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# (−)-Enantiomer EMD 57439 Antagonizes the $Ca^{2+}$ Sensitizing Effect of (+)-Enantiomer EMD 57033 on Diastolic Function but Not on Systolic Function in Rabbit Ventricular Cardiomyocytes

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ABSTRACT—EMD 53998 (5-[1-(3,4-dimethoxybenzoyl)-1,2,3,4-tetrahydro-6-quinolyl]-6-methyl-3,6-dihydro-2*H*-1,3,4-thiadiazin-2-one), the racemic mixture of (+)-enantiomer EMD 57033 and (−)-enantiomer EMD 57439, is a prototype of $Ca^{2+}$ sensitizers that act via a central and/or down-stream mechanism in cardiac E-C coupling. In rabbit ventricular cardiomyocytes loaded with indo-1/AM, EMD 53998 and EMD 57033 shifted the relationship between $Ca^{2+}$ transients and cell shortening (systolic function) to the left to the same extent as compared with that of elevation of $[Ca^{2+}]_o$. EMD 57439 did not elicit a positive inotropic effect (PIE). The PIE of EMD 57033 was associated with a more pronounced decrease in the diastolic cell length than that of EMD 53998, whereas the systolic effects of these compounds were equivalent. These results indicate that weak phosphodiesterase (PDE) III inhibition may exert a differential action on diastolic and systolic function. Thus, EMD 57439 antagonizes the $Ca^{2+}$-sensitizing effect of EMD 57033 on diastolic function with no effect on systolic function, which may lead to a decease in diastolic cell length of a lesser extent with the racemate EMD 53998 compared with (+)-enantiomer EMD 57033.

*Keywords*: EMD 53998, EMD 57033, $Ca^{2+}$ transient, $Ca^{2+}$ sensitizer, Rabbit ventricular cardiomyocyte

$Ca^{2+}$ sensitizers that elicit a positive inotropic effect (PIE) via central and/or down-stream mechanisms of cardiac E-C coupling by an increase in myofibrillar $Ca^{2+}$ sensitivity (1, 2) have a potential as cardiotonic agents used for treating the myocardial contractile dysfunction in heart failure. This is partly because $Ca^{2+}$ sensitizers do not require an increase in activation energy (3) that is associated with cardiotonic agents such as digitalis, catecholamines and phosphodiesterase (PDE) III inhibitors (e.g., amrinone, milrinone) that act via an up-stream mechanism (1). In addition, they do not have the risk of causing $Ca^{2+}$ overload in myocardial cells that could lead to cardiac arrhythmias and myocardial cell injury (4). A potential disadvantage of $Ca^{2+}$ sensitizers in clinical application could be impairment of diastolic function due to an increase in $Ca^{2+}$ sensitivity to intracellular $Ca^{2+}$ at the diastole (5). Most of the $Ca^{2+}$ sensitizers that have been developed up to now possess an inhibitory action on PDE III activity, which can antagonize the action of $Ca^{2+}$ sensitizers through an accumulation of cyclic AMP. It has not yet been elucidated how $Ca^{2+}$ sensitizing and PDE III inhibitory actions modulate the diastolic and systolic function in combination.

EMD 53998 (5-[1-(3,4-dimethoxybenzoyl)-1,2,3,4-tetrahydro-6-quinolyl]-6-methyl-3,6-dihydro-2*H*-1,3,4-thiadiazin-2-one) that is the racemic, equimolar mixture of two optical enantiomers, (+)-enantiomer EMD 57033 and (−)-enantiomer EMD 57439 (6), is a prototype of $Ca^{2+}$ sensitizers that act via the central and/or down-stream mechanism. It has been shown that EMD 57033 acts mainly via $Ca^{2+}$ sensitization, while EMD 57439 acts by PDE III inhibition in cardiac muscle in various mammalian species (6–9). However, the interaction of $Ca^{2+}$ sensitization and PDE III inhibition on systolic and diastolic function has not been studied in detail. Furthermore, the effects of EMD 53998 on the diastolic function in previous studies are contradictory. In this study, we carried out a detailed analysis of the effects of these thiadiazinone derivatives on the diastolic and systolic function in rabbit single ventricular cardiomyocytes in comparison with the effects of elevation of extracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_o$) that acts with least modulation of myofibrillar $Ca^{2+}$ sensitivity and isoproterenol that causes $Ca^{2+}$ desensitization in relation to cyclic

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AMP-mediated phosphorylation of troponin I. Namely, the concentration-dependent effects of these agents on $Ca^{2+}$ transients, systolic function and diastolic function were compared. The present study indicates that weak PDE III inhibition induced by EMD 57439 antagonizes the effect of EMD 57033 on diastolic function without affecting the effect of the compound on systolic function. A preliminary account of this study has been published as an abstract (10).

## MATERIALS AND METHODS

### *Isolation of rabbit ventricular cardiomyocytes*

Isolation of rabbit ventricular cardiomyocytes was carried out as described previously (11). Briefly, male Japanese White rabbits (1.8−2.0 kg) were given heparin (600 units/kg, i.v.) and anesthetized with pentobarbital sodium (40 mg/kg, i.v.). The heart was rapidly excised, mounted on a Langendorff apparatus and retrogradely perfused for approximately 1 min at a perfusion pressure of 80 $cmH_2O$ with HEPES-Tyrode solution consisting of 137 mM NaCl, 5.4 mM KCl, 0.53 mM $MgCl_2$, 1.2 mM $CaCl_2$, 0.33 mM $NaH_2PO_4$, 5.0 mM HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid) and 5.0 mM glucose (pH 7.4). The solution was continuously gassed with 100% $O_2$ at 37℃. The heart was then perfused with nominally $Ca^{2+}$-free HEPES-Tyrode solution for 5 min, followed by perfusion with recirculation of $Ca^{2+}$-free HEPES-Tyrode solution to which collagenase (0.6 mg/ml) and protease (0.1 mg/ml) had been added. After approximately 20 min, when the heart became homogeneously soft, the enzymes were washed out for 1 min by perfusion with HEPES-Tyrode solution containing 0.2 mM $CaCl_2$. The ventricles were then removed, minced in HEPES-Tyrode solution containing 0.2 mM $CaCl_2$ and filtered through a nylon mesh (200 $\mu$m). This suspension containing the myocytes was allowed to stand for 10 min, and then the supernatant was discarded. The myocytes were resuspended in a stepwise manner in HEPES-Tyrode solution containing 0.2, 0.4 and 0.8 mM $CaCl_2$. The myocytes were finally resuspended in HEPES-Tyrode solution containing 1.2 mM $CaCl_2$ and kept for 1 hr or longer at room temperature (24−26℃) before the loading with the acetoxymethyl ester form of the $Ca^{2+}$ sensitive fluorescence probe indo-1 (indo-1/AM).

### *Simultaneous measurements of cell length and $Ca^{2+}$ transients*

Myocytes were loaded with indo-1/AM and all the following steps were carried out at room temperature (24−26℃). The loading solution consisted of 10 $\mu$l of 1 mM indo-1/AM, 40 $\mu$l DMSO, 90 $\mu$l fetal bovine serum, 10 $\mu$l of 20% pluronic F-127 (wt/wt in DMSO) and 1 ml HEPES-Tyrode solution. The loading solution described above was sonicated for 3 min and 1 ml of cell suspension was added to it. The myocytes were allowed to load with indo-1/AM for 1 to 4 min and then centrifuged at 150 rpm for 1 min. The supernatant was discarded and the pellet was resuspended in HEPES-Tyrode solution. The myocytes were placed in a perfusion chamber on the stage of an inverted microscope (Diaphot TMD 300; Nikon, Tokyo) equipped for simultaneous recordings of cell length and indo-1 fluorescence. After 10 min, the myocytes were perfused at a rate of about 2 ml/min with bicarbonate buffer consisting of 116 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 0.81 mM $MgSO_4$, 1.02 mM $NaH_2PO_4$, 23.8 mM $NaHCO_3$, and 5.0 mM glucose. The buffer was continuously gassed with 95% $O_2$ − 5% $CO_2$ (pH 7.4). Myocytes were stimulated with square-wave pulses of 5-msec duration and a voltage of 0.5−0.7 V at 0.5 Hz by bipolar platinum electrodes placed in the perfusion chamber.

Indo-1 fluorescence was excited with the light from a xenon lamp with a wavelength of 355 nm, reflected by a 380 nm long-pass dichroic mirror, and detected by means of a fluorescence spectrophotometer (CAM-230; Japan Spectroscopic, Tokyo). Excitation light was applied to the myocyte through a neutral density filter to minimize the photobleaching of indo-1. The emitted fluorescence was collected by an objective lens (CF Fluor DL40; Nikon) and after passing through the 380 nm long-pass dichroic mirror, it was first separated by a 580 nm long-pass dichroic mirror (Omega Optical, Brattleboro, VT, USA). The fluorescence light was subsequently split by a 425 nm dichroic mirror to permit simultaneous measurements of both 405 nm and 500 nm wavelengths through band-pass filters, respectively, by use of two separate photomultiplier tubes. The fluorescence ratio (405/500 nm) was then used as an index of intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$).

The cell length was monitored simultaneously with indo-1 fluorescence using red light (>620 nm) through the normal bright field illumination optics of the microscope. The bright field image of the cell was collected by an objective lens and first separated by a 580 nm long-pass dichroic mirror (Omega Optical). This image was projected onto a photodiode array (C6294-01; Hamamatsu Photonics, Hamamatsu) scanned at every 5 msec.

Cell length and indo-1 fluorescence data were acquired by using a computer (Power Macintosh 8100/100AV; Apple Computer, Cupertino, CA, USA) with an A/D converter (MP-100A; BIOPAC Systems, Santa Barbara, CA, USA) and analyzed after the low-pass filtering (cutoff frequency of 25 Hz) and averaging of 5 successive signals. Changes in peak (systolic) cell shortening and indo-1 fluorescence ratio and resting (diastolic) levels of

both signals induced by cardiotonic interventions were defined as the difference from the baseline levels of respective signals prior to application of inotropic interventions examined.

*Experimental protocol*

Myocytes were exposed to bicarbonate buffer containing increasing concentrations of the compound. When the PIE of the compound reached a steady state, the next higher concentration was added. The cell length was continuously monitored throughout the experiment, while the indo-1 fluorescence was measured before the addition of each compound and when the response reached a steady state in the presence of the compound. In the presence of milrinone at $10^{-5}$ M and higher, a marked increase in the diastolic indo-1 fluorescence ratio was observed ($117.2\pm0.9\%$, $240.3\pm5.7\%$ and $362.0\pm14.1\%$ of the baseline value at $10^{-5}$, $10^{-4}$ and $3\times10^{-4}$ M, respectively), which is assumed to be due to an increase in autofluorescence induced by this compound. Therefore, the increase in fluorescence ratio by milrinone was calculated after subtraction of the increase in autofluorescence. EMD 53998 did not have a direct effect on autofluorescence or indo-1 fluorescence signals.

*Drugs and chemicals*

The following drugs were used: (−)-isoproterenol hydrochloride, protease (type XIV), fetal bovine serum and pluronic F-127 (Sigma Chemical Co., St. Louis, MO, USA); collagenase (class II; Worthington Biochemical, Freehold, NJ, USA); EMD 53998 and its enantiomers (E. Merck, Darmstadt, Germany); indo-1/AM and HEPES (Dojindo Laboratories, Kumamoto).

*Statistical analyses*

Experimental values are presented as the mean±S.E.M. The concentration-response curves for each compound were analyzed by one-way analysis of variance followed by application of the Bonferroni/Dunn method. The mean values in EMD 53998 and EMD 57033 groups were compared by Student's *t*-test. A value of $P<0.05$ was considered to indicate a statistically significant difference.

## RESULTS

Representative tracings of the effects of elevation of $[Ca^{2+}]_o$ and isoproterenol, a $\beta$-adrenoceptor agonist, on cell shortening and indo-1 fluorescence ratio in rabbit ventricular myocytes are shown in Fig. 1. Elevation of $[Ca^{2+}]_o$ increased cell shortening in association with an increase in indo-1 fluorescence ratio along with a decrease in the diastolic cell length and a modest increase in the diastolic fluorescence ratio at high concentrations (Fig. 1A: f and g). Indo-1 fluorescence ratio was slightly abbreviated by elevation of $[Ca^{2+}]_o$, but the duration of cell shortening was scarcely affected (Fig. 1A: i). Isoproterenol also exerted a PIE, accompanied by a pronounced concentration-dependent increase in the amplitude of $Ca^{2+}$ transients (Fig. 1B: b−f) and abbreviation of cell shortening and indo-1 fluorescence ratio (Fig. 1B: h). The changes induced by elevation of $[Ca^{2+}]_o$ and isoproterenol were reversed after washout of myocytes with drug-free bicarbonate buffer (Fig. 1A: h and B: g).

Figure 2 shows representative tracings of the effects of EMD 53998 and its enantiomers on cell shortening and indo-1 fluorescence ratio. EMD 53998 and the (+)-enantiomer EMD 57033 increased cell shortening with little change in the amplitude of indo-1 fluorescence ratio (Fig. 2A and B). The PIEs of these compounds were accompanied by a striking reduction in the diastolic cell length. These compounds prolonged the duration of cell shortening with little changes in the duration of indo-1 fluorescence ratio (Fig. 2A: g and B: g). By contrast, the (−)-enantiomer EMD 57439 elicited only small responses of cell shortening and fluorescence ratio in rabbit cardiomyocytes (Fig. 2C).

Effects of thiadiazinone derivatives on cell shortening and systolic fluorescence ratio are summarized in Fig. 3. The changes in diastolic parameters induced by elevation of $[Ca^{2+}]_o$ and isoproterenol are presented in Tables 1 and 2, respectively, and those induced by thiadiazinones, in Table 3. The diastolic cell length was significantly decreased with $[Ca^{2+}]_o$ at 10.8 mM and higher and the diastolic fluorescence ratio was significantly increased at 3.6 mM and higher (Table 1). Isoproterenol decreased the diastolic cell length to a lesser extent with a significant increase in the diastolic fluorescence ratio (Table 2).

In contrast to elevation of $[Ca^{2+}]_o$ and isoproterenol, neither EMD 53998 nor EMD 57033 increased the systolic indo-1 fluorescence ratio, when these compounds decreased the systolic cell length (Fig. 3A and B). Furthermore, EMD 53998 at $3\times10^{-6}$ M and EMD 57033 at $10^{-6}$ M and higher decreased significantly the diastolic cell length without significant changes in the diastolic fluorescence ratio (Table 3). EMD 57033 decreased the diastolic cell length significantly more than EMD 53998. EMD 57439 had a very low efficacy in the rabbit ventricular myocytes: it had an only small PIE and affected neither the diastolic cell length nor the fluorescence ratio (Fig. 3C and Table 3).

Since EMD 57439 had little effect, we examined the effect of milrinone, a prototype of PDE III inhibitors, on rabbit ventricular myocytes. Milrinone increased significantly the extent of systolic shortening in a concentration-dependent manner with an increase in the

amplitude of fluorescence ratio (Fig. 4).

Figure 5 shows the relationship between cell shortening and indo-1 fluorescence ratio during exposure to increasing concentrations of $[Ca^{2+}]_o$, isoproterenol, EMD 53998, EMD 57033 and EMD 57439. EMD 53998 and EMD 57033 shifted the relationship upwards to the left as compared with those with $[Ca^{2+}]_o$ elevation and isoproterenol. Isoproterenol shifted the relationship to the right as compared with that of $[Ca^{2+}]_o$ elevation. The relationship was unaltered during exposure to EMD 57439 because it had little inotropic effect.

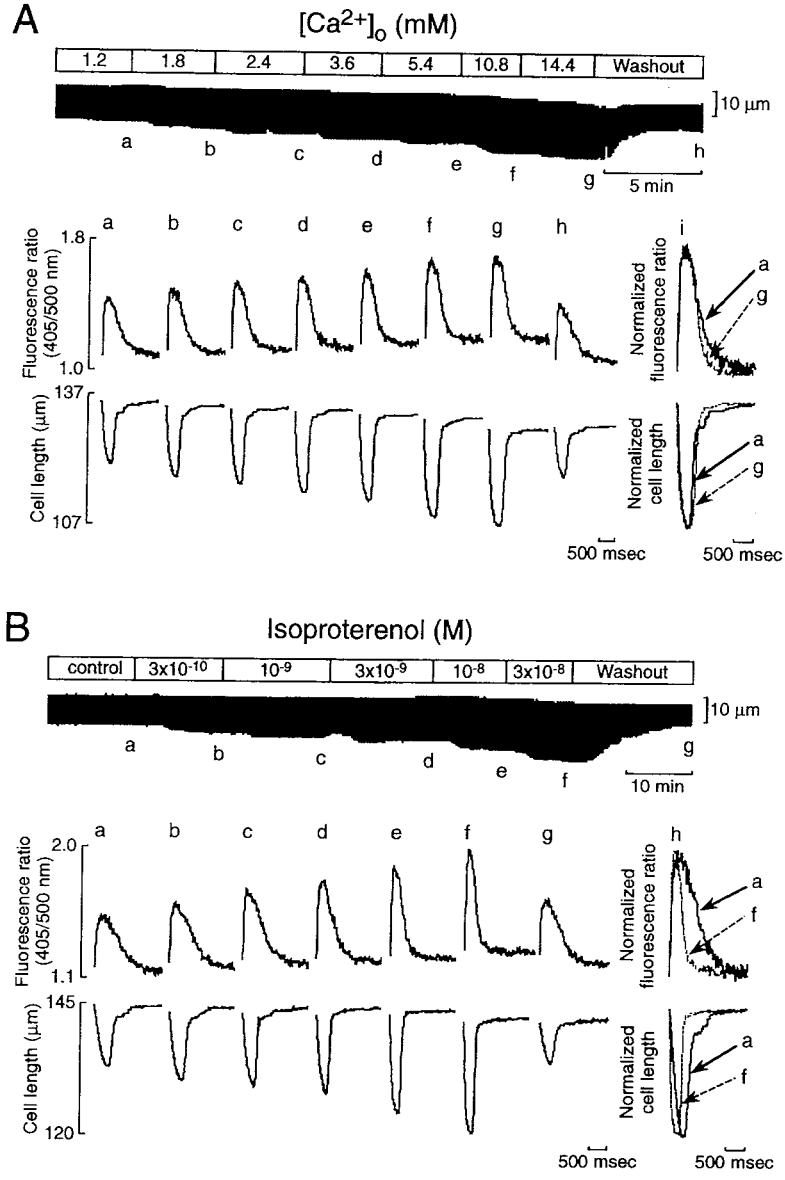


**Fig. 1.** Representative tracings of the effects of $[Ca^{2+}]_o$ (A) and isoproterenol (B) on cell shortening and $Ca^{2+}$ transients in indo-1-loaded rabbit ventricular cardiomyocytes. A: the myocyte was exposed to increasing $[Ca^{2+}]_o$ as indicated by the horizontal column in the upper row. Upper tracings: continuous recordings of cell length; middle and lower tracings: indo-1 fluorescence ratio (middle) and cell length (lower) simultaneously measured at times indicated by a–h below the continuous upper tracings; a, control ($[Ca^{2+}]_o$=1.2 mM); h, after return to control $[Ca^{2+}]_o$ of 1.2 mM; i, tracings in a and g are normalized and superimposed to facilitate comparison of the alteration of time courses of signals; a–h in middle and lower rows indicate the times corresponding to a–h in upper tracings when these high-speed recordings were performed. B: the myocyte was exposed to increasing concentrations of isoproterenol that were indicated by the horizontal column in the upper row. a, control ($[Ca^{2+}]_o$=1.2 mM) before administration of isoproterenol; g, after washout of isoproterenol; h, tracings in a and f are normalized and superimposed; a–g in middle and lower rows indicate the times corresponding to a–g in upper tracings when these high-speed recordings were performed.

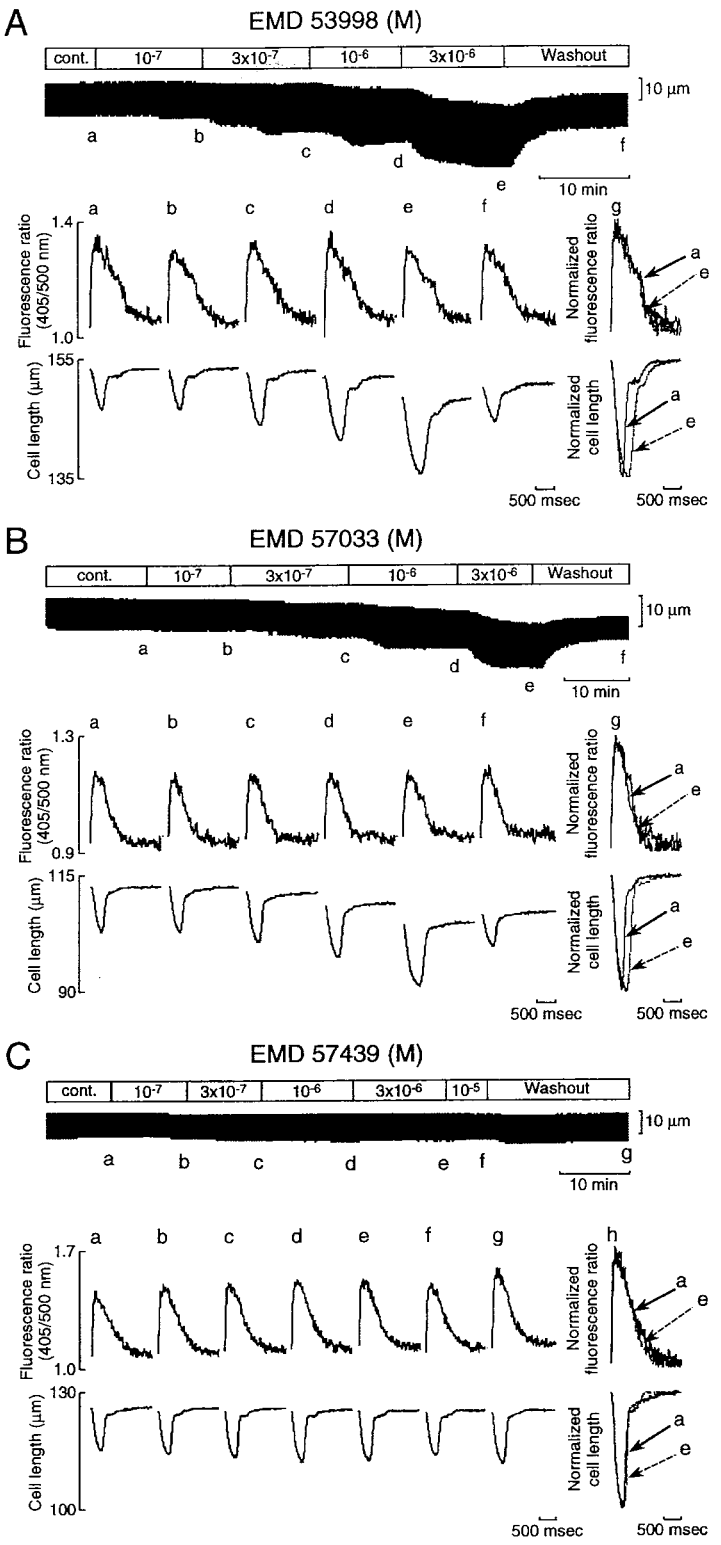


**Fig. 2.** Representative tracings of the effects of thiadiazinone derivatives, EMD 53998 (A), EMD 57033 (B) and EMD 57439 (C), on cell shortening and $Ca^{2+}$ transients in indo-1-loaded rabbit ventricular cardiomyocytes. Concentrations of thiadiazinone derivatives are presented in horizontal columns in each panel. cont.: control cell shortening ($[Ca^{2+}]_o$=1.2 mM); a, control before administration of the derivatives; f (in A and B) and g (in C), after washout of the compounds; g (in A and B) and h (in C), tracings in a and e are normalized and superimposed to facilitate comparison of the alteration of time courses of signals; a–f (in A and B) and a–g (in C) in middle and lower rows indicate the times corresponding to a–f (in A and B) and a–g (in C) in upper tracings when these high-speed recordings were performed.

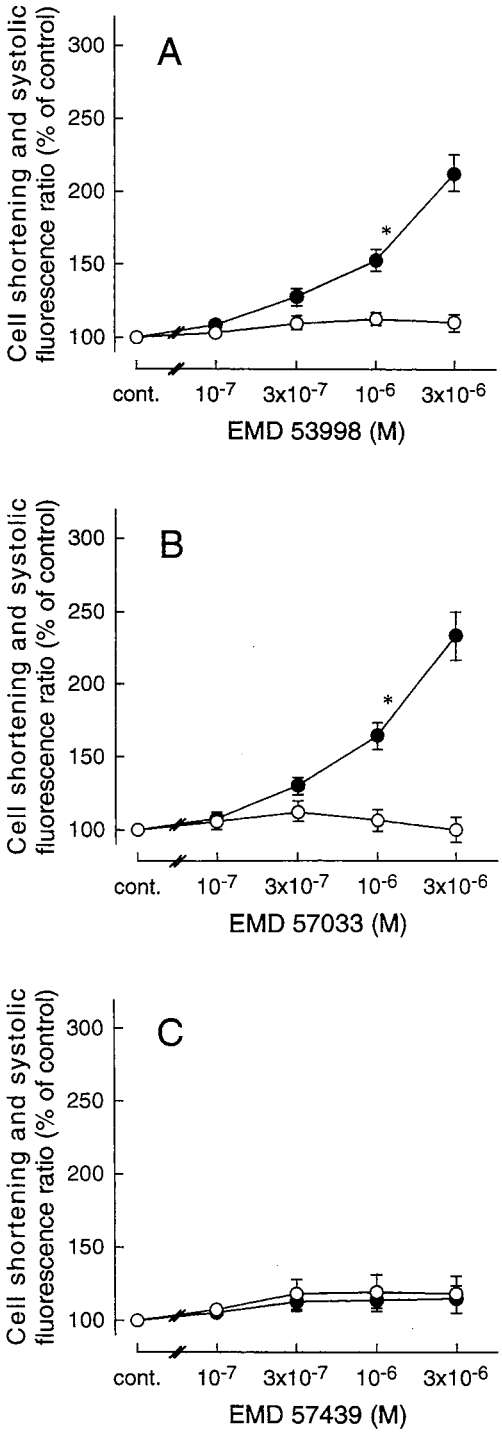


**Fig. 3.** Effects of thiadiazinone derivatives, EMD 53998 (A), EMD 57033 (B) and EMD 57439 (C), on cell shortening (closed circles) and systolic indo-1 fluorescence ratio (open circles) in rabbit ventricular cardiomyocytes. Only one concentration-response curve was determined in each cardiomyocyte. cont.: control signals before administration of each derivative were assigned as 100%. Baseline diastolic cell lengths and extents of shortening before administration of each thiadiazinone derivative: 146.3±7.3 $\mu$m and 7.43±0.76% in A (n=8); 140.3±7.7 $\mu$m and 8.07±0.93% in B (n=7); 140.5±11.3 $\mu$m and 8.46±0.89% in C (n=7). Asterisks indicate the threshold concentrations (*$P<0.05$ vs the respective baseline values).

## DISCUSSION

EMD 53998 elicited a PIE with little alteration of systolic and diastolic levels of $Ca^{2+}$ transients, an indication that the racemate acts mainly as a $Ca^{2+}$ sensitizer in rabbit ventricular cardiomyocytes. The effects of EMD 53998 on $Ca^{2+}$ transients in the rabbit are different from those in indo-1-loaded guinea pig myocytes, in which EMD 53998 induced a definite increase in the amplitude of $Ca^{2+}$ transients (8, 12). In indo-1-loaded rat myocytes (13) and in aequorin-loaded ferret papillary muscles (14), however, EMD 53998 induced a PIE with little change in $Ca^{2+}$ transients. These findings imply that in cardiac muscle of different mammalian species, the PIE of EMD 53998 shows a wide range of species-dependent variation in respect to regulation of $Ca^{2+}$ signaling.

In skinned cardiac fibers EMD 53998 shifted the pCa-tension relationship to the left with an increase in the maximum $Ca^{2+}$-activated force, indicating that the compound acts via the central and/or down-stream mechanism (12, 15, 16). In addition, EMD 53998 inhibits the activity of PDE III extracted from the guinea pig heart (12, 15). Therefore, the increase in $Ca^{2+}$ transients induced by EMD 53998 could be ascribed to the latter mechanism, which may play a crucial role in the species-dependent variation of the response of $Ca^{2+}$ transients to the racemic compound.

EMD 57033, the (+)-enantiomer of EMD 53998, elicited a PIE with a prolongation of the duration of cell shortening that was more pronounced than that with the racemate without alteration of $Ca^{2+}$ transients. Although the potency of EMD 57033 should be higher than that of the racemate EMD 53998, it could not be determined because the maximal response was not achieved even by the highest concentration of these compounds examined. It is evident, however, that EMD 57033 and EMD 53998 at equivalent concentrations decreased the systolic, but not the diastolic cell length, to an identical extent.

As the mechanism of $Ca^{2+}$ sensitization, it has been suggested that the increase in $Ca^{2+}$ binding affinity for troponin C prolongs the duration of contraction in association with abbreviation of $Ca^{2+}$ transients (17–19). Since EMD 57033 did not alter the time course of $Ca^{2+}$ transients, it may act mainly on the process subsequent to $Ca^{2+}$ binding to troponin C to exert the $Ca^{2+}$ sensitizing action; i.e., via the down-stream mechanism. Supporting this view, Solaro et al. (7) have demonstrated that EMD 57033 acts on the actin-myosin interface to lead to facilitation of crossbridge-dependent activation of thin filaments. It is a matter of course, however, that classification of the subcellular mechanism for $Ca^{2+}$ sensitization in intact myocardial cells is possible only after evaluation of pieces of evidence obtained by means of different

experimental procedures including the in vitro motility assay (2).

EMD 57439, the (−)-enantiomer, scarcely affected the cell shortening in rabbit ventricular cardiomyocytes. It has been shown that EMD 57439 shows only a weak PIE in guinea pig (6, 20) and ferret papillary muscles (9). On the other hand, in indo-1-loaded guinea pig and canine myocytes, it has been reported that EMD 57439 elicits a pronounced PIE with a marked increase in $Ca^{2+}$ transients (7, 8). We examined the inotropic effect of milrinone, a prototype of selective PDE III inhibitors, in rabbit ventricular cardiomyocytes for comparison; it increased significantly the extent of cell shortening and the amplitude of $Ca^{2+}$ transients in a concentration-dependent manner, being consistent with the previous findings in isolated canine and rabbit ventricular muscles (21, 22). Although PDE inhibitors including 3-isobutyl-1-methylxanthine (IBMX), milrinone and newly developed compounds, elicited a pronounced PIE in isolated rabbit papillary muscles, they also showed a wide range of

**Table 1.** Effects of elevation of $[Ca^{2+}]_o$ on diastolic cell length and diastolic indo-1 fluorescence ratio in rabbit ventricular cardiomyocytes

| $[Ca^{2+}]_o$ | n | 1.2 mM (control) | 1.8 mM | 2.4 mM | 3.6 mM | 5.4 mM | 10.8 mM | 14.4 mM |
|---|---|---|---|---|---|---|---|---|
| Length (%) | 7 | 100 | 99.6±0.1 | 99.2±0.2 | 98.5±0.3 | 97.7±0.4 | 96.3±0.7* | 94.7±1.5 |
| Ratio (%) | 7 | 100 | 101.6±0.5 | 102.7±0.6 | 104.3±0.7* | 106.3±0.7 | 110.0±1.1 | 112.6±1.1 |

Baseline diastolic cell length: 138.2±6.6 μm; baseline diastolic indo-1 fluorescence ratio: 1.11±0.05 (n=7, each). Asterisks indicate the threshold concentrations [*$P<0.05$ vs the respective baseline values (control)].

**Table 2.** Effects of isoproterenol on diastolic cell length and diastolic indo-1 fluorescence ratio in rabbit ventricular cardiomyocytes

| Isoproterenol | n | 0 (control) | $3\times10^{-10}$ M | $10^{-9}$ M | $3\times10^{-9}$ M | $10^{-8}$ M | $3\times10^{-8}$ M |
|---|---|---|---|---|---|---|---|
| Length (%) | 7 | 100 | 99.9±0.1 | 99.4±0.2 | 99.3±0.3 | 98.9±0.3 | 98.2±0.4* |
| Ratio (%) | 7 | 100 | 100.8±0.6 | 103.6±0.8 | 105.4±0.8* | 107.0±1.0 | 109.5±1.7 |

Baseline diastolic cell length: 140.8±7.8 μm; baseline diastolic indo-1 fluorescence ratio: 1.07±0.04 (n=7, each). Asterisks indicate the threshold concentrations [*$P<0.05$ vs the respective baseline values (control)].

**Table 3.** Effects of thiadiazinone derivatives on diastolic cell length and diastolic indo-1 fluorescence ratio in rabbit ventricular cardiomyocytes

| | n | 0 (control) | $10^{-7}$ M | $3\times10^{-7}$ M | $10^{-6}$ M | $3\times10^{-6}$ M |
|---|---|---|---|---|---|---|
| EMD 53998 | | | | | | |
| Length (%) | 8 | 100 | 99.8±0.2 | 99.3±0.3 | 98.3±0.3 | 95.1±0.9* |
| Ratio (%) | 8 | 100 | 100.6±0.8 | 101.7±1.0 | 101.8±1.3 | 100.8±1.6 |
| EMD 57033 | | | | | | |
| Length (%) | 7 | 100 | 99.6±0.2 | 98.6±0.2 | 96.9±0.4*† | 92.3±0.5†† |
| Ratio (%) | 7 | 100 | 101.4±1.1 | 101.6±1.2 | 102.6±1.5 | 101.6±1.7 |
| EMD 57439 | | | | | | |
| Length (%) | 7 | 100 | 99.8±0.1 | 99.6±0.1 | 99.4±0.1* | 99.3±0.2 |
| Ratio (%) | 7 | 100 | 100.4±0.9 | 101.6±1.1 | 101.0±1.3 | 100.0±1.6 |

Baseline diastolic cell length: 146.3±7.3 μm (n=8) for EMD 53998, 140.3±7.7 μm (n=7) for EMD 57033 and 140.5±11.3 μm (n=7) for EMD 57439. Baseline diastolic indo-1 fluorescence ratio: 1.06±0.04 for EMD 53998, 1.04±0.04 for EMD 57033 and 1.10±0.04 for EMD 57439. Asterisks indicate the threshold concentrations [*$P<0.05$ vs the respective baseline values (control)]; †$P<0.05$, ††$P<0.01$ vs EMD 53998 at the corresponding concentrations.


**Fig. 4.** Effects of milrinone on cell shortening (closed circles) and amplitude of indo-1 fluorescence ratio (open circles) in rabbit ventricular cardiomyocytes. The increase in the indo-1 fluorescence ratio was calculated after subtraction of an increase in autofluorescence. Only one concentration-response curve was determined in each cardiomyocyte. cont.: control signals before administration of milrinone were assigned as 100%. Baseline diastolic cell length and extent of shortening before administration of milrinone: 135.1±6.4 $\mu$m and 7.67±0.45% (n=7). Numbers in parentheses indicate the number of myocytes at the highest concentration, at which two myocytes, respectively, showed severe arrhythmic contractions and were therefore excluded from calculation. Asterisks indicate the threshold concentrations (*$P<0.05$ vs the respective baseline values).

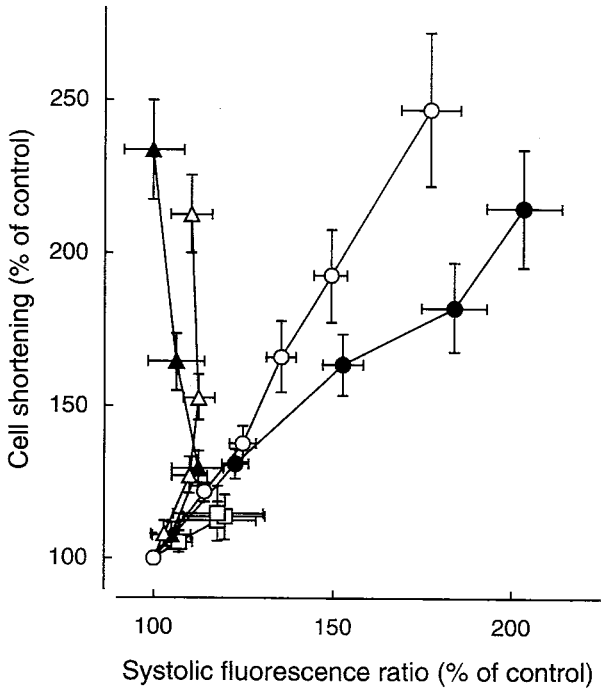


**Fig. 5.** The relationship between systolic indo-1 fluorescence ratio and cell shortening during exposure to increasing $[Ca^{2+}]_o$ (open circles: 1.8, 2.4, 3.6, 5.4, 10.8 mM) and increasing concentrations of isoproterenol (closed circles: $3\times10^{-10}$, $10^{-9}$, $3\times10^{-9}$, $10^{-8}$ M) and thiadiazinone derivatives, EMD 53998 (open triangles: $10^{-7}$, $3\times10^{-7}$, $10^{-6}$, $3\times10^{-6}$ M), EMD 57033 (closed triangles: $10^{-7}$, $3\times10^{-7}$, $10^{-6}$, $3\times10^{-6}$ M) and EMD 57439 (open squares: $10^{-7}$, $3\times10^{-7}$, $10^{-6}$, $3\times10^{-6}$ M), in indo-1-loaded rabbit ventricular cardiomyocytes. Baseline diastolic cell lengths and extents of shortening before $[Ca^{2+}]_o$ and isoproterenol: 138.2±6.6 $\mu$m and 7.58±0.94% ($[Ca^{2+}]_o$, n=7); 140.8±7.8 $\mu$m and 7.04±0.55% (isoproterenol, n=7). Data on thiadiazinone derivatives are taken from those in Fig. 3.

species-dependent variation (23). The present findings imply that EMD 57439 may act on the PDE isozyme that possesses characteristics different from those of the milrinone-sensitive PDE III in the rabbit ventricular myocardium. Supporting this postulate, it has been shown that the potency and efficacy of various PDE inhibitors, such as IBMX, milrinone, Y-20487 and OPC-18790, as positive inotropic agents, in the rabbit ventricular myocardium are quite different from those in other mammals such as the rat: e.g., in the rabbit, OPC-18790 was more potent and effective than IBMX, which is in strong contrast to the findings in the rat (22). It has generally been accepted that PDE III plays a crucial role in hydrolysis of cyclic AMP involved in regulation of the cardiovascular system in mammals (except small mammals including rats); and PDE III inhibitors, such as amrinone and milrinone, act essentially in a similar manner on the cardiac muscle of different mammalian species (24, 25). The findings with novel cardiotonic agents such as OPC-18790 and EMD 57439 in intact myocardium, however, suggest the existence of further PDE isozymes that affect species-dependent variations of these agents (22, present study); this subject awaits further studies.

A question arises about whether EMD 57439 would not possess the PDE inhibitory action on the rabbit ventricular myocardium at all since the compound affected neither the cell shortening nor the amplitude of $Ca^{2+}$ transients (Fig. 3). However, since PDE III inhibitors are able to modulate the effect of other cardiotonic agents at concentrations much lower than those at which they exert the direct inotropic effect by themselves, the absence of a direct inotropic action should not be taken as evidence for lack of PDE inhibitory action (22). It is known that it is extremely difficult to obtain definite experimental evidence for the mediator role of cyclic AMP in the PIE of PDE inhibitors and $\beta$-adrenoceptor agonists by measuring the changes in cyclic AMP levels in intact myocardium; namely, when the PIE of these agents is weak. For example, milrinone, a pure PDE III inhibitor, increases significantly the cyclic AMP level of the isolated ventricular muscle preparation first when the PIE reached approximately 80% of the maximal response. Over the lower concentration range of milrinone, the cyclic AMP content was not elevated to a detectable level even though

the PIE in association with the positive lusitropic effect strongly suggests that the PIE is mediated by cyclic AMP (21). It has been supposed therefore that the increase in cyclic AMP levels in functionally relevant intracellular compartment may be small, and it does not reach a detectable level when the cyclic AMP content of the isolated muscle preparation is measured. To overcome this difficulty, we proposed a pharmacological procedure to use the muscarinic receptor agonist to determine whether it antagonizes the PIE of the cardiotonic agent applied: a muscarinic agonist such as carbachol is useful to gain insight about the mediator role of cyclic AMP because it inhibits selectively the cyclic AMP-mediated PIE of histamine, glucagon, PDE inhibitors and $\beta$-adrenoceptor agonists, but does not affect the PIE of $[Ca^{2+}]_o$ elevation or digitalis, the action of which does not involve the cyclic AMP-mediated process (26). Since EMD 57439 scarcely affected the contractility, this procedure was not available for examining the weak PDE inhibitory action of the compound. Nonetheless, the less pronounced decrease in diastolic cell length with the racemate EMD 53998 could be explainable only by a weak PDE inhibitory action of the (−)-enantiomer EMD 57439, which has been demonstrated in mammalian cardiac muscle (6−9).

Elevation of $[Ca^{2+}]_o$ and isoproterenol at high concentrations decreased the diastolic cell length and increased the diastolic fluorescence ratio. Under these experimental conditions, the uptake capacity of the $Ca^{2+}$ pump of the sarcoplasmic reticulum (SR) and extrusion of $[Ca^{2+}]_i$ through the $Na^+/Ca^{2+}$ exchanger may not be sufficient to compensate an excessive elevation of $[Ca^{2+}]_i$ induced by these interventions at higher concentrations. On the other hand, the decrease in diastolic cell length induced by EMD 53998 and EMD 57033 was not associated with an increase in diastolic fluorescence ratio, which indicates that $Ca^{2+}$ sensitizing action of EMD 53998 and EMD 57033 is exerted even at diastolic $[Ca^{2+}]_i$ levels. The accumulation of cyclic AMP through PDE III inhibition phosphorylates phospholamban and troponin I, which results in an acceleration of the rate of $Ca^{2+}$ uptake into the SR and a decrease in myofilament affinity for $Ca^{2+}$ ions, respectively (17, 27, 28). These effects lead to acceleration of cardiac relaxation, which may be responsible for the less pronounced increase in diastolic cell length induced by the racemate than by EMD 57033. Since the systolic effects of the racemate and (+)-enantiomer were equivalent, a weak PDE III inhibition may exert a differential effect on systolic and diastolic function. Namely, EMD 57439 may antagonize the $Ca^{2+}$ sensitizing effect of EMD 57033 on the diastolic function without affecting the systolic function, which implies that the combination may be beneficial for clinical application of $Ca^{2+}$ sensitizers with respect to impairment of diastolic function. It has to be taken into consideration, however, that the single myocyte is contracting in an auxotonic manner from slack length, which is not comparable to isometric contraction of multicellular preparations such as isolated papillary muscle and is thus far from cardiac contraction in vivo. Therefore, we compared the effects of a novel cardiotonic agent, levosimendan, in rabbit ventricular myocytes loaded with indo-1/AM and in aequorin-loaded papillary muscles: levosimendan shifted the relation between the increase in contractility and peak $Ca^{2+}$ transients essentially to an equivalent extent in both preparations, an indication that the findings in single myocytes may reflect the regulation induced by the drug in a multicellular preparation (29).

The present experiments were carried out in rabbit ventricular myocytes stimulated electrically at a rate of 0.5 Hz at room temperature. It can be probable, therefore, that the frequency of contraction plays an important role in the decrease in diastolic length induced by EMD 53998 and EMD 57033 (Fig. 3, Table 3). However, the changes in cell length occurred without those of indo-1 fluorescence ratio, and other novel $Ca^{2+}$ sensitizers such as SCH00013 (11) and levosimendan (29) did not affect the diastolic cell length when these agents elicited $Ca^{2+}$ sensitization in the systolic peak shortening-$Ca^{2+}$ transient relationship. The decrease in diastolic length induced by thiadiazinone derivatives, therefore, may be due to $Ca^{2+}$ sensitization at diastolic $Ca^{2+}$ concentrations. The presence of the plateau resting level of cell length during application of thiadiazinone derivatives indicate also that it is unlikely that the frequency of contraction plays a crucial role in the decrease in diastolic cell length induced by these compounds.

It has been postulated that diastolic dysfunction induced by $Ca^{2+}$ sensitizers could constitute a hazardous problem; namely, under congestive heart failure (30, 31). Present findings support the view that weak PDE inhibition could minimize the potential disadvantage of $Ca^{2+}$ sensitizers. In addition, the $[Ca^{2+}]_i$-dependent binding of the drug molecule to troponin C has been proposed for the novel $Ca^{2+}$ sensitizer levosimendan as the mechanism for absence of impairment of relaxation (31, 32). These observations provide the possibility to avoid the relaxation impairing effect of $Ca^{2+}$ sensitizers when these agents are applied under the clinical setting (31).

In conclusion, the PIE of EMD 53998 is mainly due to its (+)-enantiomer EMD 57033 that acts by myofibrillar $Ca^{2+}$ sensitizing action. The (−)-enantiomer EMD 57439 scarcely affected the contractility by itself, but it antagonized the diastolic dysfunction elicited via the $Ca^{2+}$ sensitizing mechanism in rabbit ventricular cardiomyocytes.

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