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The effects of EMD 57033 on rigor tension in porcine skinned cardiac trabecula

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Abstract

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 (EMD 57033) is a cardiotonic agent which acts directly on the myofilaments, increasing their sensitivity to Ca^{2+}. We studied the effects of EMD 57033 on porcine cardiac skinned fibres to investigate the roles of Ca^{2+} and crossbridges in its Ca^{2+} sensitisation. EMD 57033 potentiated resting and maximum tensions and caused a leftward shift of the force-Ca^{2+} relationship. Under rigor conditions, in which fibres developed Ca^{2+}-independent force, EMD 57033 was still able to potentiate tension, provided the compound was added prior to rigor development. These results show that EMD 57033 increases force by a Ca^{2+}-independent mechanism in the intact myofilament lattice and that the Ca^{2+} sensitiser acts during the transition of crossbridge state rather than on crossbridges that are fixed in the strongly attached state corresponding to rigor.

Keywords: Ca^{2+}; Myofilament; Ca^{2+} sensitiser; EMD 57033; Skinned fiber; Rigor

1. Introduction

Much research has been directed towards development of suitably potent and specific Ca^{2+}-sensitising agents for treatment of heart failure. One such agent, the thiadiazinone derivative 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) increases the force produced by the myocardium without increasing the amplitude of the Ca^{2+} transient (Ferroni et al., 1991; Lee and Allen, 1991). EMD 53998 can be separated into its optical enantiomers and of these the (+) enantiomer, EMD 57033, is responsible for Ca^{2+} sensitisation whereas the (−) enantiomer, EMD 57439, accounts for almost all the phosphodiesterase inhibition by the racemate (Gambassi et al., 1993; White et al., 1993). The mechanism of action of EMD 57033 is independent of the regulatory complex in cardiac myofibrils and Ca^{2+} sensitisation is probably achieved through a direct effect on actomyosin interaction (Solaro et al., 1993; Strauss et al., 1992, 1994; Simnett et al., 1993; Arner et al., 1995).

Properties of crossbridges are likely to be affected by physical constraints imposed on them so agents which alter actomyosin interaction could have different effects on isolated myofibrils (Solaro et al., 1993) and on the more structurally intact skinned fibre preparations. At low concentrations of Ca^{2+}, the force produced by skinned trabeculae in the absence of ATP is Ca^{2+}-independent (Steele and Smith, 1992). The present study therefore used cardiac skinned fibres in rigor to investigate the effects of EMD 57033 on Ca^{2+}-independent mechanical activity. We concentrated on the effects of EMD 57033 on Ca^{2+}-independent force, in contrast to previous work which measured ATPase activity (Solaro et al., 1993), since EMD 57033 does not necessarily have equal effects on force and ATPase activity (Leijendekker and Herzig, 1992; Strauss et al., 1994). In addition our approach left the troponin-tropomyosin (Tn-Tm) complex intact so that possible reciprocal interactions between the complex and crossbridges were unimpeded (in contrast to Strauss et al., 1992 and Arner et al., 1995). Our results show that EMD 57033 increases the force generated by the intact cardiac myofilament lattice in a Ca^{2+}-independent

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manner. ATP was required for the Ca^{2+}-sensitising action indicating that EMD 57033 does not increase force through alteration of the conformation of cross-bridges in the force-generating state corresponding to rigor.

2. Materials and methods

2.1. Preparations

Trabeculae septomarginales were dissected from the right ventricle of porcine hearts in an abattoir and were transported to the laboratory on ice in solution X: 50% glycerol; 20 mM histidine; 5 mM EGTA; 10 mM Na azide and 1 mM dithiothreitol; pH 7.00. Subendocardial bundles of fibres were dissected out of the trabeculae and were skinned for 30 min at 4°C in solution X without glycerol and containing 0.1% triton X-100 at pH 7.30. Preparations were stored in solution X, pH 7.00, at −20°C and were used within 4 weeks. Immediately prior to an experiment a bundle of fibres was further dissected to obtain a preparation of 100–300 μm diameter and approximately 1 cm length. The skinned fibres were preconditioned with a 20 min maximal activation (see below) and were then relaxed until force was steady. Following preconditioning, the fibres were again maximally activated and, after the subsequent relaxation, fibre length was adjusted to give a resting tension of 10% peak force (sarcomere length approximately 2.2 μm as measured in a pilot study).

2.2. Apparatus

Each muscle fibre was glued with cellulose nitrate in acetone between a fixed stop and an AE801 Mesotron AG force transducer. Baths containing incubation solutions were placed on a rotating stand which was raised to submerge the fibre in the solution. The rotation of the bath ensured constant stirring of the solutions. Force was amplified and recorded on a Kontron 340 2 channel chart recorder. All experiments were performed at room temperature (22°C).

2.3. Solutions

The solutions used are detailed in Table 1. Ionic strength was 220 mM and the pH was 7.00. The solutions were stored for up to 4 weeks at −20°C. During the experiments each bathful of solution was only used once to avoid cumulative contamination with ATP or test substances.

Solution composition and free ion concentrations were calculated using a computer programme devised by Kentish (1984). The purity of the EGTA stock was measured by titrating it against $CaCl_2$ (Miller and Smith, 1984). EMD 57033 was provided by E. Merck, Darmstadt and was prepared as a stock solution of 20 mM in dimethyl sulphoxide (DMSO). This was diluted where necessary with DMSO such that the final concentration of solvent in the experimental solutions was always 0.5%. 0.5% DMSO was also added to the control solutions.

Table 1
Composition of solutions

Solution	R [a]	A [b]	GR [c]	GA
BES [d]	25	25	25	25
EGTA	10	10	10	10
ATP	10	10	–	–
KPr [e]	102	103	141	141
KCl	20	–	20	–
NaCl	–	–	20	20
$MgCl_2$	7.8	7.4	1.3	1.0
Mg^{2+}	1.0	1.0	1.0	1.0
$CaCl_2$	–	10.22	–	10.00

[a,b] R and A denote relaxing and maximal activating solutions, respectively. [c] G denotes solution without ATP. Solutions R and A were mixed to give control solutions of intermediate Ca^{2+} concentrations. Solutions GR and GA were mixed to give 'rigor' solutions of intermediate Ca^{2+} concentrations. All concentrations are given as mM. [d] BES, *N,N*-bis(2-hydroxyethyl)-2-aminoethane sulphonic acid. [e] KPr, potassium propionate.

2.4. Protocols

Effects of Ca^{2+} on rigor force

Fibres were submitted to one of two protocols. Maximum Ca^{2+}-activated force (in the presence of ATP) was measured at the start of the experiment and then after every six activations to allow for correction for the fall in peak tension during the experiment. Protocol 1 involved activating the fibres in solutions of increasing concentrations of Ca^{2+} in the presence of ATP (activating solutions) and in its absence (rigor solutions). Contractions in activating solutions were alternated with those in rigor solutions. The fibres were relaxed after every application of activating and rigor solution. Protocol 2 was similar to protocol 1 except that fibres were only relaxed in between activations at different Ca^{2+} concentrations. ATP withdrawal therefore occurred when the myofilaments were already equilibrated with Ca^{2+}.

Ca^{2+} dependence of effects of EMD 57033 on rigor and active force

Protocol 3 consisted of activating the fibres with ATP-free solutions of increasing concentrations of Ca^{2+} alternately in the presence and then the absence of EMD 57033. Fibres were relaxed after each activation. EMD 57033 was washed into the myofilaments in relaxing solution. Protocol 4 was similar to protocol 3 except that equilibration of the fibres in a rigor solu-

tion of a given Ca^{2+} concentration was followed directly by incubation in the same solution plus EMD 57033. Wash-in of the compound thus occurred in the presence of Ca^{2+} and the fibres were only relaxed in between application of different Ca^{2+} concentrations. Protocol 5 was as protocol 3 except that ATP was included in all the solutions.

Maximal Ca^{2+}-activating solution was applied after every six activations (protocols 3 and 5) or three activations (protocol 4) to allow for correction for the decline in peak tension during the experiment. The effects of EMD 57033 on maximum force, in the presence of ATP, were measured simultaneously with complete force-Ca^{2+} relationships (protocol 5).

Concentration dependence of effects of EMD 57033 on Ca^{2+}-independent rigor force

Concentration-response experiments were performed for EMD 57033 at pCa 9.00, where pCa = $-\log [Ca^{2+}]$. In these cases protocol 3 was modified such that the free Ca^{2+} concentration in the rigor solutions remained constant (pCa 9.00) throughout but the concentration of EMD 57033 increased progressively. Comparable experiments with EMD 57033 were also carried out using a modified protocol 4 (pCa again remaining constant at 9.00 in all rigor solutions and the concentration of EMD increasing as in the modified protocol 3). Contractions in the presence of 50 μM EMD 57033 were performed at the very end of the experiment and no washout was attempted (corrected maximum force was obtained by extrapolation).

2.5. Analysis

Results are expressed as means ± S.E.M. where appropriate. Ca^{2+} concentrations are given in pCa form, pCa_{50} being the pCa required to elicit 50% of maximum Ca^{2+}-activated force under given conditions. All forces are expressed relative to maximum tension in the presence of ATP under control conditions. Force-pCa curves were fit by the Hill equation. The mean results from these curve fits were used to generate the sigmoidal curves in the figures. Where appropriate, *t*-tests were used to detect significant differences ($P < 0.05$ denoting significance).

3. Results

Trabeculae had a mean diameter of 222 ± 4 μm ($n = 104$) and generated a peak tension of 25.0 ± 0.3 mN/mm^2 ($n = 104$) under control conditions.

3.1. Effects of EMD 57033 on force in the presence of ATP

At concentrations of 10 μM and above, EMD 57033 increased resting tension of cardiac trabecula in a concentration-dependent manner (Fig. 1). 50 μM EMD 57033 potentiated resting force to 32.2 ± 1.4% ($n = 5$) of control maximum force. Such generation of tension in the near absence of Ca^{2+} (pCa 9.37) indicates that EMD 57033 can promote force via a Ca^{2+}-independent mechanism. A similar conclusion can be reached from the EMD 57033-induced potentiation of maximum force, since at pCa 4.2 the Ca^{2+}-specific site of troponin C (TnC) will already be saturated with Ca^{2+}. Interestingly, at pCa 4.2 force was potentiated by concentrations of EMD 57033 (< 10 μM) which were too low to increase resting tension (Fig. 1). In addition to its effects on minimum and maximum Ca^{2+}-activated

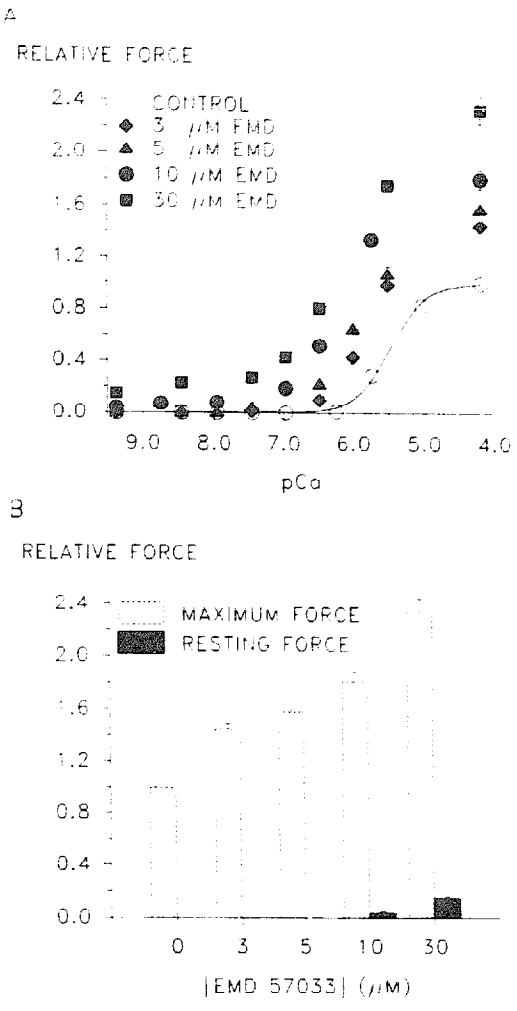

Fig. 1. Effects of EMD 57033 on force in the presence of ATP. Force-pCa curves were determined in the presence of 0–30 μM EMD 57033 (panel A). Data are plotted as means ± S.E.M. with $n = 6$ in each case except control where $n = 24$. The following parameters were obtained from the fitting of the data to the Hill equation: Control: pCa_{50} 5.48 ± 0.02, n_H 1.77 ± 0.06; 3 μM EMD: pCa_{50} 5.74 ± 0.03, n_H 1.49 ± 0.05; 5 μM EMD: pCa_{50} 5.79 ± 0.04, n_H 1.09 ± 0.09; 10 μM EMD: pCa_{50} 6.10 ± 0.04, n_H 1.22 ± 0.04 and 30 μM EMD: pCa_{50} 5.99 ± 0.06, n_H 0.87 ± 0.04. Data for resting tension (pCa 9.37) and maximum force (pCa 4.20) are replotted in panel B to facilitate comparison with subsequent figures and to emphasise the concentration-dependent potentiation of force by EMD 57033.

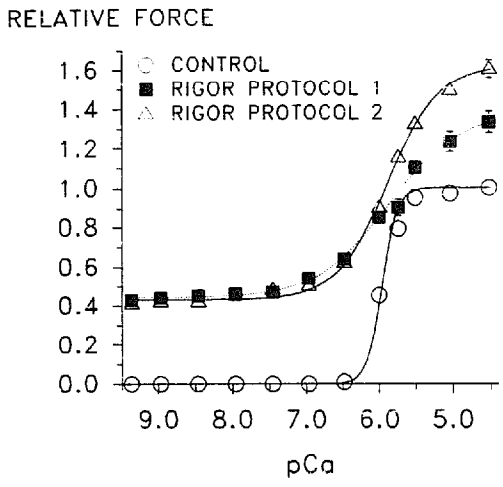

Fig. 2. Effects of Ca^{2+} on rigor force. Force-pCa relationships for porcine skinned trabecula were determined: in the presence of ATP ($n = 24$); under rigor conditions where ATP was withdrawn simultaneously with the addition of Ca^{2+} using protocol 1 ($n = 16$) and under rigor conditions where ATP was withdrawn following equilibration of the fibre with Ca^{2+} using protocol 2 ($n = 8$). Points are plotted as means ± S.E.M. Fitting the data to the Hill equation gave the following parameters: Control: pCa_{50} 5.96 ± 0.01, n_H 4.15 ± 0.69. Rigor protocol 1: pCa_{50} 5.88 ± 0.04, n_H 0.89 ± 0.04. Rigor protocol 2: pCa_{50} 5.88 ± 0.03, n_H 1.24 ± 0.09.

forces, EMD 57033 shifted the force-pCa curves leftwards in a concentration-dependent fashion.

3.2. Effects of Ca^{2+} on rigor force

Porcine cardiac fibres can generate a Ca^{2+}-independent rigor force at pCa values above 7.5, where no force is produced if ATP is present (Fig. 2). As was the case in rat myocytes (Fabiato and Fabiato, 1975), rigor tension in porcine fibres began its increase as a function of Ca^{2+} at a higher pCa (7.0) than did active force. The relationship between rigor force and pCa was sigmoidal and compared to the curve for active force had a shallower gradient and a potentiated maximum force. The shape of the rigor curve depended on the relative timings of ATP withdrawal and equilibration of the fibres with Ca^{2+}.

3.3. Effects of EMD 57033 on rigor force

5 μM EMD 57033 had no effect on force in the presence of ATP at pCa values above 7.5 (Fig. 1). This concentration of the thiadiazinone was therefore chosen to see whether it could potentiate force over this same range of low Ca^{2+} concentrations under conditions where tension was generated even in the absence of Ca^{2+} sensitiser, i.e. during rigor (Fig. 2).

When applied to the fibre prior to the simultaneous introduction of Ca^{2+} and withdrawal of ATP (protocol 3), EMD 57033 caused an increase in the subsequent rigor contraction compared to the equivalent rigor force in the absence of thiadiazinone, as shown in Fig. 3A,B. This potentiation of force occurred over the whole range of pCa values 9.5–4.5 (Fig. 4A) and was accompanied by a slight (0.47 ± 0.09 ($n = 8$) pCa unit) leftwards shift in the force-pCa relationship, its slope not being significantly changed. At pCas above 7.5, EMD 57033 caused a parallel upwards shift of the Ca^{2+}-independent rigor force. To determine whether Ca^{2+} sensitisation was the result of a change in conformation of the strongly bound rigor crossbridges (equivalent to the crossbridge state immediately prior to the

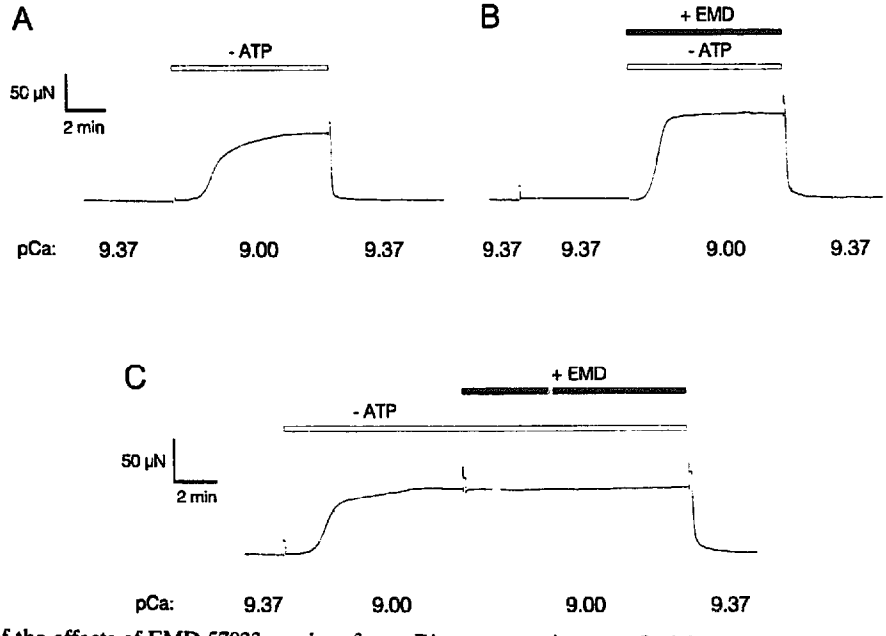

Fig. 3. Typical recording of the effects of EMD 57033 on rigor force. Rigor contractions at pCa 9.00 are shown before (panel A) and after (panel B) the application of 5 μM EMD 57033 according to protocol 3. Addition of 5 μM EMD after development of rigor tension (protocol 4) had no effect on force (panel C). All traces were recorded from the same skinned trabecula.

dissociation of actomyosin by ATP during active cycling), EMD 57033 was applied to porcine cardiac fibres which were already in a stable rigor contraction (protocol 4). As shown in Fig. 3C, rigor force was not significantly affected by the compound under these conditions. This result was the same over a wide range of Ca^{2+} concentrations (Fig. 4B).

The EMD 57033 concentration dependence of changes in rigor force at pCa 9.00 was also investigated. Clearly, whereas increasing concentrations of EMD 57033 caused increasing potentiations of force if the compound was added prior to rigor development (protocol 3), addition of the thiadiazinone to fibres already in rigor (protocol 4) had no effect at any concentration (Fig. 5). This lack of response of the fibres with protocol 4 was not an artifact of the necessarily prolonged contraction time since similar forces were produced by fibres which were activated for comparable durations but in the absence of thiadiazinone (Fig. 5). Thus a possible decrease in rigor tension with time was not masking a force increase induced by the Ca^{2+} sensitiser. The relationship between EMD 57033 concentration and rigor force at pCa 9.00 for protocol 3 was similar to that between EMD 57033 concentration and maximum force in the presence of ATP (compare Figs. 5 and 1B).

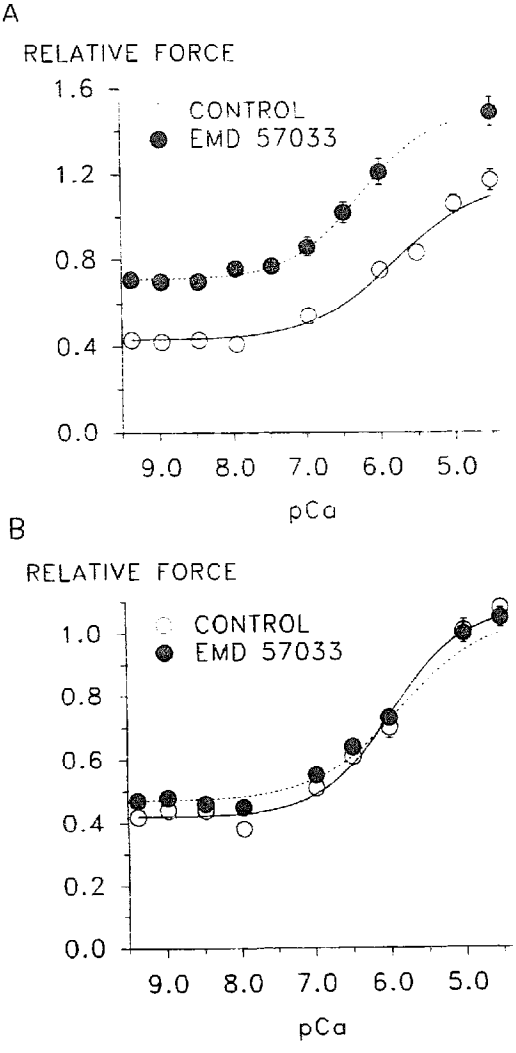

Fig. 4. Effects of EMD 57033 on rigor force. Between pCas 9.5 and 4.5 5 μM EMD potentiated rigor tension in porcine skinned trabecula when added prior to rigor development (protocol 3, panel A) but had no effect on force if added following rigor development (protocol 4, panel B). Points are plotted as means ± S.E.M. with $n = 8$ for each curve. Hill equation parameters were: panel A control, pCa_{50} 5.79 ± 0.04, n_H 0.77 ± 0.07; panel A rigor, pCa_{50} 6.26 ± 0.08, n_H 0.93 ± 0.07; panel B control, pCa_{50} 5.96 ± 0.04, n_H 0.91 ± 0.03; panel B rigor, pCa_{50} 5.85 ± 0.05, n_H 0.77 ± 0.04.

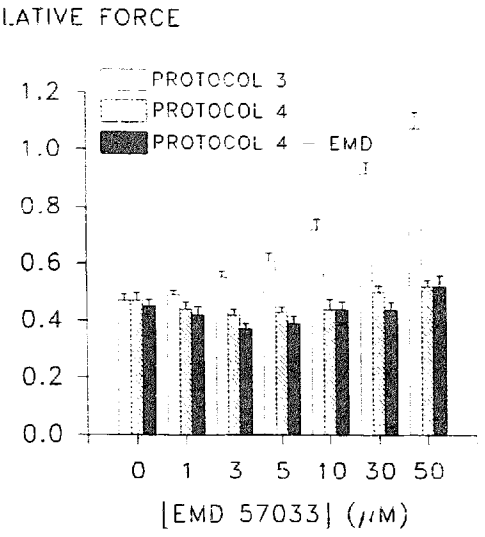

Fig. 5. Dependence of rigor force on EMD 57033 concentration at pCa 9.00. Bar graph showing effects of 0–50 μM EMD on rigor tension at pCa 9.00, achieved with either protocol 3 (open bars, $n = 16$) or protocol 4 (hatched bars, $n = 8$). Solid bars indicate rigor forces achieved using protocol 4 without the addition of EMD ($n = 6$) and are thus control data for the effects of time on rigor tension. Error bars represent S.E.M.

4. Discussion

Our results demonstrate the Ca^{2+} independence of the mechanism by which EMD 57033 increases force. At concentrations of 10 μM or more, EMD 57033 increased resting tension, in the presence of millimolar ATP, at pCa values as high as 9.5 (Fig. 1). To achieve the same potentiation of force by increasing the Ca^{2+} concentration instead of adding 30 μM EMD 57033, a drop in pCa from 9.5 to 5.8 would be necessary. This is clearly too great a change to be explained by the 0.60 unit leftwards shift of the force-pCa relationship induced by 30 μM thiadiazinone. EMD 57033 must therefore increase resting tension by a Ca^{2+}-independent mechanism. In the absence of ATP, concentrations of EMD 57033 as low as 3 μM potentiated Ca^{2+}-independent force at pCa 9.0 (Fig. 5). Further evidence that EMD 57033 does not increase tension purely by an effect on TnC is provided by the potentia-

tion of maximum force by the compound (Fig. 1) since at pCa values sufficiently low to maximally activate the myofilaments, the Ca^{2+}-specific site of TnC will be saturated with Ca^{2+}. Clearly the thiadiazinone potentiates maximum Ca^{2+}-activated force via a troponin-independent mechanism. In the presence of physiological amounts of ATP, EMD 57033 could increase resting tension (Fig. 1) by overcoming the inhibition of myofilament activation by Tn-Tm to promote formation of strongly bound crossbridges. The thiadiazinone would thus play a similar role to Ca^{2+} but would already operate at pCa values above those at which Ca^{2+} normally disinhibits the thin filament, and would act independently of the troponin complex. Following the formation of a critical number of force-generating crossbridges, EMD 57033 could also potentiate a cooperative response which facilitates further actomyosin interaction (Solaro et al., 1993).

These conclusions support the finding of Solaro et al. (1993) that EMD 57033 had no effect on total Ca^{2+} binding to troponin in skinned fibres (although a small effect could be missed using this technique which probably also measures some additional non-specific Ca^{2+} binding). In addition, EMD 57033 increased force and ATPase activity respectively in troponin I-extracted skinned fibres (Strauss et al., 1992) and troponin-depleted myofibrils (Solaro et al., 1993). A major difference between these previous studies and the present one is that we used preparations in which the myofilament lattice was left intact thereby minimizing disturbance to the geometry of the muscle proteins. This is important because interactions between crossbridges are likely to play a role in the mechanism of the EMD-induced force increase. It should also be noted that effects of EMD 57033 on ATPase activity do not necessarily indicate its effects on force since the Ca^{2+} sensitiser also changes tension cost (Leijendekker and Herzig, 1992).

Although several reports describe the effects of EMD 53998 and its enantiomers on the force-pCa relationship of skinned cardiac fibres (Beier et al., 1991; Ventura et al., 1992; Gross et al., 1993), very little attention has been directed towards their effects on resting tension. We found that the large (32% of control maximum force) resting force induced by 50 μM EMD 57033 during pilot experiments could never be completely reversed. Such results have important implications for a drug intended for treatment of heart failure since complete relaxation of the heart during diastole could be compromised by EMD 57033.

Our finding (Fig. 2) that rigor force in porcine trabecula is independent of Ca^{2+} at high pCa values (> 7.5) agrees with results from rat trabecula (Steele and Smith, 1992). At such low Ca^{2+} concentrations force is not regulated by Ca^{2+} binding to TnC, instead 'unstrained' crossbridge formation and cooperativity between rigor complexes will determine the level of tension (Kawai and Brandt, 1976). In the presence of Ca^{2+}, during development of rigor force, the number of actively cycling crossbridges falls as they are arrested in the post-energy transduction 'strained' rigor state. The ratio of unstrained and strained rigor crossbridges determines the final level of rigor tension (Kawai and Brandt, 1976) and is Ca^{2+}-dependent. The Ca^{2+} dependency of rigor arises because Ca^{2+} increases the proportion of rigor crossbridges that are in the strained state and these generate more force than the unstrained type. Withdrawal of ATP simultaneously with the introduction of Ca^{2+} (protocol 1) resulted in the myofilaments having insufficient time to reach the steady state force normally associated with a given Ca^{2+} concentration before rigor developed. The proportion of unstrained rigor crossbridges was therefore greater and the rigor tension lower than when the steady state force was achieved before induction of rigor (protocol 2). The discrepancy disappeared at high pCa values since here presumably all crossbridges were of the unstrained type (Fig. 2).

This study is the first in which the effects of EMD 57033 on rigor force have been investigated. The most important observations made were that EMD 57033 could potentiate rigor tension only when applied before ATP depletion and that the thiadiazinone had a greater effect on rigor force than on resting tension at a given pCa. Our hypothesis is that when ATP is withdrawn, EMD 57033 mimics the effects of Ca^{2+} by facilitating the formation of strained crossbridges during rigor development. During ATP withdrawal, EMD 57033 potentiates force both by overcoming the inhibition of actin by Tn-Tm and promoting cooperativity between strongly bound crossbridges. This would increase the number of strained crossbridges formed and hence increase the rigor force ultimately generated. The proportional increase in force induced by EMD 57033 during rigor development was greater at low than at high Ca^{2+} concentrations (Fig. 4). As the degree of activation rises, the proportion of functional units still inhibited by Tn-Tm would decrease so mechanisms lifting inhibition of the thin filament would decline in effect. This is compatible with the proposed effects of EMD 57033 on disinhibition of actomyosin interaction (Solaro et al., 1993).

The lack of response of trabeculae to which EMD 57033 was applied after development of a stable rigor force indicates that crossbridges need to be actively cycling for the thiadiazinone to potentiate force. During rigor the crossbridges are arrested in a strongly bound state corresponding to that immediately before ATP binds to induce detachment during the crossbridge cycle in the presence of ATP. Our results therefore show that this stage of the crossbridge cycle is not directly affected by EMD 57033. Using photorelease of

ATP, it has been shown that EMD 53998 increases the rate of activation of skinned porcine trabecula (Arner et al., 1995). This was interpreted as the result of an EMD-induced increase in the attachment rate constant, f_{app}. Although such results indicate that EMD 53998 increases the rate of transition from the detached or weakly bound crossbridge state to the strongly bound, force-generating, form, this does not preclude an additional effect of EMD 57033 on the established strongly bound crossbridge state. Our finding that EMD 57033 had no effect if added after the development of rigor (where crossbridges are arrested in the strongly bound state) shows that the Ca^{2+} sensitiser acts during the transition to force-generating crossbridges rather than on the end product. Despite the fact that crossbridge detachment is almost prohibited during rigor, the potentiation of rigor force by EMD 57033 cannot necessarily be totally ascribed to an effect of the compound on crossbridge attachment or transformation from the weakly bound to the strongly bound state. The withdrawal of ATP from the myofilaments was not instantaneous during these experiments so an ever decreasing population of actively cycling crossbridges would have been present during rigor development and EMD 57033 might also have altered force by reducing their detachment rate, although this seems unlikely (Simnett et al., 1993).

The fact that concentrations of EMD 57033 as low as 3 μM were able to potentiate rigor tension at pCa 9.0 (Fig. 5), but had no effect on resting force (Fig. 1), indicates that the magnitude of the Ca^{2+}-independent inotropic effects of the Ca^{2+} sensitiser depends on the degree of activation of the myofilaments. Although at higher concentrations EMD 57033 promoted the formation of force-generating crossbridges where none were previously present (10 μM and 30 μM EMD 57033 increased resting tension in the presence of ATP –Fig. 1), lower concentrations required actomyosin interaction to already be present before they could have an effect (most likely through increasing cooperativity between crossbridges).

The overcoming of inhibition by Tn-Tm and potentiation of cooperativity can explain at least part of the observed increase in Ca^{2+}-activated maximum force (Fig. 1) but since concentrations of EMD 57033 which have no effect on resting tension are able to potentiate maximum force, an additional mechanism might operate at low pCa values. It is important to note that even if EMD 57033 were to promote crossbridge attachment so that *all* crossbridges were strongly bound, this would increase maximum force by only approximately 30% (assuming approximately 70% are bound at maximal activation (Goldman and Simmons, 1977)). The fact that maximum force increased by more than 60% (Fig. 1) suggests that EMD may also act via a second mechanism, an increase in the force generated per crossbridge. Alternatively, the normal percentage of crossbridges bound may be lower than currently believed, i.e. only 30–40%.

In summary, the inotropic action of EMD 57033 depended on the extent of actomyosin interaction, and increasing such interaction by removing ATP promoted the potentiation of force by the thiadiazinone. In addition, since rigor force at high pCa values was independent of Ca^{2+}, the EMD 57033-induced increase in tension also confirmed the Ca^{2+} independence of the Ca^{2+} sensitisation. EMD 57033 did not potentiate force through a change in conformation of the rigor crossbridge state and, at least at high levels of myofilament activation, is likely to additionally increase tension by increasing the force generated per strongly bound crossbridge.

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References

Arner, A., J.D. Strauss, C. Svensson and J.C. Rüegg, 1995, Effects of troponin-I extraction with vanadate and of the calcium sensitizer EMD 53998 on the rate of force generation in skinned cardiac muscle, J. Mol. Cell. Cardiol. 27, 615.

Beier, N., J. Harting, R. Jonas, M. Klockow, I. Lues and G. Haeusler, 1991, The novel cardiotonic agent EMD 53998 is a potent "calcium sensitizer", J. Cardiovasc. Pharmacol. 18, 17.

Fabiato, A. and F. Fabiato, 1975, Effects of magnesium on contractile activation of skinned cardiac cells, J. Physiol. 249, 497.

Ferroni, C., O. Hano, C. Ventura, E.G. Lakatta, M. Klockow, H. Spurgeon and M.C. Capogrossi, 1991, A novel positive inotropic substance enhances contractility without increasing the Ca^{2+} transient in rat myocardium, J. Mol. Cell. Cardiol. 23, 325.

Gambassi, G., M.C. Capogrossi, M. Klockow and E.G. Lakatta, 1993, Enantiomeric dissection of the effects of the inotropic agent, EMD 53998, in single cardiac myocytes, Am. J. Physiol. 264, H728.

Goldman, Y.E. and R.M. Simmons, 1977, Active and rigor muscle stiffness, J. Physiol. 269, 55P.

Gross, T., I. Lues and J. Daut, 1993, A new cardiotonic drug reduces the energy cost of active tension in cardiac muscle, J. Mol. Cell. Cardiol. 25, 239.

Kawai, M. and P.W. Brandt, 1976, Two rigor states in skinned crayfish single muscle fibers, J. Gen. Physiol. 68, 267.

Kentish, J.C., 1984, The inhibitory effect of monovalent ions on force development in detergent-skinned ventricular muscle from guinea-pig, J. Physiol. 352, 353.

Lee, J.A. and D.G. Allen, 1991, EMD 53998 sensitizes the contractile proteins to calcium in intact ferret ventricular muscle, Circ. Res. 69, 927.

Leijendekker, W.J. and J.W. Herzig, 1992, Reduction of myocardial cross-bridge turnover rate in the presence of EMD 53998, a novel Ca^{2+}-sensitizing agent, Pflügers Arch. 421, 388.

Miller, D.J. and G.L. Smith, 1984, EGTA purity and the buffering of calcium ions in physiological solutions, Am. J. Physiol. 246, C160.

Simnett, S.J., S. Lipscomb, C.C. Ashley and I.P. Mulligan, 1993, The effect of EMD 57033, a novel cardiotonic agent, on the relaxation of skinned cardiac and skeletal muscle produced by photolysis of diazo-2, a caged calcium chelator, Pflügers Arch. 425, 175.

Solaro, R.J., G. Gambassi, D.M. Warshaw, M.R. Keller, H.A. Spurgeon, N. Beier and E.G. Lakatta, 1993, Stereoselective actions of thiadiazinones on canine cardiac myocytes and myofilaments, Circ. Res. 73, 981.

Steele, D.S. and G.L. Smith, 1992, The effects of caffeine and Ca^{2+} on rigor tension in triton-treated rat ventricular trabeculae, Pflügers Arch. 421, 343.

Strauss, J.D., C. Zeugner and J.C. Rüegg, 1992, The positive inotropic calcium sensitizer EMD 53998 antagonizes phosphate action on cross-bridges in cardiac skinned fibers, Eur. J. Pharmacol. 227, 437.

Strauss, J.D., C. Bletz and J.C. Rüegg, 1994, The calcium sensitizer EMD 53998 antagonizes phosphate-induced increases in energy cost of isometric tension in cardiac skinned fibers, Eur. J. Pharmacol. 252, 219.

Ventura, C., R. Miller, H.-P. Wolf, N. Beier, R. Jonas, M. Klockow, I. Lues, O. Hano, H.A. Spurgeon, E.G. Lakatta and M.C. Capogrossi, 1992, Novel diazinone derivatives separate myofilament Ca^{2+} sensitization and phosphodiesterase III inhibitory effects in guinea pig myocardium, Circ. Res. 70, 1081.

White, J., J.A. Lee, N. Shah and C.H. Orchard, 1993, Differential effects of the optical isomers of EMD 53998 on contraction and cytoplasmic Ca^{2+} in isolated ferret cardiac muscle, Circ. Res. 73, 61.