Force Enhancement Without Changes in Cross-Bridge Turnover Kinetics: The Effect of EMD 57033

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ABSTRACT The thiadiazinon derivative EMD 57033 has been found previously in cardiac muscle to increase isometric force generation without a proportional increase in fiber ATPase, thus causing a reduction in tension cost. To analyze the mechanism by which EMD 57033 affects the contractile system, we studied its effects on isometric force, isometric fiber ATPase, the rate constant of force redevelopment (k_{redev}), active fiber stiffness, and its effect on F_0, which is the force contribution of a cross-bridge in the force-generating states. We used chemically skinned fibers of the rabbit psoas muscle. It was found that with 50 μM EMD 57033, isometric force increases by more than 50%, whereas k_{redev}, active stiffness, and isometric fiber ATPase increase by at most 10%. The results show that EMD 57033 causes no changes in cross-bridge turnover kinetics and no changes in active fiber stiffness that would result in a large enough increase in occupancy of the force-generating states to account for the increase in active force. However, plots of force versus length change recorded during stretches and releases (T plots) indicate that in the presence of EMD 57033 the y_0 value (x axis intercept) for the cross-bridges becomes more negative while its absolute value increases. This might suggest a larger cross-bridge strain as the basis for increased active force. Analysis of T plots with and without EMD 57033 shows that the increase in cross-bridge strain is not due to a redistribution of cross-bridges among different force-generating states favoring states of larger strain. Instead, it reflects an increased cross-bridge strain in the main force-generating state. The direct effect of EMD 57033 on the force contribution of cross-bridges in the force-generating states represents an alternative mechanism for a positive inotropic intervention.

INTRODUCTION

Modulation of force generation is a major mechanism in adaptation of muscle tissue to changes in loading conditions. Different ways of such modulation have previously been demonstrated, e.g.: 1) a higher cytosolic calcium concentration in response to the action potential ($[calcium]_i$ amplitude modulation; Rüegg, 1986), and 2) a higher calcium sensitivity of the contractile system itself. If the increase in force is mediated through a higher calcium sensitivity of the contractile system instead of a higher intracellular calcium concentration, at least two different mechanisms could lead to an increase in contractility (Brenner, 1993). First, increased force can be the result of changes in calcium binding to TnC or in subsequent steps in the activation of the thin filament. In this case, at submaximum calcium concentrations, force increases for a given calcium concentration, resulting in a shift of the force/pCa relation to the left without affecting the force level at maximum activation. The second possible mechanism of increasing the calcium responsiveness of the contractile system results from the rate modulation concept for calcium regulation of muscle contraction (Brenner, 1988). That is, calcium modulates cross-bridge turnover kinetics, or more specifically, it increases the rate constant for the transition of cross-bridges from non-force-generating states to force-generating states. It was shown that interventions that affect turnover kinetics can cause not only an increase in active force at submaximum and maximum calcium concentrations, but also a shift of the force/pCa relation to lower calcium concentrations without changes in the calcium affinity of the regulatory proteins.

Here we present evidence for another mechanism that causes higher force output without changes in sarcoplasmic calcium concentration. It appears that the higher force output is due to an increase in force generated by individual cross-bridges. This mechanism was found when studying the effects of the thiadiazinon-derivative 5-(1-(3,4-dimethoxybenzoyl)-1,2,3,4-tetrahydrochinolin-6-yl)-6-methyl-3,6-dihydro-2H-1,3,4-thiadiazin-2-on (EMD 53998), a racemic equimolar mixture of two enantiomers. In several studies the effects of the mixture of the two enantiomeres, EMD 53998, were tested, and others were carried out with the (+) enantiomer, EMD 57033 (Gambassi et al., 1993; Lues et al., 1993; White et al., 1993; Solaro et al., 1993).

Analyzing the effects of EMD 53998 on different intact preparations of cardiac muscle, several investigators (Ferroni et al., 1991; Beier et al., 1991) found that EMD 53998 increases the amplitude of twitches without increasing the accompanying calcium transient. Therefore it was concluded that the positive inotropic effect of the substance might be related to an augmented sensitivity of the myofilaments to calcium. Furthermore, in intact cardiac muscle, simultaneous measurements of tension and fluorescence signals reflecting the intracellular calcium concentration (Lee and Allen, 1991; Dobrunz et al., 1995) showed a

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leftward shift of the force/pCa relation with EMD 53998 and thus supported the idea of an increase in calcium sensitivity of the thin filament. The same conclusion was derived from studies with chemically skinned cardiac muscle from different species (Beier et al., 1991; Groß et al., 1993; Leijendekker and Herzig, 1992), which also showed a shift of the force/pCa relation to the left. Measurements of energy cost of twitch force in intact cardiac muscle (Groß et al., 1993; Grandis et al., 1994) revealed a reduction in energy cost with EMD 57033. A similar effect, but only at submaximum calcium concentrations, was also observed in skinned pig trabeculae with EMD 53998 (Leijendekker and Herzig, 1992). Therefore EMD 57033 apparently exhibits a positive inotropic effect without increasing the oxygen consumption of the myocardium.

The mechanism by which EMD 57033 affects the contractile and/or regulatory proteins of cardiac muscle, however, remained unclear. Enhancement of calcium binding to TnC with EMD 57033 appeared unlikely, because EMD 53998 and EMD 57033 not only cause a leftward shift of the force/pCa relation; they also increase the force at maximum calcium concentrations and reduce the tension cost (Dobrunz et al., 1995; Beier et al., 1991; Leijendekker and Herzig, 1992; Groß et al., 1993). Both effects are unexpected for an increase in calcium binding to TnC. In agreement with that, Solaro and co-workers (1993) found no effect of EMD 57033 on calcium binding to TnC in skinned heart muscle fibers. The increase in force at maximum calcium concentration instead suggested that changes in cross-bridge turnover kinetics or even another mechanism is responsible for the effects of EMD 57033. The observed shift of the force/pCa relation, the increase in force at maximum calcium concentrations, and the lower tension cost with EMD 57033 could in principle be attributed to a change in g_{app} (rate constant for the transition of the cross-bridges from the force-generating states to the non-force-generating states) possibly associated with changes in f_{app} (rate constant for the transition of the cross-bridges into the force-generating states).

The goal of the present study was to determine the mechanism by which EMD 57033 affects force and tension cost of skeletal muscle fibers. Because most of the previous work with EMD 57033 and with the racemic mixture EMD 53998 was done on cardiac muscle preparations, a prerequisite of the present study was to demonstrate that EMD 57033 has comparable effects on single skinned skeletal muscle fibers from rabbit psoas. For the present study we focused our attention on maximum activation only.

To our surprise, measurements of isometric force and tension cost, together with additional measurements of active turnover kinetics, isometric stiffness, and cross-bridge strain, revealed that at least in maximally activated skeletal muscle fibers the increase in force with reduced tension cost in the presence of EMD 57033 cannot be accounted for by changes in cross-bridge cycling kinetics. Instead, the increase in force appears to result from a higher force generated by the cross-bridges in the force-generating states (F_0; Brenner, 1991). Specifically, it appears that EMD 57033 increases the force generated in at least the main force-generating state, possibly in all of the force-generating cross-bridge states. Such an increased force generation represents a further mechanism for a positive inotropic intervention at reduced tension cost. A preliminary account of this work has been presented earlier (Kraft et al., 1994).

MATERIALS AND METHODS

Fiber preparation

Single chemically skinned fibers from rabbit psoas muscle were isolated according to a method described earlier (Yu and Brenner, 1989). The skinning procedure, the storage of isolated muscle fibers, and the mounting of the fibers in the mechanical setup were reported in detail by Kraft et al. (1995).

Solutions

All chemicals were obtained from Sigma (Munich, Germany) except where noted. The composition of the skinning solution including protease inhibitors was the same as described recently (Kraft et al., 1995).

All solutions, except where noted, were adjusted to an ionic strength of 170 mM by adding potassium propionate.

EMD 57033 solution

EMD 57033 was kindly provided by E. Merck (Darmstadt, Germany). EMD 57033 was dissolved in propylene glycol (1,2-propanediol), and from this stock solution (2 mM) the appropriate amounts for the different EMD 57033 concentrations were added to the respective solutions. In control experiments we examined the effects of propylene glycol alone (no EMD 57033) to correct for possible propylene glycol effects. We found that the highest propylene glycol concentration used (2.5%) increases isometric force by 10% at most. All data shown in this study were corrected for effects of the respective propylene glycol concentrations.

Preactivating and activating solution

Preactivating and activating solution consisted of imidazole (10 mM), $MgCl_2$ (2 mM), MgATP (1 mM), dithiothreitol (2 mM), caffeine (10 mM), creatine phosphate (10 mM), 500 units of creatine phosphokinase/ml, and EGTA (3 mM) or CaEGTA (3 mM), respectively.

To reduce contaminating phosphate, preactivating and activating solutions also contained 20 mM sucrose and 10 units/ml sucrose phosphorylase (Boehringer, Mannheim, Germany) and were kept at room temperature (20°C) for 10 min before use (modified from Pate and Cooke, 1989).

To obtain different calcium concentrations ranging from pCa 7 to pCa 4.5, preactivating and activating solutions were added together in different proportions. The preactivating and activating solutions for varying calcium concentrations at 5°C and at 15°C (Fig. 4 *b*) contained 1 mM EGTA and 1 mM CaEGTA, respectively, and no sucrose or sucrose phosphorylase.

Relaxing solution with MgATP

Relaxing solution with MgATP consisted of 10 mM imidazole, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM ATP, and 1 mM dithiothreitol.

Solution for measurements of isometric fiber ATPase under preactivating and activating conditions

The solution for measurements of isometric fiber ATPase under preactivating and activating conditions consisted of 20 mM imidazole, 2 mM $MgCl_2$, 3 mM EGTA or CaEGTA respectively, 5 mM MgATP, 10 mM caffeine, 0.25 mM Ap_5A, 5 mM NaN_3, 50 units lactate-dehydrogenase/ml, 250 units pyruvate-kinase/ml, 2 mM phosphoenol-pyruvate, and about 0.2 mM NADH. Phosphoenol-pyruvate and pyruvate kinase are part of a coupled enzyme system that makes it possible to follow the hydrolysis of MgATP by the fibers. Pyruvate, which is one product of the rephosphorylation, was converted into lactate by lactate dehydrogenase, accompanied by oxidation of NADH to NAD^+. Production of ADP was then detected as a decrease in absorbance at 360 nm.

Solutions with MgATPγS

Solutions with MgATPγS at low Ca^{2+} consisted of 10 mM imidazole, 2 mM $MgCl_2$, 3 mM EGTA, and 10 mM MgATPγS (Boehringer Mannheim; purified by ion exchange chromatography as previously described; Kraft et al., 1992), 1 mM dithiothreitol, 0.2 mM Ap_5A, 0.5 units hexokinase/ml, and 200 mM glucose. For high calcium concentrations, EGTA was replaced by 1 mM CaEGTA.

All solutions were adjusted to pH 7.0 at the experimental temperature of 5°C or 1°C, respectively.

Experimental protocol

Isometric force was measured during a quick release of the activated fiber when force briefly dropped from its isometric value to zero. The rate constant of force redevelopment was determined after a short period of lightly loaded shortening and a subsequent restretch of the fiber to its original sarcomere length (Brenner and Eisenberg, 1986).

Isometric fiber ATPase was determined in a circulating system that included the muscle chamber and a flow cell in a fixed-wavelength monitor (UV-M II; Pharmacia LKB, Sweden), where changes in absorption at 360 nm due to the decrease of NADH concentration were measured. For each condition, the steady-state ATPase activity was recorded for at least 5 min, and the observed slopes were normalized with respect to the slope recorded without EMD 57033 to obtain the relative ATPase activities. To test whether the fiber ATPase activity measured under the different conditions could have been limited by the activity of the coupled enzyme system, we added 10 μM ADP to the activating solution after removal of the fiber. The observed slope of absorbance change in response to the ADP jump was at least 10 times larger than during the previous activation. Thus the system was certainly not limited during fiber activation.

The muscle chamber in the circulating system was designed such that, simultaneously with ATPase measurements, isometric force and the rate constant of force redevelopment could be recorded with sarcomere length feedback (Brenner and Eisenberg, 1986). In addition, it was possible to monitor the structural integrity of the fiber by light microscopy.

Fiber stiffness was measured by applying ramp-shaped stretches to one end of the fibers. Apparent fiber stiffness was defined as the ratio of force increment over filament sliding when filament sliding had reached 2 nm/half-sarcomere (chord stiffness). Stretch velocity could be varied from 10^{-1} to 5×10^3 (nm/half sarcomere)/s to obtain stiffness-speed relations (Brenner et al., 1986).

The unloaded shortening velocity (v_{max}) of the fibers was determined using the slack test (Hill, 1970). During isometric steady-state contraction, rapid length releases (5 ms) of different amplitudes were applied to slacken the fiber. The time was determined from the beginning of the release until the fiber had taken up the slack and started to redevelop force. This time was plotted as a function of the amplitude of the applied length step. V_{max} was then determined as the initial slope of this plot (cf. Fig. 1 *c*).

Plots of force versus sarcomere length change ("*T* plots"; Kuhn et al., 1979) were obtained by imposing stretches and releases on the fibers during isometric steady-state contraction (cf. Fig. 2; Brenner, 1991).

Relation between cross-bridge turnover kinetics and the measured parameters

Assuming that under the present experimental conditions such as low P_i concentration, the reverse rate constants (i.e., f_{app}^- and g_{app}^-) can be neglected, isometric force, ATPase activity, and tension cost are expected to depend on the following parameters (Huxley, 1957; Brenner, 1988):

$$\text{Isometric force } F = n \times F_0 \times f_{app}/(f_{app} + g_{app}) \quad (1)$$

$$\text{Isometric fiber ATPase } A = n \times b \times f_{app} \times g_{app}/(f_{app} + g_{app}), \quad (2)$$

whereby $f_{app}/(f_{app} + g_{app})$ represents the occupancy of force-generating states (f_{app} = rate constant for the transition into the strongly bound force-generating states, g_{app} = rate constant for the transition of the cross-bridges back into the weakly bound non-force-generating states; n = number of active cross-bridges per half-sarcomere; F_0 = force contribution by a strongly attached cross-bridge; b = number of half-sarcomeres). At low P_i concentrations, when the reverse reactions that depend on rebinding of P_i can be neglected ($f_{app}^- \to 0$), the rate constant for force redevelopment (k_{redev}) is

$$k_{redev} = f_{app} + g_{app}. \quad (3)$$

Finally, tension cost is the ratio of ATPase/force:

$$\text{ATPase/force} = (b \times g_{app})/F_0. \quad (4)$$

Note: 1) Isometric force depends on both the rate constants for cross-bridge cycling (f_{app} and g_{app}) and (F_0), the force generated by an individual cross-bridge in the force-generating state(s). 2) Tension cost depends on F_0 as well as g_{app}.

RESULTS

The effects of EMD 57033 on isometric force, k_{redev}, fiber ATPase, and v_{max} in skinned single fibers from rabbit psoas muscle

In most studies concerning the effects of EMD 57033 on the contractile system, intact and skinned preparations from heart muscle were used. For comparison of our results with data from other authors, we first tested whether EMD 57033 has similar effects on skeletal muscle fibers. We determined isometric force, the maximum unloaded shortening velocity (v_{max}), ATPase activity, and tension cost of maximally calcium-activated skinned fibers of rabbit psoas at different EMD 57033 concentrations (Fig. 1).

Fig. 1 *a* shows that the addition of up to 50 μM EMD 57033 causes an increase in active force of 50–60%. Simultaneous measurements of ATPase activity (Fig. 1 *a*) show that with 10 μM EMD 57033 the ATP turnover is about 10% higher than in the absence of EMD 57033, but there is no further increase in ATPase activity with higher EMD 57033 concentrations, in contrast to the large further increase in force generation. Consequently, the ratio of ATPase over force, i.e., the tension cost, decreases by 25% in the presence of 50 μM EMD 57033 (Fig. 1 *b*). In Fig. 1 *c* the results from slack test measurements with (30 μM) and without EMD 57033 are shown. Data recorded from

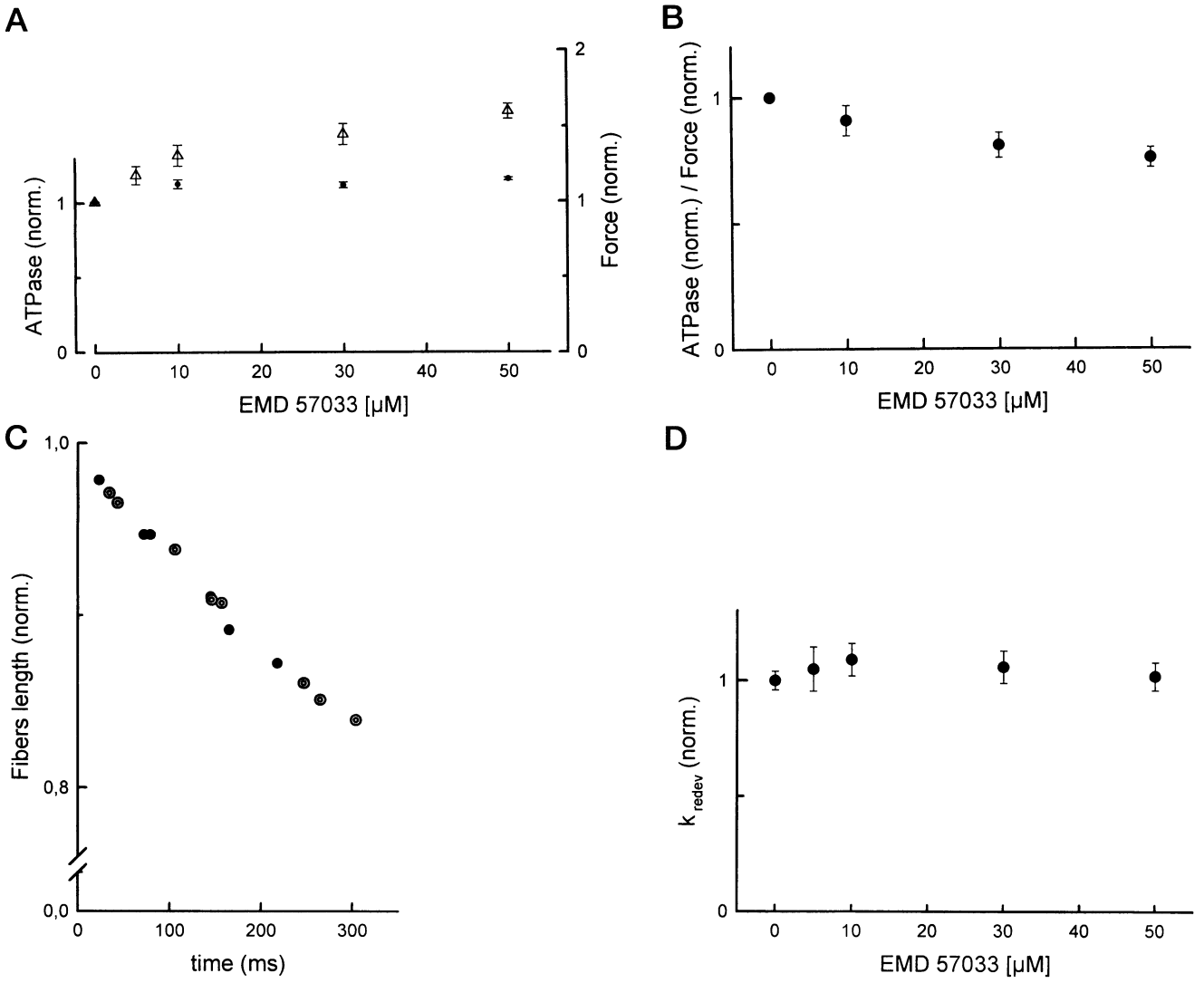

FIGURE 1 (*A*) Isometric force (△) and isometric fiber ATPase (●) of fully calcium-activated skeletal muscle fibers in the presence and absence of EMD 57033. The values are normalized to force or ATPase, respectively, without EMD 57033, n = 3–12 fibers, ± SEM. (*B*) The ratio of ATPase over force of fully calcium-activated skeletal muscle fibers as a function of EMD 57033 concentration. The values are normalized to ATPase/force without EMD 57033, n = 3–9 fibers, ± SEM. (*C*) Unloaded shortening velocity (v_{max}) of one representative muscle fiber in the absence (●) and in the presence (◎) of 30 μM EMD 57033 determined by the slack test. Fiber length is plotted as a function of time required to take up the slack (data from one fiber). The average of the initial slope (v_{max}) of 3 fibers is 1.08 ± 0.16 (μm/HS)s^{-1} without and 1.10 ± 0.1 (μm/HS)s^{-1} with 50 μM EMD 57033. (*D*) The rate constant for force redevelopment (k_{redev}) at maximum calcium activation versus EMD 57033 concentration. The values are normalized to k_{redev} without EMD 57033, n = 3–12 fibers, ± SEM.

three different fibers show that maximum unloaded shortening velocity (v_{max}) is 1.08 ± 0.16 (μm/HS)s^{-1} without and 1.10 ± 0.10 (μm/HS)s^{-1} with EMD 57033. In the concept of Huxley (1957), v_{max} is mainly determined by g_2, the rate constant for detachment when cross-bridges become unstrained during isotonic shortening. Thus our result indicates that EMD 57033 has no effect on g_2. Altogether, the effects of EMD 57033 on force, ATPase, tension cost, and v_{max} of fast skeletal muscle fibers are very similar to those found earlier for intact and skinned cardiac muscle.

The main purpose of the present study was, however, to determine the mechanism by which EMD 57033 increases force generation. According to Eq. 1 isometric force generation is, besides other parameters, controlled by the occupancy of force-generating states ($f_{app}/f_{app} + g_{app}$) and thus is sensitive to changes in turnover kinetics (f_{app} and/or g_{app}) of the cross-bridges. To obtain indications for possible changes in turnover kinetics with EMD 57033, we measured k_{redev}, the rate constant of force redevelopment after a short period of unloaded isotonic shortening and a quick restretch to isometric sarcomere length. k_{redev} represents $f_{app} + g_{app} + f_{app}^{-} + g_{app}^{-}$; under isometric conditions and low P_i, however, it is essentially $f_{app} + g_{app}$ (Eq. 3) (Brenner, 1988).

Fig. 1 *d* demonstrates that k_{redev} is essentially the same over the whole range of EMD 57033 concentrations. Only a

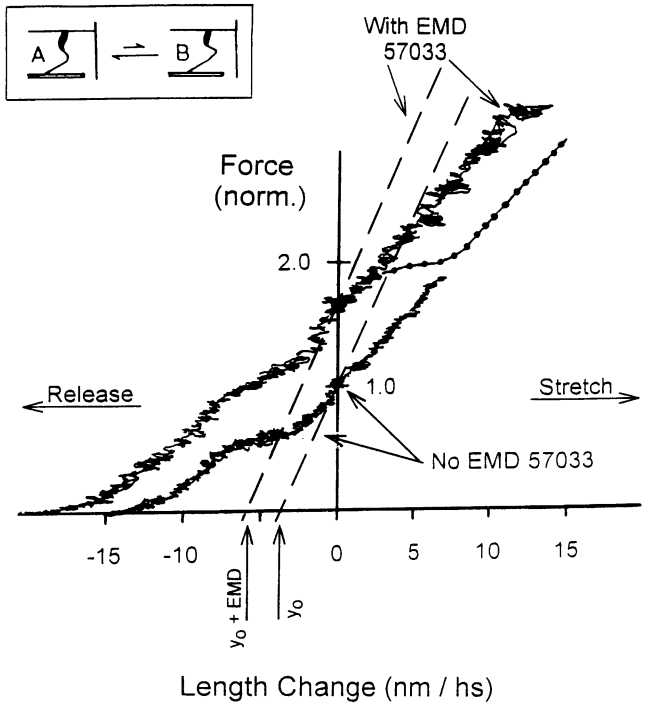

FIGURE 2 Force versus change in sarcomere length recorded during ramp-shaped stretches and releases (*T* plots). The two records with (50 μM) and without EMD 57033 were taken from one representative fiber. Speed of the applied length change was 0.5–1.2 $\times$ 10^3 (nm/HS)/s. The broken lines represent the initial slope of plots recorded from the same fiber under identical conditions but at a speed for stretch and release of 3–4 $\times$ 10^3 (nm/HS)/s. The average value for y_0 at this speed of stretch for 5 fibers is 3.6 $\pm$ 0.72 nm/HS without and 7.38 $\pm$ 1.1 nm/HS with EMD 57033. (*Inset*) Schematic representation of two attached force generating states (*A* and *B*) with different strain. The dotted line represents the expected shape of the *T* plot if in the presence of EMD 57033 more cross-bridges would occupy state *B* (cf. *inset*), i.e., a state of larger strain (for details see text).

small increase is detectable at 10 μM EMD 57033, which, however, is still within experimental error. This suggests that the increase in isometric force with EMD 57033 apparently is not due to a significant change in one of the rate constants (f_{app} or g_{app}), which determine k_{redev} under these low-P_i conditions. The data, however, cannot exclude simultaneous changes in f_{app} and g_{app} that cancel out each other.

As will be discussed below, essentially no change in k_{redev}, together with no further change in ATPase at EMD 57033 concentrations above 10 μM, indicates that the observed increase in force does not result from changes in f_{app} and/or g_{app}. Therefore, additional measurements were necessary to clarify the mechanism by which EMD 57033 increases isometric force generation.

Effects of EMD 57033 on *T* plots recorded during isometric steady-state contraction

Inspection of Eq. 1 shows that, alternatively, isometric force could be increased by an EMD 57033-induced increase in F_0, the average force generated by a cross-bridge in the force-generating states. Such an increase in F_0 could, in principle, result from 1) a different distribution of cross-bridges among force-generating states (e.g., states *A* and *B* *of the inset* in Fig. 2) favoring the state of larger strain (i.e., higher force contribution), like state *B* in the inset of Fig. 2. Alternatively, 2) higher force with EMD 57033 could result from an increase in average strain in the force-generating states (i.e., *A* and *B*).

Such a redistribution and change in strain, however, can be detected in plots of force versus length change recorded during stretches and releases (*T* plots; Kuhn et al., 1979; Brenner, 1991). Fig. 2 shows a parallel shift of the *T* plot in the presence of 50 μM EMD 57033 to higher isometric force. The deviation from a straight line (i.e., from the initial slope) upon releases indicates the transition of cross-bridges into subsequent force-generating states (e.g., from state *A* to state *B* in the *inset* of Fig. 2; cf. Huxley and Simmons, 1971; Ford et al., 1977; Brenner, 1991) and can be observed when the releases are slow enough to allow this transition. Therefore, the original records in Fig. 2 were obtained with releases and stretches of 0.5–1.2 $\times$ 10^3 (nm/HS)/s.

If an increase in occupancy of state *B* would be responsible for the observed increase in force with EMD 57033, a redistribution from state *B* to state *A* should be seen upon stretches in the presence of EMD 57033. This redistribution would show up in the *T* plots as indicated by the dotted line in Fig. 2 (Brenner, 1991). The experimental data (Fig. 2), however, give no indication of such a backward reaction in the presence of EMD 57033, not only at the stretch velocities shown in Fig. 2, but over a range of stretch velocities (from 10 (nm/HS)/s to 4 $\times$ 10^3 (nm/HS)/s; data not shown). This suggests that under isometric conditions, not only in the absence of EMD 57033 but also with EMD 57033, there is no significant occupancy of state *B* and thus for both conditions isometric force is generated essentially by cross-bridges in state *A* (cf. Brenner, 1991).

Such larger isometric force without changes in occupancy of the force-generating states or distribution between different force-generating states could be due to a larger distortion of the cross-bridges in the presence of EMD. This is expected to show up as a larger *x* axis intercept (y_0) of *T* plots recorded with the fastest releases possible. For such fast releases no transition into subsequent states occurs, and the intercept with the abscissa (y_0, y_0 + EMD 57033), obtained when extrapolating the initial parts of these plots, might be taken as a measure for average cross-bridge strain under isometric conditions. The broken lines in Fig. 2 represent the initial slope of the *T* plots obtained with the same fiber at the highest possible speeds of stretches and releases (3–4 $\times$ 10^3 (nm/HS)/s). We found that in the absence of EMD 57033 y_0 is about 3.6 $\pm$ 0.72 nm/HS, whereas with 50 μM EMD 57033 it reaches about 7.38 $\pm$ 1.1 nm/HS (n = 5 fibers).

Active and relaxed fiber stiffness with EMD 57033

With faster releases, the initial slope of the curve, i.e., the observed stiffness, increases (Fig. 2) because less detachment and reattachment occurs during the stiffness measurement. As indicated by the dashed lines, y_0 does not increase by exactly the same amount as active force, i.e., the slope of the dashed line with EMD 57033 is slightly but not significantly larger. This indicates a somewhat higher fiber stiffness in the presence of EMD 57033 and is shown in detail in Fig. 3 *a*. At 10 μM EMD 57033 stiffness increases by about 10%, and there is no further increase at higher EMD 57033 concentrations, although active force increases by another 40–50% (Fig. 1 *a*). The increase in active stiffness by 10%, which is similar to the increase in ATPase activity (Fig. 1 *a*) may, however, reflect a slightly higher (10%) occupancy of the force-generating cross-bridge states due to small changes in cross-bridge turnover kinetics leading to a small but statistically insignificant increase in k_{redev} (Fig. 1 *d*).

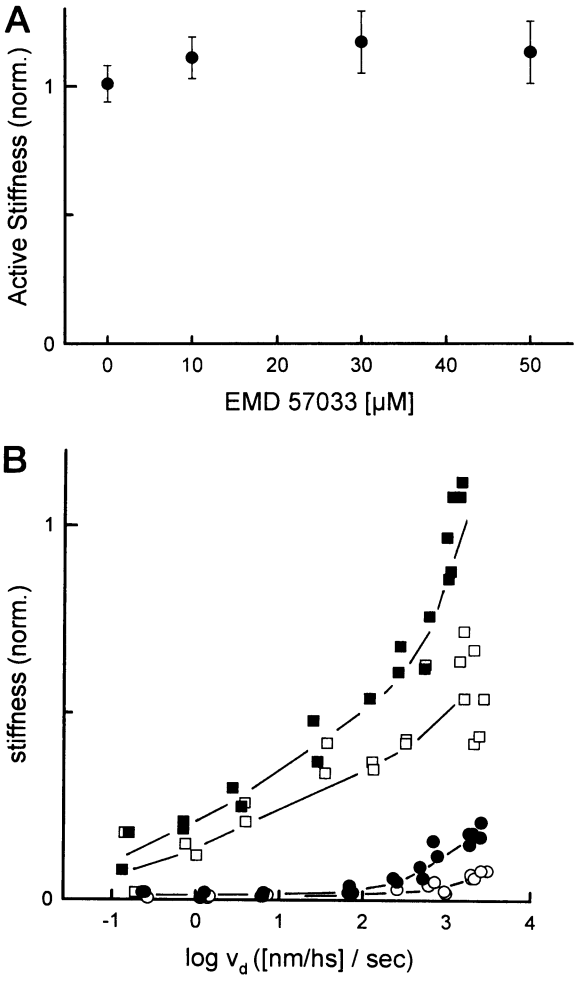

FIGURE 3 (*A*) Active fiber stiffness at maximum calcium activation as a function of EMD 57033 concentration. The values are normalized to stiffness without EMD 57033, n = 6 fibers, ± SEM, and the speed of the applied length change was 3–4 × 10^3 (nm/HS)/s). (*B*) Relaxed fiber stiffness as a function of speed of stretch in the presence of 10 mM MgATPγS at pCa 9.0 and pCa 4.5 in the absence of EMD 57033 and with 30 μM EMD 57033. The values are normalized to stiffness (average) observed in the presence of calcium and EMD 57033 at the fastest stretch velocities. Data are from one representative fiber. ○, No EMD 57033, pCa 9.0; ●, 30 μM EMD 57033, pCa 9.0; □, no EMD 57033, pCa 4.5; ■, 30 μM EMD 57033, pCa 4.5. Ionic strength = 75 mM; T = 1°C. Note that the conditions here are somewhat different from all of the other experiments presented in this paper. (Lines fitted by eye.)

To test whether the slightly higher active stiffness could indicate an increased actin affinity of weakly (and/or strongly) binding cross-bridges with EMD 57033, we also studied the effect of EMD 57033 on weakly binding cross-bridges. Because we had found that some but small active force is generated under relaxing conditions with EMD 57033, we had to use the nonhydrolyzable ATP analog ATPγS to avoid active cross-bridge cycling while measuring relaxed fiber stiffness with EMD 57033. Cross-bridges with ATPγS as nucleotide were previously shown to represent weakly binding cross-bridge states, even in the presence of calcium (Kraft et al., 1992). Fig. 3 *b* shows that 30 μM EMD 57033 causes an increase in relaxed fiber stiffness with ATPγS both in the presence and in the absence of calcium. These data are consistent with a somewhat increased actin affinity of cross-bridges in the presence of EMD 57033.

DISCUSSION

In the present study we provide evidence that the increase in force of fully calcium-activated skeletal muscle fibers with EMD 57033 is due to an increase in force contribution of a cross-bridge in the force-generating states (F_0). This increase appears to result from an increased average cross-bridge strain in each of the force-generating states. Measurements of turnover kinetics, fiber ATPase, and active fiber stiffness support the idea that cross-bridge cycling kinetics (i.e., f_{app} and/or g_{app}) are not affected by EMD 57033. From the equations that describe isometric force (Eq. 1) and tension cost (Eq. 4), it is obvious that an increase in F_0 can account not only for the increase in isometric force, but also for the decrease in ATPase/force without changes in cross-bridge cycling kinetics (f_{app}, g_{app}). In general, the effects of EMD 57033 on skinned skeletal muscle fibers, like the increase in force and the reduced tension cost, are very similar to the effects of EMD 57033 on skinned (Beier et al., 1991; Groß et al., 1993; Leijendekker and Herzig, 1992; Simnett et al., 1994) and intact cardiac muscle preparations (Groß and Daut, 1991; Dobrunz et al., 1995).

Effects on cross-bridge cycling kinetics

If the increase in force with EMD 57033 were due to changes in cross-bridge cycling kinetics resulting in a higher occupancy of force-generating cross-bridge states, i.e. a redistribution between non-force-generating and force-

generating states, these changes should 1) change the fraction of strongly bound cross-bridges and thus lead to changes in fiber stiffness and 2) appear as corresponding changes in k_{redev} and/or isometric fiber ATPase. Under the conditions used here (minimized P_i concentration and maximum calcium activation), k_{redev} is essentially the sum of f_{app} and g_{app}. Based on Eq. 1, changes in one of the two rate constants, f_{app} and g_{app}, or simultaneous changes in f_{app} and g_{app} could affect the isometric force level.

To account for a 50% increase in force (at 50 μM EMD 57033), either 1) g_{app} had to decrease by about 75% (no change in $_{fapp}$) or 2) f_{app} had to increase more than fourfold (no change in g_{app}). Such changes in g_{app} would lead to a decrease in the ratio ATPase/force by 80% and a decrease in k_{redev} by about 35%. A more than fourfold increase in f_{app} would result in an almost fourfold increase in k_{redev}. The results presented in this study (Fig. 1), however, indicate that ATPase/force decreases at most by 25%, and k_{redev} does not change significantly. Therefore both results are clearly inconsistent with the behavior expected when EMD 57033 effects would be the result of changes in f_{app} or g_{app}.

3) Another possibility is that both f_{app} and g_{app} change such that k_{redev} remains constant, while force increases more than 50%. This would require that without EMD 57033, $f_{app} \leq 1.5\ s^{-1}$, $g_{app} \geq 2.5\ s^{-1}$, and the resulting fraction of force-generating cross-bridges would be ≤ 0.375. In the presence of EMD 57033, the rate constants would have to change such that $f_{app}(+\text{EMD } 57033) \approx 2.25\ s^{-1}$, $g_{app}(+\text{EMD } 57033) \approx 1.75\ s^{-1}$. However, not only would such values for f_{app} and g_{app} without EMD 57033 be inconsistent with experimental data ($f_{app} = 3\text{–}4\ s^{-1}$, $g_{app} = 1\ s^{-1}$, occupancy of the strongly binding cross-bridge states at maximum activation $\approx 0.6\text{–}0.75$; Brenner, 1988), but fiber stiffness should also increase by 25% to 50%, depending on the contribution of filament compliance.

Note that if the occupancy of the force-generating cross-bridge states in the absence of EMD 57033 is already 0.6–0.75, an increase in force by 50–60% due to changes in the occupancy of force-generating states becomes rather unlikely.

The small change in active fiber stiffness, which is in clear contrast to the much larger increase in active force, supports this conclusion (Fig. 3 *a*). A nearly parallel increase in active stiffness and isometric force would be expected if the increase in force with EMD 57033 were due to an increase in the occupancy of the force-generating cross-bridge states, provided that stiffness is measured under conditions where non-force-generating (weakly bound) cross-bridges make little or no contribution to the observed fiber stiffness.

The findings that cross-bridge kinetics seem to be unaffected by EMD 57033 is further supported by the observation that EMD 57033 has no effect on V_{max}, the maximum unloaded shortening velocity. V_{max} is mainly determined by g_2, the rate constant for cross-bridge detachment during isotonic shortening (Huxley, 1957). This observation is consistent with results showing that EMD 57033 has no effect on the time course of cell shortening in intact cardiac muscle (Gambassi et al., 1993; Solaro et al., 1993). The fact that EMD 57033 increases isometric force generation but does not impair shortening velocity might be important for the possible role of EMD 57033 as positive inotropic agent.

A possible mechanism

As already pointed out, the increase in isometric force upon the addition of EMD 57033 seems to be due to changes in average force generated by the individual cross-bridges in the force-generating states (F_0). It was also shown that the cross-bridges presumably generate the higher force because the strain or distortion in each of the force-generating states (states *A* and *B* in the *inset* of Fig. 2) increases with EMD 57033, whereas no redistribution among the force-generating states is seen that could result in any relevant increase in isometric tension. Such an increase in strain shows up in *T* plots as a larger y_0 value in the presence of EMD 57033. However, the increase in y_0 with EMD 57033 could also result from filament compliance dominating fiber stiffness at such high force levels (Huxley, 1994; Wakabayashi, 1994). In this case, any change in force would be associated with only little change in fiber stiffness. To distinguish between the two possibilities we determined active force and active stiffness, also at submaximum force levels achieved by lowering the calcium concentration 1) in the presence and absence of EMD 57033 and 2) at 5°C and 15°C. Both the addition of EMD 57033 or raising the temperature to 15°C result in a similar increase in active force (Fig. 4, *a* and *b*). Reducing the calcium concentration in both cases causes a decrease in active stiffness which, however, is still nearly proportional to the decrease in active force as seen at low temperature in the absence of EMD. This suggests that the lack of an increase in active fiber stiffness in proportion with force upon the addition of EMD 57033 at 5°C (or raising temperature to 15°C) is not due to filament compliance dominating fiber stiffness, but rather reflects an increase in average strain of the force-generating cross-bridges. The observation that the two interventions, raising temperature or the addition of EMD 57033, show the same effects on force and fiber stiffness makes it rather unlikely that the observed behavior is mainly due to the direct effects of EMD 57033 or temperature on thin filament compliance, except when both interventions, by coincidence, would alter thin filament compliance to the same extent.

The larger strain and thus the larger F_0 with EMD 57033 might for example result from a tighter binding of these cross-bridges. Some evidence for tighter binding can be drawn from the effects of EMD 57033 on relaxed fiber stiffness with ATPγS (Fig. 3 *b*), indicating a change in actin binding kinetics, e.g., slower reversible dissociation with EMD 57033. As a consequence of higher actin affinity, cross-bridges could remain attached, even at higher strain

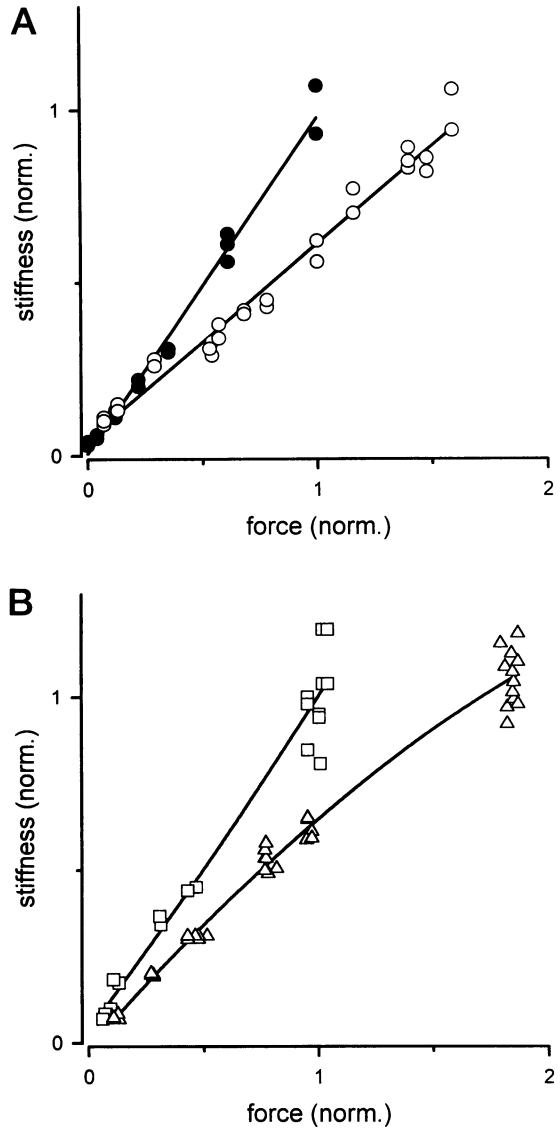

FIGURE 4 Plots of active fiber stiffness versus isometric force recorded at different calcium concentrations (pCa 7–4.5), (*A*) in the absence of EMD 57033 (●) and in the presence of 50 μM EMD 57033 (*b*) at 5°C (□) and at 15°C (△). Speed of stretch was about 4 × 10^3 (nm/HS)/s. (Lines represent linear least-squares fits (*a*) and second-order polynomial fits (*B*) to the data points.) The data in *a* and *b* show measurements of one representative fiber each. The data of each fiber were normalized to the average of all measurements made at pCa 4.5 in the absence of EMD 57033 at 5°C. The data points clustered around 1.0 represent the individual measurements at this condition. Note that for both interventions, the presence of EMD 57033, as well as increased temperature, stiffness still falls almost in proportion with tension, as it does at 5°C and without EMD 57033. Thus at all force levels fiber stiffness apparently is not dominated by filament compliance but is still sensitive to the fraction of force-generating cross-bridges.

levels, resulting in the higher F_0. It should be noted that such a mechanism does not necessarily result from binding of EMD 57033 to myosin. Our data do not allow us to determine the component of the actomyosin complex to which EMD 57033 might bind.

Relation to other studies

In several previous studies, mechanisms other than an increase in F_0 have been proposed for the EMD 57033 effects. From the observation that EMD 57033 causes a reduction in tension cost and a shift of the force/pCa relation to the left (Groß et al., 1993; Leijendekker and Herzig, 1992), it was concluded that a reduction in g_{app} could be responsible for the EMD 57033 effect. Such a conclusion can only be drawn when changes in F_0 that also affect tension cost can be excluded.

On the other hand, Simnett et al. (1994) found no effect of EMD 57033 on the relaxation rate of skinned skeletal muscle. From that, and the assumption that F_0 remains constant, they speculated that g_{app} is not affected but that EMD 57033 rather increases f_{app}. This would result in an increase in k_{redev} and would have had no effect on tension cost (Eq. 4). The opposite, however, was found in our study. Alternatively, an increase in F_0 without changes in cross-bridge cycling kinetics would also be compatible with no effect of EMD 57033 on the relaxation rate.

Findings that EMD 53998 decreases the tension cost of skinned cardiac muscle only at increased P_i concentrations (Strauss et al., 1994) and that the P_i release step is accelerated by EMD 53998 (Zhao and Kawai, 1995) led to the conclusion that EMD 53998 antagonizes the effects of high P_i concentrations on cross-bridges. This would favor force-producing cross-bridge states in the presence of high P_i concentrations and thus result in higher force levels. With such a mechanism, however, EMD 57033 would only exert an effect at high P_i concentrations. Although we cannot exclude an additional effect of EMD 57033 at high P_i levels, our results are inconsistent with the concept that EMD 57033 only reverses P_i effects. We obtained the large increase in isometric force already at very low P_i concentrations, achieved by the presence of sucrose and sucrose phosphorylase.

Several authors also observed a leftward shift of the force/pCa relation in the presence of EMD 57033 (Beier et al., 1991; Groß et al., 1993; Leijendekker and Herzig, 1992). Based on the present study, one possible explanation for such a leftward shift could be the higher actin affinity of the cross-bridges observed with EMD 57033. This could result in more pronounced activating effects of strongly attached cross-bridges on the thin filament at submaximum calcium concentrations. It may even be responsible for the activation of the thin filament at calcium concentrations sufficiently low for full relaxation in the absence of EMD 57033 (unpublished results). Hence the shift of the force-pCa relation by EMD 57033 could be due to enhanced activation potential of strongly attached cross-bridges, resulting in larger f_{app} at given nonsaturating calcium concentrations (i.e., the higher f_{app} at nonsaturating calcium concentrations would be an indirect effect of EMD 57033 via an increase in actin affinity of strongly bound cross-bridges). In agreement with that, studies on TnC-extracted cardiac myofibrils showed that EMD 57033 activates the

ATPase activity of these fibers but does not require TnC for transmission of its effect (Solaro et al., 1993). Furthermore, direct measurements of calcium binding to TnC in skinned cardiac muscle (Solaro et al., 1993) showed that EMD 57033 has no effect on the calcium binding properties of TnC.

However, it must be left to further experiments to clarify whether and to what extent a higher activation potential of strongly attached cross-bridges due to increased actin affinity may be responsible for the leftward shift of the force/pCa relation with EMD 57033 or whether additional effects are present.

Side effects

The increase in resting tension in intact cardiac muscle (Lee and Allen, 1991) and in diastolic pressure in isolated rat hearts (Ferroni et al., 1991) with EMD 53998 could well be due to the increased actin affinity of cross-bridges and possible increased activation potential that would result in a more pronounced turning on of the contractile system, even at low calcium concentrations. An increased activation potential of strongly attached cross-bridges might also be the basis for a prolongation of the time course of the twitch with EMD 57033 and EMD 53998 in intact heart muscle, which was found by several investigators (Beier et al., 1991; Ferroni et al., 1991; Lee and Allen, 1991; White et al., 1993; Groß et al., 1993; Dobrunz et al., 1995).

CONCLUSION

The results presented here indicate that the EMD 57033 effects might represent a new mechanism for positive inotropic agents that act through a direct effect on the force generated by each cross-bridge. Further support for the proposed mechanism through an increase in actin affinity must be left to additional studies. Nevertheless, it appears possible to directly affect the force contribution of a cross-bridge, which provides an additional method of positive inotropic intervention. Such a direct effect may have advantages over positive inotropic compounds that act through an increase in cytosolic calcium concentration and thus tend to produce calcium overload of the myocardium.

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