EMD 53998 Sensitizes the Contractile Proteins to Calcium in Intact Ferret Ventricular Muscle

J.A. Lee and D.G. Allen

EMD 53998 (a thiadiazinone) is an inotropic drug that produces a pronounced increase in the Ca\(^{2+}\) sensitivity of the contractile proteins in skinned cardiac fibers. The present study was undertaken to determine whether this effect on Ca\(^{2+}\) sensitivity could explain the increase in tension observed in intact ventricular muscle. The experiments were performed on isolated ferret papillary muscles that had been microinjected with aequorin to measure the intracellular Ca\(^{2+}\) concentration. Force and intracellular Ca\(^{2+}\) concentration were monitored before, during, and after application of EMD 53998. EMD 53998 (5 \(\mu\)M) increased developed tension by 230%; aequorin light transients increased by only 85%, and this increase was reduced and became insignificant in the presence of agents that prevent catecholamine release. When a similar increase in developed tension was produced by elevation of extracellular calcium, the aequorin light transients increased by 240%. Thus, EMD 53998 produces a substantial Ca\(^{2+}\) sensitization in intact ventricular muscle, and this can explain most of its inotropic effect. In addition, EMD 53998 caused a small prolongation of the time course of contraction and a small reduction of the time course of the aequorin light transient. A computer model is described that shows that both these effects can be explained by the effect of EMD 53998 on Ca\(^{2+}\) sensitivity. At much higher concentrations, EMD 53998 also caused an increase in resting tension. EMD 53998 is the first agent for which much of the inotropic effect in intact cardiac muscle can be accounted for by increased Ca\(^{2+}\) sensitivity of the contractile proteins. Inotropic agents with this mechanism of action cause increased force production with much less increase in the intracellular Ca\(^{2+}\) transients than conventional agents and, therefore, increase the energy efficiency of the myocardium and are less likely to cause Ca\(^{2+}\)-activated arrhythmias. (Circulation Research 1991;69:927–936)

The main groups of inotropic agents in current use are the cardiac glycosides and the \(\beta\)-adrenergic agents.\(^1,2\) Both of these agents have as their final common pathway an increase in intracellular free calcium (Ca\(^{2+}\)) during contraction,\(^3\) which increases calcium bound to troponin and, therefore, tension. However, both groups of drugs can cause dangerous arrhythmias at high doses, and this limits their clinical value. The arrhythmogenic effect of these compounds seems to be intimately linked to the increased levels of intracellular free calcium concentration ([Ca\(^{2+}\)]\(_i\)) that they cause. It has been shown that the cardiac sarcoplasmic reticulum (SR) releases Ca\(^{2+}\) spontaneously when overloaded with Ca\(^{2+}\)\(^4,5\) and that the resulting increases in [Ca\(^{2+}\)]\(_i\) induce a transient inward current that triggers arrhythmias.\(^6\)

Recognition of the problems caused by compounds that achieve their inotropic effects through increased calcium levels has led to an interest in drugs that increase tension by an increase in the Ca\(^{2+}\) sensitivity of the myofibrillar proteins rather than by elevation of [Ca\(^{2+}\)]. A number of the new phosphodiesterase inhibitors (e.g., sulmazole and pimobendan) seem to possess this additional Ca\(^{2+}\)-sensitizing effect in both skinned and intact cardiac muscle.\(^7\)–\(^10\) Phosphodiesterase inhibition raises cAMP and leads to protein phosphorylation\(^11\) with a resulting increase in the Ca\(^{2+}\) current, an increased SR Ca\(^{2+}\) uptake and release, and a reduced troponin binding constant for Ca\(^{2+}\). The combination of these cAMP-mediated effects plus the direct effect of these drugs on Ca\(^{2+}\) sensitivity means that the resulting effects on cellular function are complex. From the point of view of analyzing the effects of Ca\(^{2+}\) sensitization on muscle...
function and cardiac performance, it would be desirable to test agents whose main, or only, effect was Ca\textsuperscript{2+} sensitization.

EMD 53998 [5-(1-(3,4-dimethoxybenzoyl)-1,2,3,4-tetrahydro-6quinolyl)-6-methyl-3,6-dihydro-2H-1,3,4-thiadiazin-2-one], synthesized by E. Merck Pharmaceuticals, Darmstadt, FRG, is a drug that preliminary evidence suggests may have principally sensitizing actions.\textsuperscript{12-14} In skinned-fiber studies it causes a marked Ca\textsuperscript{2+} sensitization and a small increase in maximum Ca\textsuperscript{2+}-activated tension.\textsuperscript{14} EMD 53998 has also been shown to have an inhibitory effect on phosphodiesterases isolated from cardiac muscle (personal communication, Dr. M. Klockow, E. Merck, Darmstadt, FRG; 1989). The present study was undertaken both to determine the extent to which Ca\textsuperscript{2+} sensitization could explain the effects of the drug in intact ventricular muscle and to explore the consequences of Ca\textsuperscript{2+} sensitization on cardiac muscle function.

Materials and Methods

Ferrets were killed by an overdose of pentobarbital injection intraperitoneally, and the heart was removed. Thin (<1-mm-diameter) papillary muscles were removed from the right ventricle and mounted in a horizontal bath. They were superfused with Tyrode's solution containing (mM) Na\textsuperscript{+} 135, K\textsuperscript{+} 5, Ca\textsuperscript{2+} 1 or 0.5, Mg\textsuperscript{2+} 1, Cl\textsuperscript{−} 102, HCO\textsubscript{3}− 20, HPO\textsubscript{4}\textsuperscript{2−} 1, SO\textsubscript{4}\textsuperscript{2−} 1, acetate 20, and glucose 10, along with insulin 5 units/l (40 nM). The solution was equilibrated with 95% O\textsubscript{2}−5% CO\textsubscript{2} to give a pH of 7.36. The temperature was maintained at 37°C, and the muscle was stimulated at a frequency of 1 or 0.5 Hz.

[Ca\textsuperscript{2+}], was measured with the Ca\textsuperscript{2+}-sensitive photoprotein aequorin.\textsuperscript{15} Aequorin was pressure-injected into the cells from conventional glass micro-electrodes (for further details, see Reference 3). Aequorin light, an indicator of [Ca\textsuperscript{2+}], was measured by a photomultiplier tube via a Perspex light guide; consequently, aequorin light recordings are calibrated in nanoamperes of photomultiplier current. In an aequorin-injected papillary muscle, developed tension is recorded from the whole muscle, whereas Ca\textsuperscript{2+} transients are recorded from relatively few (40–100) cells that have been microinjected with the indicator. Thus, an assumption of the technique is that these cells give rise to a representative Ca\textsuperscript{2+} signal. Investigations\textsuperscript{3,8,10,16} using a variety of positive and negative inotropic interventions indicate that this assumption is valid, and the relative merits of photoproteins as Ca\textsuperscript{2+} indicators have been discussed in detail.\textsuperscript{17}

EMD 53998 was supplied by E. Merck Pharmaceuticals. It is relatively insoluble in water, and stock solutions were made in acetone, dimethyl sulfoxide, or ethanol. In most experiments a 10-mM stock solution in acetone was used; a 10-µM final concentration of EMD 53998 would produce 1:1,100 acetone in the final solution.

The possible effects of EMD 53998 on aequorin were tested in vitro. A small volume of aequorin solution was rapidly injected into 150 mM KCl, pH 7, and 1 mM CaCl\textsubscript{2}. The amplitude and time course of aequorin light emission under these conditions were unaffected by the addition of 100 µM EMD 53998 (carrier dimethyl sulfoxide) to the salt solution. The aequorin light emission from an EGTA-buffered Ca\textsuperscript{2+} solution ([Ca\textsuperscript{2+}]=0.4 µM) was also unaffected by the addition of 100 µM EMD 53998.

We were also concerned that erratic increases in aequorin light emission sometimes seen from aequorin-injected preparations after addition of EMD 53998 might be due to the carrier rather than EMD 53998 (see “Results”). On different occasions, EMD 53998 was dissolved in dimethyl sulfoxide, ethanol, and acetone, and the variable light emissions were observed with each. Carrier alone never produced these or any other detectable effects. Anoxia was produced by changing the superfusing solution from Tyrode's equilibrated with 95% O\textsubscript{2}−5% CO\textsubscript{2} to Tyrode's that had been vigorously bubbled with 95% N\textsubscript{2}−5% CO\textsubscript{2}.

Computer Model

This model simulates the Ca\textsuperscript{2+} fluxes in and out of the myoplasm of the cardiac cell, the binding of Ca\textsuperscript{2+} to troponin, and the development of tension by attachment of crossbridges (see Figure 1). The Ca\textsuperscript{2+} fluxes considered are the surface membrane Ca\textsuperscript{2+} current (K\textsubscript{1}), the surface membrane efflux (K\textsubscript{2}), the SR uptake (K\textsubscript{3}), and the SR release (K\textsubscript{4}). Ca\textsuperscript{2+} within the SR (CaSR) was able to bind (K\textsubscript{5}) and unbind (K\textsubscript{6}) to binding sites representing calsequintrin. The equations used to simulate these processes are given in the legend of Figure 1. Where possible (e.g., the surface membrane Ca\textsuperscript{2+} current), the flux has been given the magnitude and time course of the measured current. In other cases (e.g., the SR Ca\textsuperscript{2+} release), the magnitude and time course are not known in any detail; these parameters were therefore chosen so that the final time course of the change in myoplasmic [Ca\textsuperscript{2+}], is similar to that measured experimentally. The model includes troponin, at a concentration of 60 µM,\textsuperscript{20} and the three sites have been given the on and off rates similar to published values\textsuperscript{21} (only the low-affinity site is shown in Figure 1). The attachment of crossbridges is a function of Ca\textsuperscript{2+} bound to the low-affinity site of troponin, and the attachment (K\textsubscript{9}) and detachment rates (K\textsubscript{10}) were chosen so that the time course of attached crossbridges was similar to that of tension. In the version of the model shown in Figure 8, feedback between the attachment of crossbridges and the affinity of troponin for Ca\textsuperscript{2+} was included, based on experimental observations.\textsuperscript{24,25} The affinity of troponin for Ca\textsuperscript{2+} increased linearly with crossbridge attachment, so that maximum attachment increased the affinity fourfold (for details see legend to Figure 1). The general approach to the modeling was similar to that of Cannell and Allen,\textsuperscript{19} except that diffusion.
gradients were not included in the present model. The most useful aspect of the model is that it generates a Ca\(^{2+}\) transient that is similar to that measured with aequorin, and this Ca\(^{2+}\) transient causes Ca\(^{2+}\) binding to troponin and net attachment of crossbridges, which occurs with a time course similar to that of measured tension. Thus, the model can be used to examine the predicted effects of changes in various parameters, such as Ca\(^{2+}\) sensitivity on the Ca\(^{2+}\) transients and tension.

**Results**

**Effects of EMD 53998 on Tension**

EMD 53998 produced a pronounced inotropic effect when applied to isolated ferret papillary muscles. Figure 2 illustrates the effect of 10 \(\mu\)M EMD 53998. Tension increased rapidly after application of the drug, reaching a new, stable steady state within 5 minutes. Washout of the effects was slower but was typically complete within 10 or 20 minutes. Superimposed, normalized tension recordings (Figure 2B, recording iii) show that the time course of the contraction was slightly prolonged in the presence of EMD 53998. The effects of 5 \(\mu\)M EMD 53998 were examined in seven preparations; before application, the maximum developed tension was assessed, either by paired pulse stimulation or increases in extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{0}\), and then [Ca\(^{2+}\)], was reduced so that developed tension was 10–30% of maximum ([Ca\(^{2+}\)]\(_{0}\), usually either 0.5 or 1.0 mM). This was done both so that there was plenty of scope for increases in tension and so that all preparations were initially in a similar inotropic state. Under these conditions, 5 \(\mu\)M EMD 53998 increased tension by 230±70% (mean±SEM) of the control. The prolongation of the time course of contraction in the presence of EMD 53998 was quantified by two measurements: the time from stimulus to peak tension increased by 8.6±2.4% and the time from stimulus until relaxation was 50% complete increased by 11.6±2.3%.

**Figure 1.** Diagram showing the Ca\(^{2+}\) fluxes and other processes involved in the computer model. A general description of the model is given in the text; here the equations used to define these processes are described. K1 represents the inward Ca\(^{2+}\) current during the action potential (duration, 200 msec) and a small inward Ca\(^{2+}\) leak at other times. The equation used to describe the Ca\(^{2+}\) flux is

\[
[Ca^{2+}]_{o}[k_1(1-e^{-t/n})(e^{-t/n})+L]
\]

where t is the time after the start of the action potential, \([Ca^{2+}]_{o}=2\) mM, \(k_1=0.3\) sec\(^{-1}\) (the maximum membrane Ca\(^{2+}\) permeability), \(\tau_1=2\) msec (the time constant of rise of the Ca\(^{2+}\) current), \(\tau_2=20\) msec (the time constant of decline of the Ca\(^{2+}\) current), and \(L=0.004\) sec\(^{-1}\) (the membrane Ca\(^{2+}\) permeability outside the action potential). The Ca\(^{2+}\) entry during one action potential with these parameters amounted to 5 \(\mu\)mol/l cell volume, which is similar to published values. 18 K2 represents a continuous first-order pump out of the cell and can be thought of as an empirical representation of the Na\(^+\)/Ca\(^{2+}\) exchanger. The Ca\(^{2+}\) efflux was \([Ca^{2+}]_{o}k_2\), where \(k_2=30\) sec\(^{-1}\).

With the parameters of the model, the Ca\(^{2+}\) efflux produced by this pump when the action potential occurred at 1 Hz was roughly equal to the influx by K1. K3 represents a saturable pump such that sarcoplasmic reticulum (SR) uptake was \([Ca^{2+}]_{o}k_3(k_{en}+[Ca^{2+}])\), where \(k_3=600\) \(\mu\)mol/sec (the maximum pump rate) and \(k_{en}=1\) mM (the [Ca\(^{2+}\)]\(_{o}\), at which the pump rate was half maximal). Within the SR (2% of cell volume), Ca\(^{2+}\) was either free (CaSR) or bound to a calsequestrinlike protein (CaSRB) assumed to have an on rate (K5) of 1,000 M\(^{-1}\)sec\(^{-1}\) and an off rate (K6) of 1 sec\(^{-1}\). 19 The calsequestrinlike protein was present at 5 mM (in the SR), and CaSR before release was 2 mM. K4 represents Ca\(^{2+}\) release from the SR, which had an identical form to the Ca\(^{2+}\) current across the surface membrane, but with the following parameters: \(k_4=1.4\) sec\(^{-1}\), \(\tau_1=2\) msec, \(\tau_2=60\) msec, and \(L=0.003\) sec\(^{-1}\). The integrated Ca\(^{2+}\) flux from the SR during one action potential was 43 \(\mu\)mol/l cell volume. T in the model represents free troponin; total troponin was set at 60 \(\mu\)M. 20 The on rate (K7) was 10 M\(^{-1}\)sec\(^{-1}\), and the off rate (K8) was 100 sec\(^{-1}\). 21 Only the low-affinity site is depicted in the model, since only this site is thought to be involved in activation of tension. 22 X represents non-force-producing crossbridges, and XB represents force-producing crossbridges. K9 and K10 represent the attachment and detachment rates whose maximal values were 40 sec\(^{-1}\) and 10 sec\(^{-1}\), respectively. Activation was achieved by making the attachment rate a function (F) of the amount of troponin with Ca\(^{2+}\) bound. The function used was

\[
K_9 = \left[ \frac{CaT}{\text{Total troponin}} \right]^2 \times 40 \text{ sec}^{-1}.
\]

The square function was introduced to provide some of the cooperativity observed between [Ca\(^{2+}\)] and tension. 23 In some versions of the model, a further degree of cooperativity was introduced by making the troponin off rate (K8) decrease to a lower value \(K_8=K_8(1+N\cdot XB)\), where N was set to 4 so that at full tension the binding constant of troponin for calcium was increased about fourfold. Such an increase in troponin binding constant after crossbridge attachment has been experimentally demonstrated. 24, 25
Effects of EMD 53998 on Intracellular Calcium

Figure 2 also shows the aequorin light signals during the period when EMD 53998 was applied (upper tracing). In this preparation, which had been pretreated with guanethidine as described below to minimize possible catecholamine release, peak light declined slightly during the inotropic effect of EMD 53998, so it is clear that the increased tension cannot be due to an increase in \([Ca^{2+}]\). However, in most preparations there was some increase in aequorin light signals, so the possibility that the inotropic effect could be due at least in part to elevated \([Ca^{2+}]\) needs to be considered (see next section). We also attempted to determine reasons why changes in the light signal on application of EMD 53998 were variable.

We consider first applications of EMD 53998 to preparations that had not been treated with an agent designed to minimize catecholamine release. In nine preparations, the mean increase in peak aequorin light during the presence of 5 \(\mu M\) EMD 53998 was 85 ± 41% \((p \text{ lies between 0.1 and 0.05})\). EMD 53998 reduced peak aequorin light in two preparations (e.g., Figure 7, top tracings), but in the remainder, the light signal increased; this increase could take several different forms. In some preparations (e.g., Figure 3, top left panel), the transients showed a more or less smooth increase. However, in other preparations (not shown) the increases in light after application of EMD 53998 were variable, with large increases in light abruptly appearing and then returning to the previous level. In some of these preparations, resting light increased, and there were oscillations of the light signal. Characteristically, these increases in light were not accompanied by any change in the developed or resting tension. The presence of substantial and irregular increases in light in the absence of changes in tension suggested that localized elevations of \([Ca^{2+}]\) were occurring. Because of the steep relation between light and \([Ca^{2+}]\) for aequorin \([\text{light} = k([Ca^{2+}]^{2})]\), a few cells with a large elevation of \([Ca^{2+}]\) can lead to a considerable increase in the total aequorin signal but little detectable effect on tension. In aequorin-injected preparations, such changes are occasionally seen (e.g., just after the application of 2 mM \([Ca^{2+}]\) in Figure 3, bottom right panel), but they were much more frequent in the presence of EMD 53998 than with other interventions. This effect was dose related and, as noted in “Materials and Methods,” was never seen when carrier alone was applied.

One possible explanation for this phenomenon is that EMD 53998 can cause membrane breakdown in occasional, possibly damaged, cells; another possibility is that EMD 53998 can cause norepinephrine release from sympathetic terminals. If this latter effect were very localized, then it could lead to the results observed, since it is known that high concentrations of catecholamines can lead to large, oscillating increases in \([Ca^{2+}]\) in cardiac cells. To test this theory, large concentrations of \(\beta\)-blockers were applied (e.g., 10 \(\mu M\) propranolol in Figure 3), but this usually had rather little effect. Conceivably, this might be because local concentrations of norepinephrine are so high that a competitive inhibitor like propranolol is relatively ineffective. In contrast, guanethidine, which reduces norepinephrine release from adrenergic terminals, was often (two experiments out of three) quite effective at reducing the aequorin signals to control levels. Thus, in Figure 3, after the application of guanethidine, the elevation of the aequorin light signals produced by EMD 53998 was eliminated. A subsequent application of EMD 53998 in this preparation, in the continued presence of propranolol and guanethidine (Figure 6, left panel) produced an inotropic effect with very little effect on light. Five preparations were exposed to EMD 53998 in the
presence of guanethidine (and propranalol also in three preparations). In three preparations light fell, but in the other two, it showed a moderate rise. On average, the mean increase in light in these five preparations was 36±42%, which is not statistically significant. Thus, it is clear that treatment with agents that reduce catecholamine release or minimize their effects was able to abolish a large component of the erratic light increases. Since 5 μM EMD 53998 was the lowest dose that caused a substantial inotropic effect, this was used for the majority of experiments in this study to minimize these effects.

**Ca²⁺ Sensitization**

To determine the contribution that Ca²⁺ sensitization, as opposed to increased [Ca²⁺]_, made to the inotropic effect of EMD 53998, we compared the increase in aequorin light and tension produced by changes in [Ca²⁺]_ with those produced by EMD 53998. The basic assumption of this method, which has been experimentally verified, is that changes in [Ca²⁺]_ increase tension only by means of the resulting changes in the Ca²⁺_ transients. An example of the procedure is shown in Figure 3. EMD 53998 was first applied, and then a series of [Ca²⁺]_ was applied to achieve a comparable increase in tension. When the peak tension is plotted against the peak light (Figure 4), it is possible to interpolate the increase in peak light that would be required to increase tension to the level produced by EMD 53998. In this kind of plot, which can be thought of as a non–steady-state pCa/tension relation, points that lie to the left of the curve defined by changes in [Ca²⁺]_ have more tension for a given [Ca²⁺], and are therefore more sensitive to [Ca²⁺]. In the experiment shown in Figures 3 and 4, EMD 53998 initially produced an increase in tension, but nevertheless, it is still clear that there was a sensitization, since this point lies well to the left of the point defined by changes in [Ca²⁺]_. Application of guanethidine eliminated the increase in light with little change in tension so that the sensitization was apparently much greater. In seven experiments in which 5 μM EMD 53998 was compared with changes in [Ca²⁺]_, in this way, EMD 53998 increased tension to 230±70% of control and increased light by 64±29%. When the same increase in tension was produced by changes in [Ca²⁺]_, the increase in peak light was 240±60%. The difference between these two increases in light was highly significant (p<0.01 by paired t test); therefore, it is clear that EMD 53998 was having a substantial sensitizing effect. Another way of expressing this result is that, of the increase in tension caused by 5 μM EMD 53998, at most 30% could be accounted for by the increase in [Ca²⁺], whereas the remaining 70% is caused by mechanisms other than the rise in [Ca²⁺].

Potentially the increase in tension that is not due to Ca²⁺ could come about either by sensitization of the myofilaments to Ca²⁺ (leading to a parallel shift in the pCa/tension curves) or by increase in the maximum Ca²⁺-activated tension with proportionate increases at lower [Ca²⁺]. In skinned cardiac fibers, EMD 53998 (30 μM) has been shown to increase

**Figure 3.** Comparison of the effects of EMD 53998 (EMD) and changes of [Ca²⁺]_ on aequorin light and tension from an aequorin-injected ferret papillary muscle. Left panel: Continuous recordings of aequorin light (above) and tension (below). Bars show the application of 10 μM EMD 53998, 5 μM propranalol (PROP), and 5 μM guanethidine (GUAN). Right panel: Recordings showing effect of 0.5, 1, 2, and 4 mM [Ca²⁺]_. This is the same preparation as shown in the left panel but after about 2 hours later; none of the drugs shown in the left panel was present.

**Figure 4.** Plot of peak tension versus peak aequorin light for the experiment shown in Figure 3. This plot represents a non–steady-state [Ca²⁺]–tension relation for intact muscle. Circles indicate measurements of tension and aequorin light at different concentrations of extracellular calcium. Triangles indicate tension first in the presence of 10 μM EMD 53998 (EMD) and second in the additional presence of 5 μM propranalol (P) and 5 μM guanethidine (G).
maximum $\text{Ca}^{2+}$-activated tension by 25%. If the latter mechanism were important in intact preparations, then the maximum tension developed might be greater in the presence of EMD 53998 than in its absence. To test this point, $[\text{Ca}^{2+}]_o$ was increased until tension no longer increased, and 10 $\mu$M EMD 53998 was applied when tension was steady. In one experiment, the highest tension achieved (100%) occurred just after 6 mM $\text{Ca}^{2+}_o$ had been applied, although the steady-state level in 6 mM $\text{Ca}^{2+}_o$ was 85%. Application of EMD 53998 increased this tension to 93% maximum. In five experiments, it was possible to compare the maximum tension achieved by $\text{Ca}^{2+}_o$ with that occurring in the presence of a saturating concentration of EMD 53998. The tension achieved in EMD 53998 was $97.6 \pm 2.2\%$ of that in high $[\text{Ca}^{2+}]_o$. Thus, there was no evidence that EMD 53998 increased the maximum $\text{Ca}^{2+}$-activated tension in intact muscle, implying that the observed increase in tension not associated with an increase in $[\text{Ca}^{2+}]_i$ was due to $\text{Ca}^{2+}$ sensitization.

**Effects of Different Concentrations of EMD 53998**

Figure 5 shows mechanical and aequorin light measurements at a range of concentrations of EMD 53998. In this preparation the inotropic effect was first clear at 5 $\mu$M and increased up to 50 $\mu$M. The highest concentration (200 $\mu$M) caused a clear decrease in peak tension. This preparation showed no clear increase in time to peak tension, but the time to half relaxation increased progressively over the whole range tested. At the two highest concentrations, there was an additional tail of tension, which further slowed relaxation starting when relaxation was $\sim 70\%$ complete. At the three highest concentrations, an increase in resting tension that persisted throughout diastole was present (filled squares in bottom panel of Figure 5). The aequorin light signal increased over the range of concentrations tested up to 20 $\mu$M but declined again at the two highest concentrations. Increasing the concentration of EMD 53998 caused a progressive decrease in the time from stimulus to peak or 50% decline of the aequorin light signal.

**Comparison With Pimobendan**

Pimobendan is another new inotropic agent that has both phosphodiesterase-inhibiting and $\text{Ca}^{2+}$-sensitizing actions. In a previous study on pimobendan using aequorin, we found that pimobendan increased peak light about fivefold for a threefold increase in tension. Analysis of the sort shown in Figure 4 did not reveal any net sensitizing action with pimobendan in an intact preparation, although there was clear sensitization when compared with an agent such as isoprenaline, which produces its actions entirely via changes in cAMP. Thus, pimobendan and
EMD 53998 seem to have phosphodiesterase-inhibiting and calcium-sensitizing actions in rather different proportions; therefore, it was of interest to compare them directly. In addition, others\(^ {30}\) have reported only a twofold increase in aequorin light for a threefold increase in tension in the presence of pimobendan, suggesting a moderate sensitization in intact preparations. Both groups used ferret papillary muscles and a similar general approach, but one difference was the presence of a \(\beta\)-blocker (butralanol) in the experiments of one group\(^ {30}\) but not the other.\(^ {10}\) In the comparison shown in Figure 6, both guanethidine and propranolol were present throughout. Note that the inotropic effects of the two drugs were similar but that EMD 53998 had virtually no effect on the amplitude of the light signal (+17%), whereas pimobendan increased the light signal by 270% (i.e., a 3.7-fold increase). This experiment confirms our earlier observation\(^ {10}\) that pimobendan, in contrast to EMD 53998, shows little net \(\text{Ca}^{2+}\) sensitization in intact muscle.

**Action of EMD 53998 During Anoxia**

Inotropic agents will commonly be used in ischemic heart disease, in which tissue anoxia is one component. For this reason we investigated the effect of EMD 53998 on anoxic muscle (95% \(\text{O}_2\)–5% \(\text{CO}_2\) replaced by 95% \(\text{N}_2\)–5% \(\text{CO}_2\)). Figure 7 shows first a control application of 50 \(\mu\)M EMD 53998, which caused a large increase in tension and a small fall in the aequorin light signals. The \([\text{Ca}^{2+}]_i\) was then increased from 1 to 2 mM with the aim that developed tension after the application of \(\text{N}_2\) would be similar to the initial control period. \(\text{N}_2\) was then applied, and as reported previously, it reduced tension to about one third of the control but had little effect on the amplitude of the aequorin signal.\(^ {31,32}\) Because of the increase in \([\text{Ca}^{2+}]_i\), the tension in \(\text{N}_2\) was roughly the same as in the initial control period. EMD 53998 was then applied and produced a substantial increase in tension, although the maximum obtained was much lower than it was during the previous exposure. Thus, EMD 53998 is still capable of producing an inotropic effect in anoxia, but its magnitude is smaller than under aerobic conditions. A similar result was seen in one other preparation.

**Modeling**

If the sole effect of an agent was to increase the binding constant of troponin for \(\text{Ca}^{2+}\), then one would predict that, for a given release of \(\text{Ca}^{2+}\) into the myoplasm, the \(\text{Ca}^{2+}\) bound to troponin would increase, leading to an increase in tension, and that the \([\text{Ca}^{2+}]_i\) in the myoplasm would decrease. The changes in the time course of the \(\text{Ca}^{2+}\), transient and tension are not so obvious intuitively, but it has frequently been suggested\(^ {8,16,28}\) that the increased \(\text{Ca}^{2+}\) binding to troponin will prolong the time course of tension but abbreviate the time course of the \(\text{Ca}^{2+}\) transient. Because the on rate for the binding of \(\text{Ca}^{2+}\) to troponin is diffusion limited,\(^ {22}\) it seems likely that an increase in troponin binding constant will be caused by a decrease in the off rate. If this is the case, then \(\text{Ca}^{2+}\) will leave the troponin more slowly after an increase in the binding constant, and one would expect the time course of tension to be prolonged. Conversely, because \(\text{Ca}^{2+}\) remains bound to troponin longer, one would expect the SR, at least initially, to be able to lower \([\text{Ca}^{2+}]_i\) more rapidly.

These expectations are all born out by the computer model discussed in “Materials and Methods.” The results of altering the troponin binding constant are shown in Figure 8. Panel a shows the time course of aequorin light, \([\text{Ca}^{2+}]_i\), and tension generated by this model with a troponin binding constant of \(10^6\).\(^ {21}\) Note that the time course and magnitude of these model outputs are similar to the results shown in Figure 2. In panel b, the parameters of the model are initially identical, but after 20 msec, the troponin

**FIGURE 6.** Recordings comparing the effects of EMD 53998 (EMD) and pimobendan on aequorin light and tension from an aequorin-injected ferret papillary muscle. EMD 53998 (5 \(\mu\)M) (left panel) and 25 \(\mu\)M pimobendan (right panel) were added at the time shown by the bars. Recordings are from the same preparation separated by about 2 hours; 5 \(\mu\)M guanethidine and 5 \(\mu\)M propranolol were present throughout.

**FIGURE 7.** Recordings comparing the effects of EMD 53998 (EMD) during aerobic and anoxic conditions. Left panel: Aequorin light and tension from aequorin-injected ferret papillary muscle. EMD 53998 (50 \(\mu\)M) was applied as indicated by the bar ([\(\text{Ca}^{2+}]_i=1 \text{ mM}\)). Right panel: Recordings –1 hour later after washout of EMD 53998 ([\(\text{Ca}^{2+}]_i=2 \text{ mM}\)]. \(\text{O}_2\) was replaced by \(\text{N}_2\) at the time indicated by the bar. EMD 53998 (50 \(\mu\)M) was then applied as shown by the second bar. Neither guanethidine nor propranolol was present in this experiment.
binding constant was doubled by halving the off-rate constant. This approximated the change in sensitivity observed in skinned-fiber studies with 10 μM EMD 53998 and resulted in a small fall in \([\text{Ca}^{2+}]\). In the contraction that follows, all the expected changes discussed above are observed; the changes in time course are more apparent in the normalized recordings shown in panels c and d.

An interesting, and to us unexpected, feature of this modeling is that the shortening of the time course of the \(\text{Ca}^{2+}\) transient was not observed in certain other variants of this model that we tested. In particular, if the feedback between tension and the troponin binding constant is removed, then the \(\text{Ca}\) after an increase in binding constant is smaller, but its time course is prolonged rather than shortened. This arises because the major flux of \(\text{Ca}^{2+}\) into the myoplasm during the falling phase of the \(\text{Ca}^{2+}\) transient is \(\text{Ca}^{2+}\) dissociating from troponin. The intuitive explanation given above for a shortened \(\text{Ca}^{2+}\) transient in the presence of increased \(\text{Ca}^{2+}\) sensitivity was that \(\text{Ca}^{2+}\) efflux from troponin will be smaller if \(\text{Ca}^{2+}\) dissociates from troponin more slowly. This is true, but if feedback is omitted from the model, this factor is offset by increased saturation of troponin by \(\text{Ca}^{2+}\), with the net result that the combination of greater binding but slower dissociation is an increased \(\text{Ca}^{2+}\) efflux from troponin and a \(\text{Ca}^{2+}\) transient that falls more slowly. In the model shown in Figure 8, the effect of greater saturation of troponin caused by an increased \(\text{Ca}^{2+}\) sensitivity is offset by the increase in troponin binding caused by the feedback between tension and binding, which continues to increase as long as tension rises.

\[\text{Ca}^{2+}\] Sensitization

Our results show unequivocally that EMD 53998 increases the sensitivity of contractile proteins to \(\text{Ca}^{2+}\) in intact ventricular muscle. This result is clear because 1) it was observed in every muscle in which a direct comparison was made between the effects of \([\text{Ca}^{2+}]\), and EMD 53998, 2) in some preparations the increase in tension occurred at the same time as a reduction of \([\text{Ca}^{2+}]\), and 3) the increase in time course of contraction and decrease in time course of the \(\text{Ca}^{2+}\) transient are consistent with an increase in \(\text{Ca}^{2+}\) sensitivity of the contractile proteins. Although there are changes in the time course of \([\text{Ca}^{2+}]\), and tension that complicate the analysis of this type of experiment, in the present experiments there is a

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**Figure 8.** Computer model showing the effects of an increase in troponin binding constant on aequorin light, myoplasmic \([\text{Ca}^{2+}]\), and tension. Panel a: Aequorin light (above), myoplasmic \([\text{Ca}^{2+}]\) (middle), and tension (below) generated by the model for a single contraction under control conditions. Panel b: All parameters of the model identical to those in panel a except that 20 ms after the start of the simulation the troponin binding constant was increased by a factor of 2 (indicated by a small downward step in myoplasmic \([\text{Ca}^{2+}]\)). The discontinuity in the aequorin and free calcium recordings after 200 msec in panels a and b is the moment at which the action potential repolarizes and the residual \(\text{Ca}^{2+}\) current ceases. Panel c: Normalized aequorin light signals from panels a and b illustrating changes in time course. Dotted indicates recording with the increased binding constant. Panel d: Normalized tension recordings from panels a and b. Dotted indicates recording with the increased binding constant.
reduction in the time course of the Ca\textsuperscript{2+} transient that would of itself tend to reduce tension rather than increase it.

We cannot determine from the present experiments whether the effect of EMD 53998 is a direct one on the troponin binding constant for Ca\textsuperscript{2+} or an indirect one in which a constant amount of Ca\textsuperscript{2+} bound to troponin leads to a greater tension (a downstream effect in the terminology of Blinks and Endoh\textsuperscript{9}). The modeling that we have done, which qualitatively matches our results, was based on the assumption that EMD 53998 directly affects Ca\textsuperscript{2+} binding to troponin C, but in fact very similar results are obtained if, instead of changing the troponin binding constant, the feedback between tension and Ca\textsuperscript{2+} bound to troponin was increased. This represents one kind of downstream effect, so it seems that downstream effects of EMD 53998 are equally consistent with our modeling and results. A point of particular interest was that feedback between tension and the troponin binding constant\textsuperscript{24,25} was necessary in order for the model to reproduce the changes in the time course of the Ca\textsuperscript{2+} transient that are observed experimentally. Thus, this observation supports the suggestion that such feedback is active in intact muscle and may be an important factor in determining the response of cardiac muscle to agents that alter Ca\textsuperscript{2+} sensitivity.

Other Effects of EMD 53998

If the only effect of EMD 53998 were on Ca\textsuperscript{2+} sensitivity, then one would expect to have observed a reduction in the amplitude of the aequorin light signal in its presence. This is a result that we did observe in some of our preparations, but in a greater number of preparations, the Ca\textsuperscript{2+} transients showed some increase of the aequorin light in the presence of EMD 53998, and this seems likely to be the result of additional actions of the drug. Our hypothesis is that there are two additional effects. The first effect postulated is release of norepinephrine from adrenergic terminals. Judging from the effects on the Ca\textsuperscript{2+} transients, this seems to be an intermittent and variable phenomenon and was minimized by the addition of guanethidine and propranolol. However, if Ca\textsuperscript{2+} sensitization were the only effect of EMD 53998 in the presence of guanethidine and propranolol, then the Ca\textsuperscript{2+} transient ought to have fallen. For this reason, we postulate a second effect of EMD 53998: inhibition of phosphodiesterase activity. This has been observed in studies on isolated cellular enzymes (personal communication, Dr. M. Klockow, 1989). We suggest that this activity is small in intact preparations and saturates at relatively low concentrations of EMD 53998. This might account for part of the increase in Ca\textsuperscript{2+} transients seen at intermediate concentrations of EMD 53998 (e.g., see Figure 5).

**EMD 53998 as an Inotropic Agent**

Low doses of EMD 53998 (2–10 μM) lead to a pronounced inotropic effect with only minimal increase in [Ca\textsuperscript{2+}]. This means that activation of the transient inward current and associated arrhythmias should not be a problem. Furthermore, the component of energy consumption that is involved in Ca\textsuperscript{2+} movements will presumably be smaller than that for inotropes which increase [Ca\textsuperscript{2+}], so that there may be an increase in efficiency (work per unit energy consumption). This suggestion is supported by experiments indicating that heat production per unit of tension–time integral is much greater with isoprenaline than with pimobendan,\textsuperscript{33} presumably because pimobendan has dual Ca\textsuperscript{2+}-sensitizing and phosphodiesterase-inhibiting properties and produces a given inotropic effect for a much smaller Ca\textsuperscript{2+} transient than isoprenaline.\textsuperscript{10} It has now also been shown that EMD 53998 reduces heat production per unit of tension–time integral by 20% (personal communication, Dr. C. Holubarsch, Universität Freiburg; August 1990).

Addition of EMD 53998 to hypoxic muscle demonstrated that it was capable of producing an inotropic effect in this situation, clearly a desirable property. Since it is known that the myofibrillar Ca\textsuperscript{2+} responsiveness is reduced in hypoxic heart muscle,\textsuperscript{31,32} the use of a sensitizing agent such as EMD 53998 to reverse this effect would seem logical. It was noted in “Results” that the developed tension produced by EMD 53998 in the presence of hypoxia was considerably less than that produced under aerobic conditions. This is in agreement with our findings that EMD 53998 acts primarily on Ca\textsuperscript{2+} sensitivity in intact muscle and not on maximum Ca\textsuperscript{2+}-activated force. Maximum Ca\textsuperscript{2+}-activated force is expected to be depressed in hypoxic muscle because of accumulation of hydrogen and phosphate ions, and EMD 53998 would not be expected to reverse this.

Possible disadvantages of EMD 53998 and other drugs that act by increasing Ca\textsuperscript{2+} sensitivity are the prolongation of the time course of tension (seen at all concentrations) and an increase in resting tension (seen only at high concentrations, >50 μM). However, the prolongation of contraction was modest (~10%) at doses of EMD 53998 that can produce a twofold to threefold increase in tension, so it seems unlikely that this problem will prove too troublesome in practice.

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KEY WORDS • cardiac muscle • inotropic agents • calcium sensitivity • EMD 53998 • pimobendan • hypoxia
EMD 53998 sensitizes the contractile proteins to calcium in intact ferret ventricular muscle.

J A Lee and D G Allen

doi: 10.1161/01.RES.69.4.927

_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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