Novel Diazinone Derivatives Separate Myofilament Ca\textsuperscript{2+} Sensitization and Phosphodiesterase III Inhibitory Effects in Guinea Pig Myocardium

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The inotropic state of the myocardium can be enhanced via an increase in cell Ca\textsuperscript{2+} loading or in myofilament responsiveness to Ca\textsuperscript{2+}. Although different pharmacological agents combine these properties, no presently available drug acts predominantly as a myofilament sensitizer in situ. We have investigated the effects and the mechanism of action of novel diazinone derivatives, EMD 54622, EMD 53998, and EMD 54650 (developed by E. Merck, Darmstadt), on guinea pig myocardial preparations. Force- and ATPase-pCa relations in skinned fibers show differing potencies of these agents on myofilament sensitization: EMD 54622 > EMD 53998 > EMD 54650. This is in contrast to their relative potencies to inhibit isolated myocardial phosphodiesterase III: EMD 54650 > EMD 53998 > EMD 54622. In isolated hearts studied at constant coronary flow, each of the three diazinone derivatives had a positive inotropic effect. In enzymatically dissociated left ventricular myocytes loaded with the Ca\textsuperscript{2+} probe Indo-1, the positive inotropic effect of EMD 54622 occurred with no change in the amplitude of the cytosolic [Ca\textsuperscript{2+}] (Ca\textsubscript{c}) transient. In contrast, both EMD 53998 and EMD 54650 enhanced Ca\textsubscript{c} transient and twitch contraction amplitudes. Length–indo-1 fluorescence relations were analyzed to determine the effects of the three substances on myofilament responsiveness to Ca\textsuperscript{2+}. EMD 54650 had no effect on myofilament responsiveness to Ca\textsuperscript{2+}. Less uniform results were obtained with EMD 53998 (in two of five cells the myofilament responsiveness to Ca\textsuperscript{2+} was increased, whereas in three other cells it was unaltered). Our results indicate that structural changes in the diazinone molecule shift the mechanism of action for the positive inotropic effect of the diazinone derivatives in the intact cell from a predominant myofilament sensitization (EMD 54622) to an enhancement in cell Ca\textsuperscript{2+} loading and an augmentation in the Ca\textsubscript{c} transient (EMD 54650). (Circulation Research 1992;70:1081–1090)

KEY WORDS • cardiotonic agents • diazinone • calcium • Ca\textsuperscript{2+} sensitization • phosphodiesterase III

The two general mechanisms through which the contractility of the myocardium can be augmented are an increase in the amplitude of the cytosolic [Ca\textsuperscript{2+}] (Ca\textsubscript{c}) transient that causes the contraction and an increase in myofilament responsiveness to Ca\textsuperscript{2+}. An increase in myofilament Ca\textsuperscript{2+} sensitivity is related, in part, to an enhancement in the Ca\textsuperscript{2+} binding to myofilaments and in myofibrillar ATPase activity for a given [Ca\textsuperscript{2+}].2-4 This mechanism has the appealing property of a positive inotropic action that is not associated with the adverse sequelae of excessive cell Ca\textsuperscript{2+} loading, i.e., a decrease in systolic function, an increase in diastolic tonus,5 and the occurrence of arrhythmias.6-8 However, an increased myofilament Ca\textsuperscript{2+} sensitivity has the potential to prolong the time course of the twitch and to impair diastolic filling of the heart.9

Thus, studies of the effects of inotropic substances in skinned myocardial preparations and myocardial homogenates, in which it is possible to measure the force- and ATPase–pCa relations, should be complemented by measurements of the response of intact myocardial preparations.

The other mechanism to augment the contractility of the heart is an increase in Ca\textsuperscript{2+} entry into myocardial cells. One mode of enhanced cell Ca\textsuperscript{2+} loading occurs via an increase in cAMP concentration. Isoproterenol as well as other ß1-adrenergic receptor agonists increases cAMP, which leads to an increase in the transsarcolemmal Ca\textsuperscript{2+} current10-12 and increases sarcoplasmic retic-
ulum Ca\(^{2+}\) uptake.\(^{13,14}\) Thus, more Ca\(^{2+}\) is available for release from the sarcoplasmic reticulum with each subsequent action potential. The intracellular concentration of cAMP can be augmented not only by stimulating its production but also by preventing its degradation by phosphodiesterases (PDEs). Different types of PDE have been identified,\(^{15-18}\) and the inhibition of high-affinity cAMP–PDE III has been associated with effects on the amplitude and time course of the contraction and of the Ca\(^{2+}\) transient similar to those of \(\beta\)-adrenergic receptor agonists.\(^{19,20}\)

From experiments on skinned myocardial fibers, it is also apparent that sulmazole, a PDE III inhibitor, also sensitizes myofilaments to Ca\(^{2+}\).\(^{4}\) However, the predominant effect of this agent in situ is the inhibition of PDE III; thus, in intact myocardial preparations, the myofilament-sensitizing property of this drug becomes apparent only at high concentrations that exceed those required for an increase in the amplitude of the Ca\(^{2+}\) transient.\(^{21}\)

Novel diazinone derivatives (developed by Pharmaceutical Research, E. Merck, Darmstadt, FRG) vary widely in their potencies to sensitize myofilaments to Ca\(^{2+}\) and increase cAMP via their PDE III inhibitory action. We have recently reported on the effect of one of these substances, EMD 53998, in intact myocardial preparations from the rat.\(^{22,23}\) In the absence of a concomitant intervention that raises cAMP, this species is not sensitive to the effect of PDE III inhibitors.\(^{20}\) Thus, under the conditions of this study, EMD 53998 behaved as a myofilament Ca\(^{2+}\) sensitizer, and its positive inotropic action was not associated with an increase in the amplitude of the Ca\(^{2+}\) transient. In the present study we have measured the effects of EMD 53998 and of two related compounds, EMD 54622 and EMD 54650, on guinea pig myocardial preparations, which, unlike those from the rat, in the absence of \(\beta\)-adrenergic stimulation respond to PDE III inhibitors with an enhanced contraction and abbreviation of the time course of the twitch.

We have measured the effect of the three substances on force– and ATPase–pCa curves of skinned fibers and on the inhibition of separated cardiac PDE III. Additionally, we have examined their action in isolated guinea pig hearts and isolated left ventricular myocytes. Cardiac cells were loaded with the Ca\(^{2+}\) probe indo-1 to simultaneously monitor twitch contraction and indo-1 fluorescence, which is taken as an index of Ca\(^{2+}\). Thus, it could be determined whether the effects of the substances under study on contraction were mediated by changes in the Ca\(^{2+}\) transient, by altered myofilament responsiveness to Ca\(^{2+}\), or by both. Of the three substances, EMD 54650, which is a potent PDE III inhibitor, increased the amplitude and reduced the time course of the Ca\(^{2+}\) transient and twitch in intact myocytes. In contrast, EMD 54622 is the most potent myofilament Ca\(^{2+}\) sensitizer in skinned preparations; it increased the amplitude of the twitch contraction without enhancing the Ca\(^{2+}\) transient or abbreviating the time course of the twitch or indo-1 transient. Thus, in intact myocytes, EMD 54622 appears to have predominantly myofilament-sensitizing properties. The third substance, EMD 53998, which behaved as a myofilament sensitizer in a prior study with intact myocardial preparations from the adult rat heart,\(^{22}\) had effects on the guinea pig myocar-
EDTA 1, and DTE 1, pH 7.0. Myofibrillar concentration of the pellet was determined using the method of Bradford and Spector. The ATPase activity was determined by measuring the release of inorganic phosphate according to Lancet et al. The myofibrillar preparations were incubated at 30°C in a total of 250 μl in (mM) KCl 140, PIPES 20 (pH 7.0), EGTA 5.5, MgCl₂, 4, and K₂ATP 2, along with various amounts of CaCl₂ (ionic strength, 0.2 M). The diazinoines were dissolved in 1,2-propanediol; water was added to reduce the solvent concentration to 10%. The stock solutions were diluted 2.5-fold in the incubation assay; the final 1,2-propanediol concentration was 4% in the assay. Different levels of free Ca²⁺ were achieved by varying the Ca²⁺/EGTA ratio, keeping the total EGTA concentration constant. The free Ca²⁺ concentrations in the incubation buffers at levels higher than 0.1 μM were determined with a calcium-selective electrode (Radiometer, Denmark); those below 0.1 μM were calculated according to Thomas by using an apparent calcium-binding constant for ETGA, which was determined according to Harrison and Bers. The concentration range of free Ca²⁺ was between 0.01 and 30 μM. The ATPase activity was expressed as percentage of the maximal ATPase activity of the control incubations at saturating Ca²⁺ concentrations. The data were fitted to a sigmoid function by a nonlinear regression analysis.

**Experiments With Isolated Isoenzymes of PDE III**

cGMP-inhibited PDE (PDE III) was isolated from ventricular heart muscle of guinea pig, and its inhibition was determined as described. The test compounds (40 mM Tris-HCl, 50 mM MgCl₂, and 10 mM EGTA) were preincubated for 5 minutes with the PDE III isozyme at 37°C, pH 8.0. The reaction was started by the addition of 0.3 mM 2,8-[³H]cAMP. After 20 minutes at 37°C, the reaction was stopped by heating to 90°C. After treatment with a phosphatase of the venom of the king cobra, the product [³H]adenosine was separated by chromatography (anion exchanger Dowex 1 x 2) and quantified in a scintillation counter. Each determination was performed in triplicate.

**Experiments With Isolated Guinea Pig Hearts**

Isolated hearts from adult guinea pigs were retrogradely perfused at a constant flow rate (15 ml/min) with a solution of the following composition (mM): NaCl 137, KCl 5, MgSO₄ 1.2, NaH₂PO₄ 1.2, dextrose 15, HEPES 20, and CaCl₂ 1.0 (pH 7.4, 23°C). Propranolol (1 μM) was used to block the effect of endogenous catecholamines that could be released from nerve endings during electrical stimulation. Atroventricular block was effected via mechanical compression of the atroventricular nodal region, and the hearts were paced at 1 Hz via bipolar electrodes attached to the right ventricular apex. Isovolumic contractile function of the left ventricle was monitored via a balloon attached to a pressure transducer. End-diastolic pressure was set at approximately 10 mm Hg.

**Experiments With Isolated Left Ventricular Myocytes**

Left ventricular myocytes were dissociated from adult guinea pig hearts via retrograde perfusion of the aorta with a bicarbonate buffer containing collagenase and 50 μM bathing Ca²⁺ (Caₐ) according to a technique previously described. Cells loaded with the ester derivative of indo-1 (AM form) were placed in a chamber on the stage of a modified inverted microscope in which contractility and indo-1 fluorescence were monitored simultaneously as described in detail elsewhere. Myocytes were field-stimulated at 1 Hz via platinum electrodes in the bathing medium and were superfused with HEPES buffer similar to that used for the isolated heart, but without propranolol. Indo-1 fluorescence was excited by epi-illumination with 10-μsec flashes of 350±5 nm light at repetition rates of 200 Hz. The emitted light was collected by paired photomultipliers to measure simultaneously spectral windows of 391–434 and 465–507 nm. The fluorescence emission from each flash was collected by a pair of fast integrator sample-and-hold circuits under the control of a VAX 11/730 computer, which calculated the ratio of indo-1 emission at the two wavelengths as an index of Caₐ. Cell length was measured from the bright-field image of the cell by edge tracking with a photodiode array. By using red light (650–750 nm) for the bright-field image and a dichroic mirror to transmit the fluorescent light and reflect the red light, length and Caₐ measurements were obtained simultaneously without cross talk. Experiments with single cells were performed at room temperature to minimize loss of indo-1 from the myocytes. In similar experiments with non–indo-1-loaded cells, only length was monitored.

**Results**

**Effect on Force–pCa and ATPase–pCa Relations**

Figure 1 shows the structure of the three diazinoine derivatives used for this study. Figures 2 and 3 show, respectively, the effect of 10 μM of each of these substances on isometric force development in skinned fibers and on ATPase activity in myofibrillar preparations. The thiadiazinoines EMD 54622 and EMD 53998 induced a leftward shift of the force– and ATPase–pCa curves, which indicates an increase in the calcium responsiveness of the myofilaments. The Ca²⁺ concentrations required for half-maximal force development (Table 1) and ATPase activity (Table 2) were decreased by 0.6 and 0.4 pCa units, respectively, by EMD 54622 and by 0.2 and 0.3 pCa units, respectively, by EMD 53998. Furthermore, maximal calcium-activated force was increased by 32% and 18% by EMD 54622 and EMD 53998, respectively. Both compounds also enhanced maximal ATPase activity by 76% and 47%, respectively. In contrast, 10 μM of the diazinoine EMD 54650 had no or only minor effects on force and ATPase activity, and a significant leftward shift of the force–pCa curve was obtained only at concentrations >100 μM (data not shown). Thus, the order of potency for the increase in Ca²⁺ responsiveness of the myofilaments is EMD 54622>EMD 53998>EMD 54650.

**Effect on PDE III Inhibition**

The PDE III inhibitory properties of these substances is presented in Figure 4 and Table 3. The rank order of increase in the myofilament-sensitizing properties of these drugs (EMD 54622>EMD 53998>EMD 54650) noted in Figures 2 and 3 is paralleled by an opposite ordering of their ability to inhibit PDE III:
EMD 54650 > EMD 53998 > EMD 54622. This suggests that the physiological response of myocardial preparations to EMD 54650 ought to be dominated by a cAMP-dependent increase in the amplitude of the Ca transient and shortening of its time course with little or no effect on myofilament sensitization. Thus, the effect of EMD 54650 on myocardial contraction should resemble that of other agents such as milrinone. At the other end of the spectrum, EMD 54622 might be expected to behave predominantly as a "myofilament sensitizer." If this were the case, EMD 54622 might increase the amplitude of the contraction without enhancing the extent of cell Ca	extsuperscript{2+} loading or shortening the time course of the Ca transient. Finally, EMD 53998 might be expected to both increase the Ca transient and enhance myofilament responsiveness to Ca	extsuperscript{2+}, i.e., to have effects intermediate between those caused by EMD 54622 and EMD 54650. These predictions were examined in intact preparations.

**Isolated Hearts**

Intact isolated guinea pig hearts were studied at constant coronary flow during electrical stimulation at 1 Hz. Left ventricular pressures were monitored with an isovolumic balloon. Figure 5 shows the effect of incremental concentrations of each of the three diazinone derivatives on developed pressure (panel A), end-diastolic pressure (panel B), and the time from the electrical stimulus to 50% relaxation of developed pressure (panel C). All three drugs increased developed pressure (panel A), and relative to control, the effect of EMD 53998 was more marked than that of either of the other two agents. The peak increase in developed pressure was achieved at a different concentration for each of the three diazinone derivatives. The effect of EMD 54650 and EMD 54622 on developed pressure was saturated at a concentration lower than that required for EMD 53998 to achieve its peak effect. The positive inotropic action of EMD 54622 was also associated with an increase in end-diastolic pressure, which rose progressively for concentrations of the drug between 2 and 10 μM, and at the highest concentration it was approxi-
TABLE 1. Characteristics of the Force–pCa Curves of Skinned Fibers From Guinea Pig Papillary Muscle Shown in Figure 2

<table>
<thead>
<tr>
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<th>pCa50</th>
<th>Max (%)</th>
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<tr>
<td>Solvent</td>
<td>5.33±0.06</td>
<td>91±3</td>
</tr>
<tr>
<td>EMD 54650 (10 μM)</td>
<td>5.27±0.03</td>
<td>93±2</td>
</tr>
<tr>
<td>EMD 53998 (10 μM)</td>
<td>5.55±0.06</td>
<td>109±5</td>
</tr>
<tr>
<td>EMD 54622 (10 μM)</td>
<td>5.92±0.13</td>
<td>123±2</td>
</tr>
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Values are mean±SEM. EMD 54650, EMD 53998, and EMD 54622, diazino derivatives; pCa50, negative logarithm of the Ca2+ concentration required for half-maximal activation; Max, maximal Ca2+-activated force.

TABLE 2. Characteristics of the ATPase–pCa Curves of Skinned Cardiac Myofibrils From Guinea Pig Shown in Figure 3

<table>
<thead>
<tr>
<th></th>
<th>pCa50</th>
<th>Max (%)</th>
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<tbody>
<tr>
<td>Solvent</td>
<td>5.39±0.04*</td>
<td>100†</td>
</tr>
<tr>
<td>EMD 54650 (10 μM)</td>
<td>5.43±0.04*</td>
<td>115±5*</td>
</tr>
<tr>
<td>EMD 53998 (10 μM)</td>
<td>5.67±0.02*</td>
<td>147±3*</td>
</tr>
<tr>
<td>EMD 54622 (10 μM)</td>
<td>5.80±0.02*</td>
<td>176±2*</td>
</tr>
</tbody>
</table>

Values are mean±SEM. EMD 54650, EMD 53998, and EMD 54622, diazino derivatives; pCa50, negative logarithm of the Ca2+ concentration required for half-maximal activation; Max, relative maximal Ca2+-activated ATPase activity.

*Standard errors are based on the nonlinear regression analysis as shown in Figure 3.
†Solvent value was taken as 100%.

Figure 4. Graph showing concentration dependence of the effects of diazino derivatives EMD 54650 (●), EMD 53998 (▲), and EMD 54622 (□) on the relative inhibition of phosphodiesterase III activity. Each point represents the mean±SD of three parallel determinations. (See text.)

Left Ventricular Myocytes

The concentration dependence of the effect of each of the three diazino derivatives on twitch amplitude (panel A), resting cell length before the onset of each twitch (panel B), and the time from the electrical stimulus to 50% relaxation (panel C). It is apparent that in these mechanically unloaded myocytes the concentration–response curves for the effect of EMD 54622 and EMD 53998 on twitch amplitude are shifted to the left of those in the isolated and perfused guinea pig heart and that each of these drugs increased twitch amplitude by approximately two-fold (Figure 6A). Both EMD 54622 and EMD 53998, which increased myofilament Ca2+ sensitivity in isolated myofibrils (Figures 2 and 3, Tables 1 and 2), decreased diastolic cell length (Figure 6B). Reduction in the resting cell length was particularly marked with EMD 54622; this result is in agreement with the significant increase in end-diastolic left ventricular pressure observed in the isolated guinea pig hearts (Figure 5B). However, the effect of EMD 54622 and EMD 53998 on resting cell length became evident at concentrations of these substances that exceeded those required for the maximum increase in twitch amplitude in cardiac cells. In contrast, EMD 54650, even at 10 μM, a concentration that exceeded that required for the peak inotropic effect in isolated myocytes (Figure 6A) as well as in the isolated heart (Figure 5A), did not decrease diastolic cell length (Figure 6B). The effect of each of the three diazino derivatives on the time course of the contraction in isolated myocytes is given in Figure 6C. EMD

TABLE 3. Inhibition of Phosphodiesterase III Isolated From Guinea Pig Cardiac Muscle

<table>
<thead>
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<th>EMD</th>
<th>IC50 (μM)</th>
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<tr>
<td>EMD 54650</td>
<td>0.016±0.007</td>
</tr>
<tr>
<td>EMD 53998</td>
<td>0.06±0.03</td>
</tr>
<tr>
<td>EMD 54622</td>
<td>0.8±0.35</td>
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</table>

Each value (IC50) represents the mean±SD of five different preparations of cGMP-inhibited phosphodiesterase (PDE III). EMD 54650, EMD 53998, and EMD 54622 are diazino derivatives.
FIGURE 5. Graphs showing concentration dependence of the effects of diazinone derivatives EMD 54650 (●), EMD 53998 (▲), and EMD 54622 (▲) on developed pressure (panel A), end-diastolic pressure (EDP) (panel B), and time from the electrical stimulus to 50% relaxation (panel C) of isolated guinea pig hearts (n=5 for each EMD compound) studied at constant coronary flow during continuous electrical stimulation at 1 Hz in 1 mM Cao. Control values are for all hearts studied (n=15): developed pressure was 54.5±2.0 mm Hg. EDP was set at 10 mm Hg, and time to 50% relaxation was 433±14.2 (mean±SEM) msec. There was no significant difference between control values of each of the three groups.

54650 shortened the time course of the contraction to approximately 85% of the control value, and this effect was already maximal at 0.5 μM, the lowest concentration tested. In contrast, EMD 54622 had a concentration-dependent effect that prolonged the time to 50% relaxation. It is noteworthy that EMD 53998 had a dual mode of action with regard to the concentration; the time to 50% relaxation was shortened at concentrations below 2 μM and was progressively prolonged above the control value as the concentration exceeded 2 μM. At 1 μM EMD 53998, the time to 50% relaxation was approximately 80% of the control value and was back to 97% of the control value at 2 μM. However, at concentrations of EMD 53998 of 5 and 10 μM, the time to 50% relaxation was prolonged, and it was 125% of the control value at 10 μM. This dual effect on the time course of contraction in response to EMD 53998 suggests that the PDE III inhibitory action of this substance may predominate at low concentrations and that it may be offset by its myofilament-sensitizing effect as the concentration is increased. This result is consistent with the action of these drugs on myofilament sensitization in skinned fibers, PDE III inhibition, and cAMP concentration in cell homogenates.

Figures 7–9 show representative tracings of the effect of each of the three diazinone derivatives on the con-
traction and the associated Ca\textsuperscript{2+} transient in indo-1-loaded myocytes. Each cell was exposed to a concentration of the drug that had caused the maximum increase in twitch amplitude in the studies on the concentration dependence of the effect of these agents in non-indo-1-loaded myocytes (Figure 6A). EMD 54650 (Figure 7) and EMD 53998 (Figure 8) reversibly increased Ca\textsuperscript{2+} transient and twitch amplitude. In contrast, EMD 54622 had a marked effect, an increase in the amplitude of the contraction without an enhancement of the amplitude of the Ca\textsuperscript{2+} transient (Figure 9).

It is also noteworthy that the time required for the positive inotropic action for each of these agents differs. Figure 10 depicts the average time course of the increase in twitch amplitude (panel A) and indo-1 transient (panel B) for the three drugs. Both EMD 53998 and EMD 54650 had a rapid onset of action, and their maximum effect on twitch amplitude was achieved within 5 minutes of exposure (Figure 10A). This is in contrast with the effect of EMD 54622 on the twitch that developed with a slower time course (Figure 10A). The time course of the effect of EMD 53998 and EMD 54650 on the indo-1 transients (Figure 10B) paralleled that on the twitch, and the peak of the response occurred within 5 minutes of exposure. In contrast, EMD 54622 did not significantly increase the amplitude of the indo-1 transient. Figure 10 also shows that the effect of the three drugs on the contraction and the Ca\textsuperscript{2+} transient was reversible with washout (dotted lines).

The experimental results presented so far strongly suggest that an increase in myofilament responsiveness to Ca\textsuperscript{2+} represents the predominant mechanism for the positive inotropic action of EMD 54622 in intact myocardial preparations. However, it is not possible to determine whether an increase in myofilament responsiveness to Ca\textsuperscript{2+} may play a role in the positive inotropic effect of EMD 53998 and EMD 54650. Both agents increase the amplitude of the Ca\textsuperscript{2+} transient, but they may do so in conjunction with an increased myofilament Ca\textsuperscript{2+} sensitivity. Additional experiments were undertaken to determine whether EMD 54650 or EMD 53998 modulates myofilament–Ca\textsuperscript{2+} interaction. Myofilament responsiveness to Ca\textsuperscript{2+}, before and after addition of each drug, was assessed in indo-1-loaded myocardial cells. A spectrum of Ca\textsuperscript{2+} transients and twitches of different amplitudes was obtained by either changing the frequency of stimulation or resuming stimulation.

**Figure 7.** Representative example of the effect of diazinone derivative EMD 54650 on the simultaneously recorded twitch contraction and indo-1 fluorescence transient. The myocyte was exposed to the concentration of the drug that produced the most pronounced increase in the amplitude of the contraction (compare with Figure 6A). The positive inotropic effect of EMD 54650 was associated with an enhancement of the amplitude of the Ca\textsuperscript{2+} transient.

**Figure 8.** Representative example of the effect of diazinone derivative EMD 53998 on the simultaneously recorded twitch contraction and indo-1 fluorescence transient. The myocyte was exposed to the concentration of the drug that produced the most pronounced increase in the amplitude of the contraction (compare with Figure 6A). The positive inotropic action of EMD 53998 was associated with an enhancement of the amplitude of the Ca\textsuperscript{2+} transient.

**Figure 9.** Representative example of the effect of diazinone derivative EMD 54622 on the simultaneously recorded twitch contraction and indo-1 fluorescence transient. The myocyte was exposed to the concentration of the drug that produced the most pronounced increase in the amplitude of the contraction (compare with Figure 6A). EMD 54622 increased twitch amplitude without affecting the Ca\textsuperscript{2+} transient.
after a period of rest. Alternatively, $C_a$ was abruptly changed, and the resulting staircases in contraction and indo-1 transient were used for analysis.

Figure 11 shows representative examples of the effect of the three diazinone derivatives on the cell length/indo-1 ratio relation. In agreement with other experiments indicating that EMD 54622 sensitizes myofilaments to $Ca^{2+}$, this agent shifted the length–indo-1 relation to the left (panel A). EMD 54650 had no effect on this relation in any of the cells studied (panel B). Less consistent results were obtained with EMD 53998. In three of five myocytes, EMD 53998 had no effect on the length–indo-1 relation (panel C), whereas in the remaining cells it shifted this relation to the left (panel D). This is also in agreement with the results of the in vitro experiments (Figures 2–4), which showed an effect of EMD 53998 on myofilament $Ca^{2+}$ sensitivity immediately between that of the other two substances.

A criticism has been raised concerning an assessment of myofilament responsiveness to $Ca^{2+}$ that relies on the measurement of peak $C_a$ and contraction during a twitch: under these conditions, there is no steady-state relation between $C_a$ and the myofilaments.35,36 However, Figure 11 does show that the effect of each of the diazinone derivatives on the length–indo-1 relation was

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**Figure 10.** Average time course of the effects of diazinone derivatives EMD 54650 (●), EMD 53998 (▲), and EMD 54622 (▼) on twitch contraction (panel A) and indo-1 transient (panel B) amplitudes. Drug concentrations were as in Figures 7–9. EMD 53998 and EMD 54650 enhanced both $C_a$, transient and contraction amplitudes. In contrast, EMD 54622 had a marked positive inotropic action without affecting $C_a$. Statistical analysis with paired t test confirmed that EMD 54622 had no effect on $C_a$, transient and that, after 5 minutes of exposure, the increase in $C_a$, transient amplitude from the control value was significant both for EMD 53998 ($p<0.05$) and EMD 54650 ($p<0.005$).

**Figure 11.** Scatterplots showing myofilament responsiveness to $Ca^{2+}$ assessed as the relation between cell length and indo-1 ratio during the twitch and at rest. $C_a$ transients and twitches of different amplitudes in the control condition and in the presence of each of the drugs were obtained by changing the stimulation frequency, resuming stimulation after a period of rest, or changing $C_a$. Peak systolic and diastolic values were obtained for indo-1 ratio and cell length in the control condition (△, resting value; ○, peak systolic value) and in the presence of the drug (△, resting value; ○, peak systolic value). For a given indo-1 ratio value, a decrease in cell length from the control length is indicative of an enhanced myofilament responsiveness to $Ca^{2+}$. Representative examples are shown. Diazinone derivative EMD 54622 shifted the length–indo-1 relation (panel A), whereas diazinone derivative EMD 54650 had no effect on this relation (panel B). Less consistent results were obtained with diazinone derivative EMD 53998: no effect (panel C) or a shift (panel D) in the length–indo-1 relation (see text).
similar regardless of whether we consider the values obtained at the peak of the twitch and of the Ca\textsuperscript{2+} transient or those obtained in the diastolic interval for the 80 msec before the electrical stimulus (rest).

**Discussion**

Our results show that structural modifications of the diazinone molecule profoundly affect the mechanism for the positive inotropic action of its derivatives. The features of the augmentation of contractile response of the isolated hearts as well as those of intact single cardiac myocytes are in close agreement with the predictions derived from the experiments with skinned fibers and myocardial PDE III. Thus, in indo-1-loaded myocytes, the positive inotropic effect of the diazinone derivatives is associated with an increase in myofilament responsiveness to Ca\textsuperscript{2+} that parallels the leftward shift in force- and ATPase-pCa relations (EMD 54622 > EMD 53998 > EMD 54650) and is opposite that for PDE III inhibition (EMD 54650 > EMD 53998 > EMD 54622). Of the three substances tested, only EMD 54622, which shows the largest ratio of calcium sensitizing to PDE III-inhibiting activity, enhanced twitch amplitude in indo-1-loaded myocytes without a significant effect on the Ca\textsuperscript{2+} transient.

The mechanism by which the observed effect on myofilament responsiveness to Ca\textsuperscript{2+} is elicited is not known. Functional experiments of the type described in this study cannot give direct evidence about the nature of the mode of action on the regulatory proteins. However, some indications are provided that might guide further experimentation in suitable biochemical models. Our data (Figures 2 and 3) reveal three main important features: the increase in the apparent calcium sensitivity, the decreases in the steepness of the force- or ATPase-pCa relations, and the increase in the maximal calcium-activated force and ATPase activity. The increase in the apparent calcium sensitivity is believed to be the result of increasing binding of calcium to troponin C (e.g., as it was discussed for sulmazole\textsuperscript{1}). However, the increase in the apparent calcium sensitivity of the compounds reported here appears not to be caused by the binding of the compounds to troponin C itself (authors' unpublished observations, 1990), but the location of the binding domain is not yet clear. Our data also imply that the compounds have an effect on the calcium-dependent cooperative interaction in the myofilaments as indicated by the increase in the steepness of the relation of force or ATPase activity to calcium concentration. Moreover, the increases in the maximal calcium-activated activities indicate that the kinetics of the crossbridge cycle must be affected.\textsuperscript{37} The relative importance of the three effects and the underlying mechanism is still unclear and is the object of further research.

Of the three substances tested, only EMD 54622 enhanced twitch amplitude in indo-1-loaded myocytes without a significant effect on the Ca\textsuperscript{2+} transient. This represents a unique characteristic of this drug.

A potential drawback in the use of myofilament sensitizers as positive inotropic agents is the possibility that they may delay relaxation of the heart and impair diastolic filling of the ventricular cavities.\textsuperscript{2} If this were to occur, it should be reflected in a prolonged time course of the contraction and increased diastolic tonus. In myocytes, EMD 54622 had a concentration-dependent effect of prolonging the time to 50% of relaxation: at 1.25 \(\mu\)M, which had proven the most effective concentration to enhance twitch amplitude (Figure 6A), EMD 54622 prolonged the time course of contraction to 118% above the control value (100%); at 10 \(\mu\)M, which was the highest concentration that we tested, the prolongation was 151% of the control value (Figure 6C). The effect of EMD 54622 on the time course of the twitch in cells was not paralleled by changes of similar magnitude in the isolated hearts, where the time to 50% relaxation was not prolonged at 1.25 \(\mu\)M and increased to 108% of the control value at 10 \(\mu\)M (Figure 5C). This difference in the magnitude of twitch prolongation in the two experimental models may be explained by the absence of adequate restoring forces in isolated myocytes. In contrast, in the isolated heart, the presence of the intraventricular balloon may provide the necessary stretch to accelerate the return of the left ventricular pressure to its diastolic value. Physiological conditions may be more closely mimicked by the experiments with the isolated hearts and the ventricular balloon than with isolated myocytes. However, additional studies would be required in living animals to determine whether blood return to the ventricles contributes substantially to their relaxation and minimizes the impairment in diastolic filling produced by an increase in myofilament sensitivity to Ca\textsuperscript{2+}. In myocytes, EMD 54622 had a marked effect, a decrease in diastolic cell length only at concentrations above 1.5 \(\mu\)M (Figure 6B); its positive inotropic effect was saturated at 1.25 \(\mu\)M. In the isolated heart, EMD 54622, at concentrations higher than 2 \(\mu\)M, increased end-diastolic pressure without further enhancing developed pressure. Thus, it appears that concentrations of EMD 54622 between 1 and 2 \(\mu\)M would be optimal for a positive inotropic action that is not associated with detrimental changes of the diastolic properties of the myocardium.

EMD 53998 caused a leftward shift of the force- and ATPase-pCa relations, inhibited PDE III activity (Figures 2–4), and had a dual effect on the time course of the contraction (Figure 6C). In isolated myocytes, EMD 53998 abbreviated the time course of the twitch at concentrations below 2 \(\mu\)M but prolonged it at 5 and 10 \(\mu\)M (Figure 6C). Thus, in vivo there may be a balance between the effects of EMD 53998: the inhibition of PDE III activity, which may predominate at concentrations below 2 \(\mu\)M, and the enhancement of myofilament sensitivity to Ca\textsuperscript{2+}, which may be the dominant action at 5 and 10 \(\mu\)M. Taken in conjunction with the mixed effects of 1.5 \(\mu\)M EMD 53998 (the enhancement of myofilament–Ca\textsuperscript{2+} interaction [Figures 11C and 11D] and the increase in the amplitude of the Ca, transient [Figures 8 and 10]), these results suggest that even at 1.5 \(\mu\)M EMD 53998 the positive lusitropic action of an increase in cAMP, which is expected to result from PDE III inhibition, may be partially offset by the augmented myofilament sensitivity to Ca\textsuperscript{2+}. Thus, although the positive inotropic effect of EMD 53998 is likely due to both an increase in the Ca, transient and in myofilament responsiveness to Ca\textsuperscript{2+}, the relaxing effect of an increase in cAMP and the prolongation of the time course of contraction caused by an enhanced myofilament–Ca\textsuperscript{2+} interaction appear to counteract each other. In the isolated heart, the end result of these different effects of
EMD 53998 is a marked increase in developed pressure, which occurs without an increase in end-diastolic pressure or prolongation in the time course of the contraction. It is noteworthy that sulmazole, another PDE III inhibitor, has been shown to enhance myofilament-Ca\(^{2+}\) interaction.\(^4\) However, unlike EMD 53998, the effect of sulmazole on myofilament responsiveness to Ca\(^{2+}\) became evident only at concentrations higher than those required to enhance cell Ca\(^{2+}\) loading.\(^21\)

EMD 54650 had virtually no effect on myofilament Ca\(^{2+}\) sensitivity, and its positive inotropic action can be attributed uniquely to a PDE III inhibitory action. This agent is the one that more closely resembles other PDE III inhibitors, such as milrinone, which have an effect on the amplitude and time course of the contraction and the Ca\(^{2+}\) transient similar to that of isoproterenol.\(^20\)

In summary, modifications of the diazinone molecule have led to the development of novel substances that can either behave as a pure myofilament sensitizer without affecting cell Ca\(^{2+}\) loading (i.e., EMD 54622) or sensitize myofilaments to Ca\(^{2+}\) and enhance cell Ca\(^{2+}\) loading (i.e., EMD 53998). Additional animal studies are necessary to determine whether the principle of Ca\(^{2+}\) sensitization may be useful for the treatment of congestive heart failure.

### Acknowledgments

The authors thank Mr. James H. Engel for his assistance in configuring the data acquisition scheme in the measurement for the intact hearts and Ms. Miriam Glaser for excellent secretarial assistance.

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Circ Res. 1992;70:1081-1090
doi: 10.1161/01.RES.70.6.1081

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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