ pumping activity is involved in the mechanism of stunning [15, 16, 34, 35].

[+]EMD 60263 not only caused a leftward shift in the myofibrillar $pCa/Mg^{2+}$ ATPase relationship but also stimulated the maximal $Ca^{2+}$-stimulated $Mg^{2+}$-ATPase (Figs. 3 and 4; Table 2). Thus, even at the lowest $Ca^{2+}$ concentrations, myofibrillar $Mg^{2+}$-ATPase is still slightly increased by [+]EMD 60263; this may indicate the occurrence of relaxation disturbances *in vivo*. We have reported on the diastolic effects of [+]EMD 60263 in stunned porcine myocardium [39]. A bolus injection of 1.5 mg/kg returned systolic segment shortening to baseline values but had no effect on the diastolic segment function. When the dose was increased to 3.0 mg/kg, systolic segment shortening in both stunned and nonstunned regions increased beyond the baseline value. Diastolic segment lengthening which, in control animals and at lower doses of [+]EMD 60263, started to lengthen immediately after segment shortening reached minimal length, was delayed at high doses. The mean velocity of segment lengthening was unchanged compared to the baseline. However, these *in vivo* effects are observed at lower plasma concentrations of [+]EMD 60263 (approximately <1 μM) than the concentrations to which the myofibrils were directly exposed to the *in vitro* experiments. Recently, Sunderdick *et al.* [40] compared the effects of $Ca^{2+}$ sensitizers [+]EMD 60263 and [+]EMD 57033 in isolated blood-perfused rabbit heart. They used similar doses as our *in vivo* studies and reported that at the relatively high dose of 10 μM, [+]EMD 60263 had major detrimental effects on relaxation and contractile function, but not at the lower dose of 3 μM.

In summary, the results show that [+]EMD 60263, at

concentrations (1–3 μM) close to the plasma concentrations found to be effective in the *in situ* porcine model, sensitizes $Mg^{2+}$-ATPase of isolated porcine and diseased human cardiac myofibrils to $Ca^{2+}$ and has no effect on the $Ca^{2+}$-stimulated $Mg^{2+}$-ATPase of isolated porcine cardiac SR membrane vesicles. We conclude, from these data, that the positive inotropic action of the thiadiazinone [+]EMD 60263 in the *in situ* porcine model is primarily due to myofilament sensitization to $Ca^{2+}$ and that this compound may have a similar action on the diseased human myocardium.

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